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Reassessing the role of Cyclin-dependent kinase 5 in tumourigenesis

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CONTENTS

Contents i
List of figures vii
List of tables x
Abbreviations xi
Amino acid code xvii
Acknowledgements xviii
Declarations xix
Abstract xx

CHAPTER 1 1
1.1 INTRODUCING AN UNUSUAL CYCLIN-DEPENDENT KINASE 3
1.2 DISCOVERY AND CHARACTERISATION OF CDK5 5
1.3 CDK5 CATALYTIC DOMAIN STRUCTURE 6
1.4 REGULATION OF CDK5 ACTIVITY 8
1.4.1 Cdk5 activation by regulatory subunits 8
1.4.1.1 Regulatory subunit, p35 10
1.4.1.2 Regulatory subunit, p39 11
1.4.1.3 Proteolytic fragments of p35 and p39 also induce Cdk5 activity 13
1.4.1.4 The theory of aberrant Cdk5 activity following activator truncation 14
1.4.2 Regulation of Cdk5 activity by synthesis and degradation of p35 15
1.4.3 Regulation of Cdk5 activity by phosphorylation 16
1.4.4 Pharmacological inhibition of Cdk5 activity 19
1.5 FUNCTIONAL DIVERSITY OF CDK5 SUBSTRATES 19
1.6 THE ROLE OF CDK5 IN NORMAL PHYSIOLOGY 25
1.6.1 Functional implications of Cdk5 in the nervous system 25
1.6.1.1 Cdk5 activity in the developing CNS 27
1.6.1.2 Cdk5 in the mature CNS 32
1.6.1.3 The role of Cdk5 in normal adult neurophysiology 37
1.6.2 Extra-neuronal functions of Cdk5 38
1.6.2.1 Cdk5 and transcriptional regulation 39
1.6.2.2 Cdk5 and translational regulation 41
1.6.2.3 Cell migration, adhesion, and angiogenesis 41
1.6.2.4 Apoptosis 43

1.6.2.5 Endocrinology and metabolism....43
1.7 PATHOGENIC IMPLICATIONS OF CDK5 DYSREGULATION 46
1.7.1 Neurodegenerative disease....46
1.7.1.1 Alzheimer's disease....47
1.7.1.2 Parkinson's disease....48
1.7.1.3 Huntington's disease....49
1.7.1.4 Amyotrophic lateral sclerosis....50
1.7.1.5 Niemann-Pick Type C....51
1.7.2 Pathology in extra-neuronal tissues....51
1.7.2.1 Cdk5 and diabetes....51
1.7.2.2 Cdk5 and senescence....53
1.7.2.3 Cdk5 and cancer....54
1.8 AIMS OF THESIS....60
1.8.1 Consensus for substrate phosphorylation....60
1.8.2 Assessing the hyperactivity of the p25/Cdk5 complex....61
1.8.3 Validating the tools to study Cdk5 phosphorylation in cancer....61
1.8.4 Investigating the role of Cdk5 in cancerous tissue....61

***CHAPTER 2*....62**
2.1 MATERIALS....63
2.1.1 Reagents....63
2.1.2 Recombinant Proteins....65
2.1.3 Antibodies....65
2.1.3.1 In house antibodies....65
2.1.3.2 Total CRMP2 antibody....67
2.1.3.3 pCRMP2 Ser522 antibody....67
2.1.3.4 Commercial antibodies....69
2.2 METHODS....71
2.2.1 General Use Buffer Preparation....71
2.2.2 Molecular Biology....72
2.2.2.1 Agarose Gel Electrophoresis....72
2.2.2.2 Restriction enzyme digest of plasmid DNA....72
2.2.2.3 Production of Chemically Competent Bacterial Cells for DNA Transformations...73
2.2.2.4 Transformation of Bacteria....74
2.2.2.5 Plasmid DNA Purification....75
2.2.2.6 DNA Concentration Determination....76

2.2.2.7 DNA Sequencing and Analysis 76
2.2.3 Cell Culture 76
2.2.3.1 Cell Line Maintenance 76
2.2.3.2 Cell Passage 78
2.2.3.3 Cell Storage 79
2.2.3.4 Culturing Primary Cortical Neurons 79
2.2.4 Transient Transfection 81
2.2.4.1 Calcium Phosphate ($Ca_3(PO_4)_2$)/BES Transient Transfection 81
2.2.4.2 Lipofection Transient Transfection 83
2.2.4.3 Mammalian Expression Constructs 83
2.2.5 Cell Stimulations 85
2.2.5.1 Inhibitors 85
2.2.6 Confocal Microscopy 85
2.2.6.1 Primary Neuron Cell Preparation 85
2.2.6.2 Image Analysis 86
2.2.7 Protein Isolation and Assay 86
2.2.7.1 Mammalian Cell Lysis Buffer Preparation 86
2.2.7.2 Cell Protein Isolation 87
2.2.7.3 Protein Immunoprecipitation 87
2.2.7.4 Subcellular Protein Fractionation 88
2.2.7.5 Measurement of Protein Concentration 89
2.2.7.6 Sodium Dodecyl-Sulphate Poly-Acylamide Gel Electrophoresis 90
2.2.7.7 Gel Coomassie Staining 91
2.2.8 Western Blot Analysis 92
2.2.8.1 Transfer of Proteins to Nitrocellulose Membrane 92
2.2.8.2 Immunoblotting 93
2.2.8.3 Visualisation of proteins on membranes 93
2.2.8.4 Analysis and Quantification of Western blots 93
2.2.9 Phosphorylation Assay 94
2.2.9.1 Assay of proline directed kinases 94
2.2.9.2 Peptide and protein substrate assay 95
2.2.9.3 Kinase Phosphorylation Stoichiometry 96
2.2.9.4 Phosphorylation Kinetics, V_{max} and K_m Calculation 96
2.2.10 Histological Techniques 97
2.2.10.1 Tissue preparation and paraffin embedding 97
2.2.10.2 Microtomy 98

2.2.10.3 Immunohistochemistry 98
2.2.10.4 The ABC complex 99
2.2.10.5 Hematoxylin Staining 99
2.2.10.5 Slide preparation for light microscopy 100
2.2.11 Mass spectrometry 100
2.2.11.1 Phosphorylation site mapping 100
2.2.11.2 Mass Spectroscopic Fingerprint Analysis 101
2.2.12 Statistical Analysis 102

CHAPTER 3 ..... **104**
3.1 INTRODUCTION **105**
3.1.1 Chapter Aims 106
3.1.1.1 Consensus sequence that regulates Cdk5 substrate selection 106
3.1.1.2 Effect of p35 proteolytic cleavage on Cdk5 substrate affinity 107
3.1.1.3 Validation and comparison of Cdk5 substrates 107
3.2 RESULTS **108**
3.2.1 Defining Cdk5 activity for *in vitro* analysis 108
3.2.1.1 Quantification of the specific activity of commercial Cdk5 109
3.2.1.2 Investigation of the purity of the commercial Cdk5 preparations 111
3.2.2 Investigating the consensus for Cdk5 substrate phosphorylation 113
3.2.2.1 C-terminal basic residues confer Cdk5 substrate affinity 113
3.2.2.2 Comparison of Cdk5 activity towards peptide substrates with sequences related to proposed protein substrates of Cdk5 118
3.2.2.3 N-terminal residue required for Cdk5 substrate recognition 122
3.2.2.4 Establishing the optimal Cdk5 substrate motif 126
3.2.2.5 Cdk inhibitors abolish Cdk5 peptide substrate phosphorylation 129
3.2.2.6 Cdk5 substrate selection in comparison to other members of the CMGC family 131
3.2.3 Investigating peptide consensus in full-length substrates 135
3.2.3.1 Basic residues flank the phosphoacceptor site in CRMP isoforms and lack of these residues diminishes phosphorylation 135
3.2.3.2 Cdk5 in vitro tau phosphorylation site is a class 1 substrate 146
3.2.3.3 Cdk5 phosphorylation of dynamin 1 152
3.2.3.4 Phosphorylation of full-length substrates lacking basic residues 154
3.2.3.5 The role of proline residues in Cdk5 substrate recognition 159
3.3 DISCUSSION **163**
3.3.1 Redefining the Cdk5 consensus 163

3.3.1.1 Important residues that guide Cdk5 substrate selection 163
3.3.1.2 Validation of consensus in full-length substrates 165
3.3.1.3 Consensus sequence is specific to Cdk5 166
3.3.1.4 Perspectives and future directions for a Cdk5 consensus sequence 167
3.3.2 Cdk5 as a potential priming kinase for GSK3 on CRMP5 168
3.3.2.1 Cdk5 displayed phosphotransferase activity towards CRMP5 169
3.3.3 Differential inherent activities of the p25/Cdk5 and p35/Cdk5 complexes *in vitro* does not account for the role of p25/Cdk5 in the pathogenesis of neurodegenerative disease 169
3.3.3.1 Substrate profile and inherent activity is similar between complexes 170
3.3.3.2 Perspectives and future directions to delinate the effect of p35 cleavage 172
3.4 SUMMARY 173

CHAPTER 4 174
4.1 INTRODUCTION 175
4.1.2 Chapter Aims 176
4.1.2.1 Sequence determinants 176
4.1.2.2 Tools to study Cdk5 in cells and tissue 176
4.1.2.3 Cdk5 and cancer 176
4.2 RESULTS 177
4.2.1 Establishing an appropriate cell model system in which to study Cdk5 substrate phosphorylation 177
4.2.1.1 Investigating Cdk5 substrate phosphorylation within HEK293 cells 177
4.2.1.2 Investigating Cdk5 substrate phosphorylation within HeLa cells 182
4.2.1.3 Examining substrate phosphorylation following Cdk5 inhibition in primary neuronal cells 191
4.2.2 Assessing Cdk5 substrate phosphorylation in human tumours 196
4.2.2.1 Antibody Optimisation for Immunohistochemistry 196
4.2.2.2 Antigen Retrieval Optimisation 198
4.2.2.3 Optimal Antibody Concentrations 200
4.2.2.4 Validation of Phospho-Specific antibodies 202
4.2.2.5 Cdk5 substrate phosphorylation in lung carcinomas 204
4.2.2.6 Further analysis of pCRMP2 staining in squamous cell carcinoma and adenocarcinoma 209
4.2.2.7 How widespread is the abnormal pCRMP2 Ser522 in cancer? 211

4.2.2.8 Assessing phosphorylation of CRMP2 in human tumours by means of Tumour Microarray (TMA) 215
4.2.3 Does phosphorylation of CRMP2 at Ser522 occur in the nucleus of tumour cells in culture? 220
4.2.3.1 Cdk5 and its cofactor are expressed in human lung cancer cell lines 220
4.2.3.2 Fractionation of NSCLC cell lines reveals nuclear localisation of a potential CRMP2 isoform 222
4.2.3.3 Nuclear expression of CRMP2 isoform confirmed by Mass Spectroscopic fingerprint analysis 225
4.3 DISCUSSION 227
4.3.1 Cdk5 substrate phosphorylation is apparent in human tumours 227
4.3.2 CRMP2 phosphorylation is greater in squamous cell carcinoma compared to adenocarcinoma 229
4.3.3 Phosphorylated CRMP2 in other cancers 230
4.3.4 CRMP2 nuclear localisation in cancer cell lines 232
4.3.4.1 CRMP2A is observed in the nucleus of cancer cell lines 232
4.3.4.2 A novel role for the long form of CRMP1 in tumourigenesis 233
4.3.5 Developing additional readouts of Cdk5 activity *in vivo* 233
4.3.5.1 Inefficiency of the triple transfection system 234
4.3.6 Conclusions 234
4.4 SUMMARY 235

***CHAPTER 5* 236**
5.1 RESEARCH QUESTIONS SURROUNDING CDK5 237
5.2 MAIN CONTRIBUTIONS OF THE THESIS 238
5.2.1 Abnormal Cdk5 phosphorylation in cancer 238
5.2.2 Towards a Cdk5 consensus sequence and the role of p35 cleavage 239
5.3 FUTURE DIRECTIONS 241
5.3.1 Identification of novel Cdk5 substrates 242
5.3.2 Investigating the role of Cdk5 in Cancer 242
5.3.3 Mechanistic Studies 243
5.3.4 Clinical Utility 244
5.4 CONCLUSION 245

***REFERENCES* 245**

LIST OF FIGURES

CHAPTER 1 ..... **1**

Figure 1.1 Regulation of cell cycle progression by Cdk-Cyclin complexes 4

Figure 1.2 Genes encoding the Cdk5 activators, p35 and p39 9

Figure 1.3 Molecular structure of Cdk5 activators and their C-terminal fragments generated by calpain cleavage 12

Figure 1.4 Structure of the p25/Cdk5 complex cleavage 18

Figure 1.5 The multifaceted role of Cdk5 18

CHAPTER 3 ..... **104**

Figure 3.1 Comparison of the major Cdk5 complexes activity against MBP 110

Figure 3.2 MBP kinase activity of the Cdk5 preparations is dramatically decreased in vitro following treatment with purvalanol A but not harmine 112

Figure 3.3 Contribution of C-terminal basic residues to Cdk5 substrate phosphorylation in vitro 115

Figure 3.4 Optimisation of Cdk5 substrate peptides containing basic residues, phosphorylation kinetics 117

Figure 3.5 Phosphorylation of peptides with varying basic residue content by Cdk5 121

Figure 3.6 Cdk5 regulation is dependent on proline residues 125

Figure 3.7 Determination of Cdk5 optimal substrate motif 127

Figure 3.8 Determining the optimal Cdk5 peptide substrate motif 128

Figure 3.9 Inhibition of Cdk5 activity results in loss of peptide phosphorylation 130

Figure 3.10 Schematic representation of the CMCG group and selected members for substrate comparison 132

Figure 3.11 CMGC kinase screen using Cdk5 substrate peptides 134

Figure 3.12 CRMP phosphorylation sites and sequence alignment of the CRMP isoforms 136

Figure 3.13 p35/Cdk5 and p25/Cdk5 display identical CRMP isoform specificity 138

Figure 3.14 The major Cdk5 phosphorylation site is Ser522 140

Figure 3.15 Kinetic analysis of CRMP1 phosphorylation with the major Cdk5 complexes 142

Figure 3.16 Kinetic analysis of CRMP2 phosphorylation with the major Cdk5 complexes 144
Figure 3.17 Kinetic analysis of CRMP4 phosphorylation with the major Cdk5 complexes 145
Figure 3.18 Both Cdk5 complexes phosphorylate tau 147
Figure 3.19 Kinetic analysis of tau phosphorylation with the major Cdk5 complexes. 149
Figure 3.20 Validation of phospho-tau antibodies 151
Figure 3.21 Kinetic analysis of dynamin 1 phosphorylation by the major Cdk5 complexes.....153
Figure 3.22 Kinetic analysis of p53 phosphorylation by the major Cdk5 complexes.....155
Figure 3.23 Kinetic analysis of PPARγ1 phosphorylation by Cdk5 complexes.....157
Figure 3.24 Kinetic analysis of PPARγ2 phosphorylation by Cdk5 complexes.....158
Figure 3.25 Kinetic analysis of Mef2a phosphorylation by the major Cdk5 complexes 160
Figure 3.26 BSA standard curve for determining protein concentration of Mef2a preparation 162

CHAPTER 4 174
Figure 4.1 Generation of an active Cdk5 complex within a mammalian cell line ...179
Figure 4.2 Triple transfection in HEK293 cells successfully overexpresses Cdk5 and cofactor but fails to overexpress substrate 181
Figure 4.3 Kinase activity is dramatically greater following overexpression of Cdk5 and cofactors compared to cofactors alone 184
Figure 4.4 Triple transfections in HeLa cells results in Cdk5 and cofactor overexpression but overexpression of substrate is affected 186
Figure 4.5 Overexpression of Cdk5 complexes results in increased Ser522 phosphorylation in CRMP2 but not CRMP4 188
Figure 4.6 Overexpression of Cdk5 complexes results in increased phosphorylation of Tau … 190
Figure 4.7 Purvalanol A treatment reduces Cdk5 phosphorylation of CRMP2 but not CRMP4 at Ser522 193
Figure 4.8 Purvalanol A treatment reduces Cdk5 phosphorylation of tau.. 195

Figure 4.9 Schematic representation of stepwise antibody validation.....197
Figure 4.10 Cdk5 inhibition reduces pCRMP2 Ser522 immunostaining in primary cortical neurons.....203
Figure 4.11 Representative images of phosphorylated CRMP2 protein expression in lung tumour biopsies.....205
Figure 4.12 Immunohistochemistry staining scores of lung tumour biopsies 208
Figure 4.13 Phosphorylation of CRMP2 by Cdk5 is stained more intensity in squamous cell carcinoma compared to adenocarcinoma 210
Figure 4.14 Total CRMP2 antibodies show a lack of tissue specificity 212
Figure 4.15 Immunohistochemical staining of rat tissue with a phospho-specific pCRMP2 Ser522 antibody 214
Figure 4.16 Diffuse Large B-Cell Lymphoma (DLBCL) 217
Figure 4.17 Frequency of additive quick scores in TMA result subsets.....218
Figure 4.18 Immunohistochemical evaluation of pCRMP2 S522 protein expression in tissue microarrays of Diffuse Large B-Cell Lymphoma (DLBCL).....219
Figure 4.19 CRMP2 phosphorylation at Ser522 is recapitulated in NSCLC cell lines positive for CRMP2 and Cdk5 protein expression 221
Figure 4.20 Identification of phosphorylated CRMP2 in nuclear fraction 224
Figure 4.21 Identification of peptide corresponding to CRMP2A identified in HEK293 nuclear fraction 226

LIST OF TABLES

CHAPTER 1 1

Table 1.1 Identified Cdk5 substrates and their functional categories 21

CHAPTER 2 62

Table 2.1 In-house antibodies 66

Table 2.2 Phosphospecific CRMP antibodies 68

Table 2.3 Commercial antibodies.....70

Table 2.4 $Ca_3(PO_4)_2$/BES transfection mix volumes.....82

Table 2.5 Mammalian expression constructs 84

CHAPTER 3 104

Table 3.1 Peptides designed to investigate the role of basic residues for Cdk5 phosphorylation.....119

Table 3.2 Further investigation of the role of basic residues in Cdk5 substrate phosphorylation.....123

Table 3.3 Phosphorylation kinetic parameters of Cdk5 substrates and comparison between the major Cdk5 complexes 171

CHAPTER 4 174

Table 4.1 Antibodies to assess Cdk5 substrate phosphorylation by IHC require antigen retrieval by heat induced epitope retrieval (HIER) 199

Table 4.2 Optimised antibody concentrations selected for IHC 201

Table 4.3 Evaluation of immunohistochemistry 207

ABBREVIATIONS

Aβ	β amyloid
Abl	c-Abelson
AChR	acetylcholine receptor
AD	Alzheimer's Disease
ADC	adenocarcinoma
ALS	Amyotrophic lateral sclerosis
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ape1	apurinic/apyrimidinic endonuclease 1
ATM	ataxia telangiectasia mutated
ATP	adenosine 5' triphosphate
APP	amyloid precursor protein
Bax	Bcl-2-associated X protein
Bcl-2,	B-cell CLL/lymphoma 2
BDNF	brain-derived neurotropic factor
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BPDK	brain proline-directed kinase
BSA	bovine serum albumin
Cables	Cdk5 and Abl enzyme substrates
CAMKII	Ca^{2+} /calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CASK	calcium/calmodulin-dependent serine protein kinase
$Ca_v1.2$	L-type voltage-gated calcium channel
$Ca_v2.1$	P/Q-type voltage-gated calcium channel
Cdc	Cell division cycle
Cdh1	cadherin-1
Cdk	cyclin-dependent kinase
Cdk5-AD	Cdk5 activation domain
CKI	cyclin kinase inhibitor
Clk	Cdk-like kinase
CNS	central nervous system
CRMP	collapsin response mediator protein

c-Src	non-receptor tyrosine kinase
C-terminal	carboxy terminal
°C	degrees celsius
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein, M_r 32 kDa
DCX	doublecortin
DISC1	Disrupted in Schizophrenia 1
Dixdc1	Dix-domain containing 1
DLBCL	diffuse large B-cell lymphoma
DMSO	dimethyl sulphoxide
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DRG	dorsal root ganglion
DTT	dithiothreitol
DYRK	dual specificity tyrosine phosphorylation regulated kinase
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
ePK	eukaryotic protein kinase
EPRS	glutamyl-prolyl-tRNA synthetase
ErbB3	receptor tyrosine-protein kinase erbB3
Erk	extracellular signal-regulated kinase
E-syt	extended synaptotagmins
FAK	focal adhesion kinase
FBS	foetal bovine serum
fEPSP	field excitatory postsynaptic potential slope
FFAs	free fatty acids
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
GFP	green fluorescent protein
GLUT4	glucose transporter type 4
GR	glucocorticoid receptor
GSK3	glycogen synthase 3

GST	gluthathione S transferase
GTP	guanosine triphosphate
HD	Huntington's Disease
HDAC	histone deacetylase
HEK	human embryonic kidney
HMGB1	high mobility group box 1
HSF2	heat shock factor 2
hrs	hours
htt	huntington protein
IHC	immunohistochemistry
IL	interleukin
IP	immunoprecipitation
IPTG	isopropyl β-D-1-thiogalactopyranoside
JNK3	c-Jun N-terminal kinase 3
kDa	kilodalton
kb	kilobase
KO	knockout
LB	liquid broth
LH	luteinising hormone
LTD	long-term depression
LTP	long-term potentiation
M	milli
M	molar
MAP	microtubule-associated protein
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MBP	myelin basic protein
mDab1	mouse disabled
MEF	myocyte enhancer factor
MEK	MAP kinase kinase-1
Mg-ATP	magnesium adenosine 5'-triphosphate complex
mib1	mind bomb 1
min	minute

mol	mole
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MT	microtubule
MTC	medullary thyroid carcinoma
Munc18	mammalian uncoordinated 18
myt1	myelin transcription factor 1
N	nano
nclk	neuronal cdc-2-like kinase
NF	neurofilament
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFT	neurofibrillary tangle
NGF	nerve growth factor
NMDA	N- methyl D-aspartate
NMJ	neuromuscular junction
NPC	Niemann-Pick type C
NR2A	N- methyl D-aspartate receptor subunit 2A
NR2B	N-methyl D-aspartate receptor subunit 2B
NRG	neuregulin
NSCLC	non-small cell lung cancer
NSF	N-ethylmaleimide sensitive factor
N terminal	amino terminal
NUDEL	nuclear distribution protein nude-like
P	pico
p27(kip1)	cyclin-dependent kinase inhibitor
p35	regulatory activator of cyclin-dependent kinase 5, M_r 35 kDa
p39	regulatory activator of cyclin- dependent kinase 5, M_r 39 kDa
p53	tumor protein 53
PAGE	polyacrylamide gel electrophoresis
PAK1	p21-Activated Kinase
Paxillin	focal adhesion- associated adaptor protein
PBS	phosphate buffered salines
PCR	polymerase chain reaction
PCTAIRE1	cyclin-dependent kinase 16 (cdk16)

PD	Parkinson's disease
PFA	paraformaldehyde
PHF	paired helical filament
PI3K	phosphoinositide 3-kinase
PIPKI	phosphatidylinositol(4) phosphate 5 kinase type I gamma
PKA	cyclic AMP-dependent protein kinase
PLD2	phospholipase D2
Plk2	polo- like kinase 2
poly-Q	polyglutamine
PP-1	protein phosphatase-1
PPARγ	peroxisome proliferator-activated receptor gamma
PRE	prion-related encephalopathies
Prx2	peroxiredoxin 2
PSA	prostate-specific antigen
PSD-95	postsynaptic density protein 95
RasGRF	ras guanine nucleotide releasing factor 2
Rb	retinoblastoma protein
RNA	ribonucleic acid
RNAi	RNA interference
ROCK	Rho-associated kinase
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SCC	squamous cell carcinoma
S6K1	S6 kinase 1
SDS	sodium dodecyl sulfate
sec	second
SEM	standard error of the mean
Sema3	semaphorin3
Sept5	septin 5
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SNAP-25	synaptosomal-associated protein 25
SNARE	soluble N-ethylmaleimide-sensitive fusion attachment receptor
SNP	single-nucleotide polymorphism

SPAR	spine-associated Rap guanosine triphosphatase activating protein
stAR	steroidogenic acute regulatory protein
STAT3	signal transducer and activator of transcription 3
SVE	synaptic vesicle endocytosis
T2DM	Type 2 Diabetes mellitus
TBS	tris buffered saline
TBST	tris buffered saline tween
TCF	transcription factor
TH	tyrosine hydroxylase
TNF-α	tumour necrosis factor-alpha
TPKII	tau protein kinase II
TrkB	tropomyosin receptor kinase B
tRNA	transfer ribonucleic acid
TRPV-1	transient receptor potential vanilloid-1
U	unit
VAMP	vesicle-associated membrane protein
v/v	volume to volume
w/v	weight to volume
WFS1	Wolfram syndrome 1
WT	wild type

AMINO ACID CODE

Amino Acid Code	Three Letter Code	Single Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ilo	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenyalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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DECLARATIONS

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is of my own composition. Work other than my own is stated with reference to the researcher or their publications. This body of work has not been previously presented for a higher degree.

Nicola Grant

I certify that Nicola Grant has spent the equivalent of at least nine terms in research work in the Medical Research Institute (MRI), School of Medicine, University of Dundee. She has fulfilled the conditions of the Ordinance General No. 14 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Dr. Calum Sutherland

ABSTRACT

The discovery of Cyclin-dependent 5 (Cdk5) added a new member to the mammalian Cdks, appearing uncharacteristic of its family members despite strong sequence homology. In contrast to the mitotic Cdks, Cdk5 exerts no apparent role in cell cycle regulation therefore its potential role in cancer is subject to much controversy.

Cdk5 is heavily depicted as a neuronal-specific kinase, due to its association with neuronal activators, and dysregulation of Cdk5 activity is thought to contribute to the pathogenesis of several neurodegenerative diseases, including Alzheimer's disease. Nonetheless, the physiological functions of this kinase are being complicated by an upsurge in potential substrates that implicate Cdk5 activity in extra-neuronal functions and diseases such as cancer. However, the predicted substrates fail to meet a general consensus sequence and are yet to be fully validated *in vivo*. Furthermore, the involvement of Cdk5 in pathophysiological pathways is only partially understood. Adding further complexity, truncation of the Cdk5 cofactor, p35 is thought to increase the catalytic ability of the Cdk5 complex and alter its substrate specificity profile, although little evidence has been provided to support this claim.

In the present study, *in vitro* peptide analysis was performed in an attempt to identify a consensus sequence that regulates Cdk5 substrate phosphorylation, as a consensus for Cdk5 substrate selection is generally considered degenerative. Despite this theory, Cdk5 displayed high peptide selectivity that was shown to be distinct from other members of the CMGC family of proteins to which it belongs. While Cdk5 showed a preference for basic residues, interestingly, it was the presence of a proline residue located N-terminally (-2) relative to the phosphoacceptor residue that appeared to

dictate substrate phosphorylation. Further *in vitro* analysis using full-length proteins confirmed a correlation between substrates possessing the optimal consensus residues and substrate phosphorylation; those containing these residues were phosphorylated to a greater extent *in vitro* compared to those lacking these residues. Furthermore, both Cdk5 complexes displayed similar substrate profiles and inherent catalytic ability, as determined by comparison of phosphorylation kinetic parameters between complexes, despite the notion of a hyperactive complex with altered substrate specificity following activator truncation.

Following manipulation of Cdk5 activity in cell lines and intact tissue, tau and CRMP2 were confirmed as Cdk5 targets. Subsequently, a panel of total and phospho-specific antibodies to each substrate was assessed for compatibility with the immunohistochemistry protocol to serve as a surrogate for examining Cdk5 activity in tumours. For the first time, this study presents abnormal CRMP2 phosphorylation at a Cdk5 sensitive site in the nuclei of lymphoma, lung, and breast tumour cells, implicating Cdk5 activity in tumourigenesis. Furthermore, this is the first implication of long-form CRMP2 (CRMP2A) in the nucleus and potentially the isoform associated with cancer.

Overall, the data presented in this study provides a platform in which to improve mechanistic insight into tumourigenesis in specific forms of cancer, which will potentially provide new diagnostic and therapeutic strategies for cancer in the near future.

CHAPTER 1

Introduction

Protein phosphorylation, mediated by protein kinases and opposed by protein phosphatases, is one of the most widespread regulatory mechanisms in eukaryotes, as well as being the major molecular mechanism through which protein function is regulated in response to extracellular stimuli (Newman *et al*., 2014). As a consequence, protein kinases comprise one of the largest families of evolutionarily related proteins and comprise one of the most abundant gene families in humans, encompassing ~600 members (Mannin, 2002). Phosphorylation of target substrates dynamically regulates protein activity, changes in protein complexes, subcellular localisation, marks proteins for degradation, and serves to orchestrate the activity of almost all cellular processes (Peck, 2006). Kinases are heavily involved in signal transduction and coordination of complex functions such as the cell cycle (Rauch *et al*., 2011).

To ensure signalling specificity in response to a given environmental stimulus, phosphorylation networks must be precisely coordinated in cellular space and time (Newman *et al*., 2014). This involves regulation at several different levels, including the levels of protein kinase expression, substrate specificity and selection, and spatiotemporal regulation of enzymatic activity. Dysregulation can have profound effects and contribute to the pathophysiology of many pervasive diseases, including cancer (Guha *et al*., 2008; Deschenes-Simard *et al*., 2014), diabetes (Guo, 2014; Mackenzie & Elliott, 2014; Ortsater *et al*., 2014), and heart disease (Kooij *et al*., 2014; Sciarretta *et al*., 2014). Therefore, understanding the regulation and effects of manipulating kinase activity and isolating the physiological implications of protein kinases is of ultimate importance as a therapeutic option. Consequently, therapies based on this fundamental mechanism are one of the largest and fastest growing areas of drug discovery.

1.1 INTRODUCING AN UNUSUAL CYCLIN-DEPENDENT KINASE

The Cyclin-dependent kinases (Cdks) are a large family of proline-directed serine-threonine kinases that are well known for their role in the regulation of the eukaryotic cell cycle (***Figure 1.1***). The family has been highly conserved throughout evolution, reflecting its key roles in cell growth and division. The Cdk family consists of nine small (30 - 35 kDa) kinases, numbered according to their discovery, from Cdk1 to Cdk9 (Morgan, 1997). The biological function of these kinases ranges from a fundamental role in the control of mitosis through to regulation of key cellular processes, such as differentiation, senescence, and apoptosis (Tannoch *et al*., 2000). In proliferating cells, Cdk dysregulation is associated with tumour formation, whereas their disappearance/inhibition in neuronal precursors coincides with terminal differentiation (Okano *et al*., 1993).

Cdk activity generally requires association with regulatory subunits, the best known of which are the cyclins. Specific Cdks are associated with different phases of the cell cycle however their activities can sometimes overlap depending on their binding with different cyclins (Morgan, 1997; Nguyen *et al*., 2002; Nigg, 2001).

Despite strong sequence homology to mouse Cdk1 and human Cdk2 (Hellmich *et al*., 2002), Cdk5 is considered the atypical member of the Cdk family of proteins. In direct contrast to the mitotic Cdks, Cdk5 exerts no apparent role in cell cycle control and is most active in postmitotic neurons (Lew *et al*., 1992). This is attributed to high expression of its specific activators in the nervous system and together the Cdk5 complex plays an important role in neuronal development and neurotransmission. The dysregulation of Cdk5 activity is thought to contribute to the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD) (Cruz & Tsai, 2004).

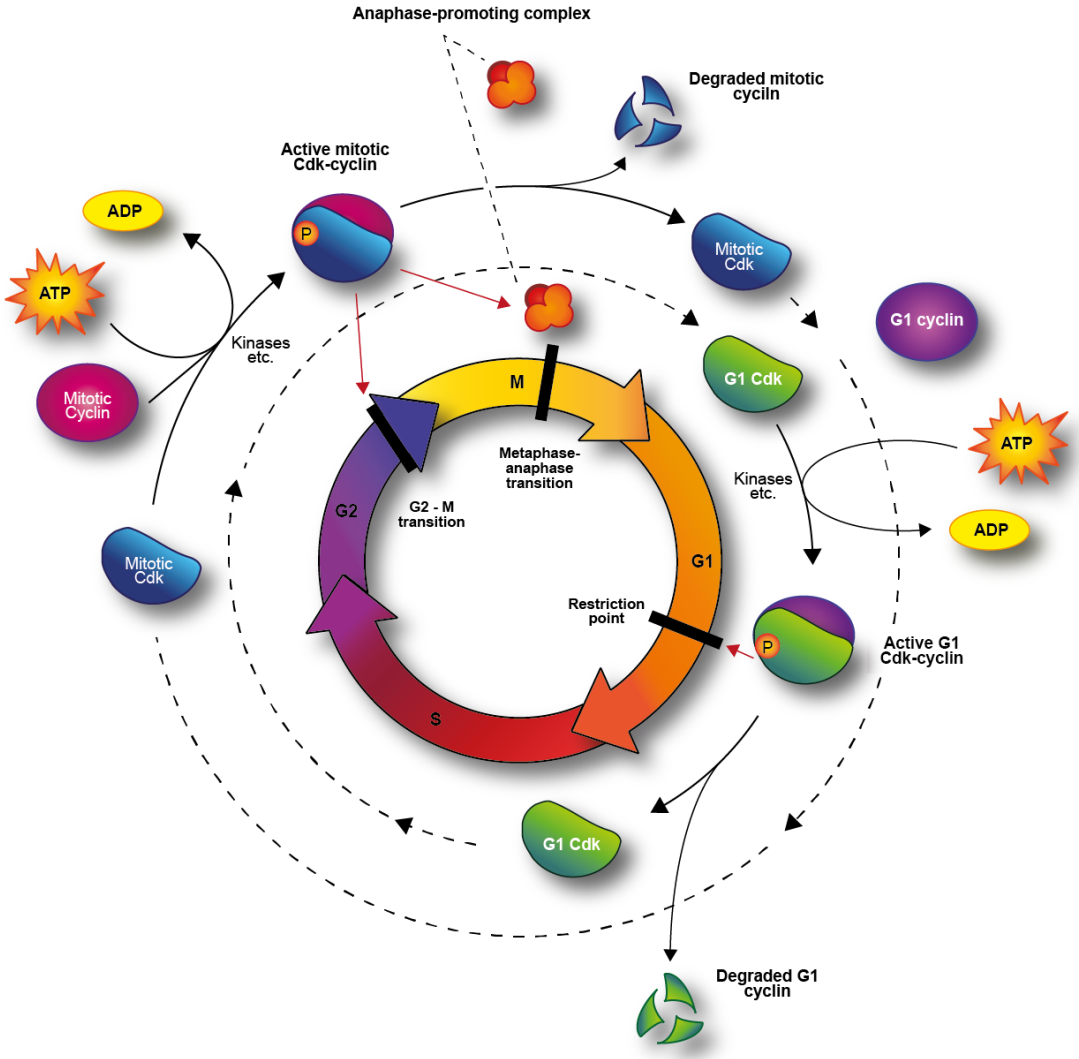


Figure 1.1. ***Regulation of cell cycle progression by Cdk-Cycln complexes***. As a cell progresses though the cell cycle, successive waves of Cdk activity rise and fall as cyclins are synthesised and then degraded by the proteasome after ubiquitylation. In response to mitogenic signals, D-type cyclins are synthesised and subsequently direct Cdk4 (and/or Cdk6) to phosphorylate pocket proteins such as the retinoblastoma (Rb) tumour suppressor. At the G1/S transition, the cyclin E/Cdk2 complex functions to trigger chromosomal DNA replication and to initiate centrosome duplication. The cyclin A/Cdk2 complex collaborates with cyclin E/Cdk2 to regulate DNA replication both positively and negatively to ensure that DNA is only replicated once. Finally, cyclin B/Cdk1 activity reorganises the cell for mitosis (Moore, 2013).

1.2 DISCOVERY AND CHARACTERISATION OF CDK5

In the early 1990s, a novel Cdk family member was described by four independent groups: firstly, an activity termed brain proline-directed kinase (BPDK), was purified from bovine brain based on the ability to phosphorylate a synthetic peptide containing the phosphorylation consensus sequence for yeast $p34^{cdc}$ kinase, a protein kinase integral to the eukaryotic cell cycle (Lew *et al*., 1992); simultaneously, a sequence termed PSSALARE (alpha C helix) was identified using reverse-transcriptase polymerase chain reaction (RT-PCR) of HeLa cell mRNA with degenerate primers corresponding to conserved regions of Cdc2 (Meyerson *et al*., 1992); thirdly, a cDNA was cloned from a rat brain cDNA library as an enzyme that could phosphorylate lysine-serine-proline motifs of neurofilaments and was, subsequently, termed neuronal cdc-2-like kinase (nclk) due to strong sequence homology to mouse Cdk1 (58%) and human Cdk2 (61%) and its high expression in terminally differentiated neurons no longer in the cell cycle (Hellmich *et al*., 1992); finally, a kinase that associated with microtubules and phosphorylated the Alzheimer's related protein Tau (Tau protein kinase II (TPKII)) was identified (Ishiguro *et al*., 1992). Following the identification of the 33 kDa protein non-cyclin regulatory subunit and the confirmation of the high homology with Cdks, the consensus nomenclature for these four activities became Cdk5 (Kobayashi *et al*., 1993).

The chromosomal location of the Cdk5 gene was mapped to 7q36 using a genomic clone encoding human Cdk5 (Demetrick, 1994). The Cdk5 protein is encoded by a 3.95kb stretch of DNA with 12 exons encoding for the 987bp kinase, a regulatory subunit of molecular mass 33 kDa that displays enzymatic activity when it becomes bound to activators.

The transcription factor ΔFosB regulates the transcription of Cdk5. Elevated Cdk5 mRNA and protein levels are seen following dopamine or cocaine administration, both of which are known to increase ΔFosB. Furthermore, an elevation in the levels of Cdk5 mRNA and protein levels is witnessed in transgenic mice that overexpress ΔFosB (Bibb *et al*., 2001; Cyr *et al*., 2003). Cdk5 is found at the highest expression levels in the brain of adult mice and in low or undetectable levels in all other tissues (Tsai *et al*., 1994).

1.3 CDK5 CATALYTIC DOMAIN STRUCTURE

Along with the other members of the Cdk family, Cdk5 belongs to the CMGC group of eukaryotic protein kinases (ePKs) (Hanks & Hunter, 1995; Kannan & Neuwald, 2004; Manning *et al*., 2002). The CMGC group, whose name is derived from an acronym of Cdk, MAP kinase, GSK3, and Cdk-like kinase (Clk), principally consists of proline-directed kinases that phosphorylate serine or threonine (S/T) residues in close proximity to a proline (P) residue. As is the case for the Cdk and Erk (MAP) kinase family, this requirement take the form of a phosphate residue situated N-terminally adjacent to a proline residue. For GSK3, however, the requirement for a proline residue is not absolute and co-exists with an additional phosphate-priming mechanism (Dajani *et al*., 2001; ter Haal *et al*., 2001).

The presence of a proline residue located immediately C-terminal to either a serine or threonine residue is a critical requirement for phosphorylation by all members of the Cdk family. The presence of a hydrophobic pocket near the catalytic site accommodates the proline residue, formed as a result of the unusual peptide backbone (at valine 164, *numbering in Cdk2*), and dictates the strong preference for S/TP

motifs. The structure alleviates the requirement for the peptide substrate to provide a hydrogen bond donor and correctly positions the side chain of the phosphoacceptor residue for phosphate transfer (Brown *et al*., 1999).

In addition to the proline requirement, Cdk5 is reported to favour substrates that contain a basic residue, either lysine (K), arginine (R), or histidine (H), following the phosphoacceptor residue at the +3 position, defining the current Cdk5 consensus as (S/T)PX(K/R/H), where X is any amino acid (Beaudette *et al*., 1993; Shetty *et al*., 1993). Despite this consensus, a plethora of substrates has been identified for Cdk5 that fail to meet the requirements of this consensus sequence and, as a result, a consensus sequence that governs Cdk5 substrate selection is generally considered degenerative.

At present, it remains unclear how Cdk5 specifically recognises and discriminates between substrates. Recently, biochemical and structural studies have revealed subtle differences in substrate preferences between the Cdk family members (Echalier *et al*., 2010). For example, Cdk2 shows a very strong preference for a basic residue at the +3 position (Brown *et al*., 1999) whereas Cdk4 will phosphorylate SPXX motifs with similar efficiency to SPXK/R motifs (Takiki *et al*., 2009). This information suggests that differences in substrate recognition between the Cdks lies within the primary sequence surrounding the phosphoacceptor residue and therefore needs to be examined more thoroughly.

1.4 REGULATION OF CDK5 ACTIVITY

While the regulation of classical Cdks has been investigated extensively, regulation of Cdk5 activity is yet to be elucidated. However, it is clear that Cdk5 requires a regulatory subunit, and at least two distinct genes, namely p35 and p39, are known. Thus the synthesis and degradation of these subunits could be a major mechanism for regulation of Cdk5 activity. Similarly, pathways that regulate the interaction of Cdk5 with regulatory subunits, or alter inherent activity (e.g. through phosphorylation of Cdk5), or modify the association of the regulatory subunits with membranes could all contribute to Cdk5 control. In addition to these physiological regulations of Cdk5 activity, dysregulation of Cdk5 activity can occur subsequent to pathological p35 cleavage leading to hyperactivation/mis-localisation of Cdk5 (Dhavan & Tsai, 2001).

1.4.1 Cdk5 activation by regulatory subunits

Cdk5 is the catalytic subunit of an active heterodimeric complex consisting of Cdk5 bound to either p35 or p39, two largely similar Cdk5 cofactors encoded for by two different genes (Tsai *et al*., 1994; Tang *et al*., 1995) (***Figure 1.2***). These regulatory subunits are, at most, distantly related to the cyclins, with little sequence similarity, however they do possess three-dimensional domains similar to the Cdk-binding motif of cyclins (Tang *et al*., 1997; Morgans, 1995) and are selective in their binding of Cdk5 (Poon *et al*., 1997). It is likely that p35 folds into a similar rigid tertiary structure and interacts with Cdk5 through both N-terminal and C-terminal domains (Tang *et al*., 1995). This complex is predominantly found in post-mitotic cells and astrocytes but absent in mitotically active cells. Protein expression and mRNA studies indicate that p39 is most abundant in the posterior part of the brain and appears with a delay after p35 in the forebrain (Zheng *et al*., 1998; Takahashi *et al*., 2003).

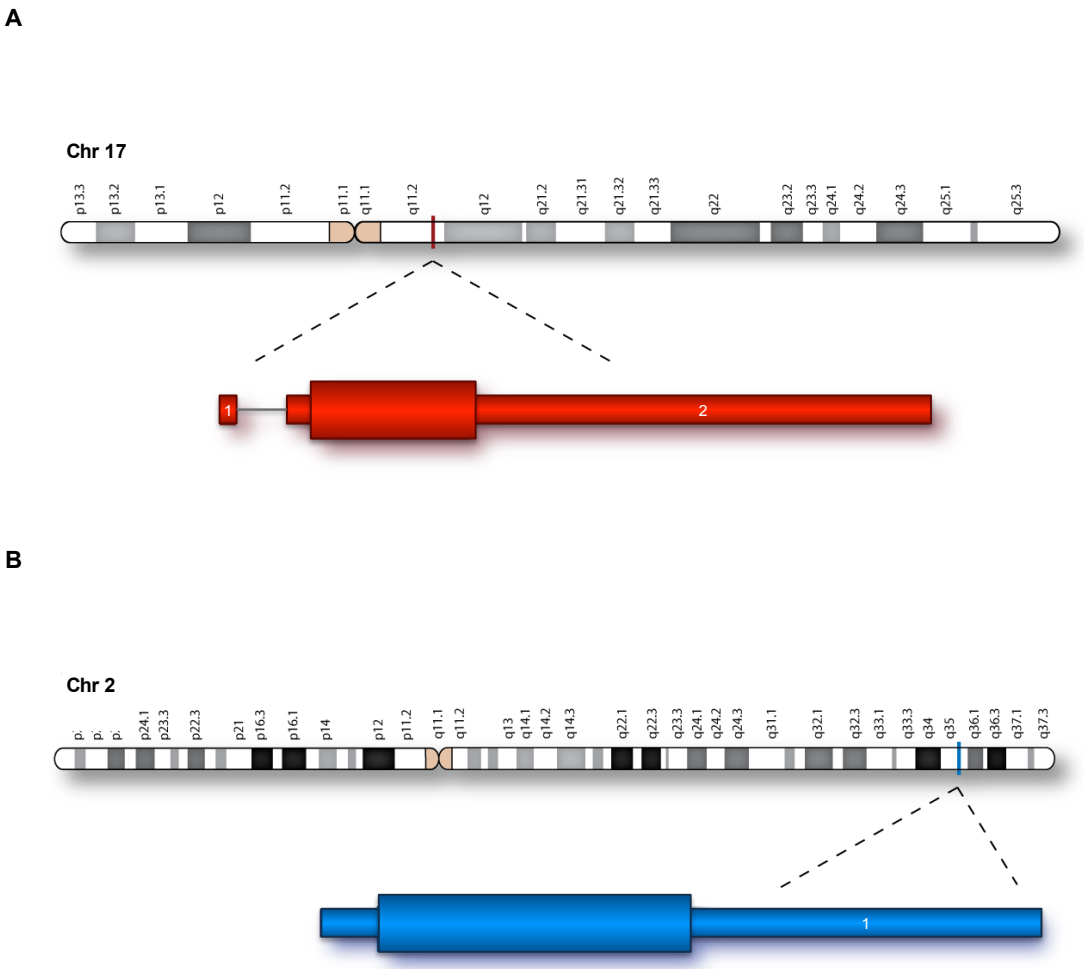


Figure 1.2. ***Genes encoding the Cdk5 activators, p35 and p39***. Schematic representation of the genes encoding the Cdk5 activator proteins. Each numbered box represents a coding exon. A) The gene encoding CDK5R1 (p35) is located on chromosome 17. B) The gene encoding CDK5R2 (p39) is located on chromosome 2.

1.4.1.1 Regulatory subunit, p35

Identification of the regulatory subunit activating Cdk5 kinase activity followed shortly after the discovery of the catalytic subunit (Tsai *et al*., 1994). The Cdk5 cofactor termed p35, which shares no homology to cyclins, was cloned and characterised by virtue of its association and regulation of Cdk5 activity. Subsequently, p35 was shown to exhibit a neuronal-specific pattern of expression *in vivo*, with expression of p35 mRNA identified in postmitotic cells and developing axonal tracts in the brain (Patrick *et al*., 1998). Transcription of p35 is stimulated by laminin in cerebellar macroneurons (Paglini *et al*., 1998), and p35 is a short-lived protein with a half-life ($t_{1/2}$) of 20 to 30 minutes (Patrick *et al*., 1998).

The minimum region of p35 that can induce Cdk5 activity spans from residues 138 – 291, and has an approximate molecular weight of 16 kDa (Amin *et al*., 2002). Within this region, three separate domains (N-, C-, and central domain) associate and interact with Cdk5, each of which is essential for Cdk5 activation (Amin *et al*., 2002) as the absence of any one of these domains prevents p35 induction of Cdk5. Furthermore, polypeptides that lack these domains can also behave as dominant negative inhibitors of Cdk5 activity (Amin *et al*., 2002).

Within the cell, timely activation of Cdk5 kinase activity at the precise site of substrate localisation is critical for the precise functioning of Cdk5. Cdk5 kinase activation is controlled through signalling by various changes in the cellular and external medium and the role of p35 is pivotal in maintaining the spatial activation of Cdk5. Association with p35 tethers Cdk5 to the membrane by hanging through a myristoyl residue linked to the N-terminal glycine and facilitates access of Cdk5 to membrane protein targets. Direct interaction of p35 with the substrates of the kinase is

not common. In addition, myristoylation of p35 regulates the access of Cdk5 to the nucleus by increasing the amount of kinase retained by the cytoplasmic and plasma membranes (Asada *et al*., 2008).

1.4.1.2 Regulatory subunit, p39

The discovery of another Cdk5 activator, p39 was made based on its high sequence homology to p35 (Tang *et al*. 1995). The p39 isoform contains around 150 amino acids in the Cdk5 activation domain (Cdk5-AD) in its C-terminal two-thirds, sharing ~70% sequence homology with p35 (Tang *et al*., 1995; Zheng *et al*., 1998) (***Figure 1.3***). In contrast, the N-terminal one-third displays a low sequence homology (37%) with p35, except for the extreme nine N-terminal amino acids that include a myristoylation signal (Patrick *et al*., 1999) and the lysine cluster in amino acids 75-85. At the C-terminus following the Cdk5-binding region, p39 has a domain that interacts with muskelin, a protein regulating cytoskeleton organisation in adherent cells (Ledee *et al*., 2005).

Isolating active p39/Cdk5 from brain extracts or cultured cells has proved problematic therefore purification of p39/Cdk5 from Sf9 cells using Ni-beads was used to characterise the kinase properties of Cdk5 complexed to p39 (Yamada *et al*., 2007). The p39/Cdk5 complex showed similar specific activity towards histone H1 when directly compared to p35/Cdk5. Additionally, both complexes phosphorylated tau, MAP2, neurofilament H subunit, inhibitor 1, mDab1, p35, and p39 (Ohshima *et al*., 2005; Yamada *et al*., 2007). It is perhaps not surprising that p35 or p39 have similar substrate preferences given the high homology of possible substrate-docking sites in both activators (Tarricone *et al*., 2001), however it is remains to be established whether either complex has alternative selective substrate targeting mechanisms.

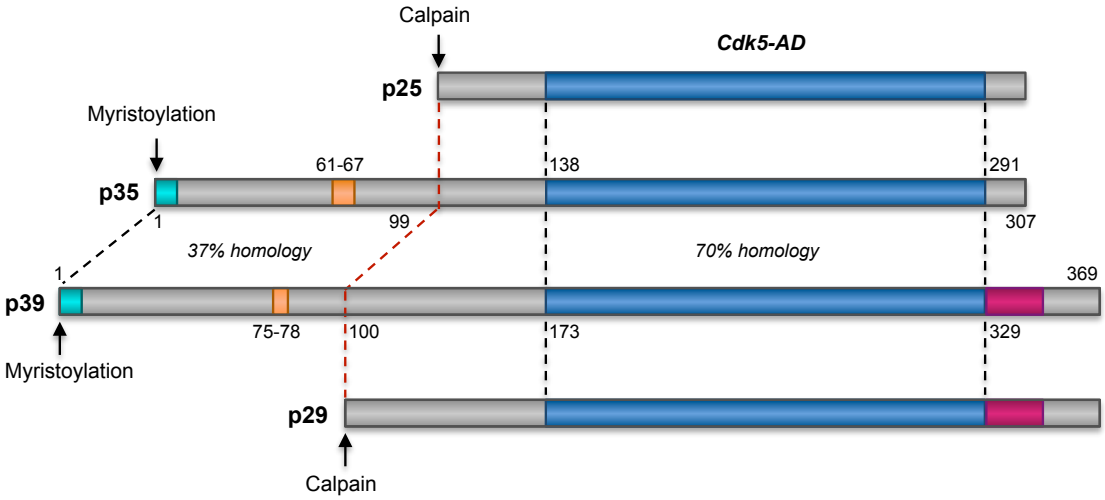


Figure 1.3. ***Molecular structure of Cdk5 activators and their C-terminal fragments generated by calpain cleavage.*** Comparison of the structure of p35 (307 amino acids) and p39 (369 amino acids). Both 35 and p39 contain a myristoylation site at Gly2 in the N-terminal common 9 amino acids (Green), and a lysine cluster at residues 61-67 in p35 and at residues 75-85 in p39 (Orange). Calpain cleaves p35 between residues 98 and 99 to generate p25, and cleaves p39 between residues 99 and 100 to generate p29. The Cdk5 activation domain (Cdk5-AD) is present in the C-terminal two-thirds encompassing about 150 amino acids (Blue). An insertion after the Cdk5 activation domain (Purple) is present in p39.

1.4.1.3 Proteolytic fragments of p35 and p39 also induce Cdk5 activity

Under normal physiological conditions, Cdk5 activity is tightly regulated by p35 and p39. However, under conditions such as oxidative stress, Aβ toxicity, inflammation, and others that led to increased levels of intracellular calcium (Ca^{2+}), proteolytic cleavage of both activators occurs and results in truncated fragments that can bind to and induce Cdk5 activity.

Elevation of intracellular Ca^{2+} induces activation of the calcium-dependent protease, calpain which then cleavages p35 between residues 98 and 99 to generate a 25 kDa N-terminally truncated proteolytic fragment (p25) and a 10 kDa proteolytic fragment (p10) (***Figure 1.3***). Calpain directly cleaves p35 *in vitro* to release a fragment with relative molecular mass 25, 000 daltons and the sequence of the calpain product corresponds precisely to that of p25 isolated from cells; hypoxic stress, excitotoxins, and Ca^{2+} influx promotes production of p25 in primary cortical neurons; furthermore, cleavage of p35 to p25 was promoted in fresh brain lysates treated with Ca^{2+} and prevented by specific inhibitors of calpain activity (Lee *et al*., 2000). More recent research has implicated calpastatin as a critical regulator of calpain activity and, hence, the truncation of p35 into p25 (Sato *et al*., 2011).

The proteolytic cleavage of p35 results in the release of a more stable protein expressing a half-life that is 5-10 fold greater than that of p35, which has a half-life of 20-30 minutes (Patrick *et al*., 1998; Patrick *et al*., 1999). Additionally, the p25 cofactor is not readily degraded and therefore considered to maintain Cdk5 in a state of hyperactivation. Furthermore, regulation of enzymatic activity by p25 reportedly alters substrate specificity and cellular localisation of Cdk5 (Patrick *et al*., 1999).

Analogous to the cleavage of p35, calpain is additionally responsible for the generation of p29, a truncation of p39 (Patzke *et al*., 2003) (***Figure 1.3***). *In vitro*, calpain cleavage of p39 generates a C-terminal p29 fragment, and high levels of calpain activity lead to the production of p29 *in vivo*. Consistent with the increased stability of p25, the p29 fragment is also more stable than p39. Moreover, p29 activation of Cdk5 results in the redistribution of Cdk5 in primary cortical neurons (Patzke *et al*., 2003).

The prolonged activation of Cdk5 following association with p25 coupled with the reported mislocalisation of the Cdk5 complex is often associated with neurotoxicity and has been implicated as a potential regulatory mechanism underpinning several neurodegenerative disorders, including Alzheimer's disease (AD). The alteration in subcellular localisation of the p29/Cdk5 complex together with a prolonged activity suggests that the conversion of p39 to p29 may also contribute to neurotoxic injury although this remains to be proven.

1.4.1.4 The theory of aberrant Cdk5 activity following activator truncation

In addition to the evidence that p35 truncation promotes hyperactivation of Cdk5 with potential altered substrate specificity and cellular localisation, the pathophysiological significance of p25/Cdk5 is largely supported by the fact that p25 accumulates in AD brains (Patrick *et al*., 1999; Tseng *et al*., 2002).

Neurofibrillary tangles (NFTs) are present in some neurons in AD and p25, but not p35, was found in neurons containing NFTs as demonstrated by AT8 antibody counterstaining (Augustinack *et al*., 2002). Hyperphosphorylation of tau in AD tangles has been attributed, at least in part, to p25/Cdk5 activity, reducing the ability

of tau to associate with microtubules (MT) (Hashiguchi *et al*., 2002). Consistent with this hypothesis, p25/Cdk5 overexpression in primary neurons results in cytoskeletal disruptions and, ultimately, apoptotic cell death evident by fragmented nuclei (Patrick *et al*., 1999).

These studies highlight a potential association between p25 and neurodegenerative disease by suggesting that the unique properties associated with p25/Cdk5 hyperactivation may exert a critical role in the pathogenesis of NFTs and neuronal death. However, despite being heavily depicted as a 'hyperactive' complex when bound to p25, very few, if any, studies have actually demonstrated that the p25/Cdk5 complex has inherent higher specific activity, or even enhanced activity toward specific substrates *in vitro*.

1.4.2 Regulation of Cdk5 activity by synthesis and degradation of p35

The kinase activity of Cdk5 relies primarily on the availability of p35 or p39, evident by the excess of Cdk5 relative to cofactor in brain tissue proposing p35 or p39 availability as the limiting factor in determining Cdk5 activity (Qi *et al*., 1995; Zhu *et al*., 2005). The level of p35 or p39 is controlled through a balance between synthesis and degradation.

In the brain, Cdk5 kinase activity starts to increase, along with neuronal differentiation, at about day 13 (E13) when the synthesis of p35 initiates (Tsai *et al*., 1994; Wu *et al*., 2000). The p35 and p39 genes are reported targets for heat shock factor 2 (HSF2) during cortical development (Chang *et al*., 2006), and the expression of p35 is induced by brain-derived neurotropic factor (BDNF) in cultured cortical neurons and medium-sized spiny neurons (Tokuoka *et al*., 2000; Bogush *et al*., 2007).

The increase in p35 expression in medium-sized spiny neurons is prevented by phosphatidylinositol 3-kinase (PI3K) inhibitors suggesting that BDNF signals to the gene promoter through the PI3K pathway (Bogush *et al*., 2007).

Inactivation of Cdk5 activity is achieved through degradation of Cdk5 regulatory subunits by the proteasome (Patrick *et al*., 1998; Saito *et al*., 1998; Patzke & Tsai, 2002) and this is, in part, mediated by autoregulation. Cdk5 phosphorylates p35 resulting in its ubiquitination and degradation by the proteasome (Patrick *et al*., 1998). In p35 mutants that lack the Cdk5 phosphorylation sites, Ser8 and Thr138, p35 becomes more stable, with an extended half-life, suggesting a negative feedback loop and implicating Cdk5 phosphorylation of p35 as essential component for the ubiquitination of the regulatory subunit (Kamei *et al*., 2007).

1.4.3 Regulation of Cdk5 activity by phosphorylation

Consistent with the majority of eukaryotic protein kinases, Cdk5 possesses a catalytic domain flanked with additional domains essential for activation. The catalytic domain is comprised of an ATP binding site between an N-terminal lobe of β-sheet and a α-helical C-terminal (C-lobe) domain (Manning *et al*. 2002). The α-helical domain is termed PSAALRE based on the polypeptide sequence. The activation loop consists of 20 amino acids between the N-terminal and C-terminal domains and acquires a conformation competent of phosphate transfer. Despite sharing structurally similarities with the Cdks, the activation of Cdk5 is atypical from the other family members (Mapelli & Musacchio, 2003).

Regulation of Cdk5 activity can be modulated through specific phosphorylation events. Three distinct phosphorylation sites on Cdk1, Thr14, Tyr15, and Thr161, are

conserved in Cdk5 (Thr159 in Cdk5 corresponds to Thr161 in Cdk1). Despite this, the effect of Cdk5 phosphorylation at these sites differs entirely from the other members of the Cdk family. Cdk1 requires T-loop phosphorylation at Thr161 to induce the active conformation however the crystal structure of p25/Cdk5 (***Figure 1.4***) indicates that T-loop phosphorylation of Thr159 on Cdk5 is not essential for activation of the kinase. The association with p25 forces the activation loop to adopt an extended conformation, typical of active proline-directed kinases, in the absence of loop phosphorylation (Tarricone *et al*., 2001).

Phosphorylation of Thr14 and Tyr15 in Cdk1 by Wee1 or Myt1 reduces its activity (Fattaey & Booher, 1997). Conversely, Cdk5 kinase activity is stimulated following its phosphorylation at Tyr15 by nonreceptor tyrosine kinases, c-Abelson (Abl) and Fyn, or receptor-type tyrosine kinases, Eph4 and TrkB (Zukerberg *et al*., 2000; Sasaki *et al*., 2002; Fu *et al*., 2007; Cheung *et al*., 2007). Cables, a Cdk5-binding substrate, stimulates phosphorylation of Cdk5 by Abl at Tyr15 (Zukerberg *et al*., 2000). Phosphorylation of Tyr15 is detected when a growth cone encounters Sema3, a repellent cue (Sasaki *et al*., 2002). Ephrin A1 stimulates Eph4 receptor kinase activation and subsequent phosphorylation of Cdk5 at Tyr15 is observed (Fu *et al*., 2007). Furthermore, following BDNF stimulation, Cdk5 is recruited to the TrkB receptor and subsequently phosphorylated at Tyr15 by TrkB which results in enhanced Cdk5 activity that promotes the phosphorylation of TrkB at Ser478 (Cheng *et al*., 2007). In contrast, phosphorylation of Thr14 has an inhibitory effect on Cdk5 (Matsuura & Wang, 1996). A 43 kDa protein kinase was purified that phosphorylated Cdk5, as well as Cdk1 and Cdk2, and inhibited Cdk5 kinase activity (Matsuura & Wang, 1996).

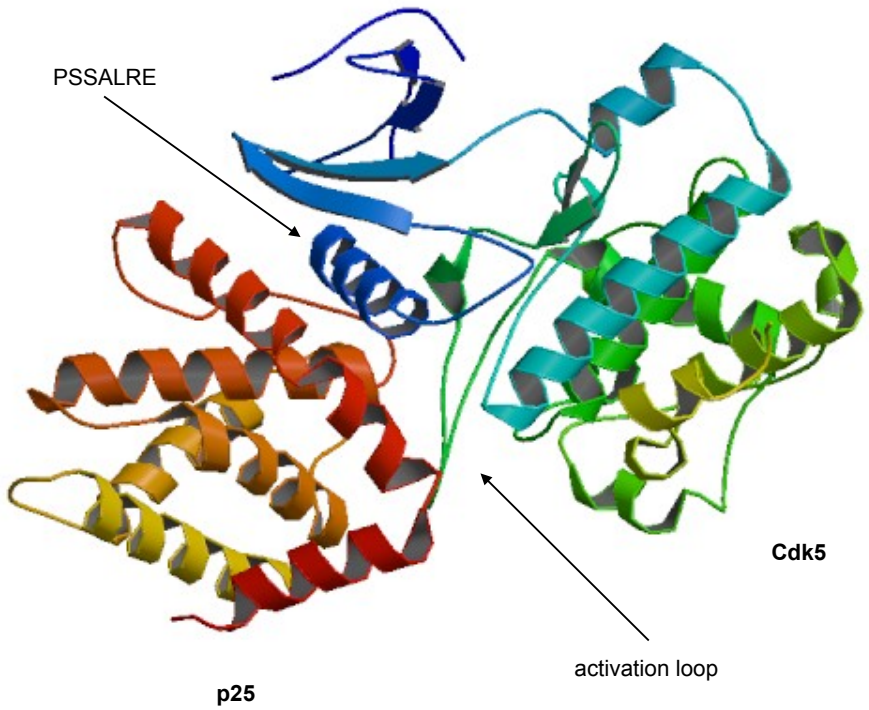


Figure 1.4. ***Structure of the p25/Cdk5 complex. cleavage.*** Cdk5 (green – pale blue) binds Cdk5 (yellow – red) in the region of the PSSALRE helix (dark blue) in the kinase small lobe. There are extensive interactions with the activation loop. Image taken and adapted from the RCSB PDB (www.rcsb.org) of PDB 1H4L (Tarricone, C., Dhavan, R., Peng, J., Areces, L.B., Tsai, L.H., Musacchio, A (2001) Structure and regulation of the CDK5-p25(nck5a) complex Mol.Cell 8:657) created with PQS software.

Finally, the cyclin kinase inhibitors (CKIs), p21 and p27 inhibit mitotic Cdks by inactivating the cyclin Cdk complex, however they have little or no effect on Cdk5 activity (Lee *et al*., 1996). This, again, provides a contrast in the regulation of Cdk5 to that of the classical Cdk family members, and suggests that there is no co-ordination of Cdk5 activity with that of Cdk1 or Cdk2.

1.4.4 Pharmacological inhibition of Cdk5 activity

Olomoucine (Vesely *et al*., 1994) and roscovitine (Meijer *et al*., 1997) are both ATP competitive inhibitors of Cdk activity, however they do not discriminate between individual family members. Whilst these inhibitors do remove Cdk5 activity from cells, more selective approaches are required in order to dissect the functional role of Cdk5 over other Cdks. For example, overexpression of a dominant-negative mutant of Cdk5 or silencing Cdk5 via siRNA should be used in addition to the available pharmacological tools.

1.5 FUNCTIONAL DIVERSITY OF CDK5 SUBSTRATES

The functional implications of kinases arise largely due to the identification of substrates that have been identified as direct targets of that kinase. Since the initial identification of Cdk5 as a brain kinase (Lew *et al*., 1992) that directly phosphorylates tau (Ishiguro *et al*., 1992), the past two decades have seen a dramatic increase in the number of substrates that are reportedly regulated by Cdk5. Cdk5 has been reported to phosphorylate a large number of proteins *in vitro*, however it is not clear how many of these proposed substrates are regulated by Cdk5 *in vivo* and, furthermore, these substrates fail to meet a common consensus.

Currently, a compendium of substrates exists for Cdk5, each with varying functional significance, and their number is continually increasing (***Table 1.1***). As a result, the physiological functions of Cdk5 are becoming more complex, extending its functions beyond the nervous system and into extra-neuronal tissues. In addition, the involvement of Cdk5 in pathophysiological pathways is only partially understood. The physiological and pathophysiological implications of Cdk5 are discussed in greater in ***sections 1.6 and 1.7***.

In order to fully understand the function and significance of Cdk5, particularly under pathophysiological conditions, proposed substrates must be confirmed as *bona fide* Cdk5 substrates and greater understanding of Cdk5 substrate selection is required to identify novel substrates of Cdk5. This, in turn, will contribute to the understanding of the regulatory signalling networks involved, help dissect the consequences of manipulating Cdk5 activity, and increase interest in Cdk5 as a therapeutic target in drug development.

	Substrate	Functional Outcome	Phosphorylation Sites	References
Neuronal Migration	β- Catenin	N-cadherin-mediated cell adhesion, binding following dissociation	Multiple	Kwon *et al*., 2000 Kesavapany *et al*., 2001
	Disabled 1	Control of neuronal positioning, reelin interaction	S491	Keshvara *et al.*, 2002
	Dixdc1	Allows interaction between DISC1 and NUDEL	S250	Singh *et al*., 2010
	DISC1	Switch between progenitor proliferation and migration	S710	Ishizuka *et al*., 2011
	Doublecortin	Lowered affinity to microtubules, reduced effect on polymerization	S297	Tanaka *et al*., 2004
	FAK	Nuclear translocation through microtubule fork	S732	Xie *et al*., 2003
	NUDEL	Regulation of dynein and axonal transport	S198, T219, S231	Niethammer *et al*., 2000 Sasaki *et al*., 2000
	p27(kip1)	Actin organisation, cortical neuronal migration	S10, T187	Kawauchi *et al*., 2006
Neurite Outgrowth	Cables	Dissociation of Cdk5/Cables/c-Abl complex, neurite outgrowth	Multiple	Zukerberg *et al*., 2000
	CRMP2	Semaphorin 3A-induced growth cone collapse, axonal growth and development	S522	Brown *et al*., 2004 Cole *et al*., 2004
	c-Src	Neural development, neurite outgrowth, function	S75	Kato & Maeda, 1999
	MAP1B	Regulation of axonal formation and elongation	Multiple	Pigino *et al*., 1997 Paglini *et al*., 1998
	p39	Regulation of actin cytoskeletal dynamics	Multiple	Humbert *et al*., 2000
	PAK1	Actin polymerisation, cytoskeletal reorganization	T212	Nikolic *et al*., 1998
	RasGRF2	Altered RasGRF2, microtubule protein accumulation	S737	Kesavapany *et al*., 2004
	TrkB	BDNF-stimulated dendritic outgrowth	S478	Cheung *et al*., 2007
Synaptic Vesicle Cycle	Amphiphysin 1	Regulation of synaptic vesicle endocytosis	S262, S272, S276, S285, T310	Floyd *et al*., 2001 Liang *et al*., 2007
	Dynamin 1	Clathrin-mediated synaptic vesicle endocytosis	S774, S778	Tomizawa *et al*., 2003 Tan *et al*., 2003
	Munc18	Modulation of synaptic vesicle exocytosis	T574	Shuang *et al*., 1998 Fletcher *et al*., 1999
	PCTAIRE1	synaptic vesicle exocytosis via NSF interaction	S95	Cheng *et al*., 2002
	Septin 5	Reduction of sept5 binding to	S17	Taniguchi *et al*., 2007

		syntaxin		
	Synapsin 1	Impact on cytoskeletal components, actin bundling	S551, S553	Matsubara *et al*., 1996
	Synaptojanin 1	Interaction with other endocytosis components	S1144	Lee *et al*., 2004
Synaptic transmission and plasticity	CASK	Promotion of synaptogenesis, presynaptic proteins	S51, S395	Samuels *et al*., 2007
	$Ca_v1.2$	Decreased calcium influx upon glucose stimulation	S783	Wei *et al*., 2005a
	$Ca_v2.1$	Synaptic plasticity and neurotransmitter release	aa724-981	Tomizawa *et al*., 2002
	DARPP-32	Signal transduction modulation in striatal neurons	T75	Bibb *et al*., 1999
	δ-Catenin	Dendritic morphogenesis, synaptic activity	S300, S357	Poore *et al*., 2010
	Kalirin-7	Formation, regulation of dendritic spine protrusion	T1590	Xin *et al*., 2008
	NR2A	NMDA current, LTP, ischemia-mediated cell death	S1232	Li *et al*., 2001 Wang *et al*., 2003
	NR2B	Surface expression levels of NR2B and endocytosis	T1472	Zhang *et al*., 2008
	PP-1	Signalling pathways involving PKA, calcineurin	S6, S67	Nguyen *et al*., 2007
	PP-1 inhibitor	Multiple cellular functions and signalling pathways	T72	Agarwal-Mawal & Paudel, 2001
	PSD-95	Clustering size of K^+ channels at dendritic spines	T19, S25	Morabito *et al*., 2004
	SPAR	Plk2-mediated degradation during synaptic scaling	S1328	Seeburg et al., 2008
	TH	Presynaptic component in dopamine synthesis	S31	Moy & Tsai, 2004 Kansy *et al*., 2004
Neurodegenerative Disease	APP	Potential localisation of the β-amyloid protein	T668	Iijima *et al.,* 2000
	Huntington	Reduction in Huntington aggregation and formation	S434, S1181, S1201	Luo *et al*., 2005 Anne *et al*., 2007
	MEF2D	Inhibition of transcriptional factors and cell death	S444	Smith *et al*., 2006
	NF	Regulation of axonal transport of NF	Multiple	Lew *et al*., 1994 Ackerley *et al*., 2003 Gong *et al*., 2003
	Parkin	Modulation of synphilin-1/α-synuclein inclusions	S131	Avraham *et al*., 2007
	Prx2	Tuning sensitivity of neuron to oxidative stress	T89	Qu *et al*., 2007 Rashidian *et al*., 2009
	Tau	Accumulation and disruption of axonal transport	Multiple	Baumann *et al*., 1993 Kobayashi *et al*., 1993

Other	Ape1	Involvement in regulation of DNA damage, cell death	T232	Huang *et al*., 2010
	ATM	DNA damage, ATM activation, neuronal death	S794	Tian *et al*., 2009
	β-2 syntrophin	Regulatory mechanism of insulin secretion	S75	Schubert *et al*., 2010
	Bcl-2	Neuroprotective effect, neuronal survival	S70	Cheung *et al*., 2008
	Cdh1	Mediates cyclin B1 accumulation in excitotoxicity	S40, T121, S163	Maestre *et al*., 2008
	ErbB3	Nrg-induced AChR expression at NMJ		
	GR	Regulates glucocorticoid receptor transcriptional activity	S203, S211, S232, S246 and others	Kino *et al*., 2007 Adzic *et al*., 2009
	JNK3	Inhibition of JNK activity and reduced apoptosis	T131	Li *et al*., 2002
	MEF2A	Regulates apoptosis	S408	Gong *et al*., 2003
	MEK1	Down-regulation of MAPK signalling	T286	Sharma *et al*., 2002
	mSds3	HDAC transcriptional co-repressor complex	S228	Li *et al*., 2002
	p35	Promotion of ubiquitin-mediated proteolysis	S8, T138, S170, T197	Tsai *et al*., 1994 Patrick *et al*., 1998
	p53	Increased cell cycle arrest or cell death genes	S33, S315	Zhang *et al*., 2002
	Paxillin	Involvement in oligodendrocyte differentiation	S244	Miyamoto *et al*., 2007
	PIPKIγ	Blocked PI(4,5)P2 synthesis at synapses and at focal adhesions	S650	Lee *et al*., 2005
	PPARγ	Obesity-linked phosphorylation in fat cells	S273	Choi *et al*., 2010
	Rb	Cellular growth, differentiation, apoptosis	Multiple	Lee *et al*., 1997 Hamdane *et al*., 2005
	S6K1	Regulation of S6K1 catalytic activity	S411	Hou *et al*., 2007
	STAT3	Transcription of target genes c-fos and junB	S727	Fu *et al*., 2004
	Talin	Regulation of Smurf1-mediated talin ubiquitination	S425	Huang *et al*., 2009
	TRPV-1	Modulation of nociceptive signalling in DRG	T407	Pareek *et al*., 2007

Table 1.1. ***Identified Cdk5 substrates and their functional categories***. Abbreviations: AChR, acetylcholine receptor; Ape1, apurinic/apyrimidinic endonuclease 1; APP, amyloid precursor protein; ATM, Ataxia telangiectasia mutated; Bcl-2, B-cell CLL/lymphoma 2; BDNF, brain-derived neurotropic factor; Cables, Cdk5 and Abl enzyme substrates; CASK, calcium/calmodulin-dependent serine protein kinase; $Ca_v1.2$, L-type voltage-gated calcium channel; $Ca_v2.1$, P/Q-type voltage-gated calcium channel; Cdh1, cadherin-1; CRMP2, collapsing response mediator protein 2; c-Src, non-receptor tyrosine kinase; DARPP-32, Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa; DISC1, Disrupted in Schizophrenia 1; Dixdc1, Dix-domain containing 1; DRG, dorsal root ganglion; ErbB3, receptor tyrosine-protein kinase erbB3; FAK, focal adhesion kinase; GR, glucocorticoid receptor; HDAC, histone deacetylase; JNK3, c-Jun N-terminal kinase 3; LTP, long-term potentiation; MAP1B, microtubule-associated protein 1B; MEK, MAP kinase kinase-1; MEF, myocyte enhancer factor; Munc18, mammalian uncoordinated 18; NF, neurofilament; NMJ, neuromuscular junction; NR2A, N- methyl D-aspartate receptor subunit 2A; NR2B, N-methyl D-aspartate receptor subunit 2B; Nrg, neuregulin; NSF, N-ethylmaleimide sensitive factor; NUDEL, nuclear distribution protein nude-like; p27(kip1), cyclin-dependent kinase inhibitor; p35, regulatory activator of cyclin-dependent kinase 5 Mr 35 kDa; p39, regulatory activator of cyclin- dependent kinase 5; p53, tumour protein 53; PAK1, p21-Activated Kinase; paxillin, focal adhesion- associated adaptor protein; pctaire1, cyclin-dependent kinase 16 (cdk16); PIPKI, phosphatidylinositol(4) phosphate 5 kinase type I gamma; PPARγ, Peroxisome proliferator-activated receptor gamma; Plk2, polo- like kinase 2; PP-1, protein phosphatase-1; Prx2, peroxiredoxin 2; PSD-95, Postsynaptic density protein 95; RasGRF, ras guanine nucleotide releasing factor 2; Rb, retinoblastoma protein; S6K1, S6 kinase 1; SPAR, spine-associated Rap guanosine triphosphatase activating protein; STAT3, signal transducer and activator of transcription 3; TH, tyrosine hydroxylase; TRPV-1, Transient Receptor Potential Vanilloid-1.

1.6 THE ROLE OF CDK5 IN NORMAL PHYSIOLOGY

Cdk5 has been extensively described as a multifaceted kinase exerting a diversity of functions on brain physiology, ranging from neuronal development and migration, through to synaptic plasticity and pain signalling (***Figure 1.5***). More recently, studies have extended these investigations to include key functional roles for Cdk5 in extra-neuronal tissues.

1.6.1 Functional implications of Cdk5 in the nervous system

Transfection of p35 and p39 into cortical cultures and immortalised hippocampal cells stimulated neurite outgrowth, and, conversely, dominant-negative mutants of Cdk5 or antisense p35 and p39 constructs inhibited neurite outgrowth (Nikolic *et al*., 1996; Xiong *et al*., 1997). These results, taken together with the severe disruption of the cytoarchitecture of the brain cortex in both Cdk5 knockout (KO) and double KO p35/p39 null mice (Chae *et al*., 1997), implicated Cdk5 activity as a key component of the central nervous system (CNS). Therefore, the CNS has formed the basis of many of the physiological systems investigated when studying Cdk5 cellular functions. Subsequently, identification of a vast array of potential Cdk5 substrates has added complexity to the role of Cdk5 in the nervous system but most likely explains the severe phenotype of the Cdk5 KO animals.

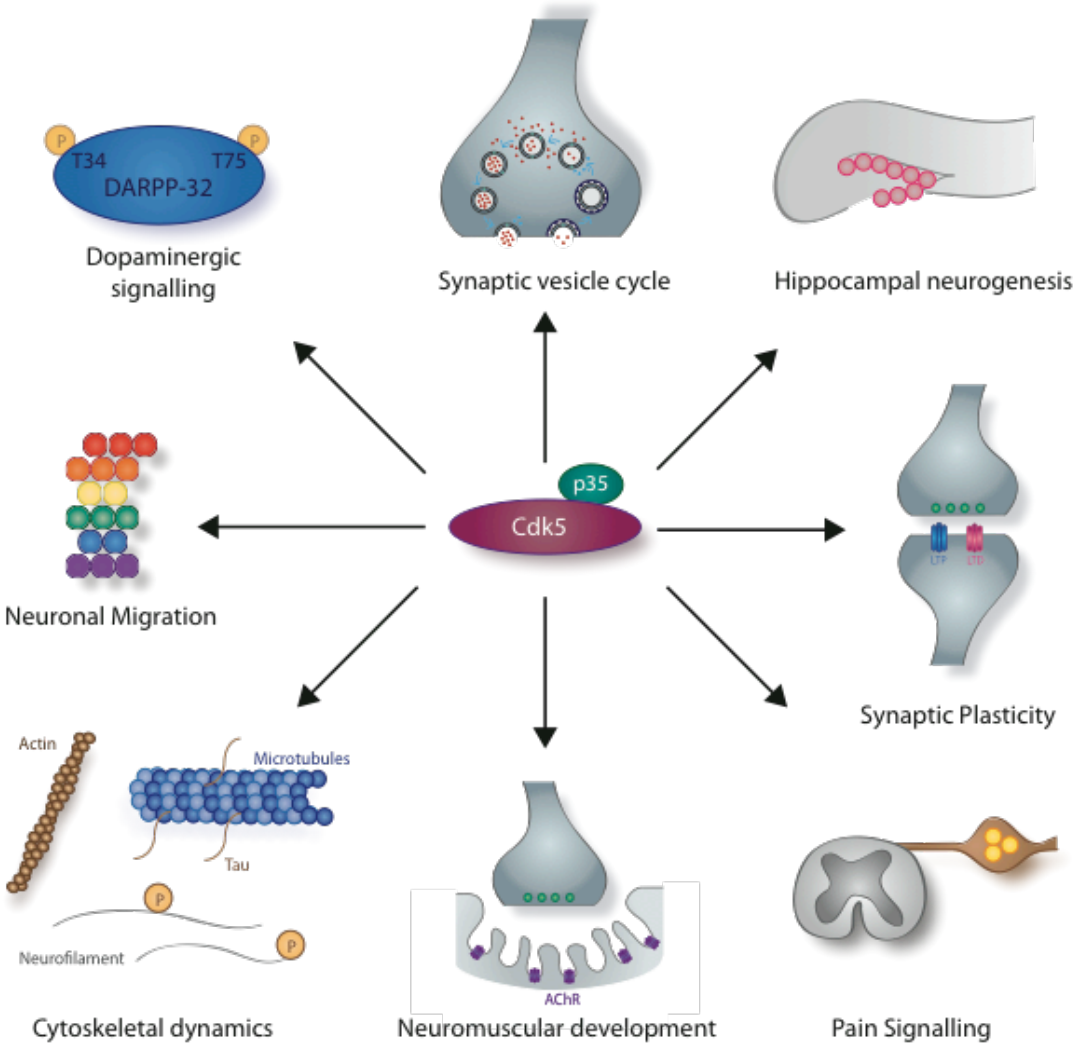


Figure 1.5. ***The multifaceted role of Cdk5***. Currently, Cdk5 is reported to play a role in a large number of physiological functions and impacts various cellular processes. Following activation by 35, Cdk5 regulates multiple events including neuronal migration, neurite outgrowth, cytoskeletal dynamics, in addition to roles in a number of pathways such as pain signalling, neuromuscular development, and adult neurogenesis (Su & Tsai, 2011).

1.6.1.1 Cdk5 activity in the developing CNS

1.6.1.1.1 Cdk5 exerts a critical role in neuronal cytoarchitecture

Whole body Cdk5 knockout mice (Cdk5 -/-) display perinatal lethality owing to CNS lesions (Ohshima *et al*., 1996). In particular, there are neuronal migration deficits in multiple brain compartments including the cerebral cortex, hippocampus, and cerebellum: the laminar organisation of neurons within the cerebral cortex is inverted with a superficial ectopic subplate; the pyramidal neurons of the hippocampus are not organised in a discrete layer; the cerebellum lacks foliation and tripartite layering; and, motor neurons of the spinal cord and cranial nerve nuclei display enlarged perikarya characteristic of chromatolytic changes (Ohshima *et al*., 1996, 1999; Gilmore *et al.*, 1998).

Interestingly, mice deficient in the regulatory subunit p35 are viable and fertile. They are, however, prone to seizures and lethality in adulthood. While these mice only exhibit mild disruptions in the hippocampus and possess fairly normal cerebella, they express lamination defects in the cerebral cortex as well as alterations in cell orientation, and dendrite and axon trajectories (Chae *et al*., 1997; Kwon *et al*., 1998; Wenzel *et al*., 2001).

The difference in phenotypic severity between the Cdk5 and p35 KO mice is likely due to compensation by p39. Mice that are deficient in p39 do not display detectable abnormalities in neuronal positioning in the CNS. However, p35 and p39 double-null deletion mice express identical phenotypes to Cdk5 (-/-) mice including severe disruption of various central nervous system components, an absence of Cdk5 kinase activity, lamination defects, and perinatal lethality (Ko *et al*., 2001). These mice highlight p35 and p39 as critical, and potentially the sole, regulators of Cdk5 activity

in the CNS. Additionally, mice deficient in the transcription factors Brn-1 and Brn-2 lack p35 and p39 mRNA in migrating neurons in the cortex and hippocampus. These animals display a similar cortical lamination phenotype to Cdk5-null mice linking Brn-1 and Brn-2 to regulation of Cdk5 function in the CNS through the regulation of p35 and p39 expression (Smith *et al*., 2001).

Taken together, these gene-targeting experiments demonstrate the critical nature of Cdk5 in the CNS and highlight Cdk5 and the cofactors, p35 and p39, as necessary components for establishing accurate and fundamental neocortical lamination.

1.6.1.1.2 Neurite development, neuronal migration, and axonal elongation

Neuronal migration and axon guidance are fundamental mechanisms underlying the wiring of the brain. The nervous system has acquired the ability to grow in size and complexity, using cell migration as a strategy to position cell types from different origins into specific coordinates, enabling the generation of brain circuitries.

Cdk5 expression is ubiquitous however it is most abundant in neuronal cells (Tsai *et al*., 1993). During development of the nervous system, Cdk5 and its associated kinase activity increases progressively as increasing number of cells exit the proliferative phase of the cell cycle. Cdk5 activity is expressed solely in postmitotic neurons (Paglini *et al*., 2001b) and astrocytes (Gao *et al*., 2001; Gao *et al*., 2002) but not in mitotically active cells (Paglini *et al*., 2001b). The p35/Cdk5 complex was found to colocalise with axonal growth cones (Nikolic *et al*., 1996), and Cdk5 activity is present in neurite outgrowths (Lin *et al*., 2003).

In situ hybridisation examining the temporal and spatial distribution of p35 mRNA in the developing and adult mouse brain demonstrated that young post-mitotic neurons begin to express p35 mRNA as soon as they leave the cell cycle and begin to migrate to their final destination (Delalle *et al*., 1997). Additionally, p35 mRNA is also found to be present in newly formed axonal bundles. Immunohistochemical studies located p35 at the cell periphery in lamellipodial and filiopodial structures, both of which are important for neurite outgrowth (Nikolic *et al*., 1996). Postnatally, p35 is relatively abundant in most areas of the forebrain, in particular, the pyriform cortex and pyramidal layer of the hippocampus where high levels of neuronal plasticity are retained (Liu & Green, 2001; Tsai *et al*., 1994; Delalle *et al*., 1997; Ino *et al*., 1994; Zheng *et al*., 1998; Paglini *et al*., 2001a; Paglini *et al*., 2001b). Expression of p39 mRNA is found in the developing and adult CNS with highest expression occurring postnatally suggesting that p39/Cdk5 may mediate functions distinct from those involving p35/Cdk5 during neurodevelopment. Subcellular distribution studies indicate both distinct and overlapping features (Humbert *et al*., 2000).

In immortalised hippocampal cells, p35 is sufficient but not essential for inducing neurite outgrowth. The majority of anti-sense oligonucleotide studies suggest that the p35/Cdk5 complex acts as a modulator of axonal elongation, rather than having an essential role in process formation (Ko *et al*., 2001; Dhavan & Tsai, 2001). One hypothesis proposes that these effects are essentially the result of neurofilament phosphorylation and subsequent slowing of their transport through the cytoplasmic extension (Sharma *et al*., 1999; Grant *et al*., 2001).

There are many proposed targets of Cdk5 that could mediate its regulation of neurite outgrowth. For example, following Tyr15 phosphorylation of Cdk5 when it forms a

complex with the tyrosine kinase c-Abl and Cables (Zukerberg *et al*., 2000). Additionally, active Cdk5 hyperphosphorylates PAK1 in a Rac-dependent manner that results in the downregulation of PAK1 kinase activity (Nikolic *et al*., 1998). Since the Rho family of GTPases and the PAK kinases are implicated in actin polymerisation, Cdk5 phosphorylation of PAK1 is likely to have an impact on the dynamics of the reorganisation of the actin cytoskeleton in neurons, thereby promoting neuronal migration and neurite outgrowth. Cdk5 phosphorylation of the BDNF receptor tyrosine kinase TrkB modulates BDNF-stimulated dendrite outgrowth (Cheung *et al*., 2007), and phosphorylation of ephexin and WAVE1 modulates ephrin-A1 mediated spine retraction (Fu & Ip, 2007). Cdk5 interacts with the ubiquitin ligase mind bomb 1 (Mib1) during neurite morphogenesis (Choe *et al*., 2007). Finally, Cdk5 may affect neurite outgrowth via phosphorylation of Microtubule Associated Protein 1B (MAP1B) (Smith, 2003; Grant *et al*., 2001; Nikolic *et al*., 1996). Therefore multiple players in the regulation of neurite initiation, growth and retraction are coordinated by Cdk5 mediated phosphorylation.

Harada and colleagues placed Cdk5 downstream of the Erk-1/2 signalling pathway in NGF-dependent neurite outgrowth since activation of Erk-1/2 by nerve growth factor (NGF) in PC12 cells induced p35 production (Harada *et al*., 2001). More recently, S-nitrosylation of Cdk5 was shown to influence the regulation of neurite growth and branching (Zhang *et al*., 2010), however the upstream regulation of Cdk5 by multiple factors that control neurite outgrowth remains poorly defined.

There are a number of additional proposed Cdk5 targets that play key roles in neuronal migration. These include the tyrosine kinase FAK, which regulates an MT fork to guide proper neuronal migration through nuclear translocation (Xie *et al*.,

2003); NUDEL, a Lis-1-interacting protein that cooperates with dynein, an MT-interacting motor protein (Niethammer *et al*., 2000; Sasaki *et al*., 2000); doublecortin (Dcx), which regulates cytoskeletal dynamics during neuronal migration (Tanaka *et al*., 2004); Dix-domain containing 1 (Dixdc1), which interacts with the psychiatric illness risk factor gene Disrupted in Schizophrenia-1 (DISC1) and NUDEL during migration (Singh *et al*., 2010); and DISC1, which mediates neural progenitor migration (Ishizuka *et al*., 2011). Additional cytoskeletal substrates of Cdk5 include NF proteins and the microtubule (MT)-associated protein tau (Baumann *et al*., 1993; Lew *et al*., 1994; Paudel *et al*., 1993). Tau phosphorylation by Cdk5 modulates the rate of MT polymerisation (De Vos *et al*., 2011).

Work in embryonically derived primary neurons from the $Cdk5^{-/-}$ mice implicates Cdk5 in axonal elongation and this is supported by the high degree of temporal correlation in p35 expression and Cdk5 activation with the formation of axonal tracts in the developing brain (Paglini *et al*., 2001; Smith & Tsai, 2002; Delalle *et al*., 1997). During terminal neuronal differentiation, both Cdk5 and p35 localise to axonal pathways, such as the corpus callosum and external capsule (Tsai *et al*., 1993).

Semaphorins are a class of secreted and membrane proteins that act as axonal growth cone guidance molecules. Sema3A (collapsin-1) was the first identified vertebrate semaphorin and acts primarily as a repulsive axon guidance cue, and can cause dramatic collapse of the growth cone lamellipodium (Schmidt & Strittmatter, 2007). The collapsin response mediator protein (CRMP) family, composed of five cytosolic phosphoproteins (CRMP1-5) of similar molecular size (60-66 kDa) and high (50-75%) sequence homology, was originally identified as mediators of Sema3A signalling and neuronal differentiation (Goshima *et al*., 1995). Phosphorylation of

these proteins at the C-terminal region is considered to be important in Sema3A signalling. Members of the class A plexins, PlexA1 and A2 are constitutively bound to the src family tyrosine kinase, fyn, in a kinase-independent manner. Stimulation with Sema3A results in activation of fyn and leads to the recruitment of Cdk5 into the complex and activates it kinase activity (Sasaki *et al*., 2002). The blockade of Cdk5 kinase activity attenuates dorsal root ganglion (DRG) growth cone collapse in response to Sema3A (Sasaki *et al*., 2002; Brown *et al*., 2004; Uchida *et al*., 2005). Cdk5 phosphorylates CRMP1, 2, and 4 at Ser522 and this can occur in response to Sema3A *in vitro* and *in vivo* (Brown *et al*., 2004; Uchida *et al*., 2005). The growth cones of neurons overexpressing a mutant CRMP2, in which Ser522 is replaced by alanine (S522A), fail to collapse when Sema3A is applied (Brown *et al*., 2004; Uchida *et al*., 2005). Furthermore, the phosphorylation of CRMPs at Ser522 by Cdk5 allows for the subsequent phosphorylation of CRMP2 and CRMP4 at Ser518, Thr509, and Thr514 by the serine/threonine kinase GSK3β (Brown *et al*., 2004; Uchida *et al*., 2005; Cole *et al*., 2007; Yoshimura *et al*., 2005). Further studies indicate that the functions of CRMPs are not restricted to the transduction of a single extracellular signal but may be involved in multiple cellular and molecular events in apoptosis/proliferation, cell migration, and differentiation.

1.6.1.2 Cdk5 in the mature CNS

In the mature central nervous system (CNS), Cdk5 regulates multiple steps critical for neurotransmitter synthesis, synaptic vesicle exocytosis, vesicle fusion with the presynaptic membrane, and endocytosis. Again, this occurs through phosphorylation of a variety of substrates associated with neurotransmitter release and synaptic plasticity (Benavides & Bibb, 2004; Bibb, 2003; Lagace *et al*., 2008; Zheng *et al*., 1998).

1.6.1.2.1 Cdk5 as a critical regulator of presynaptic function

At the presynaptic terminal, vesicles containing neurotransmitters undergo docking and fusion, or exocytosis, at the active zone to enable neurotransmitter release across the synaptic cleft (Murthy & De Camilli, 2003; Sudhof, 2004). A presynaptic influx of calcium triggers vesicle fusion and exocytosis by the SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment receptor) complex proteins, consisting of v-SNARES (synaptobrevin or VAMP) and t-SNARES (SNAP-25 and syntaxin). The recycling of synaptic vesicle proteins occurs via clathrin-mediated synaptic vesicle endocytosis (SVE). Components of SVE include the accessory protein AP-2, dynamin, and amphiphysin proteins to pinch off the vesicle from the active zone using GTP hydrolysis; synaptojanin 1 to assist in synaptic vesicle uncoating; and other dephosphin proteins. The phosphatase calcineurin removes the phosphate groups from the dephosphins in response to calcium influx and enables synaptic vesicle exocytosis, and rephosphorylation by protein kinases, including Cdk5, is crucial for continued SVE (Nguyen & Bibb, 2003).

In both endocytosis and exocytosis, Cdk5 phosphorylation of presynaptic proteins is required for the synaptic vesicle cycle. *In vitro*, p35/Cdk5 forms a complex with Munc18 and syntaxin1A, thought to be an essential complex in vesicle function, that is dissociated upon addition of ATP suggesting Cdk5 phosphorylation may allow syntaxin 1A to form a SNARE complex and promote neurotransmitter release (Fletcher *et al*., 1999; Shuang *et al*., 1998). The p35/Cdk5 complex also regulates the interaction between syntaxin 1A and Sept5, another protein implicated in exocytosis (Taniguchi *et al*. 2007).

The presynaptic protein, CASK becomes phosphorylated by Cdk5 to promote synapse development (Samuels *et al*., 2007) and the P/Q-type voltage-gated calcium channel to inhibit its interaction between SNAP-25 and synaptotagmin (Tomizawa *et al*. 2002). The field excitatory postsynaptic potential (fEPSP) slope and glutamate release from synaptosomes are increased following incubation of acute hippocampal slice preparations with roscovitine, suggesting that inhibiting Cdk5 activity results in a long-term potentiation (LTP) of synaptic strength. However, roscovitine is not a specific Cdk5 inhibitor, indeed it also acts on the extracellular domain of $Ca_v2.1$ to slow channel deactivation. Therefore further studies are required to confirm that Cdk5 regulates synaptic transmission (Yan *et al*., 2002).

Cdk5 is proposed to phosphorylate several components of synaptic vesicle endocytosis, including amphiphysin (Floyd *et al*. 2001), dynamin I (Tan *et al*. 2003; Tomizawa *et al*. 2003), synapsin I (Matsubara *et al*. 1996), and synaptojanin I (Lee *et al*. 2004), however the role of Cdk5 in SVE is subject to debate. Whereas one report claims that Cdk5 is essential for SVE (Tan *et al*. 2003), another suggests that Cdk5 disrupts SVE (Tomizawa *et al*. 2003). Complicating matters further, Cdk5 is involved in the activation of PCTAIRE1, a key component in the phosphorylation and regulation of *N*-ethylmaleimide sensitive fusion protein, which is essential for disassembling the SNARE complex (Cheng *et al*., 2002). It is proposed that Cdk5 is required for the activity-dependent slow component of SVE (Evans & Cousin, 2007).

1.6.1.2.2 Postsynaptic transmission

Cdk5 is described as an important regulator of the activity of at least two major neurotransmitter systems, the cholinergic and the glutamatergic (Fu *et al*., 2001; Fu *et al*., 2005; Hawasli *et al*., 2007; Li *et al*., 2001).

Initially, colocalisation of Cdk5 and p35 with the nicotinic acetylcholine receptor (AChR) was reported on the postsynaptic muscle membrane, where Cdk5 was proposed to regulate the trafficking of these receptors (Fu *et al*., 2001). Subsequently, Cdk5 was activated by acetylcholine (ACh) agonists and required for ACh-induced dispersion of AChR clusters that had not been stabilised by agrin (Lin *et al*., 2005). In agrin mutants, genetic elimination of Cdk5 or blocking ACh production prevented the dispersion of AChR clusters. Therefore, Cdk5 was proposed to negatively regulate the formation of synapses at the neuromuscular junction.

Regulation of synaptic function by Cdk5 is additionally associated with the glutamatergic neurotransmitter system through modulation of N-methyl-d-aspartate (NMDA) receptor activity. The conditional Cdk5 knockout results in enhanced synaptic plasticity through an increase in the amount of NMDA receptors containing NR2B subunits and in the subsequent excitatory postsynaptic currents (Hawasli *et al*., 2007). Furthermore, Cdk5 phosphorylation of the NR2A subunit of NMDA increases the activity of the receptor (Li *et al*., 2001).

The dopaminergic signalling pathways are also a prominent area of Cdk5 research. Cdk5 controls dopaminergic signalling through postsynaptic phosphorylation of 32 kDa dopamine cyclic-AMP regulated phosphoprotein DARPP-32 (Bibb *et al*., 1999), and presynaptic phosphorylation of the N-terminal domain of the dopamine-synthesising enzyme, tyrosine hydroxylase (Moy & Tsai, 2004). Cdk5 phosphorylates DARPP-32 on Thr75 permitting DARPP-32 to become a protein kinase A (PKA) inhibitor and, therefore, a protein phosphatase 1 activator, promoting dephosphorylation of specific targets (Bibb *et al*., 1999). Conversely, if DARPP-32 is solely phosphorylated at Thr34 by PKA, following its activation by dopamine D1

receptor signalling, it inhibits protein phosphatase 1. The mRNA and protein levels of p35/Cdk5 are upregulated following chronic cocaine exposure in medium spiny neurons and ΔFosB mice (Bibb *et al*., 2001). PKA activation during acute cocaine exposure downregulates phosphorylation of Thr75 however following chronic exposure, Cdk5 becomes activated to reduce the locomotor response as a part of a novel homeostatic mechanism and the subsequent phosphorylation of Thr75 decreases D1/PKA signalling.

1.6.1.2.3 Synaptic plasticity

Synaptic plasticity describes the ability of synapses to strengthen or weaken subsequent responses following synaptic activity. The molecular mechanisms underlying synaptic plasticity occur both pre- and post-synaptically and involve the regulation of vesicle cycling, changes in ion channel conductance and synaptic expression, and modulation of both kinase and phosphatase activities (Dineley *et al*., 2001; Vautrin & Barker, 2003). Events outside the synapse that influence plasticity include extracellular signalling, cell adhesion, and gene transcription.

The initial evidence implicating Cdk5 in synaptic plasticity came from transgenic models. Long-term potentiation (LTP) induced by tetanic stimulation was found to be normal in p35 KO mice however impairment of hippocampal long-term depression (LTD) was observed in these mice (Ohshima *et al*., 2005). LTP is thought to correlate with memory formation whereas LTD may play a role in weakening synapses and perhaps blocking memory formation (Malenka & Bear, 2004). Subsequently, hippocampal-dependent spatial memory and contextual fear memory was enhanced in a Cdk5 KO line and LTP was greater than that of control mice (Hawasli *et al*., 2007). The evoked excitatory post-synaptic current indicated that the NMDA, but not AMPA

(α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), receptor-mediated current is enhanced in these mice which is attributed to a reduction in NMDA receptor degradation and greater surface NR2B levels.

1.6.1.3 The role of Cdk5 in normal adult neurophysiology

1.6.1.3.1 Learning and memory

Involvement of Cdk5 in the early stages of memory formation became apparent after treatment with the Cdk5 inhibitor, butyrolactone 1 impaired contextual, but not cued, fear conditioning in two different strains of mice (Fischer *et al*., 2002). Furthermore, Cdk5 inhibition in the hippocampus profoundly impaired associative learning and memory (Fischer *et al*., 1997; Fischer *et al*., 2005). This effect on hippocampal learning and memory is partially supported by an absence of spatial memory learning strategy in the Morris water maze in p35 null mice (Ohshima *et al*., 2005). However, it should be considered that developmental defects in p35 null mice might result in confounding effects on behaviour.

The molecular basis of learning and memory are proposed to involve phosphorylation and dephosphorylation of proteins. The glutamate receptor, N-methyl-D-asparate (NMDA) is well-characterised calcium channel that is key for the development of long-term potentiation (LTP) of neurons associated with learning and memory. *In vitro*, Cdk5 has been reported to regulate the NMDA receptor by phosphorylating its subunit, NR2A on Ser1232. Suppression of this phosphorylation *in vivo* prevented the LTP of hippocampal neurons (Cheng *et al*., 2003; Fischer *et al*., 2005).

1.6.1.3.2 Cdk5 and pain perception

Pain is perceived through stimulation of specialised receptors known as 'nociceptors'. The MAPK and Calcium calmodulin kinase pathway (CAMKII) are two important signal transduction pathways involved in nociception and the reported interactions of Cdk5 with each of these pathways provides a rationale for how Cdk5 could influence pain regulation (Pareek & Kulkami, 2006). In comparison to wild-type mice, there is a delayed response to painful thermal stimulation in p35 KO mice, which have reduced Cdk5 activity, and mice overexpressing p35 are sensitive to thermal stimulation (Pareek *et al*., 2006). Furthermore, inhibition of Cdk5 activity using roscovitine in rats attenuates formalin-induced nocireceptive response (Wang *et al*., 2005). This data places Cdk5 activity within the pathway that senses pain. Cdk5 is proposed to regulate mitogen-activated protein kinase kinase 1/2 (MEK1/2) activity through a negative feedback loop during the peripheral inflammatory response therefore it can regulate the Erk1/2 pathway. Moreover, a differential nociception response is observed after chronic morphine exposure in p35-/- and p35-overexpressing mice suggesting that Cdk5 activity is important for opioid tolerance (Pareek & Kulkami, 2006). In addition, during pain sensation, ΔFosB, a transcriptional regulator of Cdk5, is upregulated resulting in subsequent upregulation of the kinase. These results suggest molecular roles for Cdk5 in pain signalling and opioid tolerance implicating Cdk5 as a potential target for analgesic drug development.

1.6.2 Extra-neuronal functions of Cdk5

The extensive role of Cdk5 in the CNS and its apparent enriched expression in the brain has led to Cdk5 being considered a neuronal specific kinase. However Cdk5 activity can be detected in virtually all tissues (Rosales *et al*., 2006) suggesting that Cdk5 has functions beyond the nervous system. The presence of Cdk5 in different

organs and cell types (Daval *et al*., 2001, Feldmann *et al*., 2010; Lin *et al*., 2009; Pallari *et al*., 2011; Shimomura *et al*., 2011) has expanded the search for Cdk5 substrates into non-neuronal cells, and an increasing number of novel substrates that mediate unique and critical extra-neuronal functions have since been identified (***Table 1.1***). As a result, a new dimension has been added to the functional implications of Cdk5 and greater diversity to the physiological function of Cdk5. Although it must be noted that most of the studies detailed below claim Cdk5 functions based on the effect of chemical inhibitors lacking absolute specificity.

1.6.2.1 Cdk5 and transcriptional regulation

Cdk5 has been implicated as a critical regulator of gene expression in both neuronal and non-neuronal cells and, either directly or indirectly, regulates phosphorylation of multiple transcription factors (Contreras-Vallejos *et al*., 2012). Indeed, Cdk5-mediated transcription regulation appears critical for many cellular functions and deregulation can lead to multiple disease-related pathologies.

Cdk5 reportedly phosphorylates signal transducer and activator of transcription (STAT3) at Ser727, altering its activity, and this has been linked to the control of the proliferation of medullary thyroid and colorectal cancer cells (Chen *et al*., 2007; Courapied *et al*., 2010; Fu *et al*., 2001). In muscle cells, Cdk5 phosphorylation of STAT3 is enhanced by neuregulin (NRG) 1, a critical neuregulin family protein that acts on the EGFR family of receptors and is essential for development of multiple organs. This promotes the expression of STAT3 target genes including c-fos, junB, fibronectin, and others (Fu *et al*., 2004).

Cdk5 directly phosphorylates tonicity-responsive enhancer-binding protein/osmotic

response element-binding protein (TonEBP/OREBP), an osmotic stress-responsive transcription factor, at Thr135 in renal cells under high osmolarity (Gallazzini *et al*., 2011). Phosphorylation results in accumulation of TonEBP/OREBP in the nucleus and transcription of osmo-protective target genes, thereby contributing to the protection of renal cells from hyperosmotic stress. Cdk5 also modulates transcription through phosphorylation of Ser180 of acetylated high mobility group box 1 (HMGB1) (Ugrinova *et al*., 2011). HMGB1 is a ubiquitous and abundant protein in nucleated eukaryotic cells that interacts with multiple transcription factors to increase their affinity for DNA elements, primarily regulating cellular processes critical for glucose homeostasis and inflammation (Thomas & Travers, 2001).

Inhibition of Cdk5 activity by roscovitine blocks NF-κB activation in leukocytes and in lung carcinomas (Berberich *et al*., 2011), while Cdk5 phosphorylates and regulates the activity of the androgen receptor (Hsu *et al*., 2011). In pancreatic β-cells, p35 expression increases with elevated extracellular glucose (post-prandial), and the resultant increase in Cdk5 activity is associated with increased transcription of the insulin gene promoter (Daval *et al*., 2011).

Cdk5 can also negatively regulate transcription. Sds3, a constituent of the mSin3-histone deacetylase (HDAC) co-repressor complex, is phosphorylated by Cdk5 at Ser228, which enhances mSds3-mediated transcriptional repression (Li *et al*., 2004). As a result, Cdk5 is linked to multiple transcription factors with a diverse range of cellular outcomes from endocrine/nutrient responses through to cell growth and stress protection.

1.6.2.2 Cdk5 and translational regulation

Cdk5 can also regulate translation (Arif *et al*., 2011). Phosphorylation of glutamyl-prolyl tRNA synthetase (EPRS) at Ser886 results following interferon-gamma-mediated activation of Cdk5. EPRS becomes part of the interferon-gamma-activated inhibitor of translation complex, which suppresses the expression of pro-inflammatory genes in myeloid cells (Mukhopadhyay *et al*., 2009; Arif *et al*., 2009). In response to inflammatory stimulus, EPRS can recognise and directly bind to target mRNAs transcribed in response to the stimulus. EPRS is released from the multi-synthetase complex following Cdk5 phosphorylation, which enhances the binding of EPRS to the interferon-gamma-activated inhibitor of translation complex to inhibit translation of mRNAs associated with the inflammatory response (Arif *et al*., 2009).

1.6.2.3 Cell migration, adhesion, and angiogenesis

Angiogenesis involves the formation of new capillaries and plays a physiological role in healing injured tissues and in the formation of the placenta. In the development of cancer, there is a large requirement for nutrient supply from the blood so angiogenesis is vital for tumour growth. Hence, the balance between angiogenic and anti-angiogenic factors is critical in determining the rate of growth of a tumour (Tonini *et al*., 2003). Inhibition or silencing of Cdk5 prevents angiogenesis implicating Cdk5 as a new target for anti-angiogenic therapies (Liebl *et al*., 2010; Liebl *et al*., 2011). Pharmacological inhibition of Cdk5 with roscovitine reduces endothelial cell migration in a dose-dependent manner, *in vitro*. Additionally, roscovitine treatment *in vivo* produces anti-angiogenic effects, which are reproducible using siRNA or shRNA to silence Cdk5. Overexpression of the Cdk5 dominant negative mutant D145N reduces endothelial cell migration. Inhibition of Cdk5 also reduces phosphorylation of focal adhesion kinase (FAK) at Ser732 without altering the formation of focal

adhesions or changing microtubule dynamics. Nonetheless, the overall structure of the actin cytoskeleton is differentially organised after Cdk5 inhibition with a subsequent increase in RhoA and decrease in Rac1 activities, implicating Cdk5 in controlling the actin cytoskeleton in endothelial cells that supports migration and angiogenesis (Liebl *et al*., 2010).

Correspondingly, a correlation between the function of small GTPases and Cdk5 activity has been described in lens epithelial cells (Tripathi & Zelenka, 2009; Tripathi & Zelenka, 2010). Cdk5 suppresses Src activity, resulting in decreased p190RhoGAP phosphorylation and subsequent activation of RhoA. Src activity becomes increased following inhibition of Cdk5 with olomoucine and decreases Rho-ROCK signalling, which is responsible for the phosphorylation of myosin and maintenance of the stress fibers that are necessary for cell adhesion. Local activation of Rho is consistent with the colocalisation of Cdk5 with contractible stress fibers (Tripathi & Zelenka, 2009). In agreement, olomoucine increases the expression of matrix metalloprotease-9, which is required for corneal epithelial cell migration, most likely through activation of Src to increase the migration of these cells (Tripathi & Zelenka, 2010).

Cdk5 can regulate epithelial cell adhesion and migration in the lens and cornea of the eye (Tripathi & Zelenka, 2009; Tripathi & Zelenka, 2010). In these cells, migration is inhibited by overexpression of Cdk5 whereas a dominant-negative Cdk5 mutant enhanced motility. In a mouse model overexpressing Cdk5, corneal wound closure was delayed however wound healing was accelerated following treatment with olomoucine (Tripathi & Zelenka, 2009). Activation and localisation of Src was found to be dependent on Cdk5 activity and determined as a downstream target of Cdk5 during epithelial cell migration (Tripathi & Zelenka, 2010).

1.6.2.4 Apoptosis

Consistent with the majority of information regarding Cdk5 activity, evidence for a role for Cdk5 in apoptosis comes mainly from work in the CNS, however evidence of a similar role in non-neuronal cells does exist (Rosales & Lee, 2006). Inhibition of Cdk5 activity by either genetic or pharmacological manipulation promotes susceptibility to cell death in several cell types (Sharma *et al*., 2004; Wu *et al*., 2008; Brinkkoetter *et al*., 2010). In retinal pigment epithelial cells treated with roscovitine, expression of pro-apoptotic Bax increased while expression of anti-apoptotic Bcl-2 decreased (Wu *et al*., 2008). Under stress conditions, apoptosis is increased significantly in immortalised podocytes derived from p35 KO mice in comparison to controls. Furthermore, transfection of p35 siRNA into wild-type podocytes results in increased apoptosis. The increased apoptosis observed in podocytes from p35 KO mice could be rescued by expression of p35 (Brinkkoetter *et al*., 2010). Finally, stimulation of proliferation of bovine aortic endothelial cells by basic FGF is blocked by roscovitine and this treatment ultimately induces apoptosis (Sharma *et al*., 2004).

1.6.2.5 Endocrinology and metabolism

Besides the brain, highest expression of Cdk5 is observed in the testis (Tsai *et al*., 1993). Cdk5 activity is observed in both Sertoli- and Leydig cells (TM4 and TM3, respectively), in addition to the developing rat testis (Musa *et al*., 1998). The expression and activity of Cdk5 was hormonally regulated (by luteinising hormone (LH), follicle stimulating hormone (FSH), and epidermal growth factor (EGF)) in Leydig, but not Sertoli, cells (Musa *et al*., 2000). *In vivo*, Cdk5 activity was affected by hypophysectomy, which causes a reduction in secretion of LH and FSH (Musa *et al*., 2000). Human chorionic gonadotropin also regulates Cdk5 expression and activity, in a time- and concentration-dependent manner, in a pathway that utilises

cyclic-AMP (cAMP) to promote phosphorylation and activation of Cdk5 and the subsequent phosphorylation of steroidogenic acute regulatory protein (StAR), which then accumulates in Leydig cells (Lin *et al*., 2009). StAR is critical for androidogenesis, and in keeping with regulation by Cdk5, testosterone production was reduced when Cdk5 activity was silenced (Lin *et al*., 2009).

In pancreatic β-cells Cdk5 translocates from the membrane to the cytoplasm upon glucose stimulation, coinciding with the resultant increase in insulin secretion and insulin gene induction. Simultaneous treatment with roscovitine reduces glucose induced insulin secretion (Lilja *et al*., 2001). Subsequently, p35 and p39 expression was confirmed in pancreatic β-cells (Lilja *et al*., 2004). However, p39, rather than p35, was the key regulator of insulin secretion, and Munc 18-1, a key protein in vesicle trafficking, was proposed as the Cdk5 target (Lilja *et al*., 2004). In contrast, however, Ubeda and colleagues found that p35 expression and Cdk5 activity were regulated in β-cells by glucose, and the main action of this complex was to induce the insulin gene promoter (Ubeda *et al*., 2004). Although, in later work, the same group found that Cdk5 inhibition reduces glucotoxicity in INS-1 cells (Ubeda *et al*., 2006). The regulation of insulin secretion by Cdk5 may involve phosphorylation of phospholipase D2 (PLD2) and β2-syntrophin. EGF-dependent insulin secretion is modulated by PLD2 (Lee *et al*., 2008a), and activation of PLD2 by EGF requires Cdk5 phosphorylation of PLD2 at Ser134 (Lee *et al*., 2008b). In β-cells, β-syntrophin is a central adaptor protein that is phosphorylated at Ser75 and Ser90 by Cdk5 (Schubert *et al*., 2010).

The referenced data suggests that there may be at least two distinct mechanisms by which Cdk5 contributes to control of glucose-stimulated insulin secretion; firstly,

during first phase insulin release it regulates secretion and secondly, it enhances production of new insulin polypeptide for second phase insulin release and/or replenishing insulin secreting vesicles. In contrast, chronic hyperglycemia associated glucotoxicity may involve dysregulated activation of Cdk5.

Insulin stimulates glucose transport into adipocytes by inducing translocation of the glucose transporter GLUT4 to the cell membrane. Cdk5 and p35 are expressed in differentiated 3T3L1 adipocytes and insulin stimulation of these cells causes rapid and transient phosphorylation of Cdk5 at Tyr15 by the upstream kinase Fyn, paralleled by an increase in Cdk5 activity (Okada *et al*., 2008; Lalioti *et al*., 2009) Cdk5 and p35 overexpression reduces translocation of GLUT4 while Cdk5 silencing, or inhibition, prevented glucose uptake in adipocytes, suggesting a vital role for Cdk5 in this process (Okada *et al*., 2008). TC10α is a Rho family GTPase important for vesicle trafficking and GLUT4 translocation to the membrane in adipocytes. Cdk5 phosphorylates TC10α at Thr197, and this phosphorylation regulates TC10α function (Okada *et al*., 2008). Other work proposes that the synaptotagmin analog E-Syt1, as opposed to TC10α, is the substrate of Cdk5 that links it to the control of glucose transport. Insulin treated 3T3-L1 adipocytes stimulates Cdk5 activity and E-Syt1 was subsequently phosphorylated at Ser314 promoting association with GLUT4 transporters (Lalioti *et al*., 2009). Both E-Syt1 phosphorylation and GLUT4 association are inhibited by roscovitine.

Cdk5 is implicated in other adipocyte processes as it phosphorylates PPARγ, a nuclear receptor of great importance for adipogenesis, at Ser273 both *in vitro* and *in vivo* (Choi *et al*., 2010). Phosphorylation at this site reportedly alters the transcription of certain genes including adiponectin, leptin, and adipsin. Hyperinsulinemia induced

by fat feeding mice was accompanied by an increase in PPARγ phosphorylation in adipocytes. Additionally, anti-diabetic drugs of the thiazolidinedione class prevented phosphorylation of PPARγ *in vitro* and in subcutaneous biopsies of fat tissue taken from drug treated animals. Mutation of Ser273 to Ala altered the transcriptional profile of adipocytes suggesting that phosphorylation of this residue modifies the profile of PPARγ mediated transcription rather than simply enhancing or inhibiting PPARγ (Choi *et al*., 2010). The same group proposed that obesity induced the processing of p35 to p25, thereby activating Cdk5 to modify PPARγ function, and that interfering with this process would have similar benefits to PPARγ agonists on metabolic disease without the known side effects of the glitazone drugs.

1.7 PATHOGENIC IMPLICATIONS OF CDK5 DYSREGULATION

Cdk5 knockout studies in mice have demonstrated that development and maintenance of the central nervous system relies on the precise functioning of Cdk5 activity. Therefore, dysregulation of this activity has been implicated in the pathogenesis of several neurodegenerative diseases including Alzheimer's disease (AD), prion-related encephalopathies (PRE), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) or acute neuronal injury caused by ischemia/stroke (Alvira *et al*., 2008; Lopes *et al*., 2007; Lopes *et al*., 2010; Nguyen & Julien, 2003; Slevin & Krupinski, 2009; Tsai *et al*., 2004). The emerging functions of Cdk5 beyond the nervous system now suggest that Cdk5 dysregulation may have wider health implications, such as in the development of cardiovascular disease, diabetes and cancer.

1.7.1 Neurodegenerative disease

The etiology of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) is unknown. Each disease is characterised by signature pathological hallmarks with different brain regions initially, and preferentially, affected however, as a result of disease progression, cognitive impairments are profound and neuronal death is severe. The generation of p25/Cdk5 in response to neurotoxic agents and speculation regarding its heightened activity has implicated Cdk5 as a common culprit in the mechanisms underlying neurodegeneration.

1.7.1.1 Alzheimer's disease

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disease that causes dementia, and eventually death, in affected individuals. AD is clinically characterised as late-onset, age-dependent cognitive decline as a result of neuron degeneration in the cortex and hippocampus. The pathological corollary of these symptoms is the formation of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). Accumulation of oligomeric amyloid beta (Aβ) results in the formation of senile plaques due to the amyloidogenic processing of the amyloid precursor protein (APP) by various secretases. NFTs are produced due to hyperphosphorylation of cytoskeletal proteins such as tau, neurofilaments, and CRMP2, all proposed substrates of Cdk5, and these are the main components of the paired helical filament (PHF), an integral part of neurofibrillary tangles. The PHF associates with Cdk5 in AD brain where Cdk5 can be found in the intracellular neurofibrillary tangles in neurons of the elderly and in AD (Liu *et al*., 1995). These data implicates Cdk5 in the pathophysiology of Alzheimer's disease, and formed the basis for future investigations into the association between Cdk5 and AD.

1.7.1.2 Parkinson's disease

Manifestation of tremors, muscle rigidity, and postural instability is symptomatic of Parkinson's disease (PD), a neurodegenerative disorder characterised by α-synuclein-containing Lewy body inclusions, loss of dopaminergic neurons in the substantia nigra pars compacta, and impaired motor skills and cognition (Olanow & Tatton, 1999). Examination of post-mortem PD brains has revealed elevated levels of p35/Cdk5 (Brion & Couck, 1995; Nakamura *et al*., 1997; Takahashi *et al*., 2004) associating abnormal Cdk5 regulation in the pathogenesis of the disease.

Cdk5 inhibition confers neuroprotection in animal models of PD (Smith *et al*., 2003). The pathological consequences of PD can be induced in animal models via application of the chemical compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Cdks including Cdk2 or Cdk4 are unaffected by MPTP treatment however MPTP injection or treatment with MPP^+ enhanced p25 levels and Cdk5 activity (Wong *et al*., 2007; Smith *et al*., 2003; Smith *et al*., 2006; Qu *et al*., 2007: Huang *et al*., 2010). Additionally, neuronal death is attenuated subsequent to Cdk5 inhibitor treatment or dn-Cdk5 virus application. MPTP treatment also increases levels of ΔFosB, a transcriptional regulator of Cdk5 that is upregulated during drug addiction.

Parkin, the gene most frequently implicated in familial PD, is a Cdk5 interaction partner and substrate (Avraham *et al*., 2007). Parkin confers neuroprotection through its function as an ubiquitin ligase, with substrates including α-synuclein, synphilin-1, and p38MAPK. Phosphorylation of Parkin at Ser131 by Cdk5 reduces the auto-ubiquitination activity of parkin, which in turn modulates synphilin-1/α-synuclein inclusion formation.

Cdk5 was also found to interact with and phosphorylate Prx2, another neuroprotective protein, resulting in down-regulation of its activity (Qu *et al*., 2007). Reducing Prx2 in neurons (both in MPTP mouse models of PD and in cultured cells) promotes accumulation of reactive oxygen species, specifically in dopaminergic neurons of the substantia nigra pars compacta, resulting in neuronal death (Hu *et al*., 2011). Furthermore, an increase in phosphorylated Prx2 is observed in human PD post-mortem brain suggesting Cdk5 may have contributed to the development of the pathology.

1.7.1.3 Huntington's disease

Huntington's disease (HD) is an autosomal-dominant disorder caused by an expansion in the number of CAG-repeats in the Huntingtin gene, leading to abnormally large polyglutamine (poly-Q) repeats in the huntingtin (htt) protein (Cattaneo *et al*., 2001). Symptomatic manifestations of HD include involuntary tremors, known as chorea, physical impairments, and irreversible cognitive decline. The mutant and poly-Q containing htt contains multiple caspase cleavage sites and the processing of htt by caspase precedes the development of aggregation and neurodegeneration. This implies that enhanced processing of htt by caspase may contribute to the disease process.

In contrast to the evidence suggesting a detrimental role of Cdk5 in AD and PD, the literature supports a protective role for Cdk5 in HD. Cdk5 phosphorylates the htt protein at Ser434 in cytoplasmic fractions resulting in reduced htt cleavage at residue 513 by caspase, thus attenuating aggregate formation and toxicity (Luo *et al*., 2005). In the brain of HD transgenic mice, the activity of Cdk5 is reduced (Luo *et al*., 2005). Correspondingly, Cdk5 activity is reduced in late-stage HD patients and the stability of the p35/Cdk5 interaction is impaired by mutant htt. This might suggest a late-stage

compensation mechanism as reduced Cdk5 activity is in direct contrast to observations made in PD patients and mouse models of ALS.

Cdk5 phosphorylation of htt at Ser1181 and Ser1201 promotes a neuroprotective effect (Anne *et al*., 2007). Phosphorylation of these sites can be induced by DNA damage both *in vitro* and *in vivo*. The state of htt phosphorylation is a critical regulator of neuronal cell death; absence of phosphorylation at Ser1181 and Ser1201 confers toxic properties to wild-type htt in a p53-dependent manner in striatal neurons and accelerates neuronal death induced by DNA damage, whereas phosphorylation of these sites protects against polyQ-induced toxicity. Furthermore, Cdk5 suppresses the formation of mutant htt inclusion bodies in cell lines and primary neurons by disrupting the microtubule network (Kaminosono *et al*., 2008). Collectively, these findings suggest that Cdk5 activity is required to limit mutant htt toxicity and may, therefore, serve a protective role in HD.

1.7.1.4 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is an adult onset, rapid, progressive neurodegenerative disorder that is characterised by selective demise of motor neurons in the brain and spinal cord (Cleveland, 1999) which manifests symptomatically as muscle atrophy, paralysis, and ultimately death due to cachexia or respiratory failure (Walling *et al*., 1999). The accumulations of neurafilaments in the cell body and proximal axons of the motor neurons hallmark the disease. Hyperphosphorylation of tau and neurofilaments, namely the heavy chain subunit (NF-H) is evident in the disease, at sites that may be Cdk5 targets, while Cdk5 activity appears enhanced in ALS (Nguyen *et al*., 2001)(Bajaj *et al*., 1999).

1.7.1.5 Niemann-Pick Type C

Niemann-Pick type C (NPC) is a lysosomal storage disease associated with mutations in the NPC gene and is an autosomal-recessive neurodegenerative disorder characterised by distorted lipid storage in neurons, dendritic and axonal abnormalities, demyelination, and neuronal death (Elleder *et al*., 1985). NFTs, comprised of PHFs with phosphorylated tau are a key feature of NPC disease (Auer *et al*., 1995).

Enhanced Cdk5 activity was reported in one NPC KO mouse (Bu *et al*., 2002), but not a second (Sawamura *et al*., 2001). Additionally, Cdk5 inhibitors, roscovitine and olomoucine, attenuated tau and neurofilament hyperphosphorylation, cytoskeletal lesion formation, and motor defects in the NPC KO model with high Cdk5 activity, suggesting that Cdk5 activity is required for the neuropathology and subsequent motor impairment in that model (Zhang *et al*., 2004). More recently, silencing of the Cdk5 gene in NPC KO mice significantly attenuated tau hyperphosphorylation, delayed neuronal death, and ameliorated motor defects implying a neuroprotective effect of the reduction of Cdk5 activity in this model (Hao *et al*., 2009).

1.7.2 Pathology in extra-neuronal tissues

1.7.2.1 Cdk5 and diabetes

The discovery that Cdk5 promotes insulin secretion from β-cells (Lilja *et al*., 2001; Wei & Tomizawa, 2007) and regulates the insulin gene (Ubeda *et al*., 2004; Wei *et al*., 2005) prompted investigations into the importance of Cdk5 dysregulation in the development of Type 1 and Type 2 diabetes. In addition, the links between Cdk5 and glucotoxicity suggested inhibition of Cdk5 might protect pancreatic β-cells in hyperglycaemic states such as Type 2 diabetes (Ubeda *et al*., 2006).

Type 2 diabetes mellitus (T2DM) results as a consequence of the inability of cells to adequately respond to insulin produced by the pancreas, and is characterised by an initial phase of hyperinsulinemia followed by progressive loss of β-cell function. The incidence of T2DM is correlated with more than 60 genetic polymorphisms including CNJ11, which encodes a potassium rectifying channel, and the transcription factors peroxisome proliferator-activated receptor gamma (PPARγ), hepatic transcription factor 2 (TCF2), and Wolfram syndrome 1 (WFS1) (Ferguson, 2008). Of note, a single nucleotide polymorphism (SNP) in intron 5 of the Cdk5 regulatory subunit-associated protein 1-like 1 (Cdkal1) gene was also linked to increased risk of Type 2 diabetes. Cdkal1 is a 65 kDa protein that shares considerable amino acid and domain homology with Cdk5RAP1, an inhibitor of Cdk5 activity. This polymorphic variant of Cdkal1 is associated with decreased insulin secretion, and one group has hypothesised (without any experimental evidence) that this is a result of abnormal Cdk5 activity regulation (Chistiakov *et al*., 2011).

In obese animals, adipocyte secretion of cytokines and adipokines (cytokine-like molecules) is altered. These agents modulate glucose homeostasis and deposition of free fatty acids (FFAs) in multiple tissues and may contribute to the development of insulin resistance and T2DM (Cusi, 2010). Several cytokines, including tumour necrosis factor-alpha (TNF-α) (Utreras *et al*., 2009), can enhance Cdk5 activity through the transcriptional up-regulation of p35. PPARs are a family of structurally related nuclear receptors that control the expression of several genes and physiological processes such as insulin secretion and adipogenesis (Brun et al., 1997; Sharma & Staels, 2007). Cdk5 activation by TNF-α, interleukin (IL)-6, and FFAs is reported to promote phosphorylation of PPARγ at Ser273 in 3T3-L1 adipocytes (Choi *et al*., 2010). This phosphorylation is proposed to modulate the transcriptional activity

of PPARγ toward specific genes such as the fatty acid transporter CD36, adiponectin, adipsin, and leptin potentially by modulating PPARγ interaction with transcriptional co-regulators. Importantly, treatment of cells and animals with the anti-diabetic drug, rosiglitazone (a PPARγ agonist) inhibits Cdk5-dependent phosphorylation of PPARγ (Choi *et al*., 2010). The authors therefore suggest that drugs targeting the Cdk5 regulation of PPARγ could invoke the anti-diabetic potential of the PPARγ agonists without some of the side effect problems that are reducing the clinical use of this class of drug.

1.7.2.2 Cdk5 and senescence

Cellular senescence describes the phenomenon of cell growth arrest due to telomere shortening however this term may also be applied to the attrition process in response to cellular damage produced by endogenous or exogenous stimuli such as DNA damage, oxidative stress, or oncogene activation (Campisi & Fagagna, 2007). In response to such noxious stimuli, cells enter a state of cell arrest that eventually results in senescence as an anti-proliferation mechanism.

The retinoblastoma protein (pRB) and tumour suppressor protein p53 are important regulators of senescence, and mutations that inhibit activation of these proteins enable abnormal proliferation (Mao & Hinds, 2010). In human osteosarcoma SAOS-2 cells, ectopic expression of pRB induces Cdk5 activity during senescence thereby promoting the phosphorylation of ezrin and suppression of Rac activity, resulting in acquisition of a pRB-induced senescent phenotype (Yang *et al*., 2003; Alexander *et al*., 2004). Also, p35 expression is essential for Cdk5 activation in pRB-induced SAOS-2 senescent cells. Conversely, reducing p35 levels with shRNA antagonises development of the senescent phenotype in SAOS-2 cells. Notably, the p35 promoter

seems to contain no regulatory elements responsive to pRB therefore it seems unlikely that RB directly regulates the p35 gene promoter (Mao & Hinds, 2010).

1.7.2.3 Cdk5 and cancer

Cancer is a genetic and epigenetic disease arising from a single cell that has acquired the hallmarks of cancer (Barber *et al*., 2014). These hallmarks comprise a series of distinctive and complementary biological capabilities acquired during the multistep development of tumours, enabling tumour growth and metastatic dissemination (Hanahan & Weinberg, 2011). The hallmarks constitute an organising principle for rationalising the complexities of neoplastic disease and include: (1) *Sustaining proliferative signalling*. Arguably, the most fundamental hallmark of cancer cells involves their ability to sustain chronic proliferation. In contrast to normal cells which carefully manage the production of growth promoting or inhibiting factors to ensure a tight control of cell number, tissue architecture and function, tumour cells show deregulated signalling cascades that enable them to be more or less independent of proliferation which results in unlimited growth. Cancer cells sustain proliferation through autocrine proliferative signals, overexpression of cell-surface receptors and/or constitutive activation of signalling pathway components. (2) *Evading growth suppressors*. Normal cells are maintained in a non-dividing state by antigrowth signals, which cancer cells evade through genetic changes; (3) *Activating invasion and metastasis*. Cancers cells are involved in the inactivation of a whole series of controls that confines a cell to the site and tissue of normal growth enabling tumour cells to invade local tissues and metastasise to distal sites. This requires alterations in cell morphology, decreased attachment to other cells and the extracellular matrix, and an increase in cell motility; (4) *Enabling replicative immortality*. Most mammalian cells can replicate a finite number of times due to progressive shortening of telomeres

however cancer cells proliferate indefinitely by activating telomerase. This enzyme extends the telomeres enabling cancer cells to progress through multiple cycles of growth and division without concern of the imminent collapse of its telomeres; (5) *Inducing angiogenesis*. The process of angiogenesis is critical for almost all cancers. In tumour progression, there is an 'angiogenic switch' that results in quiescent vasculature to promote growth of new blood vessels into the tumour mass, thereby providing the tumour with nutrients and glucose and oxygen and evacuating metabolic waste and carbon dioxide, sustaining tumour growth; (6) *Resisting cell death*. Although cancer cells are under continual stress as the result of DNA damage and oncogenic signalling associated with hyperproliferation, they have devised mechanisms to evade apoptosis resulting in the accumulation of aberrant cells (Hanahan & Weinberg, 2000).

The potential links between Cdk5 activity and tumourigenesis remain shrouded in considerable controversy. Firstly, Cdk5 is considered the atypical member of the Cdk family, a family whose role in cancer is well documented, due to an absent role in cell cycle regulation and mitotic function coupled with enriched expression in postmitotic neurons and glial cells. Additionally, the activity of Cdk5 is regulated by neuronal activators, and its extensive role in the development of the central nervous system opposes the potential implications of Cdk5 in cancer. However neuronal migration during development of the nervous system and cancer cell migration are mediated by quite similar processes (Kasemeier-Kulesa *et al*., 2008), suggesting that the involvement of Cdk5 in cancer may not be that surprising. Furthermore, the identification of Cdk5 activity in malignancy and expression of Cdk5 and cofactors in extra-neuronal tissues is suggesting that role of Cdk5 in tumourigenesis should perhaps be reconsidered.

Evidence for an association between Cdk5 dysregulation and cancer was first found in leukaemia cells (Chen *et al*., 1999; Sandal *et al*., 2002). 1alpha, 25-dihydroxyvitamin D3 (1,25D3), the biologically active form of vitamin D, induced Cdk5 activity in human leukaemia cells (Chen *et al*., 1999).

More recently, Cdk5 activation promoted medullary thyroid carcinoma (MTC) in a conditional MTC mouse model (Pozo *et al*., 2013). Furthermore, signalling via the retinoblastoma protein (Rb) was a critical regulator of MTC tumourigenesis and progression. In addition, retinoic acid can modify Cdk5 activity in thyroid cancer cells through a mechanism involving the sodium/iodide transporter, a plasma membrane glycoprotein that stimulates the uptake of iodide (Jeong *et al*., 2006). These findings suggest that Cdk5 activity may function as a potential molecular diagnostic or therapeutic target for the treatment of thyroid cancer.

In the breast cancer cell lines MCF-7 and MDA-MB321, decreased proliferation was observed in the presence of either roscovitine or siRNA specific for Cdk5. Additionally, application of carboplatin, a known chemotherapeutic drug used in the treatment of breast cancer, induced Cdk5 activation by increasing ERK activity in response to DNA damage (Upadhyay *et al*., 2008). An increase in Cdk5 activity stimulates p53 stability that can ultimately lead to cell death in response to DNA damage caused by carboplatin. This implies that tight regulation of Cdk5 activity may be important for normal cell physiology and that local and temporal loss or gain of function might result in abnormal cell proliferation.

The p35/Cdk5 complex was active in both nTERT-HPNE and MIAPaCa-2 pancreatic carcinoma cells while inhibition of Cdk5 activity using either dominant negative

Cdk5 (dn-Cdk5) or pharmacological inhibitors of Cdk5 activity decreased the polarity and migration of both cell lines (Feldmann *et al*., 2010). Cdk5 also regulated cell invasion through modulation of the formation of invadopodia (Quintavalle *et al*., 2011). In correlation with these effects, inhibition of Cdk5 activity by RNA interference (RNAi) reduces tumour growth and metastasis in pancreatic cancer cells (Feldmann *et al*., 2010). The monomeric GTPases RalA and RalB play a fundamental role in the development of pancreatic cancer cells (Lim *et al*., 2006). Blocking RalA signalling with KRAS2 mutants inhibited the tumourigenicity of pancreatic cancer cells, while expression of dn-Cdk5 in MIAPaCa-2 cells decreased levels of RalA-GTP and RalB-GTP (Feldmann *et al*., 2010). Co-expression of constitutively active forms of either RalA or RalB overcame the effect of dn-Cdk5 suggesting that Ral proteins are downstream of Cdk5. By controlling the function of RalGEFs and RalGAP, Cdk5 may regulate the activation of RalA and RalB implicating Cdk5 as a potential target for the treatment of pancreatic cancer (Feldmann *et al*., 2010). Additionally, increased Cdk5 kinase activity was also observed in immortalised human pancreatic nestin-expressing (HPNE) cells expressing a mutant form of K-Ras (G12D) compared with HPNE cells expressing native K-Ras (Eggers *et al*., 2011). Mutant K-Ras elevated p35 cleavage to p25, whereas p25 protein levels were decreased following inhibition of the signalling cascade downstream of mutant K-Ras which involves mitogen-activated protein/extracellular signal-regulated kinase, phosphoinositide 3-kinase, or Cdk5. This suggests that mutant K-Ras acts in concert with Cdk5 and its activators to increase malignant progression, migration, and invasion of pancreatic cancer cells (Eggers *et al*., 2011).

In cervical carcinoma caused by human papilloma virus (hPV), Cdk5 can inhibit cell proliferation through phosphorylation of p53 at Ser43 and Ser20, however hPV can

overcome this mechanism by inducing p53 degradation. HeLa cells infected with hPV do not undergo apoptosis or cell cycle arrest even when p53 is overexpressed, however modifying the phosphorylation state of p53, either by promoting the activity of protein kinases or inhibiting phosphatases, can induce apoptosis (Ajay *et al*., 2010). In this context, Cdk5-dependent phosphorylation of p53 could increase stability and transcriptional activation of p53 (Zhang *et al*., 2002).

In contrast, Cdk5 activity was essential for control of motility and the metastatic potential of prostate cancer cells (Strock *et al*., 2006). Phosphorylation of the androgen receptor at Ser81 by p35/Cdk5 regulates the stability of this protein and increases the accumulation of androgen receptors in the nucleus, thereby regulating the proliferation of cancer cells (Hsu *et al*., 2011). Additionally, Cdk5 can increase both the expression and secretion of prostate-specific antigen (PSA), perhaps by regulating exocytosis, suggesting that Cdk5 may play a critical role in the development of prostate cancer (Hsu *et al*., 2011).

In PC12 pheochromocytoma cells, talin, a β-integrin tail-binding protein required for integrin activation, was identified as a Cdk5 substrate under physiological conditions. Cdk5 phosphorylates talin at Ser425, which prevents binding to the ubiquitin ligase SMURF1 and halts its degradation. In SHSY5Y cells, the talin mutant tal(S425A) is resistant to Cdk5 phosphorylation and is intensively ubiquitinylated by SMURF1, resulting in an increased focal adhesion turnover and inhibition of cell migration (Huang *et al*., 2009). In glioma and glioblastoma cells, annexin-2 is a gliotropic factor upregulated in these cells that contributes to glioma malignancy (Zhai *et al*., 2011). Treatment of the cells with roscovitine abrogated annexin-2-induced migration.

In some patients with non-small cell lung cancer there is a significant correlation between the expression of p35/Cdk5 and the degree of differentiation and metastasis to lymph nodes (Liu *et al*., 2011). Polymorphisms in the Cdk5 promoter increase the risk of lung cancer in a specific Korean population (Choi *et al*., 2009). Also, overexpression of Cdk5 induced by gene amplification in lung cells can enhance signalling through the EGF receptor pathway (Lockwood *et al*., 2008). Decreased methylation of the Cdk5 gene has been found in mantle cell lymphoma, a subtype of B cell lymphoma, and this is associated with an increase in Cdk5 mRNA levels (Leshchenko *et al*., 2010). However the mechanism of generation of this cancer and the link with Cdk5 remains unknown.

Further evidence in support of Cdk5 contributing to cancer initiation and progression comes from the study of probable Cdk5 substrates in cancerous tissue. Although considered to be neuron-enriched proteins, the CRMP family (Cdk5 targets) have now been found in several malignant tumours unrelated to the CNS. For example, downregulation of the CRMP1 isoform is associated with poor outcome in lung cancer (Shih *et al*., 2001), while upregulation of CRMP2 is found in colorectal cancer (Wu *et al*., 2008). Phosphorylation of both of these isoforms by Cdk5 is associated with enhanced function in neurons (Schmidt & Strittmatter, 2007), however the role of phosphorylation of CRMPs by Cdk5 in cancer has not yet been studied. Additionally, the CRMP4 isoform functions as a metastasis suppressor in prostate cancer (Gao *et al*., 2010). This isoform may not be a physiological substrate for Cdk5 (Cole *et al*., 2004) however it can be phosphorylated by Cdk5 *in vitro* and this may provide a means to upregulate its activity in disease states.

In addition, Cdk5 phosphorylation of STAT3 at Ser727 in medullary thyroid

carcinoma cells results in increased cell proliferation and promotion of tumourigenesis (Fu *et al*., 2004). In Cdk5 null mice, tumour growth was delayed in a STAT3 phosphorylation-dependent manner (Lin *et al*., 2007).

Therefore, there is evidence of a potential role of Cdk5 in cancer progression and/or as a treatment option. Enhancing Cdk5 action on specific substrates may reduce progression/metastasis of specific tumours, while inhibition has potential to antagonise others. Similarly, Cdk5 substrates may offer diagnostic or prognostic value. It is therefore vital to further our understanding not only of how Cdk5 dysfunction could occur to affect tumourigenesis but what specific downstream substrates confer Cdk5 action (positive or negative) to each specific form of cancer.

1.8 AIMS OF THESIS

The overall main objectives of this thesis were to further explore, and validate where possible, a number of proposed Cdk5 substrates as physiological targets. This information would be used to reassess the role of Cdk5 activity in tumourigenesis.

1.8.1 Consensus for substrate phosphorylation

The primary sequence requirements for Cdk5 substrate phosphorylation have not been fully established and are generally considered degenerative. This thesis will aim to explore the primary amino acid sequences of proposed Cdk5 substrates and generate peptides to study the substrate selection profile of Cdk5 based on the primary amino acid sequence surrounding the phosphoacceptor residue. This will contribute towards establishing a consensus for the targeting of Cdk5 phosphorylation. This consensus

will aid in identifying the physiological substrates of Cdk5 and provide a substrate profile to investigate *in vivo*.

1.8.2 Assessing the hyperactivity of the p25/Cdk5 complex

The current consensus surrounding Cdk5 dysregulation is associated with the cleavage of p35 by calpain resulting in a hyperactive Cdk5 complex. Therefore, this thesis aims to compare substrate specificities and phosphorylation kinetics of the p35/Cdk5 and p25/Cdk5 complexes in order to assess whether different substrate phosphorylation rates are likely to occur following cleavage of p35 to p25 in disease.

1.8.3 Validating the tools to study Cdk5 phosphorylation in cancer

Following *in vitro* validation of Cdk5 consensus and substrates, these will be investigated using a mammalian cell line system to assess substrate phosphorylation in intact cells. Tools to monitor regulation of conferred substrates will be developed and optimised for the study of Cdk5 substrate phosphorylation in human cancer.

1.8.4 Investigating the role of Cdk5 in cancerous tissue

Cdk5 is commonly described as an atypical member of the Cdk family, a large gene family that contains specific members that are heavily associated with cancer and tumour progression. The proposed atypical nature of Cdk5 will be explored in the final part of this thesis by investigating Cdk5 substrate phosphorylation within a range of human tumours. The information gathered throughout this thesis will contribute to the investigation of abnormal Cdk5 activity in cancer, with a view to develop useful diagnostic or prognostic reagents, and identify possible avenues for novel therapeutics.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

2.1.1 Reagents

Invitrogen (Groningen, The Netherlands): Streptomycin/penicillin, NuPAGE Bis-Tris 4-12% pre-cast gels, pCR TOPO 2.1 cloning kit, NuPAGE buffers for SDS-PAGE, SeeBlue molecular weight standards, Western blotting Mini Cell apparatus, Lipofectamine™ 2000, PureLink PCR purification kit, PureLink® Quick Plasmid Miniprep Kit, PureLink® HiPure Plasmid Filter Maxiprep Kit, SilverQuest™ staining kit, LDS sample buffer.

Sigma-Aldrich (Dorset, United Kingdom): Glycine, Tris-base, imidazole, kanamycin sulphate, Tween®20, DMSO, Brilliant Blue G-Colloidal Concentrate, NP-40, BES, ethidium bromide, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), MOPS, ampicillin, orthophosphoric acid, myelin basic protein (MBP), glycerol, Triton X-100, Brij® L23 solution, Igepal CA-630, sodium vanadate, β-mercaptoethanol.

Thermo Fischer Scientific (Massachusetts, United States): Agarose, sodium hydroxide, sodium dodecyl sulphate (SDS), calcium chloride, paraformaldehyde, Subcellular Protein Fractionation kit for Cultured Cells, Kodak® Biomax MS autoradiography film, Ambion® nuclease free water.

Gibco (California, United States): 0.05% (w/v) trypsin-EDTA, 2.5% (w/v) trypsin-EDTA, penicillin-streptomycin.

Lonza (Switzerland): Dulbecco's Modified Eagle's Medium (DMEM) high glucose without sodium pyruvate.

Sera Laboratories International (United Kingdom): foetal bovine serum (FBS).

PerkinElmer ((Massachusetts, United States)): Ultima Gold™ scintillation fluid, [γ-32P] adenosine triphosphate (specific activity, 3000Ci/mmol).

Corning (New York, United States): polypropylene tubes.

NUNC (Roskilde, Denmark): tissue culture flasks and plates.

Promega (United Kingdom): All restriction nuclease enzymes, 10 kb DNA ladder.

Bio-Rad (California, United States): Bio-Rad protein assay dye reagent concentrate.

Merck Millipore (Massachusetts, United States): p25/Cdk5 active complex, p35/Cdk5 active complex, purvalanol A, roscovitine.

Roche Molecular Biochemicals (Lewes, United Kingdom): complete™ protease inhibitor tablets (EDTA free).

GE Healthcare (New Jersey, United States): Hybond™ Extra-C, protein G-sepharose beads.

Vector Laboratories (California, United States): VECTASTAIN® ABC Reagent

VWR International: p81 phosphocellulose paper, microscope slides and coverslips.

Whatman International (Maidstone, United Kingdom): 3 MM chromatography paper.

Kind Gifts: pCMV5 Cdk5, pCMV5 p35, pCMV5 p25 (Dr. Margareta Nikolic, Imperial College London). Anti-PPARγ antibody (Professor Colin Palmer, University of Dundee), Dynamin 1 constructs (Professor Mike Cousin, University of Edinburgh).

2.1.2 Recombinant Proteins

The Division of Signal Transduction Therapy (DSTT), University of Dundee was responsible for the production of all bacterially expressed recombinant proteins. Briefly, *E. coli* were transformed with a plasmid expression vector pGEX6p, containing the cDNA of the protein of interest fused to a purification tag (e.g. glutathione S transferase (GST) and placed under the control of a bacterial promoter. The cells were grown in liquid broth and protein expression was increased using isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were then lysed, the insoluble material removed, and the remaining lysate passed through an affinity purification column containing the ligand of the tag of interest (e.g. glutathione). The protein bound to the column was then washed several times to remove contaminating proteins followed by elution by competition or pH change.

2.1.3 Antibodies

Antibodies used throughout this thesis were obtained either commercially or were produced in-house by the Division of Signal Transduction Therapy (DSTT), University of Dundee.

2.1.3.1 In house antibodies

The Division of Signal Transduction Therapy (DSTT), University of Dundee provides an antibody generation service to staff members at the University. Aliquots of all antibodies previously generated are made available to colleagues within the University after the PI who raised the antibody has published. ***Table 2.1*** lists the antibodies used in this thesis that were generated by Division of Signal Transduction Therapy, University of Dundee for the Sutherland lab.

Antibody	Species	Dilution	Supplier
anti-CRMP2	Sheep	1:1000	DSTT, University of Dundee
anti-pCRMP2 Ser522	Sheep	1:1000	DSTT, University of Dundee
anti-pCRMP4 Ser522	Sheep	1:1000	DSTT, University of Dundee
anti-PPARγ Ser273	Rabbit	1:1000	DSTT, University of Dundee

Table 2.1. ***In-house antibodies.*** The following antibodies were generated by the Division of Signal Transduction Therapy (DSTT), University of Dundee.

2.1.3.2 Total CRMP2 antibody

An antibody recognising both phosphorylated and non-phosphorylated forms of CRMP2 was generated in sheep following injection with full-length recombinant GST-CRMP2 (Sutherland lab in collaboration with DSTT). Following preclearing on GST-sepharose, antiserum was affinity purified on GST-CRMP2-sepharose. Due to the high amount of homology between the CRMP isoforms this antibody was further purified using GST-CRMP1 and GST-CRMP4 proteins bound to gluthathione-sepharose in order to remove antibodies that recognised CRMP1 and CRMP4. Each purified CRMP2 antibody preparation was routinely tested against GST-CRMP1, GST-CRMP2 and GST-CRMP4 protein to ensure efficient purification.

2.1.3.3 pCRMP2 Ser522 antibody

For generation of a phosphospecific antibody that recognises CRMP2 phosphorylated at Ser522, a phosphopeptide based on the sequence surrounding Ser522 (CASSAKTpSPAKQQA where pS is phosphoserine) was synthesised (Sutherland lab in collaboration with DSTT). This was conjugated separately to both bovine serum albumin (BSA) and keyhole limpet hemocyanin and then injected into a sheep host (Diagnostics Scotland, Penicuik, UK). Antiserum was collected and affinity-purified on a CRMP2 Ser522 phosphopeptide antigen sepharose column. Isoform specificity and sensitivity was then assessed by SDS-PAGE and Western blotting using WT and S522A mutant CRMP isoforms (recombinant phosphorylated and over-expressed protein), and was found to be specific for CRMP2 phosphorylated at S522 (Adam Cole, personal communication). Additional CRMP phosphospecific antibodies recognising other phosphorylation sites were generated by a similar method (***Table 2.2***). For immunoblotting, each phospho-specific antibody was diluted in TBS containing 5% (w/v) skimmed milk and 1 μM non-phosphorylated peptide.

Isoform	Epitope Sequence	Antibody Description	Western blotting Dilution
CRMP2	CEVSVpTPKTVpTPAS	pThr514/509	1:1000
CRMP2	CEVSVpTPKTVTPAS	pThr514	1:1000
CRMP4	GSARGpSPTRPNP	pSer522	1:1000
CRMP4	FDLTTpTPKGGTPAG	pThr509	1:1000

Table 2.2. ***Phosphospecific CRMP antibodies.*** Peptide sequences used to generate CRMP antibodies.

2.1.3.4 Commercial antibodies

Primary and secondary antibodies were sourced commercially. For immunoblotting, each primary antibody was diluted (at the concentration specified in ***Table 2.3***) in Tris-buffered saline (TBS) containing 5% (w/v) skimmed milk or 5% (w/v) bovine serum albumin (BSA), dependent on antibody requirements, and incubated overnight at 4°C. Secondary antibodies containing a fluorescent conjugate were diluted in TBS containing 5% (w/v) skimmed milk and incubated for 1 hr at room temperature.

For immunofluorescence, antibodies were diluted 1:50 in phosphate-buffered saline (PBS) containing 5% (w/v) bovine serum albumin (BSA). Incubation time was an hour at room temperature followed by secondary antibody incubation at 1:250 for an hour. The secondary antibodies used for immunocytochemistry were Cys-3 conjugated donkey anti-sheep IgG (H+L) and Cys-3 conjugated donkey anti-rabbit IgG (H+L) (*Jackson ImmunoResearch Laboratories, Inc*). Secondary antibodies were diluted in PBS containing 5% (w/v) BSA for 1 hr at R.T.

For use with immunohistochemistry (IHC), primary antibodies were diluted in 5% (v/v) normal serum in PBS containing 5% (v/v) biotin at overnight at 4°C. Biotinylated secondary antibodies (*Vector Laboratories, CA, USA*) were diluted 1:250 in 5% (v/v) normal serum and incubated for 30 min at R.T., followed by streptavidin complexed with biotinylated peroxidase (Vectastain ABC kit: *Vector Laboratories, CA, USA*) for 30 min at R.T.

Antibody	Species	Dilution	Supplier	Catalogue No.
anti-Cdk5 (total)	Rabbit	1:1000	Santa Cruz	sc-173
anti-p35/p25	Rabbit	1:1000	Santa Cruz	sc-820
anti-CRMP2 (total)	Rabbit	1:1000	Cell Signaling	9393
anti-TUC4/CRMP4	Rabbit	1:1000	Merck Millipore	AB5454
anti-Tau (total)	Rabbit	1:1000	Dako	A002401-2
anti-pTau S235	Rabbit	1:1000	Bioworld	BS4193
anti-pTau S231	Rabbit	1:1000	Merck Millipore	AB9668
anti-pTau S205	Rabbit	1:1000	Invitrogen	44-738G
anti-pTau S202	Rabbit	1:1000	Cell Signaling	11834
anti-FLAG	Mouse	1:500	Sigma-Aldrich	F3615
anti-Dynamin 1 S778	Mouse	1:1000	Abcam	ab18101
anti-STAT3 S727	Rabbit	1:1000	Cell Signaling	9134
anti-Mef2a S408	Rabbit	1:1000	GeneTex	GTX79018
anti-PAK1 T212	Rabbit	1:1000	Abcam	ab75599
anti-Lamin A/C	Rabbit	1:1000	Cell Signaling	2032
anti-Histone H4	Rabbit	1:1000	SeroTec	AHP413
anti-GAPDH	Rabbit	1:1000	Abcam	ab37168
anti-ANT	Mouse	1:1000	Calbiochem	AP1034

Table 2.3. ***Commercial antibodies.*** List of antibody dilutions and species used for Western blot analysis. Antibodies were diluted to the specific dilutions in 5% (w/v) Milk/TBST or 5% (w/v) BSA/TBST dependent on antibody specifications.

2.2 METHODS

2.2.1 General Use Buffer Preparation

PBS (pH 7.4) (1L)

Sodium chloride (NaCl)	8 g
Potassium chloride (KCl)	0.2 g
Disodium phosphate (Na_2HPO_4)	1.44 g
Monopotassium phosphate (KH_2PO_4)	0.24 g
ddH_2O	Fill up to 1L
	Autoclave to sterilise

TBS 10 x Stock Solution (1L)

Sodium chloride (NaCl)	87.66 g
Tris base	12.11 g
ddH_2O	800 ml
	Adjust pH to 8.0
	Fill up to 1L with ddH_2O

1M Tris Buffer (1L)

Tris base	121.1 g
ddH_2O	800 ml
	Adjust pH accordingly with HCl
	Fill up to 1L with ddH_2O and autoclave to sterilise

0.5M EDTA (pH 8.0) (1L)

Na_2EDTA	186.1 g
ddH_20	500 ml
	Adjust pH to 8.0 with 10 M NaOH
	Fill to 1L with ddH_2O and autoclave to sterilise

0.5M EGTA (pH 8.0) (1L)

Na_2EGTA	190.2 g
ddH_20	500 ml
	Adjust pH to 8.0 with 10 M NaOH
	Fill to 1L with ddH_2O and autoclave to sterilise

TBE 10 x Stock Solution

Tris base	108 g
Boric acid	55 g
0.5M EDTA	40 ml
	Fill to 1L with ddH_2O and autoclave to sterilise

2.2.2 Molecular Biology

2.2.2.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis separates DNA based primarily on molecular size and was used during analysis of DNA digestion patterns by restriction enzymes. An agarose gel of 0.8% was used for most applications; a higher percentage gel would be used to separate relatively small DNA constructs (e.g. <1 kbp), whereas a lower percentage gel was used for relatively large constructs (e.g. > 10 kbp). To prepare a 100 ml, 0.8% gel, 0.8g of agarose was dissolved in 100ml 1 x TAE buffer by heating in a microwave oven. Upon cooling, 2 μg of the intercalating agent ethidium bromide was added to enable visualisation of the DNA under ultraviolet (UV) light. The solution was then poured into a gel cast with a well separating comb and left to set at room temperature. Once set, the gel was placed into a suitable gel tank where it was submerged in 1 x TAE buffer. Finally, samples and DNA molecular mass ladder were loaded onto the gel and run for 60 min at 60V before examining the gel under UV. The ethidium bromide interacts with the DNA in each sample and emits fluoresces when exposed to UV allowing the DNA bands to be visualised. The Qiaquick gel purification kit (*Qiagen*) was used as the manufacturer's instructions for the purification of DNA bands from the agarose gel.

2.2.2.2 Restriction enzyme digest of plasmid DNA

A standard restriction enzyme digest contained 1 Unit of endonuclease enzyme, 1 μl of 10 x compatible buffer and 0.2 mg/ml of DNA in a final volume of 10 μl. The endonuclease was added last before placing the reaction at 37°C for 2 hrs, unless otherwise dictated by specific instructions for the enzyme in question.

Plasmid DNA	1 µl (0.2 mg/ml)
Endonuclease	1 µl (1 Unit)
10 x Endonuclease buffer	1 µl
ddH_2O	7 µl

2.2.2.3 Production of Chemically Competent Bacterial Cells for DNA Transformations

A starter culture of 5 ml liquid broth (LB) was inoculated with an XL-1 blue glycerol stock and incubated on a shaker overnight at 37°C. Subsequently, 1 ml was removed from this culture and added to 50 ml LB. This was then incubated at 37 °C for 1-2 hrs until an optical density between 0.45 and 0.55 at 550 nm was obtained to ensure bacteria were in the log phase of growth. The cells were pelleted via centrifugation at 4000 x g for 5 min at 4°C, the supernatant discarded, and the pellet resuspended in 10 ml ice cooled Transformation Buffer 1 (TFB1) buffer for 5 min on ice, followed by the addition of a further 70 ml for 5 min. Once again, the cells were pelleted via centrifugation at 4000 x g for 5 min at 4°C and then resuspended in 8 ml ice cooled Transformation Buffer 2 (TFB2) buffer and stored on ice for 15 min. The cells were then aliquoted into pre-cooled 1.7 ml microcentrifuge tubes in 100 µl volumes, snap frozen in liquid nitrogen, and stored at -80°C.

TFB1 Buffer

Potassium acetate (KoAc)	30 mM
Rubidium chloride ($RbCl_2$)	100 mM
Calcium chloride ($CaCl_2$)	10 mM
Manganese chloride ($MnCl_2$)	50 mM
Glycerol	15% (v/v)
	Adjust pH to 5.8 with acetic acid
	Sterile filtration

TFB2 Buffer

MOPS	10 mM
Calcium chloride ($CaCl_2$)	75 mM
Rubidium chloride ($RbCl_2$)	10 mM
Glycerol	15% (v/v)
	Adjust pH to 6.5 with HCl
	Sterile filtration

2.2.2.4 Transformation of Bacteria

Plasmids that contain a bacterial origin of replication are recognised by the bacterial cell DNA polymerases, allowing replication and amplification, and subsequent expression of proteins encoded elsewhere on the plasmid.

Routinely, approximately 1-2 μl of plasmid DNA was added to 50 μl of chemically competent XL-1 blue cells, avoiding excessive pipetting and disturbance of the cells, then incubated on ice for a minimum of 30 min. The cells were then heat shocked at 42°C for 90 seconds to facilitate DNA uptake and then placed back on ice for a further one minute. To allow for the recovery of the bacteria and the expression of antibiotic resistance genes, 1 ml of liquid broth (LB) was added to the cells that were then incubated and allowed to grow for a minimum of one hour at 37°C. Finally, under a burning blue flame of a Bunsen burner to avoid contamination of the samples, approximately 50 μl of bacterial culture was plated on agar plates containing either 50 μg/ml ampicillin or 30 μg/ml kanamycin, dependent on whether the vector contained a gene encoding for ampicillin or kanamycin resistance. The plates were placed overnight in an incubator set at 37°C. The following day, confirmation of the uptake of DNA was confirmed by the growth of colonies.

LB Broth (1L)

Bacto-tryptone	10 g
Yeast extract	5 g
NaCl	10 g
ddH_2O	800 µl
	Adjust pH to 7.5 with NaOH
	Fill up to 1L with ddH2O and sterile by autoclaving

LB Agar (1L)

Bacto-tryptone	8 g
Yeast extract	5 g
NaCl	10 g
ddH_2O	800 µl
	Adjust pH to 7.5 with NaOH

Agar

Agar	15 g
	Melt in microwave
	Fill up to 1L with ddH2O and sterile by autoclaving

2.2.2.5 Plasmid DNA Purification

For plasmid DNA purification, 5ml of LB, containing the appropriate antibiotic, was inoculated with bacteria containing the plasmid of interest and incubated at 37°C overnight in a shaker rotating at 250 rpm. For larger DNA purification, a larger volume of 200 ml LB was inoculated with bacteria. In order to pellet the cells, the 5 ml cultures were centrifuged at 5000 x g for 10 min. Plasmid DNA was extracted from the bacteria using the PureLink™ mini prep kit (Invitrogen) following the manufacturer's instructions. Plasmid DNA was eluted from the anion exchange resin using 50 µl nuclease free water. For 200 ml cultures, a J-25 Avanti centrifuge

(*Beckman*) was set at 7000 x g for 10 min to pellet the cells and plasmid DNA was extracted from the bacteria using the PureLink® HiPure Plasmid Maxiprep Kit (*Enzo Life Sciences*) following the manufacturer's instructions. The purified plasmid DNA was reconstituted with 500 µl of 5 mM Tris pH 8.5 for long-term storage.

2.2.2.6 DNA Concentration Determination

The optical density of DNA was measured using a Nanodrop® 2000 spectrophotometer, which calculated the sample absorbance at 260 nm (A_{260}). The purity of the DNA was determined by calculating the A_{260}/A_{280} ratio, which should be between 1.8 and 2.0. Ratios smaller than 1.8 indicated major protein contamination whereas ratios larger than 2.0 suggested significant RNA contamination.

2.2.2.7 DNA Sequencing and Analysis

All sequencing reactions were preformed by The Sequencing Service, School of Medicine, Ninewells Hospital, University of Dundee. Raw sequencing data was analysed using FinchTV (version 1.4) whereas Clustal (www.ebi.ac.uk/clustalw/) (version 2.0) was used as an alignment program against Basic Local Alignment Search Tool (BLAST) sequences.

2.2.3 Cell Culture

2.2.3.1 Cell Line Maintenance

Prior to use, all cell culture reagents were warmed for 15 min in a water bath set to 37°C. Cells were incubated at 37°C in a 5% water saturated atmosphere until passage. Once cells reached around 70-90% confluency (usually every 2-3 days) cell passage was performed. Cells were seeded at 1 x 10^5 cells per 10 cm diameter culture dish with supplemented Dulbecco's modified Eagle's medium (DMEM) (as described

below for individual cell lines). All cell culture techniques were preformed in aseptic conditions.

2.2.3.1.1 *HEK293 Cell Maintenance*

The Human Embryonic Kidney (HEK) 293 cell line is derived from transformed human embryonic kidney cells. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose supplemented with 10% (v/v) fetal bovine serum (FBS) and a penicillin (100 units/ml)/ streptomycin (100 μg/ml) mix. Cells were passaged using trypsin-EDTA (0.05% w/v).

2.2.3.1.2 *SHSY5Y Cell Maintenance*

The SHSY5Y cell line is one of three serially isolated neuroblast clones (SHSY, SHSY5, and SHSY5Y) of the human neuroblastoma cell line SKNSH, which was established from a metastatic bone tumour (Biedler *et al*., 1978; Biedler *et al*., 1973). SHSY5Y cell lines were cultured and passaged as for the HEK293 cell line.

2.2.3.1.3 *HeLa Cell Maintenance*

The human cervix adenocarcinoma HeLa cell lines were cultured and passaged as for the HEK293 cell line.

2.2.3.1.4 *EBC-1 Cell Maintenance*

EBC-1 is a human lung squamous cell carcinoma cell line (Watanabe *et al*., 1985) obtained from the Japanese Collection of Research Bioresources (JCRB), Tokyo, Japan. Cells were maintained in Eagle's medium (MEM) containing 4.5 g/l glucose supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin (100 units/ml)/ streptomycin (100 μg/ml) under standard cell culture conditions at 37°C

and 5% CO_2 in a humid environment. Cells were passaged using trypsin-EDTA (0.25% w/v).

2.2.3.1.5 *A549 Cell Maintenance*

A549 cells are adenocarcinoma human alveolar basal epithelial cells. The A549 cell line was first developed in 1972 by D. J. Giard, *et al*. through the removal and culturing of cancerous lung tissue in the explanted tumor a of 58-year-old Caucasian male (Giard *et al*., 1973). Cells were originally sourced from ATCC and provided by Dr Colin Henderson, University of Dundee. Cells were passaged and maintained as for the HEK293 cell line.

2.2.3.1.6 *H460 Cell Maintenance*

A.F. Gazdar and associates derived the NCI-H460 cell line in 1982 from the pleural fluid of a patient with large cell cancer of the lung (Banks-Schlegel *et al*., 1985). Cells were originally sourced from ATCC and were provided by Dr Colin Henderson, University of Dundee. Cells were passaged and maintained as for the HEK293 cell line.

2.2.3.2 Cell Passage

During cell passage, culture media was removed and cells washed with 10 ml PBS to remove the serum and prevent inhibition of trypsin. PBS was completely removed prior to addition of 2 ml of trypsin/EDTA and cells were then incubated at 37°C for 5 min to allow them to detach from the flask. Subsequently, 8 ml of supplemented DMEM was added to the flask and the cells were triturated ten times. The cells were diluted 1/10 at each passage and re-incubated at 37°C and 5% CO_2 until required. Cells were maintained in culture until passage 30.

2.2.3.3 Cell Storage

For cell storage, cells were passaged as normal while around 70-80% confluent so that the cells were still in the growth phase. Cells were then pelleted; media removed, and resuspended in 10% (v/v) DMSO, 40% (v/v) serum and 50% (v/v) media. Cells were then placed in the -80°C freezer overnight before being transferred into liquid nitrogen for longer-term storage. To revive cells, cells were rapidly thawed in pre-warmed media and left overnight before media change. Media was changed daily until passage where cells were then split as normal.

2.2.3.4 Culturing Primary Cortical Neurons

Primary cortical neuron cultures were kindly prepared and supplied by Dr. Ritchie Williamson, (now at the University of Bradford). The day prior to culturing neurons, coverslips were placed in plates and coated with 2 ml poly-D-lysine (PDL) (50 µl in 10 ml sterile ddH_2O) per 6 well or 1 ml per 12 well. Embryos were dissected from a pregnant rat and brains removed and submerged in Hanks Balanced Salts Solution (HBSS) (w/o Ca and Mg). The cerebellum, optic nerve, and meninges were then removed under a dissecting microscope. The cortex was opened out and the midbrain removed then transferred to a fresh small dish with HBSS and taken to tissue culture. Subsequently, a trypsin/HBSS solution was prepared using 0.5 ml 2.5% trypsin plus 4.5 ml HBSS w/o Ca and Mg. Tissue pieces plus HBSS from the dish were pipetted and transferred to a fresh 15 ml tube and the tissue left to settle. The media was then aspirated off and replaced with trypsin/HBSS solution before placing in a water bath set at 37°C and mixing occasionally for 20-30 min. Meanwhile, PDL was washed off the coverslips/plates that were then rinsed with fresh sterile ddH_2O prior to addition of fresh neurobasal media (1 ml per 12 well, 2 ml per well of 6 well), before returning to the incubator. Subsequently, 10 mg/ml DNase in HBSS was added to the trypsin-

tissue mix, the tube inverted gently twice and then the tissue left to settle for 10 min. The media was aspirated off, 0.5 ml titrating solution was added and the tissue gently pipetted up and down 10-15 times with previously flamed glass pipettes. Cells were resuspended in neurobasal media so that the suspension was approximately 1 x 10^6 cells per ml. For the purpose of immunostaining, 2-5 x 10^6 cells/well were added.

Media

Neurobasal Medium (*Gibco*)	500 ml
B27 Supplement (*Invitrogen*)	10 ml
Pen/Strep (*Sigma*)	5 ml
L-glutamine (*Sigma*)	5 ml

Poly-D-Lysine (0.01% w/v)

Poly-D-Lysine	5 mg
ddH_2O	5 ml
	Store at -20°C
	Use at final concentration of 20 µg/ml

DNase I stock solution

DNase I	7.5 mg
HBSS (with Ca and Mg	750 ml
	Vacuum filter and store at -20°C

Trituring Solution

HBSS (with Ca and Mg)	100 ml
Trypsin Inhibitor (*Sigma*)	50 g
Albumax (*Invitrogen*)	1 mg
DNase I (*Sigma*)	1 mg
	Vacuum filter and store at -20°C

Dissection Solution

HBSS (without Ca and Mg)	500 ml
1M HEPES (pH 7.3)	3.5 ml
1M $MgCl_2$	5 ml
Penicillin/Streptomycin	5 ml

2.2.4 Transient Transfection

2.2.4.1 Calcium Phosphate ($Ca_3(PO_4)_2$)/BES Transient Transfection

Typically, a DNA precipitation mix consisted of plasmid DNA made up to volume using sterile RNAse free water, plus 2 x BES and mixed gently into 14 ml polypropylene plastic tubes. Subsequently, 2.5 M $CaCl_2$ (final concentration 0.15 M) was added slowly and drop-wise to the mixture, which was then allowed to precipitate at room temperature for 20 min (see table for transfection mix volumes). The precipitation mix (volume dependent of dish size) was then added drop-wise and evenly across the cells after which the dish was gently swirl-mixed to ensure homogeneity. The cells were then incubated for 4 hrs at 37°C in a 5% CO_2 water saturated atmosphere. At the time of transfection, plated cells were ~50% confluent.

	6 well plate (3 cm)	6 cm plate	10 cm plate
2 x BES	150 µl	200 µl	250 µl
Nuclease free H_20	Up to 135 µl	Up to 177.5 µl	Up to 220 µl
Plasmid DNA	1 µg	2 µg	3 µg
$CaCl_2$	15 µl	22.5 µl	30 µl
Total Volumes	**300 µl**	**400 µl**	**500 µl**

Table 2.4. ***$Ca_3(PO_4)_2$/BES transfection mix volumes*****.** A minimum volume of 500 µl was made and a maximum volume of 1500 µl in a single transfection mix.

2.2.4.2 Lipofection Transient Transfection

Lipofectamine^{TM} 2000 (*Invitrogen*) transfections involved the formulation of 3 transfection mixes. Mix 1 consisted of DMEM media (serum and antibiotic free) plus 1 μg plasmid DNA (200 μl total volume per 3 cm diameter culture dish). Mix 2 consisted of DMEM media (serum and antibiotic free) Lipofectamine^{TM} 2000 (1 μl) (200 μl total volume). These were mixed gently and incubated at room temperature for 5 min. Mix 1 and Mix 2 were combined (Mix 3), mixed gently and incubated for a further 20 min at R.T. 1600 μl of DMEM (10% (v/v) FBS with no antibiotics) was then added to Mix 3 (final volume 2000 μl), then added to cells to be transfected (previous media removed) and incubated for a total of 48 hrs at 37°C in 5% CO_2 water saturated atmosphere. At the time of transfection cells were ~ 50% confluent.

2.2.4.3 Mammalian Expression Constructs

The cDNA encoding full-length hCRMP2 was amplified by PCR from Image clone #6177866 using the primers 5′-GGATCCGCCACCATGGACTACAAGGACGACG-ATGACAAGTCTTATCAGGGGAAGAAAAATATTCCACGC-3′ and 5′-GAATT-CTTAGCCCAGGCTGGTGATGTTGGC-3′. The cDNA encoding full-length hCRMP4 was amplified by PCR from Image clone #5725550 using the primers 5′-GAATTCGCCACCATGGACTACAAGGACGACGATGACAAGTCCTACCAAGG CAAGAAGAACATCCCG-3′ and 5′-GAA-TTCTTAACTCAGAGATGTGATAT-TAGAACGGCCG-3′. The PCR products were subcloned into pCMV5 vectors for mammalian expression. Expression constructs for CDK5, p35 and p25 were generated by Dr. Margareta Nikolic, Imperial College, London. All other expression constructs were obtained from MRC Protein Phosphorylation Reagents, University of Dundee. Constructs used in this study are shown in ***Table 2.5***.

Construct	Expression System	Promoter	Bacterial Resistance	Features
pCMV5 FLAG-Mef2a WT	Mammalian	CMV	Ampicillin	FLAG-tagged
pCMV5 FLAG-PAK1 WT	Mammalian	CMV	Ampicillin	FLAG-tagged
pCMV5 FLAG-STAT3 WT	Mammalian	CMV	Ampicillin	FLAG-tagged
pCMV5 FLAG-Dynamin-1 WT	Mammalian	CMV	Ampicillin	FLAG-tagged
pCMV5 FLAG-CRMP2 WT	Mammalian	CMV	Ampicillin	FLAG-tagged
pCMV5 FLAG-CRMP4 WT	Mammalian	CMV	Ampicillin	FLAG-tagged
pCMV5 Cdk5 WT	Mammalian	CMV	Ampicillin	None-tagged
pCMV5 p35 WT	Mammalian	CMV	Ampicillin	None-tagged
pCMV5 p25 WT	Mammalian	CMV	Ampicillin	None-tagged

Table 2.5. ***Mammalian expression constructs.***

2.2.5 Cell Stimulations

All Cdk5 inhibitor treatments were performed in serum-free media therefore prior to treatment serum-containing media was removed from cells and replaced with pre-warmed serum-free media.

2.2.5.1 Inhibitors

Stock solutions of roscovitine (*Calbiochem*) and purvalanol A (*Calbiochem*) were prepared in DMSO (10 mM). Cells were treated with either purvalanol A or roscovitine (10 μM final concentration) or vehicle (0.1% DMSO) for 3 hrs, unless stated otherwise in figure legends.

2.2.6 Confocal Microscopy

2.2.6.1 Primary Neuron Cell Preparation

Following 6 days *in vitro* (6 DIV), primary cortical neurons were fixed in ice-cold 4% (w/v) paraformaldehyde (PFA) (in 1.0% (w/v) PBS) for 10 min at 4°C prior to washing twice in PBS followed by two washes in 50 mM TBS. Subsequently, neurons were permeabolised with 0.1% (v/v) Triton x-100 in TBS for 3 min at room temperature, washed thrice in 50 mM TBS, and blocked with 1% (w/v) BSA in TBS containing 0.005% (v/v) Tween-20 in for 1 hr at room temperature. Fixed cells were then incubated in primary antibodies diluted 1:50 in PBS containing 5% (w/v) BSA for 1 hr at room temperature prior to three washes in 50 mM TBS. Secondary antibodies conjugated to Cy3-fluorophores were diluted 1:250 in PBS containing 5% (w/v) BSA and incubated with neurons for 1 hr at room temperature. Cells were then washed three times in 50 mM TBS and subsequently counterstained with 0.5 μg/ml DAPI solution (*Invitrogen*).

2.2.6.2 Image Analysis

Image acquisition was performed on a Leica TSC SP-5 laser scanning confocal microscope imaging system (version 2.0.2). Images were subsequently viewed using Volocity 3D Image Analysis Software (version 6.3) (*PerkinElmer*) and this software was used to insert scale bars into each image.

2.2.7 Protein Isolation and Assay

2.2.7.1 Mammalian Cell Lysis Buffer Preparation

Ions are essential for the function of many enzymes (including most proteases, kinases and phosphatases). During cell lysis, a selection of ion chelators are used to sequester enzyme cofactors in order to halt cellular activity and therefore ensure protein modifications and integrity (e.g. phosphorylation status) is maintained during isolation. As such, cell lysis buffer was prepared to contain EDTA to chelate divalent cations (+2 charge), such as magnesium, manganese, zinc, and calcium (Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+}, respectively), whereas EGTA was used to chelate Ca2+ ions. In addition to these reagents, sodium fluoride and sodium pyrophosphate were included in order to inhibit serine/threonine phosphatases as well as sodium orthovanadate to inhibit protein tyrosine kinases. Sodium orthovanadate was prepared by carrying out several rounds of boiling and cooling on ice. This was continued until the pH remained stable at pH 10, subsequent to boiling. This boiling and pH adjustment ensures that sodium orthovanadate is monomeric and will therefore effectively inhibit tyrosine phosphatases. Furthermore, β-mercaptoethanol was used as a reducing agent in the cell lysis buffer and protease inhibitor cocktail tablets were added to prevent protein degradation.

Lysis Buffer

EGTA	0.1 mM
Triton X-100	0.1 mM
Sucrose	0.27 M
Tris- HCL, pH 7.4	50 mM
NaF	50 mM
Na_3VO_4	1 mM
$Na_2P_2O_7$	5 mM
β-mercaptoethanol	0.1% (v/v)
Protease inhibitor tablets (*Gibco*)	1 per 10 ml

2.2.7.2 Cell Protein Isolation

Cell transfections were terminated by washing cells twice with 2 ml ice-cold PBS in order to remove excess media. Once the PBS was totally removed a small volume (typically 50-500 μl) of ice-cold mammalian lysis buffer was added, followed by cell scraping using a plastic scraper on ice. Cell lysates were transferred to ice-cooled eppendorf microcentrifuge tubes and left on ice for 20 minutes to allow the proteins to solubilise. They were then centrifuged at 13,000 x g for 10 minutes in order to remove cellular debris. The supernatant (lysate) was removed and snap frozen in liquid nitrogen and stored at -80°C until required.

2.2.7.3 Protein Immunoprecipitation

In order to couple an antibody to protein G-sepharose, beads were washed 3 times in lysis buffer followed by centrifugation at 5000 x g for 1 minute. Antibody was then incubated with a 50% (v/v) protein G bead slurry in 1 μg: 10 μl antibody to slurry ratio, and incubated on a shaking platform for 1 hour at 4°C. The beads are then washed x 3 with lysis buffer in order to remove unbound antibody and stored as a 50% (v/v) slurry at 4°C until required.

Typically, proteins were immunoprecipitated following incubation of a cell lysate (1 mg/ml, 100 µl) with antibody bound agarose beads (10 µl of 50% (v/v) slurry) for 16 hrs at 4°C on a shaking platform. Sepharose beads were pelleted by centrifugation at 2000 x g and then washed twice with cell lysis buffer and once with lysis or assay buffer dependent on whether they were for SDS-PAGE or kinase assay. Immunoprecipitated proteins/agarose beads were either incubated in 1 x SDS sample buffer and heated at 95°C for 10 min in preparation for SDS-PAGE, or retained in kinase buffer at 4°C for subsequent kinase assay reactions.

2.2.7.4 Subcellular Protein Fractionation

Adherent cells were harvested with trypsin-EDTA then pelleted in the centrifuge at 500 x g for 5 minutes. The cell pellet was then suspended in ice-cold PBS in order to wash the cells. Then, 1 – 10 x 10^6 cells were transferred to a 1.5 ml microcentrifuge tube and spun at 500 x g for 5 minutes to pellet the cells. The supernatant was carefully removed and discarded, leaving the pellet as dry as possible. The fractionation protocol was scaled depending on cell pellet volume, maintaining the volume ratio of Cytoplasmic Extraction Buffer: Membrane Extraction Buffer: Nuclear Extraction Buffer: Pellet Extraction Buffer (CEB:MEB:NEB:PEB, respectively) reagents at 200:200:100:100µl, respectively. Following addition of CEB containing protease inhibitors to the cell pellet, the tube was incubated at 4°C for 10 minutes with gentle mixing before being centrifuged at 500 x g for 5 minutes. The supernatant (containing the cytoplasmic extract) was then immediately transferred to a clean pre-chilled eppendorf on ice. Ice-cold MEB containing protease inhibitors was added to the pellet before vortexing the tube for 5 seconds on the highest setting and then incubating at 4°C for 10 minutes with gentle mixing. This was followed by

centrifugation at 3000 x g for 5 minutes. The resulting supernatant (containing the membrane extract) was transferred to a clean pre-chilled eppendorf on ice. In order to isolate the soluble nuclear fraction, ice-cold NEB containing protease inhibitors was added to the pellet followed by vortexing on the highest setting for 15 seconds. The tube was then incubated at 4°C for 30 minutes with gentle mixing before being centrifuged at 5000 x g for 5 minutes. The supernatant (containing the soluble nuclear extract) was transferred to a clean pre-chilled eppendorf on ice. The chromatin-bound extraction buffer was prepared by adding 5µl of 100mM CaCl2 and 3µl of Micrococcal Nuclease (300 units) per 100µl of NEB at room temperature. The tube was vortexed on the highest setting for 15 seconds after adding this extraction buffer to the pellet. The tube was then incubated at room temperature for 15 minutes followed by further vortexing for 15 seconds. The tube was centrifuged at 13,000 x g and the resultant supernatant transferred to a clean pre-chilled tube on ice. Finally, PEB containing protease inhibitors was added to the pellet and then vortexed on the highest setting for 15 seconds, prior to incubation at room temperature for 10 minutes before centrifuging at 13,000 x g for 5 minutes. The supernatant (containing the cytoskeletal extract) was then transferred to a new tube. For same-day use, fractions were maintained on ice for downstream applications and analysis. For long-term storage, fractions were stored at -80°C.

2.2.7.5 Measurement of Protein Concentration

Sample protein concentration was determined using the method described by M. Bradford in 1976 (Bradford 1976). The Coomassie brilliant blue G-250 dye in the Bradford reagent binds to arginine, aromatic amino acids and histidine containing proteins. When Coomassie binds to proteins in solution its absorbance shifts from 465

nm to 595 nm. An increase in protein concentration correlates with an increase in 595 nm absorbance.

Briefly, protein lysates were diluted 1:20 in water, and 20 μl was then transferred to a plastic cuvette. 1 ml of Bradford reagent was added, vortex mixed and incubated at room temperature for 10 minutes, vortex mixed again and the absorbance read at 595 nm. A serial dilution linear bovine albumin (BSA) standard curve was used to find the gradient coefficient value from which an unknown protein concentration can be determined. The assay was blanked using 20 μl of water in 1 ml of Bradford reagent incubated for 10 min alongside samples.

2.2.7.6 Sodium Dodecyl-Sulphate Poly-Acylamide Gel Electrophoresis

Samples were prepared for Sodium Dodecyl-Sulphate Poly-Acylamide Gel Electrophoresis (SDS-PAGE) using SDS sample buffer supplemented with 10 mM dithiothreitol (DTT) and heated to 70°C for 10 minutes. SDS is an anionic molecule that coats proteins with a negative charge in a uniform charge to mass ratio. The negatively charged sulphate groups disrupt amino acid hydrophobic interactions while DTT reduces disulphide bonds between cysteine residues therefore denaturing any secondary structure of the proteins within the sample. Samples were subjected to 1 dimensional SDS-PAGE (*Bio-Rad*) using prepoured 4-15% NuPAGE gels (*Invitrogen*). Upon loading of samples onto the gel, a current of 100V for 10 minutes was applied followed by 180V for 60 minutes. Negatively charged SDS residues greatly outnumber charged amino acid side chain groups of the protein, therefore proteins move towards the positively charged electrode and the rate of movement through the polyacrylamide matrix is directly related to molecular mass independently

of inherent protein charge. SeeBlue molecular weight markers are pre-stained proteins of known molecular mass that provide a standard to determine the apparent molecular weight of proteins of interest within the gel.

2.2.7.7 Gel Coomassie Staining

Following SDS-PAGE, protein bands can be visualised using Coomassie stain. The gel is immersed in Coomassie Brilliant Blue (CBR-250) stain, heated in a microwave for 10 seconds, and incubated on a shaking platform for 10 min at room temperature. The gel is de-stained by immersion in 40% methanol (v/v) and 10% acetic acid (v/v). De-stain is changed regularly until the gel background is reduced and protein bands become visible. The methanol/acid immersion dehydrates the gel, to prevent cracking during drying, a final wash with 50 ml distilled water containing 2 ml 50% (v/v) glycerol is performed. The gel is placed onto 3 mm Whatman filterpaper, covered with clingwrap and placed into a Slab gel dryer at 70°C for 90 min.

Coomassie Stain

Methanol	40 % (v/v)
Acetic Acid	10 % (v/v)
Brilliant Blue R-250	200 mg/L (w/v)

Coomassie Destain

Methanol	40 % (v/v)
Acetic Acid	10 % (v/v)

2.2.8 Western Blot Analysis

2.2.8.1 Transfer of Proteins to Nitrocellulose Membrane

Following SDS-PAGE, the gel was assembled into a gel membrane sandwich as follows: nylon sponge, 3 mm Whatman paper, polyacrylamide gel containing protein, 0.45 mm nitrocellulose membrane (Hybond™ C-Extra), 3 mm Whatman filterpaper, and then filled with nylon sponges until the transfer cassette was tightly packed. Each sandwich component was pre-soaked in transfer buffer prior to transfer sandwich assembly. Once placed into the transfer tank, the inner chamber was filled with transfer buffer and the outer chamber filled with water. A current of 35 V was applied across the sandwich for 2 hrs, facilitating the transfer of proteins from the gel to the nitrocellulose. After transfer, the nitrocellulose membrane was stained with reversible Ponceau S protein staining solution (*Sigma Aldrich*) for 1 min to assess transfer efficiency. Ponceau S staining was removed by washing the membrane in TBST.

NuPAGE® MOPS Running Buffer (20X)

MOPS	50 mM
Tris bas	50 mM
EDTA (pH 7.7)	1 mM
SDS	0.1% (w/v)

NuPAGE® Transfer Buffer (20X)

Bicine	25 mM
Bis-Tris (free base)	25 mM
EDTA (pH 7.2)	1 mM

TBST (1L)

10 x TBS stock solution	100 ml
Tween 20	500 µl
ddH_2O	900 ml

2.2.8.2 Immunoblotting

Immunoblotting of membranes enables visualisation of specific proteins or protein modifications (such as phosphorylation) in a complex mixture, and is a semi-quantitative technique dependent on the quality of antibodies for the target of interest. The nitrocellulose membrane from above was blocked for 1 hr at R.T. in TBST blocking buffer in order to block non-specific binding of the antibody to the membrane. Membranes were incubated with primary antibody (diluted in TBST with appropriate blocking agent) overnight at 4°C on a rolling mixer and then washed 3 x 10 min with TBST at R.T. Primary antibody bound to the target protein was visualised by incubation with an appropriate fluorophore-conjugated secondary antibody (in TBST plus 1% (w/v) milk) for 1 hr at R.T.

2.2.8.3 Visualisation of proteins on membranes

Membranes were washed 5 x 10 min in TBST at room temperature before protein-antibody-dye conjugates were visualised using a LI-COR Odyssey® Infrared Imaging System (*LI-COR Biosciences, Lincoln, NE*).

2.2.8.4 Analysis and Quantification of Western blots

Densitometric analysis was performed using the LI-COR Odyssey® Infrared Imaging System software. In order to determine changes in protein phosphorylation, total protein levels corrected for any variation in protein loaded between samples (loading control), therefore a ratio between phosphorylated and total protein for each sample could be calculated (phosphorylated protein value divided by total protein, from the same sample). An inert protein (such as actin) was used as a loading control when assessing potential changes in the levels of a specific protein between two samples.

2.2.9 Phosphorylation Assay

2.2.9.1 Assay of proline directed kinases

The inherent specific activity of purified CGMC protein kinases was assessed using myelin basic protein (MBP) as a non-specific substrate. Briefly, the kinase of interest was incubated in kinase buffer with MBP (0.3 mg/ml) final concentration) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) (50 µl final reaction volume) for 10 min at 30°C. Reactions were then terminated by absorption onto phosphocellulose p81 paper followed by immediate immersion in 75 mM phosphoric acid. Papers underwent 5 x 5 min washes in orthophosphate to remove any unincorporated negatively charged [γ-32P] Mg-ATP. A final wash in acetone was performed to dry papers before they were placed 2 ml scintillation fluid and phosphate incorporation measured by scintillation counting in a Wallace 1409 scintillation counter. MBP kinase specific activity was used to match activities of different kinases in subsequent comparisons with protein substrates.

MgATP (10 x stock solution)

Magnesium chloride	100 mM
ATP	1 mM

Kinase Buffer (2 x stock solution)

MOPS buffer (pH 7.5)	50 mM
EDTA	0.5 mM
Brij-35	0.01% (v/v)
Glycerol	10% (v/v)

One unit (U) of kinase activity is the amount required to transfer one nanomole of phosphate to substrate in one minute at 30°C.

2.2.9.2 Peptide and protein substrate assay

Protein kinases were assayed by quantifying their ability to transfer radioactive ^{32}P from [γ-^{32}P] ATP into a protein/peptide substrate. A reaction mixture, typically, contained purified kinase, protein/peptide substrate , 10 mM $MgCl_2$, 0.1 mM ATP (cold or radiolabelled [γ-^{32}P]) and kinase buffer in a total volume of 50 µl. All assays were initiated by the addition of 5 µl of a 10 x MgATP (100 mM $MgCl_2$ and 1 mM ATP (for radioactive assays specific activity of ATP was around 0.5 x 10^6 CPM/nmole) solution and incubated on an IP shaker at 30°C for the times indicated in the figure legend.

For protein substrates, reactions were stopped by the addition of an equal volume of 2 x SDS loading buffer (containing 10 mM DTT), heated at 100 °C for 10 minutes and then subjected to SDS-PAGE. For peptide substrates, 40 µl of the kinase reaction (50µl total assay volume) was spotted onto 2cm^2 phosphocellulose P81 paper and immediately immersed in 75 mM phosphoric acid. The peptides contain a positively charged Arginine residue at the C-terminus, which promotes binding to the negatively charged P81 paper. Papers were then washed 5 times for 5 min using 75 mM orthophosphoric acid which removes any unincorporated negatively charged [γ-^{32}P] ATP. After a final wash in acetone, followed by air drying, the papers were then individually placed into labelled tubes and 2 ml of scintillation fluid added. The radioactivity of the sample was measured by Scintillation counting in a Wallace 1409 scintillation counter. Substrate proteins such as myelin basic protein (MBP) were also analysed using this method due to their high content of basic amino acids promoting interaction with the p81 paper. An aliquot of the 10 x MgATP [γ-^{32}P] was also

counted in order to calculate the exact specific activity and to permit quantification of the molar incorporation of phosphate into the substrate.

2.2.9.3 Kinase Phosphorylation Stoichiometry

Kinase phosphorylation stoichiometry was calculated as follows: 1µl of 10 x [γ-^{32}P] MgATP (100 mM $MgCl_2$ and 1 mM ATP) stock contains 1 nmole of ATP. Therefore, the counts per minute (CPM) in 1 µl provide the CPM per nmole (specific activity) of the ATP. As the kinase transfers the radiolabelled γ-phosphate the CPM of the substrate at the end of the reaction provides the nmoles of phosphate transferred to the substrate. The ratio of nmoles of phosphate transferred to nmoles of substrate in the reaction gives the phosphorylation stoichiometry (mol/mol). The nmoles of phosphate transferred into substrate over time provides the velocity of the reaction and can be used to calculate affinity of the substrate for a given enzyme.

2.2.9.4 Phosphorylation Kinetics, V_{max} and K_m Calculation

V_{max} is the maximal velocity that the reaction can reach (all the enzyme catalytic sites are occupied) and K_m is the concentration at which half of all the enzyme active sites contain substrate (a measure of affinity of the enzyme for the substrate). The Michaelis-Menten equation provides a Lineweaver-Burk plot that will give a straight line (reciprocal of both substrate concentration and velocity) where the line that intercepts the Y axis will give a value for $1/V_{max}$ and the line intercept on the X axis will give a value for $-1/K_m$. These laws are only applicable to linear kinetic reaction velocities obtained using a constant concentration of enzyme in the reaction whereas the substrate concentration is increased.

The phosphorylation kinetics, V_{max} and K_m were calculated using the Michaelis-Menton and Lineweaver-Burk equations:

Michaelis-Menton Equation: $v = V_{max}[S]/K_m + [S]$

Lineweaver-Burk Equation: $1/v_o = 1/V_{max} + ((K_m/V_{max}) \times (1/[S]))$

(Linear transformation of the Michaelis-Menton equation)

2.2.10 Histological Techniques

2.2.10.1 Tissue preparation and paraffin embedding

The aim of tissue processing is to remove water from the tissue and replace it with a solidifying medium, mostly commonly used is paraffin wax, to enable cutting of sufficiently thin sections for subsequent analysis of morphology and composition. Tissue preparation and paraffin embedding was performed by Dr. Phil Coates, Tayside Tissue Bank, using the following procedure: Samples that were fixed in formalin were loaded into cassettes and processed to wax using a Tissue-Tek Vacuum Infiltration Processor (*Sakura Finetek Europe, The Netherlands*). Briefly, cassettes were dehydrated in graded concentrations of ethanol (70%, 90%, 100%, 100% (v/v), each for 2 hrs; *Fisher Scientifc, United Kingdom*), then cleared in xylene (two changes, each for 2 hrs; *Fisher Scientific, United Kingdom*) and, finally, immersed in molten paraffin wax at 60°C (three changes, each for 2 hours, *Thermo-Shandon, United Kingdom*). Each step was preformed under vacuum and with agitation to accelerate infiltration of solvent/wax. After processing, samples were positioned in molten paraffin wax-filled embedding moulds and cooled rapidly on a Tissue-Tek Cryo-console (*Sakura Finetek Europe, The Netherlands*) to form solid blocks.

2.2.10.2 Microtomy

Sections were prepared by Dr. Phil Coates and Dr. Susan Brey, Tayside Tissue Bank using the following procedure: Paraffin blocks were cut into sequential 4 μm thick sections using a Leica RM 2135 microtome (*Leica Microsystems, Germany*). Immediately after sectioning, ribbons of sections were allowed to float on top of a water bath (*Sakura Finetek Europe, The Netherlands*) set at 40°C to allow the sections to expand and smooth prior to mounting on Superfrost Plus electrostatically-coated microscope slides (*VWR International, United Kingdom*). Slides were then incubated at 37°C overnight to ensure adherence of the sections to the slides.

2.2.10.3 Immunohistochemistry

Immunohistochemistry (IHC) staining is an antibody-antigen reaction that can be used to identify tissue origin such as with tumours of unknown origin that have metastasized or are poorly differentiated. The antibody-antigen interaction can be visualised by fluorescence or enzymatic development of a chromogenic substrate, usually following several amplification steps.

Firstly, slides were incubated in histosol for 10 min to remove the paraffin wax. This was followed by an additional 10 min incubation in histosol. The samples were then hydrated by incubation in methylated spirits for 10 min. In order to block endogenous peroxidase, specimens were treated with methanol containing 0.5% (v/v) hydrogen peroxidase before being washed in distilled water. For antigen retrieval, each specimen was incubated for 15 minutes in boiling 10 mM citrate buffer, pH 6. Slides were left to cool at room temperature prior to blocking. Specimens were then blocked in normal serum diluted to 5% (v/v) in PBS containing azide, 1% (v/v) NP-40, and a 5% (v/v) avidin solution for 30 min at room temperature. The specimens were then

washed in PBS before the addition of primary antibodies. Antibodies were diluted in normal serum diluted to 5% (v/v) in PBS containing azide plus 5% (v/v) biotin solution and incubated at 4°C overnight. Primary antibodies were removed by 3x 5 minute washes in PBS. The specimens were then incubated in biotinylated secondary antibody at a dilution of 1/250 in normal serum diluted to 5% (v/v) in PBS containing azide for 30 minutes at room temperature. This was followed by 3x 5 min washes in PBS to remove the secondary antibodies.

2.2.10.4 The ABC complex

The avidin-biotin complex (ABC) method allows for the amplification of detection due to the high affinity between avidin and biotin. It involves biotinylated secondary antibody and an avidin conjugated enzyme such as horseradish peroxidase (HRP). The mechanism of this method entails first the binding of the primary antibody to the target antigen in the specimen. Then, the biotinylated secondary antibody binds to the primary antibody. This is followed by the enzyme conjugate avidin binding to the biotin. Lastly, a substrate chromogen solution is added for colour detection. The specimen is incubated in avidin-HRP for 30 minutes at R.T. followed by 3x 5 minute washes in PBS and incubation in 3’ diaminobenzidine tetrahydrochloride (DAB) solution (peroxidase substrate solution) for 10 minutes at room temperature to turn staining brown. The sample is then washed with distilled water.

2.2.10.5 Hematoxylin Staining

Hematoxylin is isolated from an extract of logwood, Hematoxylin campechuanun Linnaeus, whose oxidation product, hematein, produces a blue/purple colour when combined with a mordant metallic ion such as aluminium salt. The positively charged aluminium hematein complex combines with negatively charged phosphate groups of

nucleic acid producing the characteristic blue/purple colour characteristic of hematoxylin stains, and providing a simple method to visualise cell nuclei. Following incubation in DAB, specimens were counterstained with hematoxylin for 80 seconds at room temperature before they were washed in water and placed in PBS for 10 minutes.

2.2.10.5 Slide preparation for light microscopy

Specimens were dehydrated in methylated spirits for 10 minutes at R.T. before secondary 10 min dehydration in methylated spirits. An additional 10 minute incubation in methylated spirits was performed before placing the specimens in isopropanol for 10 minutes at R.T. Finally, specimens were incubated in histoclear for two ten minute periods before mounting the coverslips with desferrioxamine (DPX). The DPX was allowed to set for 24 hrs before detection under the light microscope.

2.2.11 Mass spectrometry

2.2.11.1 Phosphorylation site mapping

Prior to phosphorylation site mapping, 0.5 μM hTau (WT) and 1 mU of either p35/Cdk5 or p25/Cdk5 were incubated at 30°C in buffer containing 25 mM MOPS (pH 7.5), 0.05% (v/v) Brij-35, 0.25 mM EDTA, 5% (v/v) glycerol, 10 mM $MgCl_2$ and 100 μM [γ-^{32}P] ATP in a total reaction volume of 60 μl. This was repeated using 1 mU of either p35/Cdk5 or p25/Cdk5 that had been pre-incubated with either 10 μM purvalanol A or 10 μM roscovitine prior to assay. Duplicate samples were assayed using radiolabelled [γ-^{32}P] for autoradiography purposes to enable localisation of the phosphoprotein. The reaction was stopped at the 5, 20 and 60 min intervals by removing 20 μl and adding dithiothreitol (DTT) and sample buffer (*Invitrogen*) to give final concentrations of 10 mM DTT and 1 x sample buffer. Samples were then

heated at 70°C for 10 min before being allowed to cool R.T. for 30 min. Subsequently, 6 µl 250 mM iodoacetamide was added to the 30 µl reaction mixture and samples were placed in the dark for 30 min. Samples were then separated by SDS-PAGE and stained using Brilliant Blue G-Colloidal Concentrate (*Sigma Aldrich*). The destained and alkylated ^{32}P labeled proteins were excised and the gel pieces were digested with either endoproteinase Lys-C (50 µl, 2 µg/ml) or 100 µl 2 µg/ml trypsin in 50 mM TEAB overnight. Digests were extracted with 5 µl acetonitrile, supernatants dried and redissolved in 50 µl 5% (v/v) acetonitrile (ACN)/0.1% (v/v) trifluoroacetic acid (TFA). Subsequently, 10/50 µl was injected onto Biosphere C18 150 x 0.1 mm column (*Nanoseparations, Holland*) and analysed using the CapC1_45minTop10_MSA method. Subsequently, data was searched against Contaminant database using Proteome Discoverer 1.3 with either Lys-C or trypsin as the enzyme.

Phosphorylation site mapping was performed by Dr. Nicholas Morrice, The Beatson Institute for Cancer Research, Glasgow.

2.2.11.2 Mass Spectroscopic Fingerprint Analysis

Cell lines (EBC-1, HeLa, and HEK293) were fractionated as described in ***section 2.2.7.4***. Duplicate samples for each cell line were prepared in LDS sample buffer (*Invitrogen*) and DTT to a final concentration of 10 mM DTT and 1 x sample buffer. Samples were heated at 70°C for 10 min before being allowed to cool R.T. for 30 min. Subsequently, 5 µl 250 mM iodoacetamide was added to the 25 µl reaction mixture and samples were placed in the dark for 30 min. Samples were then separated by SDS-PAGE and the gel was subsequently cut into two halves. One half of the gel was subjected to immunoblotting with total CRMP2 and pCRMP2 Ser522 antibodies

to confirm presence and protein position. The other half of the gel was stained using Brilliant Blue G-Colloidal Concentrate (*Sigma Aldrich*). The destained and alkylated bands equivalent to the CRMP2 bands were excised and digested by trypsin to generate peptides for Mass Spectroscopic fingerprint analysis. Peptide samples were analysed on the Velos Pro (Thermo Scientific Fisher, Boston) Orbitrap mass spectrometer coupled to an Ultimate 3000 RSLC nanoflow HPLC system (Dionex, Sunnyvale California). Sample volumes of 5 µl were eluted over a 100 min run on a 15 cm C18 column (75 µm × 15 cm nanoviper column, Thermo Scientific Boston) using a 2–40% linear gradient of solvent A (0.1% (v/v) formic acid; 5% (v/v) ACN): solvent B (80% (v/v) ACN with 0.08% (v/v) formic acid) at a flow rate of 0.3 µl/min. The initial precursor scan (mass range 335–1800, resolution 60,000 and a tolerance of 10 ppm) was measured in the Orbitrap. The samples were run with an inclusion list to search for peptides from CRMP2A and included the N-terminal peptide for the truncated version (CRMP2B), as well as the peptide relating to the site that is recognised by the pCRMP2 Ser522 antibody.

Mass spectroscopic fingerprint analysis was performed by Mr. Douglas Lamont, Fingerprint Proteomics Facility, College of Life Sciences, University of Dundee.

2.2.12 Statistical Analysis

All statistical analysis was performed using Prism 6.0 software (GraphPad software, CA, USA). Calculation of the mean was used to determine central tendency and standard error of the mean was calculated to quantify the precision of the mean. For comparison of *in vitro* phosphorylation between peptides (***Chapter 3***), statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's post hoc test. For comparison of *in vitro* peptide substrate phosphorylation following

inhibitor treatment (***Chapter 3***), statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's post hoc test vs. untreated. For comparison of substrate phosphorylation following transfection of p35/Cdk5 and p25/Cdk5 with untransfected control (***Chapter 4***), statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's post hoc test. A p value of <0.05 was considered significant and p values are expressed in relevant figures using asterisks where * represents <0.05, ** represents <0.001, and *** represents <0.0001. In all *in vitro* and mammalian cell studies, n refers to the number of experiments from which data were acquired.

CHAPTER 3

In vitro analysis of Cdk5 substrate phosphorylation

3.1 INTRODUCTION

Since its discovery, Cdk5 has been associated with a continually increasing compendium of substrates, each with varying physiological significance, implicating Cdk5 as a fundamental regulator of multiple cellular processes, in multiple tissues throughout the body, ultimately defining Cdk5 as a highly versatile protein kinase with diverse physiological function. Despite being credited as the phosphorylating kinase of these substrates, very few have undergone full substrate validation or meet a common consensus. This is further complicated by the existence of different Cdk5 containing complexes and the potential for altered substrate selection. For that reason, a defined consensus sequence for each complex and identification of the true physiological substrates of Cdk5 is necessary to validate its physiological functions and potential contribution to pathophysiology. Furthermore, this information is crucial to develop Cdk5 as a therapeutic target in the treatment of disease.

In this chapter, the consensus substrate sequences that confer Cdk5 substrate selection and subsequent phosphorylation will be examined in peptides designed around the proposed Cdk5 substrates in order to determine the residues that influence Cdk5 phosphorylation rate and define a Cdk5 substrate consensus. On obtaining a consensus sequence motif for Cdk5 activity, this sequence will be investigated in full-length human proteins to confirm proposed substrates of Cdk5 and predict likely Cdk5 targets. Additionally, the relative hyperactivity and substrate profile of the p25/Cdk5 complex will also be addressed by directly comparing phosphorylation kinetics with p35/Cdk5.

3.1.1 Chapter Aims

The collective focus of this chapter is to generate a greater understanding of the fundamental mechanisms that shape Cdk5 function in health and disease through application of a variety of *in vitro* techniques. There are three major objectives of this chapter: firstly, identifying a consensus peptide sequence that could validate proposed physiological Cdk5 substrates and also aid in the identification of novel Cdk5 substrates; secondly, to investigate whether there are basic differences in catalytic activity of the two major Cdk5 complexes; and finally, confirming this consensus in a selection of proposed Cdk5 substrates, including performing a comparison of the phosphorylation kinetics of the best Cdk5 substrates that could allow the development of screening tools to accurately access Cdk5 activity *in vivo* (in particular, in diseases such as cancer).

3.1.1.1 Consensus sequence that regulates Cdk5 substrate selection

The literature depicts Cdk5 as a highly promiscuous protein kinase with a large number of proposed substrates, each with varying primary sequences around the Cdk5 phosphorylation sites. This raises the possibility that Cdk5 can phosphorylate proteins with little requirement for specific consensus primary sequence around the phosphorylation site or, alternatively, that many of the proposed substrates are relatively weak and not physiologically relevant. Currently, a consensus for Cdk5 substrate selection is not yet defined and is generally thought to be highly degenerate. The focus of this section of the thesis is to investigate the importance of amino acids flanking the phosphoacceptor residue and determine a consensus that is essential or even preferred for Cdk5 activity (when complexed to p35 or p25). This sequence information would aid in validating truly physiological targets as well as identifying novel *in vivo* targets of Cdk5.

3.1.1.2 Effect of p35 proteolytic cleavage on Cdk5 substrate affinity

As described in ***Chapter 1***, it is widely accepted that proteolytic cleavage of p35 to produce p25 enhances Cdk5 activity and thus alters substrate phosphorylation profile. However, there is limited experimental evidence to suggest that the activity of Cdk5 is increased following p25 generation. This chapter aims to investigate whether Cdk5 activity towards substrates *in vitro* is altered when p35 is replaced by p25 by establishing the phosphorylation kinetics for potential Cdk5 substrates with each of the major Cdk5 complexes, p35/Cdk5 and p25/Cdk5.

3.1.1.3 Validation and comparison of Cdk5 substrates

The final part of this chapter sets out to assess the consensus peptide sequence in predicting Cdk5 substrates. A selection of Cdk5 substrates will be chosen to investigate whether the consensus peptide sequence found *in vitro* translates into better phosphorylation of full-length proteins by comparing phosphorylation kinetic parameters for a selection of proposed Cdk5 substrates with diverse primary sequence around the phosphoacceptor site.

3.2 RESULTS

3.2.1 Defining Cdk5 activity for *in vitro* analysis

Robust analysis of physiological phosphorylation events related to a specific protein kinase can be difficult due to the transient nature of many phosphorylation events, the often low stoichiometry of phosphorylation at a given site, the low copy number of phosphoprotein per cell and the large number of protein kinases present in every cell (Goodlett *et al*., 2000). Despite the improvement in global phosphoproteomic technology and the availability of animal and cell models with conditional genetic ablation of specific kinases, these limitations still necessitate the use of *in vitro* assays using recombinant protein in order to provide fundamental information on kinase-substrate interactions. In addition, *in vitro* approaches allow simple direct comparison of the rates of phosphorylation of a given substrate with related kinases. Hence, detailed enzyme kinetic analysis *in vitro* is a useful first step in the investigation of the kinase-substrate interactions that are likely to occur *in vivo*.

Here, Cdk5 substrate phosphorylation was investigated in an *in vitro* assay system using radiolabelled ^{32}P and defined assay conditions in order to accurately calculate phosphate transfer from ATP into substrate. In order to directly compare the major Cdk5 complexes, p35/Cdk5 and p25/Cdk5, the activity of each complex was assessed against a generic substrate and then an equivalent amount of enzyme activity was used in all studies. Thus, kinetic information obtained for each substrate was relative to that generic substrate.

3.2.1.1 Quantification of the specific activity of commercial Cdk5

The major Cdk5 complexes, p35/Cdk5 and p25/Cdk5, were sourced commercially (*Merck Millipore*). Both Cdk5 complexes were produced by co-transfection of Sf21 insect cells with two baculoviruses: one that codes for full-length human Cdk5 with an N-terminal His6-tag; and, a second that codes for recombinant full-length human p35 or p25 containing an N-terminal GST-tag. A complex of recombinant kinase was then purified from the cell suspension using Ni-NTA agarose. Upon arrival, the commercial kinases were routinely aliquoted, to minimise freeze thaw cycles, and stored at -80°C. The certificate of analysis accompanying each complex contained an estimate of kinase activity as measured against histone H1 and kinase preparations were diluted to 50 mU/µl based on the reported histone H1 kinase activity. The specific activity of both kinases was re-measured against myelin basic protein (MBP) *in vitro*. MBP is readily phosphorylated by numerous kinases and contains multiple proline-directed phosphorylation sites hence it is often used to assess the specific activity of CDKs. Kinase activity was re-assessed with every aliquot on the day of use in case the activity of one complex degraded at a different rate to the other.

Each complex was incubated with 0.3 mg/ml MBP in the presence of MgATP [γ32-P] for 10 min (***Figure 3.1***). The [γ32-P] incorporation into MBP was assessed as described in ***Chapter 2.2.9.1***. Consistently, the measured activity of p25/Cdk5 was relatively higher than p35/Cdk5 activity when assessed against MBP suggesting that p25/Cdk5 has a relative preference for MBP over histone H1 compared to p35/Cdk5. Alternatively the p35/Cdk5 complex may be more unstable to transport or freeze thaw compared to p25/Cdk5. In the subsequent experiments, this MBP measure of activity was used to match the kinase preparations prior to assay. In general, a 1:1.7 dilution was preformed on p25/Cdk5 to match the specific activity prior to *in vitro* analysis.

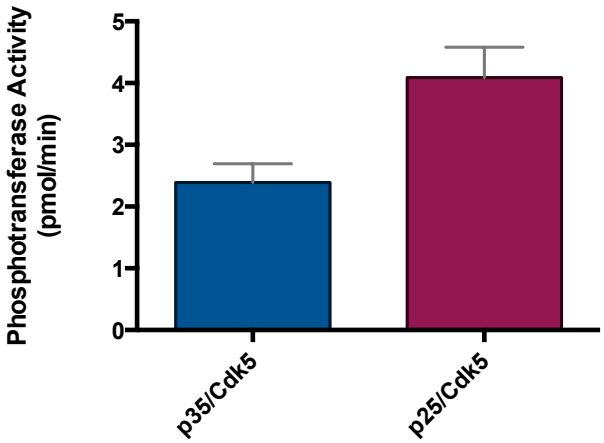


Figure 3.1. ***Comparison of the major Cdk5 complexes activity against MBP.*** Myelin basic protein (MBP), containing serine residues immediately followed by a proline, was used as a standard substrate to compare and subsequently match Cdk5 activity. Specific activity of each complex was assessed by incubating either p35/Cdk5 or p25/Cdk5 with MBP (0.3 mg/ml final concentration) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for 10 min followed by scintillation counting to measure phosphate transfer. Data expressed as mean ± SEM, n = 12.

3.2.1.2 Investigation of the purity of the commercial Cdk5 preparations

As the commercial Cdk5 preparations would be used for detailed kinetic comparisons of Cdk5 activity, both kinase preparations were assessed for the presence of other contaminating kinase activities that could influence the *in vitro* analysis data. For example, dual-specificity tyrosine-regulated kinase (DYRK) 1A possesses an inherent His-tag sequence capable of binding to nickel and, as a result, often contaminates commercial kinase preparations prepared using Ni-agarose purification. This is of particular importance to analysis of Cdk5 activity as DYRKs have an overlapping consensus sequence (proline-directed) and are therefore likely to target many of the same substrates *in vitro*.

Inhibitors selective for either Cdk or DYRK were used to establish their ability to repress the commercial Cdk5 activity. The Cdk5 complexes were pre-incubated with either 10 μM purvalanol A, an inhibitor of Cdk activity, or 10 μM harmine, an inhibitor of DYRK activity, for 30 min prior to *in vitro* phosphorylation assay with MBP. Kinase activity was determined by incubating inhibitor treated complexes, in direct comparison with vehicle, with MBP in the presence of MgATP [γ32-P] for 10 min, as described in ***Chapter 2.2.9.1***.

Following treatment with harmine, no apparent reduction in the specific activity of either p35/Cdk5 or p25/Cdk5 was observed confirming that DYRKs were not isolated with the Cdk5 complexes during Ni-agarose purification (***Figure 3.2A)***. Incubation with purvalanol A resulted in almost complete ablation of p35/Cdk5 and p25/Cdk5 specific activities confirming that the Cdk5 complexes were the major, if not only, kinase in the preparations (***Figure 3.2B)***.

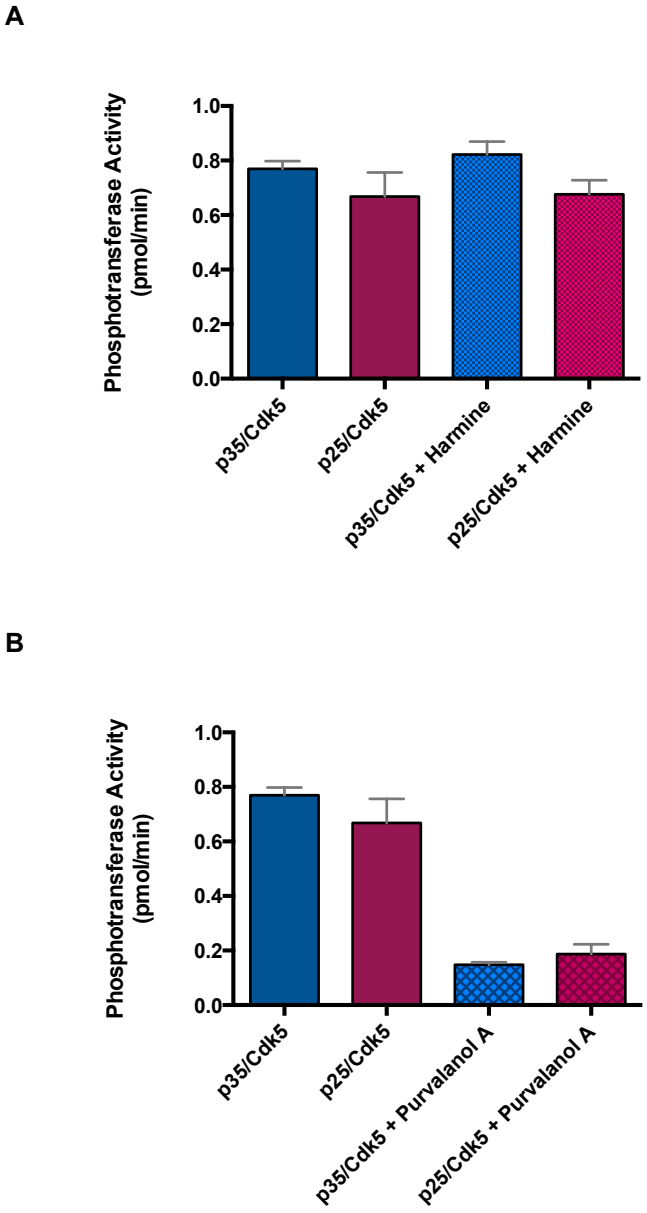


Figure 3.2. ***MBP kinase activity of the Cdk5 preparations is dramatically decreased in vitro following treatment with purvalanol A but not harmine.*** A) Both p35/Cdk5 and p25/Cdk5 were pre-incubated with or without 10 μM harmine for 30 min prior to performing an MBP kinase assay. Phosphate transfer (pmol/min) was determined by scintillation counting. B) Both p35/Cdk5 and p25/Cdk5 were pre-incubated with or without 10 μM purvalanol A for 30 min prior to performing an MBP kinase assay. Phosphate transfer (pmol/min) was determined by scintillation counting. Data expressed as mean ± SEM, n = 2.

3.2.2 Investigating the consensus for Cdk5 substrate phosphorylation

The consensus proposed for p35/Cdk5 phosphorylation is defined as (S/T)PX(K/H/R) (Beaudette *et al*., 1994; Shetty *et al*., 1999) however many of the proposed *in vivo* substrates of Cdk5 do not fit this consensus. Additionally, proteolytic cleavage of p35 to generate p25 reportedly alters Cdk5 substrate specificity (Patrick *et al*., 1999). Therefore, the substrate consensus for Cdk5 was investigated further in order to examine the importance of certain residues flanking the phosphoacceptor residue on the rate and extent of phosphorylation by each Cdk5 complex.

3.2.2.1 C-terminal basic residues confer Cdk5 substrate affinity

The current substrate consensus indicates that a negatively charged residue positioned three residues C-terminal to the phosphoacceptor residue (+3) is required for Cdk5 substrate recognition however not all of the proposed Cdk5 protein substrates include this specific residue, although most have basic residues within 5 positions in either direction of the phosphorylated residue. As an initial investigation of the significance of the +3 basic residue, the phosphorylation of a series of synthetic peptides based on the Cdc2-kinase/Cdk2 substrate peptide, PKTPKKAKKL (originally derived from Cdk2 *in vitro* phosphorylation of histone H1) were compared directly for phosphorylation by each Cdk5 complex. The sequences of the peptides compared to the original Cdk2 peptide were as follows: PK**TPKAA**KKL, PK**SPKAR**KKL, and PK**TPKKR**KKL. The peptides are referred to as TPKAA, SPKAR, and TPKKR in figures for simplicity.

Both p35/Cdk5 and p25/Cdk5 (1 mU) were independently incubated with three peptides, TPKAA, SPKAR, and TPKKR, (50 μM final concentration) in the presence of MgATP [γ32-P] for up to 20 min prior to measuring phosphate transfer to each

peptide (pmol/min). All three peptides were phosphorylated to some extent by both Cdk5 complexes (***Figure 3.3***). The phosphotransferase activity with each peptide was not dramatically different between the Cdk5 complexes and the relative peptide preference profile was also the same between the two major complexes. Both Cdk5 complexes exhibited lowest phosphotransferase activity towards TPKAA, the peptide containing the least basic residues. Substituting Ala (+4) for a basic residue (SPKAR) resulted in an increase in Cdk5 phosphotransferase activity (~3-fold), although a contribution from changing the Thr for a Ser cannot be ruled out (peptides were gifted from Professor Sakamoto therefore TPKAR was not compared in this analysis). The increase is most likely due to the addition of the basic residue however as substituting both Ala residues for basic residues (TPKKR) dramatically increased phosphorylation of the peptide substrate (~10-fold). This is consistent with the basic residue at the +3 position having a profound effect on Cdk5 activity, although comparing phosphorylation of additional sequences where the +3 Lys was the only basic residue in the peptide in the peptide would indicate whether the effect was synergistic with the basic residues at +2 and +4.

These results confirm that the presence of C-terminal basic residues enhances the rate that Cdk5 will phosphorylate peptide substrates *in vitro*, and proposed substrates of Cdk5 lacking such basic residues need to be scrutinised very carefully.

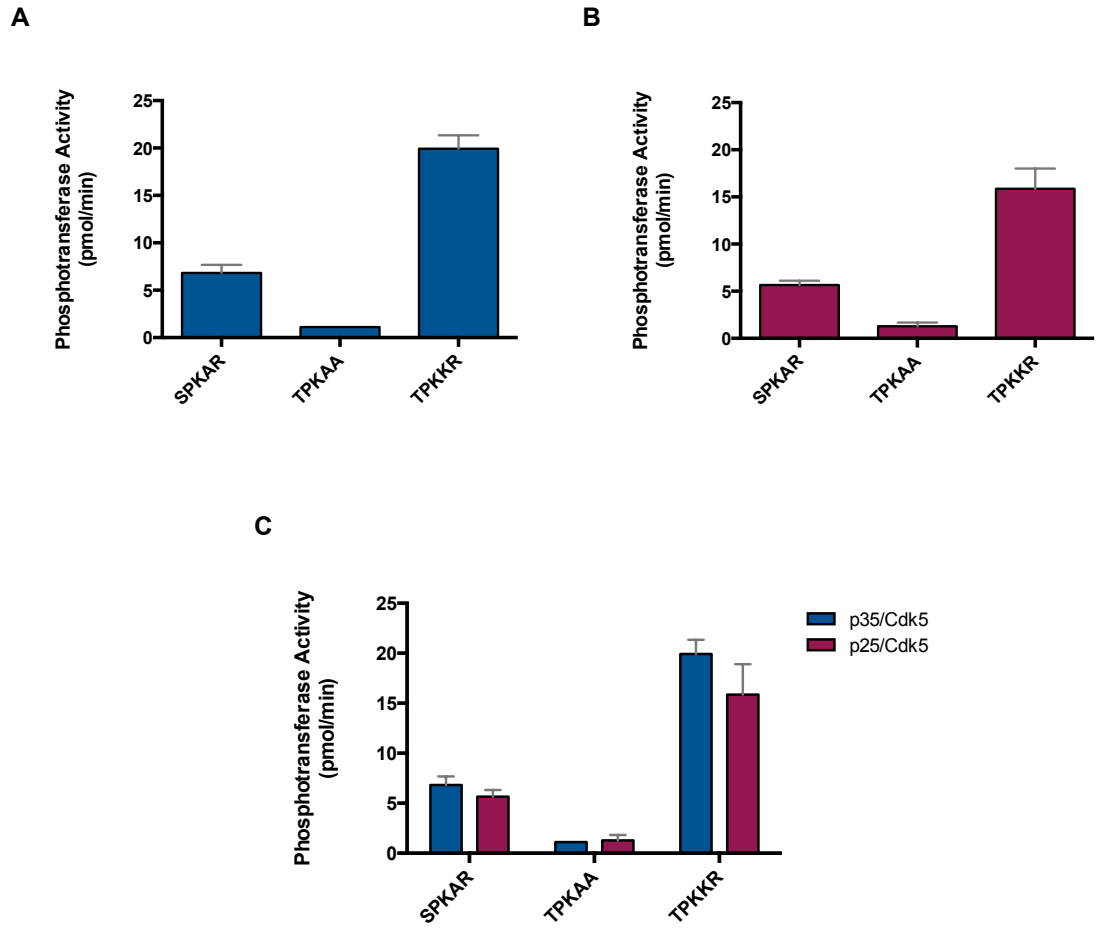
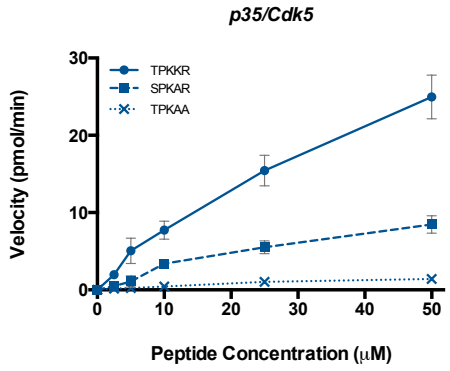


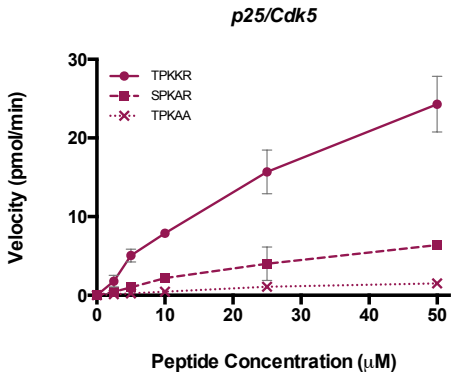
Figure 3.3. ***Contribution of C-terminal basic residues to Cdk5 substrate phosphorylation in vitro***. The influence of C-terminal basic residues to peptide substrate recognition and phosphorylation for each Cdk5 complex was assessed by incubating 1 mU A) p35/Cdk5 or B) p25/Cdk5 with the indicated peptides (50 μM final concentration) for 20 min and measuring phosphate transferred to each peptide. C) Phosphotransferase activity (pmol/min) of each complex was directly compared in a single assay. Data expressed as mean values ± SEM, n = 2.

To determine whether the basic residues increased phosphorylation by altering the K_m of the substrates, the Cdk5 complexes (1 mU) were incubated with each of the different peptides in the presence of MgATP [γ32-P] at varying concentrations of peptide (0 μM, 2.5 μM, 5 μM, 10 μM, 25 μM, 50 μM final concentration) for 20 min. Lineweaver-Burke plots were generated for both complexes against each peptide and V_{max} and K_m values calculated for each peptide (***Figure 3.4A-B***). As expected, the values for p35/Cdk5 were very similar to those obtained for p25/Cdk5 indicating that these two complexes do not have inherently different kinetic properties based on immediate sequences around the phosphoacceptor sites of the substrate. Interestingly, the presence of a basic residue at +3 in the sequence did not alter K_m as dramatically as it altered V_{max} (***Figure 3.4C***). This may indicate that the rate of phosphorylation of the more basic peptides is higher without a large change in affinity for the substrate peptide and requires further investigation.

A



B



C

	p35/Cdk5			*p25/Cdk5*		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
TPKAA	2.164	45.2	0.048	2.901	62.66	0.046
SPKAR	12.69	42.8	0.296	19.04	120.3	0.158
TPKKR	39.99	51.8	0.77	31.99	54.5	0.59

Figure 3.4. ***Optimisation of Cdk5 substrate peptides containing basic residues, phosphorylation kinetics***. Substrate saturation curves were established by incubating 1 mU A) p35/Cdk5 or B) p25/Cdk5 with the indicated peptides (0 μM, 2.5 μM, 5 μM, 10 μM, 25 μM, 50 μM final concentration) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated and measuring phosphate transfer to each peptide. C) Phosphorylation kinetics, V_{max} (pmol/min) and K_m (μM), were determined for each Cdk5 complex for the indicated peptides. Data expressed as mean ± SEM, n = 2.

3.2.2.2 Comparison of Cdk5 activity towards peptide substrates with sequences related to proposed protein substrates of Cdk5

Evidently, a basic residue at +3 relative to the phosphoacceptor site of substrates influences Cdk5 phosphorylation, however many proposed substrates do not have this important sequence requirement. Therefore, the position and number of basic residues relative to the phosphoacceptor site was used to classify a spectrum of reported Cdk5 substrates (***Chapter 1, Table 1.1***). Proteins that contained basic residues on both the N-terminal and C-terminal (within $\pm$5) side of the phosphoacceptor residue were designated as Class 1 substrates. Substrates containing basic residues only towards the C-terminus (within +5) were defined as Class 2, while substrates containing basic residues only towards the N-terminus of the phosphoacceptor residue (within -5) were classified as Class 3. Absence of basic residues $\pm$ 5 residues of the phosphoacceptor residue defined the substrates as Class 4.

Five peptides were designed to provide a representation of these variations in amino acid sequence (± 5 residues), the core sequence being based on an amalgamation of the sequence of a number of the best characterised Cdk5 substrates, allowing variation only in the position and number of the basic residues (***Table 3.1***). In short, the peptides were each twelve amino acids long containing a central serine residue directly followed by a proline residue, flanked on both sides by 5 amino acids. Two additional lysine residues were placed at the N-terminus of each peptide sequence to ensure binding to the negatively charged p81 paper during *in vitro* phosphorylation assays. Although not comprehensive, this study was designed to provide a general comparison of the contribution of the position of basic residues in substrates on the regulation of phosphorylation by each Cdk5 complex.

Designation	Peptide Sequence	Basic Residues
Class 1.1	KKASAKK**SP**RKPRS	N-terminal, C-terminal
Class 1.2	KKASAPK**SP**RKPRS	N-terminal, C-terminal
Class 2	KKASAPV**SP**PRDRK	C-terminal
Class 3	KKPKSRR**SP**PSIPT	N-terminal
Class 4	KKENNVL**SP**LPSQA	None

Table 3.1. ***Peptides designed to investigate the role of basic residues for Cdk5 phosphorylation.*** Five peptides were designed to provide a representation of variations in amino acid sequence around proposed Cdk5 substrates (± 5 residues), the core sequence being based on an amalgamation of the sequence of a number of the best characterised Cdk5 substrates, allowing variation only in the position of the basic residues. Basic residues are highlighted in blue.

Both complexes were assayed against each peptide (***Table 3.1***) to quantitatively compare the effect of the primary sequence differences on the rate of phosphorylation by each of the Cdk5 complexes. Recombinant Cdk5 with a specific activity of 1 mU was incubated with each of the five peptides at the following concentrations: 0 μM, 2.5 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM in the presence of MgATP [γ32-P] for 20 min. The [γ32-P] incorporation into each peptide was assessed as detailed in ***section 2.2.9.2***.

Surprisingly, Cdk5 exhibited a highly selective peptide substrate profile, preferentially phosphorylating only one of the five peptides (***Figure 3.5***). This profile was identical for both Cdk5 complexes. Class 3 and Class 4 peptide substrates do not contain a basic residue at +3; indeed they lack any C-terminal basic residues, which may explain the lack of phosphorylation of these peptides. This, again, indicates the importance of C-terminal basic residues within the substrate sequences to Cdk5 activity and suggests that this cannot be replaced by the presence of N-terminal basic residues (Class 3 peptide had basic residues at -1, -2 and -4). More surprising was the low activity towards peptides KKASAKKSPRKPRS (1.1) and KKASAPVSPPRDRK (2). The Class 2 peptide contains a basic residue at +3 and this indicates that a proline at +1 and a basic residue at +3 are not sufficient to direct phosphorylation by Cdk5. Although, the lack of phosphorylation may be related to the presence of the proline residue at +2 or the asparate at +4, both of which would introduce major structural changes. The Class 3 peptide also possesses a proline residue at +2. It is more difficult to understand the inability of Cdk5 to phosphorylate Class 1.1 as it only differs from the Class 1.2 peptide by a single amino acid (proline at -2 in 1.2 instead of lysine at -2 in 1.1). This suggests that the N-terminal sequence of substrates also has a major contribution to Cdk5 substrate specificity.

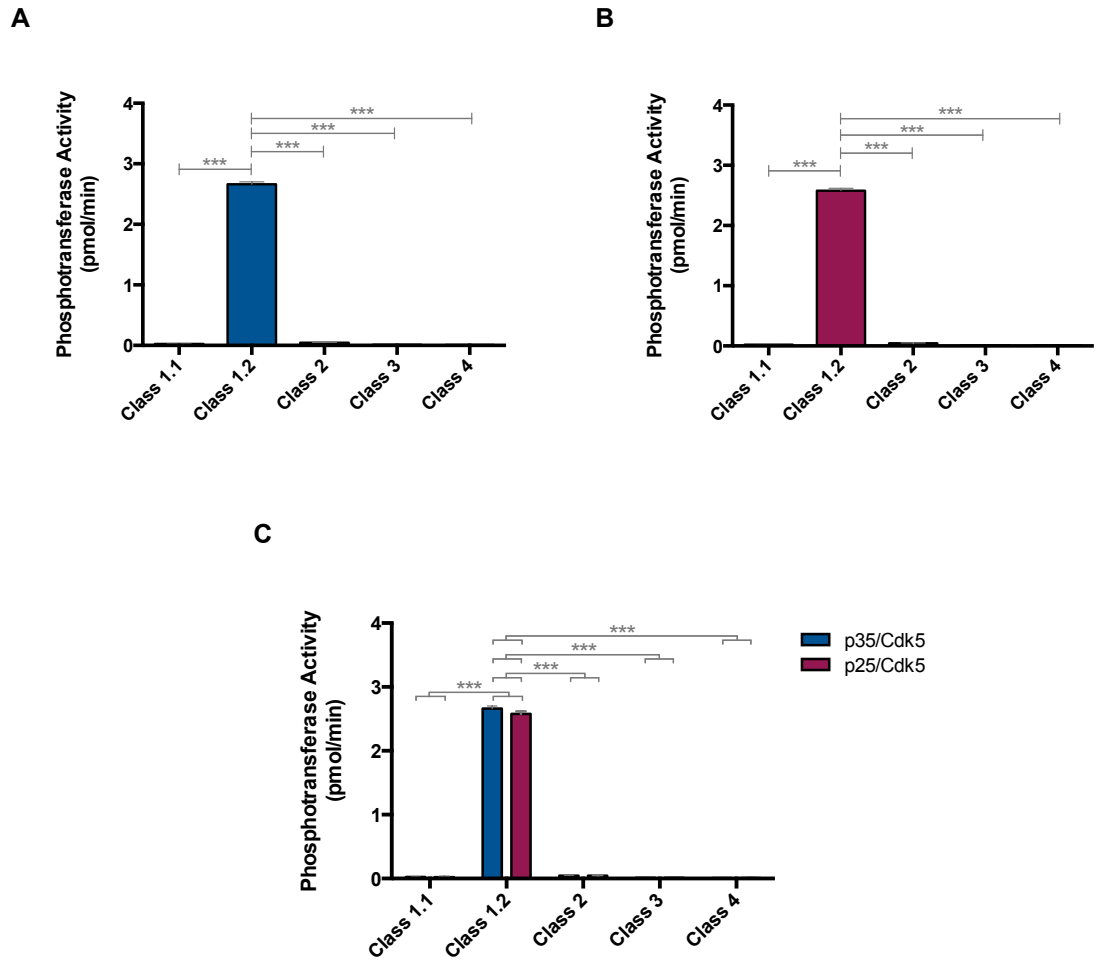


Figure 3.5. ***Phosphorylation of peptides with varying basic residue content by Cdk5***. The contribution of basic residues to peptide substrate recognition and phosphorylation was assessed by incubating 1 mU A) p35/Cdk5 or B) p25/Cdk5 with the indicated peptides (100 μM final concentration) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for 20 min and measuring phosphate transfer to each peptide. C) The substrate profile between complexes was compared in a single assay. Data expressed as mean ± SEM, n = 3. Statistical analysis was performed by one-way ANOVA with Tukey's post hoc test, ***p<0.001.

3.2.2.3 N-terminal residue required for Cdk5 substrate recognition

The data in ***Figure 3.5*** suggested that Cdk5 may exhibit a preference for substrates with a proline residue located at the -2 position yet prefer not to have a proline at the +2 position relative to the phosphoacceptor residue (compare Class 1.1 with Class 1.2, and Class 1.2 with Class 2).

To further elaborate on the importance of these proline residues (+2 and -2), an additional set of peptides were generated with minor deviations from the Class 1.2 peptide KKASAPKSPRKPRS (1.2) in order to assess the key residues of this peptide for Cdk5 phosphorylation (***Table 3.2***). Again, the new peptides were twelve amino acids long containing a central serine residue directly followed by a proline residue, along with two additional N-terminal lysine residues to aid interaction with p81 paper during assay.

The rate of phosphorylation of peptides *KK*SSAPK**SP**ARPRS (Class A), *KK*SSAPV**SP**RKPRS (Class B), *KK*ASAAK**SP**PKPRS (Class C), and *KK*ASAPK**SP**PRPRS (Class D) by Cdk5 was assessed by incubating each of the peptides (100 μM final concentration) with either p35/Cdk5 or p25/Cdk5 at a specific activity of 1 mU in the presence of MgATP [γ32-P] for 20 min (***Figure 3.6***). The [γ32-P] incorporation into each peptide was assessed as described in ***section 2.2.9.2***.

Designation	Peptide Sequence	Basic Residue Changes
Class 1.2	*KK*ASAPK**SP**RKPRS	Parent peptide
Class A	*KK*SSAPK**SP**ARPRS	Reduced C-terminal
Class B	*KK*SSAPV**SP**RKPRS	Reduced N-terminal
Class C	*KK*ASAAK**SP**PKPRS	Reduced C-terminal plus removed N-terminal Pro
Class D	*KK*ASAPK**SP**PRPRS	Reduced C-terminal plus added C-terminal Pro

Table 3.2. ***Further investigation of the role of basic residues in Cdk5 substrate phosphorylation.*** Four further peptides were designed to investigate the role of additional basic residues in Cdk5 phosphorylation. Amino acid variations from Class 1.2 are highlighted in red.

Once more, the rate of phosphorylation of these peptides by p35/Cdk5 and p25/Cdk5 was identical. Consistent with the previous result, Cdk5 phosphorylates the peptides that contain a proline residue situated at -2 position (Class A and Class B) whilst failing to phosphorylate the peptide that contains an alanine residue at this position (Class C) (***Figure 3.6***), indicating the importance of this proline residue. There appears to be little difference in the phosphotransferase activity observed with peptides Class A and B. The basic residue at the +2 position in Class 1.2 was substituted to an alanine in Class A and the basic residue at the -1 position in Class 1.2 was substituted to a valine in Class B. Altering the amino acid either side of the SP motif does not appear to alter Cdk5 phosphorylation as dramatically as substituting the proline residue in the -2 position. Interestingly, despite containing a proline residue at the -2 position, Cdk5 failed to phosphorylate peptide D. This peptide contains a single amino acid difference compared to Class A, a proline residue at the +2 position instead of an alanine. This suggests that the presence of this proline residue has a negative impact on Cdk5 phosphorylation.

These findings suggest that the presence of proline residues may be important in aiding Cdk5 substrate recognition and subsequent phosphorylation. In addition to the proline residue that is already known to be critical to Cdk5 phosphorylation (+1), the presence of an N-terminal proline residue (-2) appears to guide Cdk5 phosphorylation whereas the presence of an additional proline residue (+2) appears to abolish Cdk5 phosphorylation. Evidently, the amino acid sequence directly surrounding the phosphoacceptor site is critical to Cdk5 substrate recognition and phosphorylation.

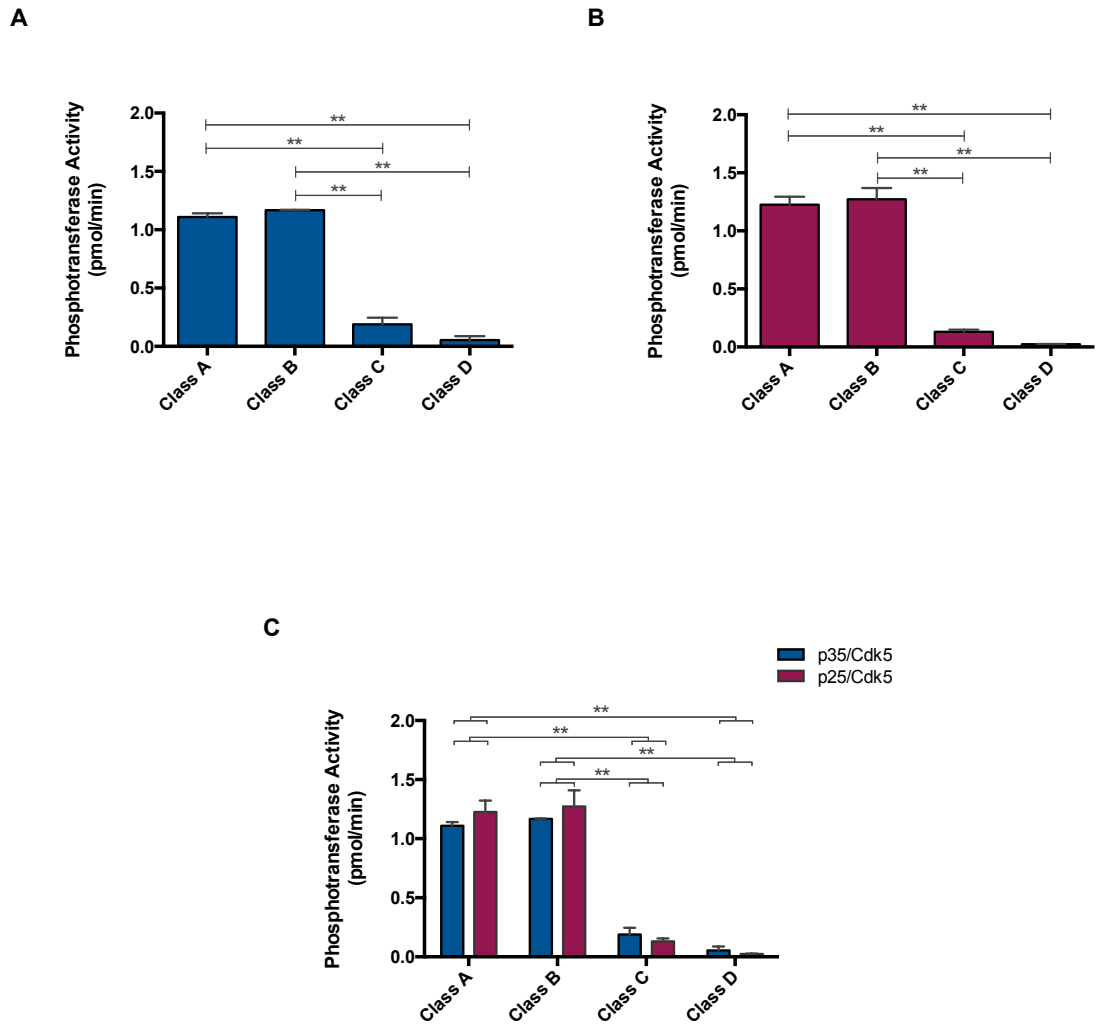


Figure 3.6. ***Cdk5 regulation is dependent on proline residues.*** The contribution of proline residues to peptide substrate recognition and phosphorylation was evaluated by incubating 1 mU A) p35/Cdk5 or B) p25/Cdk5 with the indicated peptides (100 μM final concentration) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for 20 min and measuring phosphate transfer to each peptide. C) The substrate phosphorylation profile of each complex was directly compared in a single assay. Data expressed as mean ± SEM, n = 3. Statistical analysis was performed by one-way ANOVA with Tukey's post hoc test, $^{**}p<0.01$.

3.2.2.4 Establishing the optimal Cdk5 substrate motif

In order to obtain more information regarding the influence of the sequence surrounding the phosphoacceptor site on the rate of substrate phosphorylation by Cdk5, phosphorylation kinetic parameters were determined and directly compared between the peptides optimally phosphorylated by Cdk5 (Class 1.2, A, and B).

Peptides (0 μM, 2.5 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM final concentration) were incubated with recombinant p35/Cdk5 and p25/Cdk5 (1 mU) in the presence of MgATP [γ32-P] for 20 min. The [γ32-P] incorporation into each of the peptides was assessed as described in ***section 2.2.9.2*** and allowed quantification of the number of moles of phosphate incorporated per unit time (***Figure 3.7***). Substrate saturation curves were fitted by non-linear regression to the Michaelis-Menton model (***Figure 3.8***). Subsequently, reciprocal values were plotted and phosphorylation kinetic parameters, V_{max} (pmol/min) and K_m (μM) calculated using the Lineweaver-Burke.

Both Cdk5 complexes display highest phosphotransferase activity towards the Class 1.2, being ~2-fold greater in comparison to Class A (reduced C-terminal) or Class B (reduced N-terminal) (***Figure 3.7***). In this case, increased phosphorylation seems to be due to a lower K_m when basic residues are present upstream *and* downstream of the phosphoacceptor site as substitution of either of these basic residues reduces phosphotransferase activity to a similar magnitude and affinity does not differ greatly suggesting that an N-terminal basic residue at -1 is as important for Cdk5 phosphorylation as a C-terminal basic residue at +2. The data presented here extends the understanding of the sequence determinants for optimal Cdk5 phosphorylation of substrates, where we can suggest that the optimal consensus would be P-K-**S**-**P**-R-K.

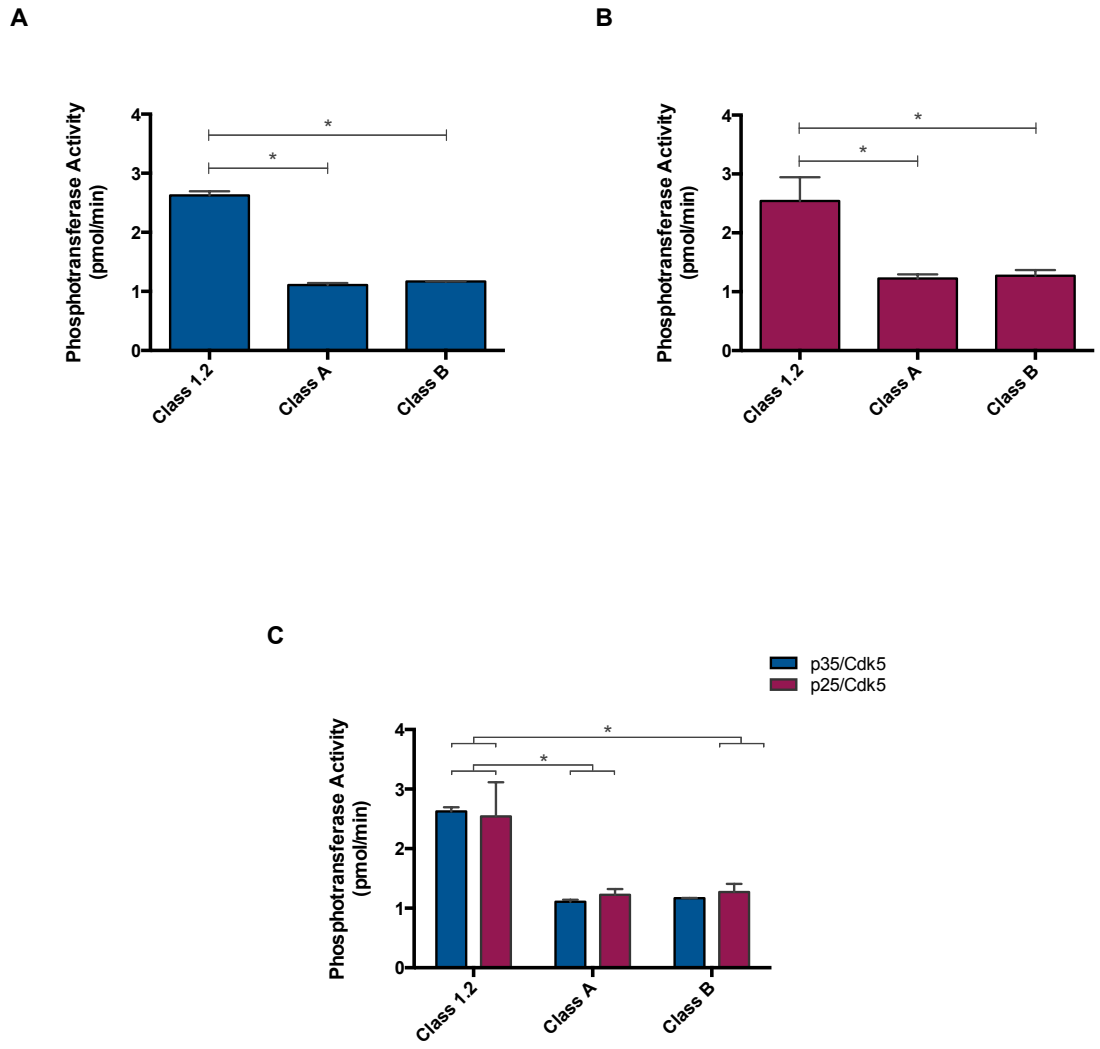
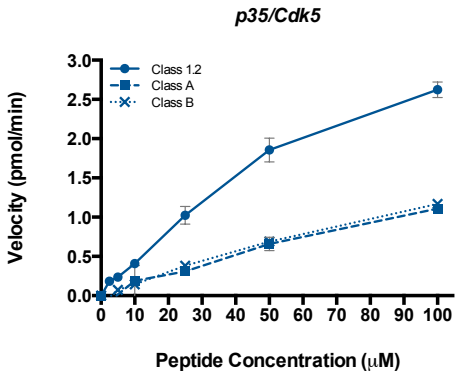
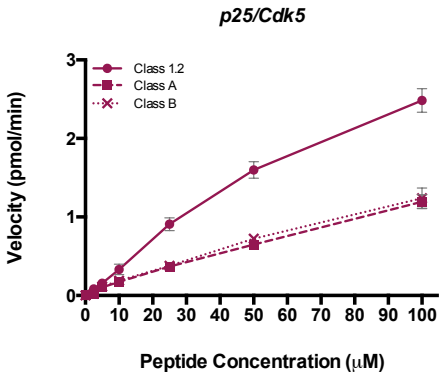


Figure 3.7. ***Determination of Cdk5 optimal substrate motif.*** The optimal residues for Cdk5 recognition and phosphorylation was examined by incubating 1 mU A) p35/Cdk5 or B) p25/Cdk5 with the indicated peptides (100 μM final concentration) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for 20 min and measuring phosphate transfer to each peptide. C) The substrate profile between complexes was compared in a single assay. Data expressed as mean ± SEM, n = 3. Statistical analysis was performed by one-way ANOVA with Tukey's post hoc test, *p<0.05.

A



B



C

	p35/Cdk5			*p25/Cdk5*		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
Class 1.2						
KKASAPKSPRKPRS	5.321	10.05	0.529	6.074	14.35	0.423
Class A						
KKASAPKSPARPRS	4.079	26.7	0.153	5.356	35.03	0.153
Class B						
KKSSAPVSPRKPRS	3.927	23.59	0.166	4.368	25.24	0.173

Figure 3.8. ***Determining the optimal Cdk5 peptide substrate motif.*** Substrate-velocity graphs were established by incubating 1 mU A) p35/Cdk5 or B) p25/Cdk5 with the indicated peptides (0 μM, 2.5 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM final concentration) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated and measuring phosphate transfer to each peptide. C) Phosphorylation kinetics, V_{max} and K_m, were determined for each Cdk5 complex for the indicated peptides. Data expressed as mean ± SEM, n = 3.

3.2.2.5 Cdk inhibitors abolish Cdk5 peptide substrate phosphorylation

To confirm that the observed phosphorylation of peptides KKASAPKSPRKPRS (1.2), KKASAPKSPARPRS (A), and KKSSAPVSPRKPRS (B) was due to the enzymatic activity of Cdk5, both major complexes of Cdk5 were pre-incubated with either 10 μM purvalanol A or 10 μM roscovitine for 30 min prior to assay. Peptides (50 μM final concentration) were then incubated with treated Cdk5 complexes in the presence of MgATP [γ32-P] for 30 min. The [γ32-P] incorporation into each peptide is expressed as the number of moles of phosphate incorporated into each peptide substrate per min (***Figure 3.9***).

As expected, incubation with Cdk5 resulted in a similar phosphate incorporation profile to that observed previously. However, pre-incubation with puravalanol A robustly reduced all p35/Cdk5 activity (>95%) towards Class 1.2 ($p < 0.001$), Class A ($p = 0.002$) and Class B ($p = 0.005$) peptides. A similar decrease in peptide phosphorylation was observed with roscovitine pre-incubation which resulted in a significant reduction in the phosphorylation of peptide Class 1.2 ($p < 0.0001$), Class A ($p = 0.0003$), and Class B ($p = 0.0007$) by p35/Cdk5. Similarly, purvalanol A treatment significantly reduced all p25/Cdk5 activity (>95%) towards peptide Class 1.2 ($p < 0.0001$), Class A ($p = 0.0002$), and Class B ($p = 0.0005$). Roscovitine treatment also decreased peptide Class 1.2 ($p < 0.0001$), Class A ($p = 0.0003$), and Class B ($p = 0.0007$) phosphorylation by p25/Cdk5 by >90%.

This data confirms Cdk5 is responsible for the observed kinase activity in each preparation and the differences in kinetics with each substrate are not influenced by a contaminating activity.

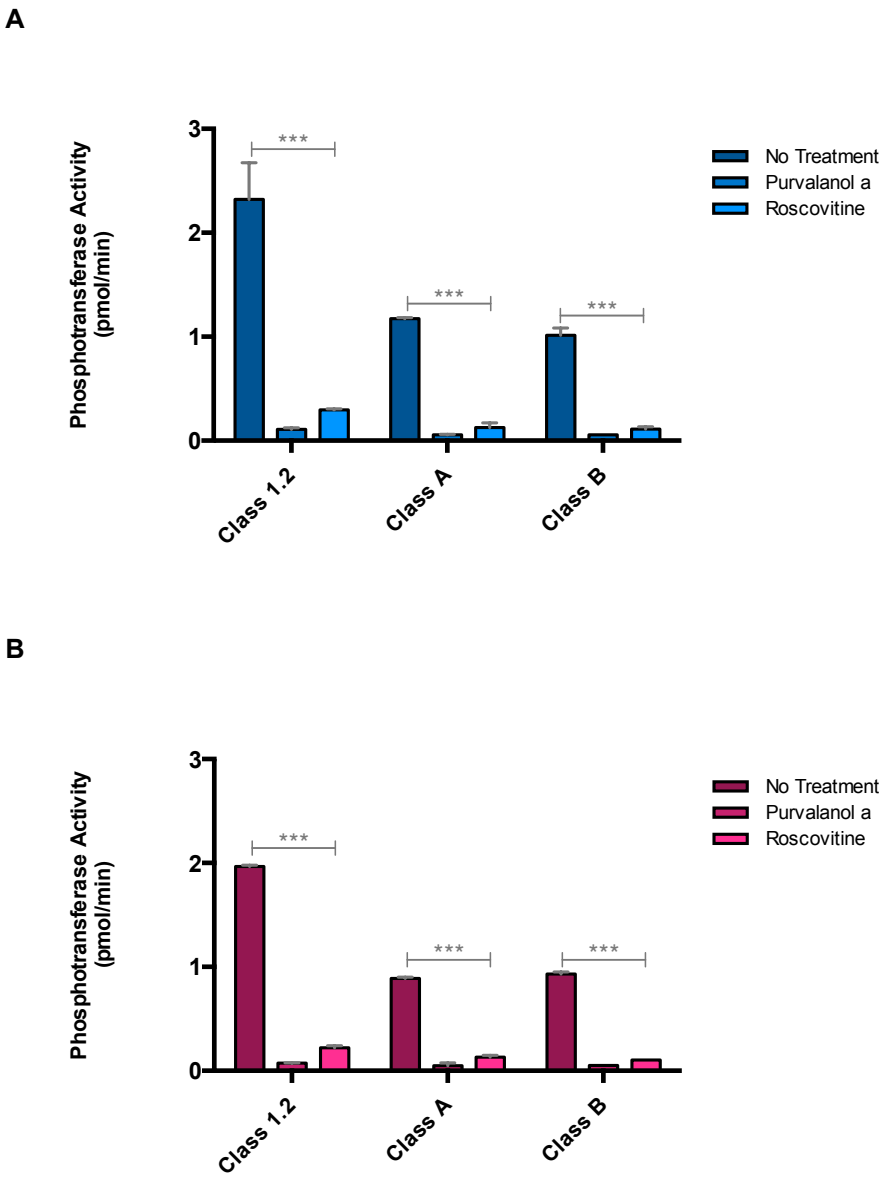


Figure 3.9. ***Inhibition of Cdk5 activity results in loss of peptide phosphorylation.*** Prior to kinase assay, 1 mU A) p35/Cdk5 and B) p25/Cdk5 was pre-incubated with or without 10 μM purvalanol A or 10 μM roscovitine for 30 min before being incubated with the indicated peptides (50 μM final concentration) for 30 min and measuring the phosphate transferred to each peptide. Data expressed as mean ± SEM, n = 3. Data analysed by two-way ANOVA with Dunnett's post hoc test vs. no treatment, ***p<0.0001.

3.2.2.6 Cdk5 substrate phosphorylation in comparison to other members of the CMGC family of protein kinases

The CMGC family is a large and important group of protein kinases that are present in all eukaryotes, and include the Cdk-like kinases, Mitogen-activated protein kinases (MAP kinases), Glycogen synthase kinases (GSK), and the Cyclin-dependent kinases, of which Cdk5 is a member.

The majority of the CMGC family prefer substrates to contain a proline residue at the +1 position relative to the phosphoacceptor site, although the degree of requirement varies across the family. As described previously, Cdk5 exhibits a high degree of selectivity for the presence of specific residues around the phosphoacceptor site in addition to the proline residue at +1. To determine if the other aspects of selectivity determined in the previous experiments were also important for other members of the CMGC group, peptide preference was examined for a selection of kinases, chosen to represent different branches of this protein kinase family (***Figure 3.10, selected kinases shown in red***).

The peptides selected for comparison included the three peptides that were previously shown to be phosphorylated by Cdk5 *in vitro* (KKASAPKSPRKPRS (1.2), KKASAPKSPARPRS (A) and KKSSAPVSPRKPRS (B)), as well as the peptide KKASAPKSPPRPRS (D) that was not phosphorylated by Cdk5 despite possessing the critical proline residue at +1 and an Arg at +3. Prior to the kinase comparison assay, the specific activity of all kinases was matched against MBP. Subsequently, peptides (50 μM final concentration) were incubated with 1 mU CLK2, PCTAIRE, HIPK1, DYRK2, SPRK1, SAPK4, JNK3, Cdk5, or ERK8 in the presence of MgATP [γ32-P] for 30 min and phosphate incorporation measured (***Figure 3.10***).

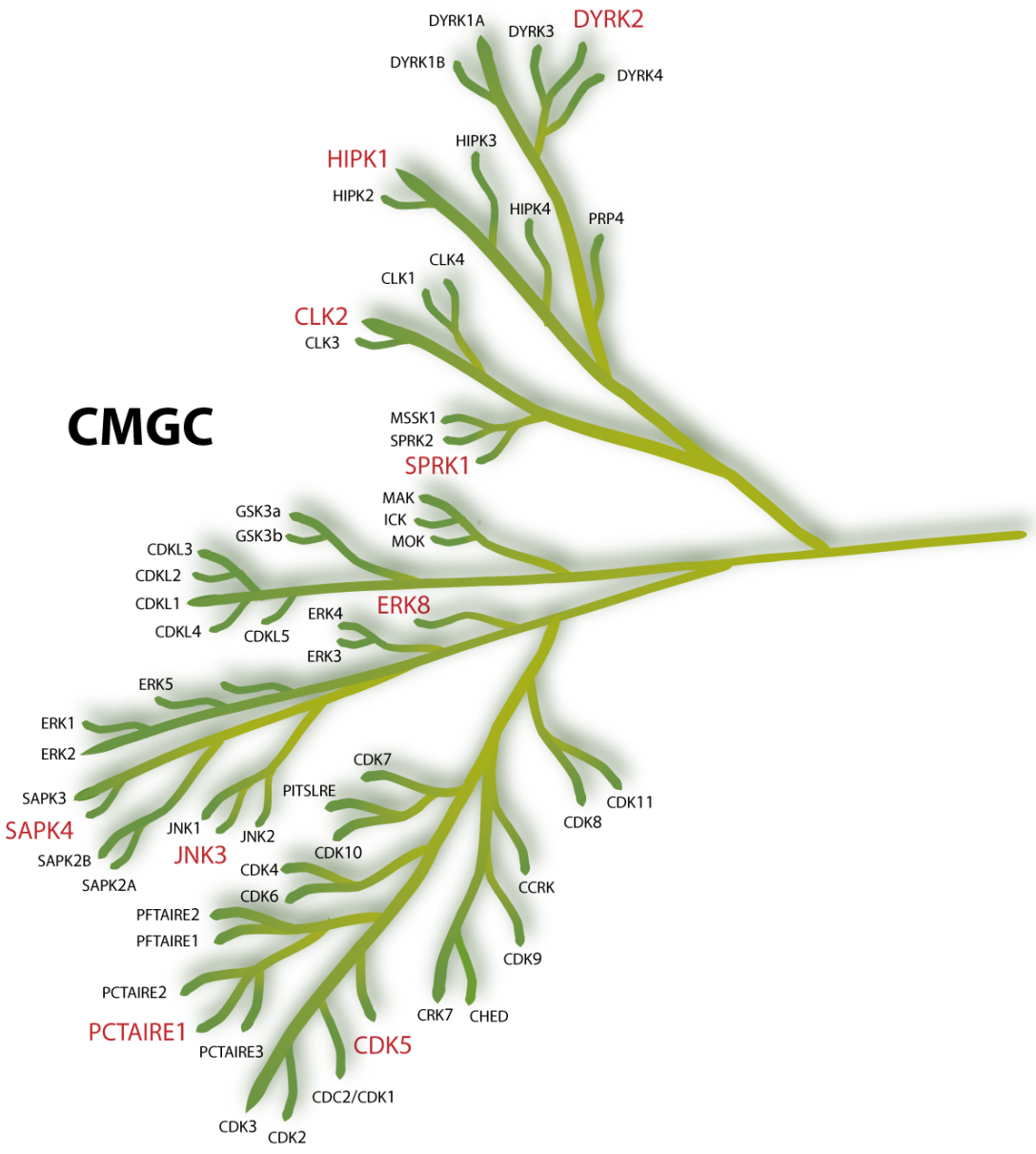


Figure 3.10. ***Schematic representation of the CMCG group and selected members for substrate comparison.*** Kinases highlighted in red indicate the kinases selected for comparison against Cdk5 in peptide analysis screen.

Once more, Cdk5 phosphorylated peptides KKASAPKSPRKPRS (1.2), KKASAPKSPARPRS (A), and KKASAPVSPRKPRS (B) but failed to phosphorylate peptide KKASAPKSPPRPRS (D). Interestingly, none of the peptides targeted by Cdk5 (Class 1.2, A and B) were phosphorylated to an appreciable extent by any of the other CMGC kinases tested, at least under these conditions where their activities were matched against MBP. This suggests that these peptide sequences are highly specific for Cdk5 phosphorylation. In contrast, one kinase (PCTAIRE) was able to phosphorylate KKASAPKSPPRPRS (D) at a much higher rate than Cdk5, confirming that this peptide was soluble. PCTAIRE did weakly phosphorylate all the peptides but to <15% of that seen with Cdk5 and the relative preference for the peptides was distinct from that of Cdk5 (Cdk5: 1.2>B=C>>>D, while PCTAIRE: 1.2 = D = A = B).

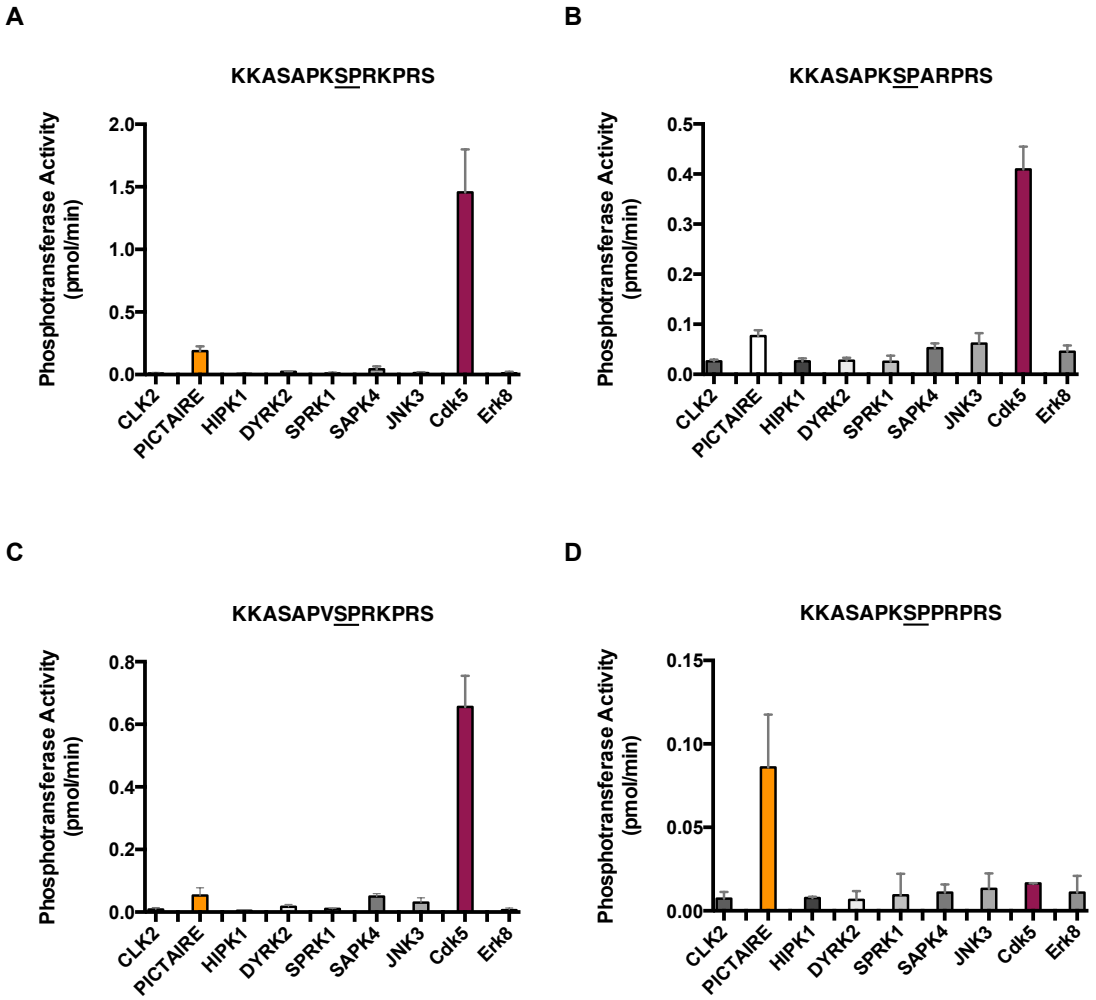


Figure 3.11. ***CMGC kinase screen using Cdk5 substrate peptides*****.** The rate of phosphorylation of the indicated peptides by selected members of the CMCG kinase family was compared by incubating each peptide (50 μM final concentration) with 1 mU (as assessed against the generic substrate MBP) CLK2, PCTAIRE, HIPK1, DYRK2, SPRK1, SAPK4, JNK3, Cdk5 and ERK8 in the presence of radiolabelled ATP for 30 min and measuring phosphate transfer. Data expressed as mean ± SEM, n = 2. Graphs are formatted to a scale that best displays the variation across the kinase panel.

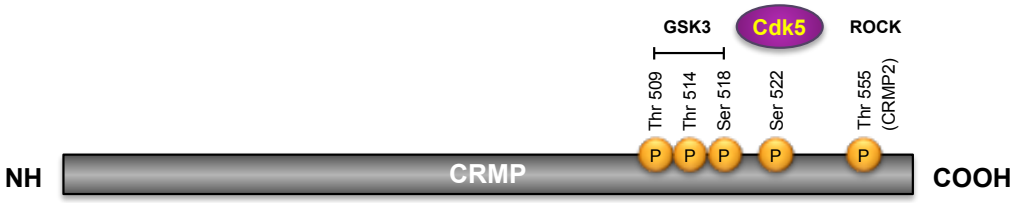
3.2.3 Investigating peptide consensus in full-length substrates

There is a clear preference for particular amino acids surrounding the phosphoacceptor residue to regulate the rate that Cdk5 phosphorylates short peptides. Basic residues flanking the phosphorylatable residue were found to enhance recognition by Cdk5 whereas other inputs were found to be antagonistic (e.g. proline at +2 or the aspartate at +4). In an attempt to further investigate the importance of such sequences to Cdk5 function in health and disease, Cdk5 phosphorylation was further examined in a range of full-length protein substrates.

3.2.3.1 Basic residues flank the phosphoacceptor site in CRMP isoforms and lack of these residues diminishes phosphorylation

Previous work within the lab established that Cdk5 phosphorylates CRMP1, CRMP2, and CRMP4 at Ser522 *in vitro* converting the CRMP isoforms into substrates for GSK3, which then phosphorylates at Ser518, Thr514 and Thr509 (Cole *et al*., 2004) (***Figure 3.12A***). These isoforms share reasonable homology surrounding the Ser522 site (***Figure 3.12B***). In contrast, CRMP3 contains no SP motif at 522, only a single TP motif at Thr84 surrounded by no basic residues (PVLGM**TP**ADDFC) and a single SP motif at Ser304 containing a proline at +2 and flanked by non-basic residues (AAFVT**SP**PVNPD) suggesting that CRMP3 would be a very weak substrate of Cdk5, if phosphorylated at all. Likewise, CRMP5 lacks an equivalent Ser522 residue. Upstream of this residue, there are three TP motifs: RGVDR**TP**YLGDV (Thr490), PLAD**TP**TRPVTR (equivalent to Thr514 in CRMP2) and KKEMG**TP**LADTP (equivalent to Thr509 in CRMP2). In CRMP2 Thr509 and Thr514 are phosphorylated by GSK3 rather than Cdk5. However, Thr514 in CRMP5 has a basic residue at +3, not present around Thr514 in CRMP1, CRMP2 or CRMP4 therefore it is possible that Cdk5 primes CRMP5 at Thr514 allowing GSK3 to phosphorylate at Thr509.

A



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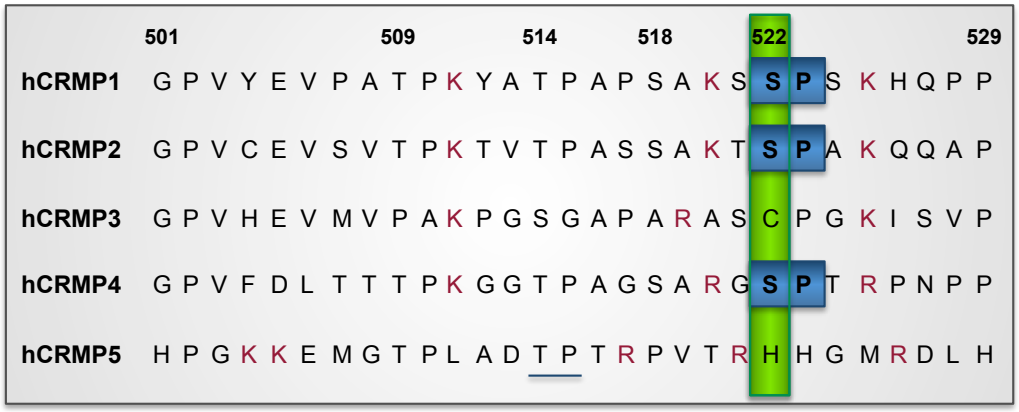


Figure 3.12. ***CRMP phosphorylation sites and sequence alignment of the CRMP isoforms.*** A) Schematic representation of CRMP1, CRMP2, and CRMP4 phosphorylation sites and the phosphorylating kinases. B) Sequence alignment of residues 501-529 of human CRMP1, CRMP2, CRMP3, CRMP4, and CRMP5. Residues Ser518, Thr514, and Thr509, on CRMP1, CRMP2, and CRMP4 are phosphorylated by GSK3 following Cdk5 phosphorylation of the Ser522 priming site, which is outlined in green. The Cdk5 required SP motifs are outlined in blue. Basic residues surrounding the Ser522 phosphorylation site are highlighted in red. A potential Cdk5 site on CRMP5 is underlined in blue and basic residues that may confer Cdk5 recognition are in red.

In direct comparison, 1 mU p35/Cdk5 and 1 mU p25/Cdk5 were independently incubated with recombinant human WT GST-CRMP1, 2, 3, 4 or 5 (0.5 μM final concentration) in the presence of MgATP [γ32-P] for the times indicated (***Figure 3.13***). The [γ32-P] incorporation into each substrate was assessed as detailed in ***section 2.2.9.2***.

Incubation of p35/Cdk5 with each of the CRMP isoforms resulted in a similar maximal stoichiometry (0.5-0.6 mol/mol) for CRMP1, 2, and 4 however CRMP5 (~0.2 mol/mol) and CRMP3 (<0.1 mol/mol) were relatively weakly phosphorylated by p35/Cdk5 (***Figure 3.13A***). Despite showing slightly lower stoichiometry than p35/Cdk5, the isoform specificity of p25/Cdk5 was identical to that of p35/Cdk5. Again, similar phosphorylation stoichiometries were reached for CRMP1, 2, and 4 after 60 min and a relatively lower stoichiometry was reached for CRMP3 and CRMP5, with CRMP3 very weakly phosphorylated by p25/Cdk5 (***Figure 3.13B***).

These results show that the optimal CRMP isoforms were those that contained basic residues on both the N- and C-terminal side of the phosphoacceptor residue. Interestingly, CRMP1, 2 and 4 isoforms contain a basic residue positioned at -2 and +3, the sites found to be important by the peptide analysis. The degree of phosphorylation increased when a lysine was substituted for an arginine at both of these positions (compare CRMP4 with CRMP1 and CRMP4 with CRMP2). It would appear that a basic residue at the -2 position rather than a proline does not prevent Cdk5 phosphorylation and it may be interesting to investigate whether a Lys520Pro mutant CRMP2 would be a better substrate for Cdk5. Meanwhile, previous work in the lab had established that a Lys520Ala CRMP2 mutant was a weaker substrate for Cdk5 in comparison to wild-type CRMP2 (Cole *et al*., 2006).

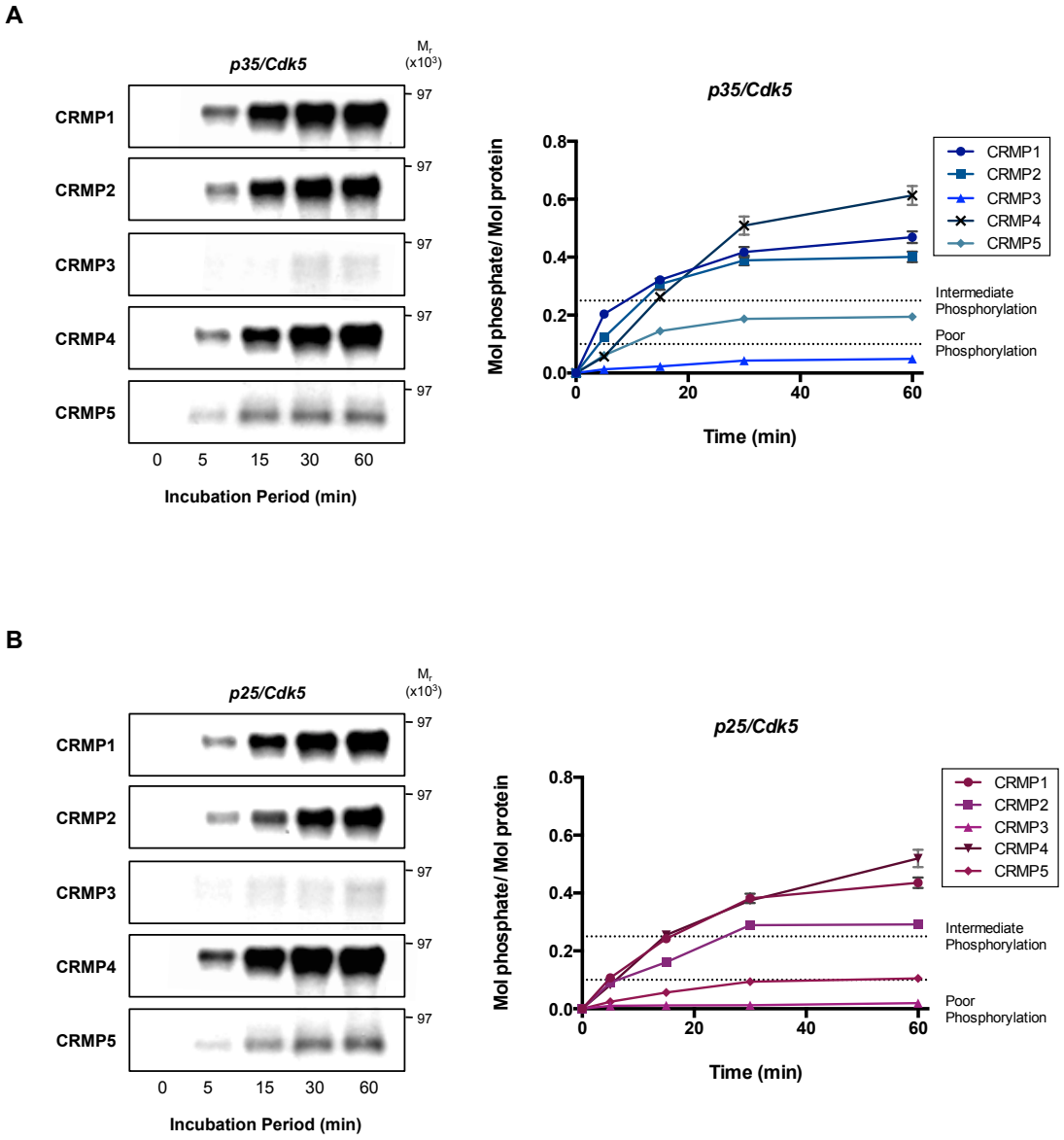


Figure 3.13. ***p35/Cdk5 and p25/Cdk5 display identical CRMP isoform specificity.*** Recombinant WT GST-hCRMP-1, -2, -3, -4, or -5 (0.5 μM final concentration) was incubated with 1 mU of A) p35/Cdk5 or B) p25/Cdk5 in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film. The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting. Data expressed as mean ± SEM, n = 2.

3.2.2.1.1 Ser522 is the major Cdk5 site *in vitro*

CRMP isoforms 1, 2, and 4 were better substrates for Cdk5 compared to CRMP3 and CRMP5. Due to the relatively poor phosphorylation observed for CRMP3 and CRMP5, these substrates were not included in the subsequent kinetic analysis. Firstly, to confirm that Cdk5 phosphorylated CRMP primarily at Ser522 the phosphorylation of a Ser522Ala mutant CRMP was also studied *in vitro*.

Recombinant human WT GST-CRMP1, 2, and 4 (0.5 μM final concentration) was independently incubated with p35/Cdk5 or p25/Cdk5 (1 mU) in the presence of MgATP [γ32-P] for 30 min. In direct comparison, recombinant human CRMP1 S522A, CRMP2 S522A, and CRMP4 S522A (0.5 μM final concentration) was independently incubated with p35/Cdk5 and p25/Cdk5 (1 mU) in the presence of MgATP [γ32-P] for 30 min. Following the assay, samples were subjected to SDS-PAGE and then autoradiography (***Figure 3.14***).

The degree of phosphate incorporated into each wild-type CRMP with both p35/Cdk5 and p25/Cdk5 was substantially greater (>10-fold) than the amount of phosphate incorporated into the cognate CRMP S522A mutant. These results confirm that the major p35/Cdk5 and p25/Cdk5 phosphorylation sites on wild-type human CRMP1, CRMP2, and CRMP4 is Ser522 during *in vitro* phosphorylation assays.

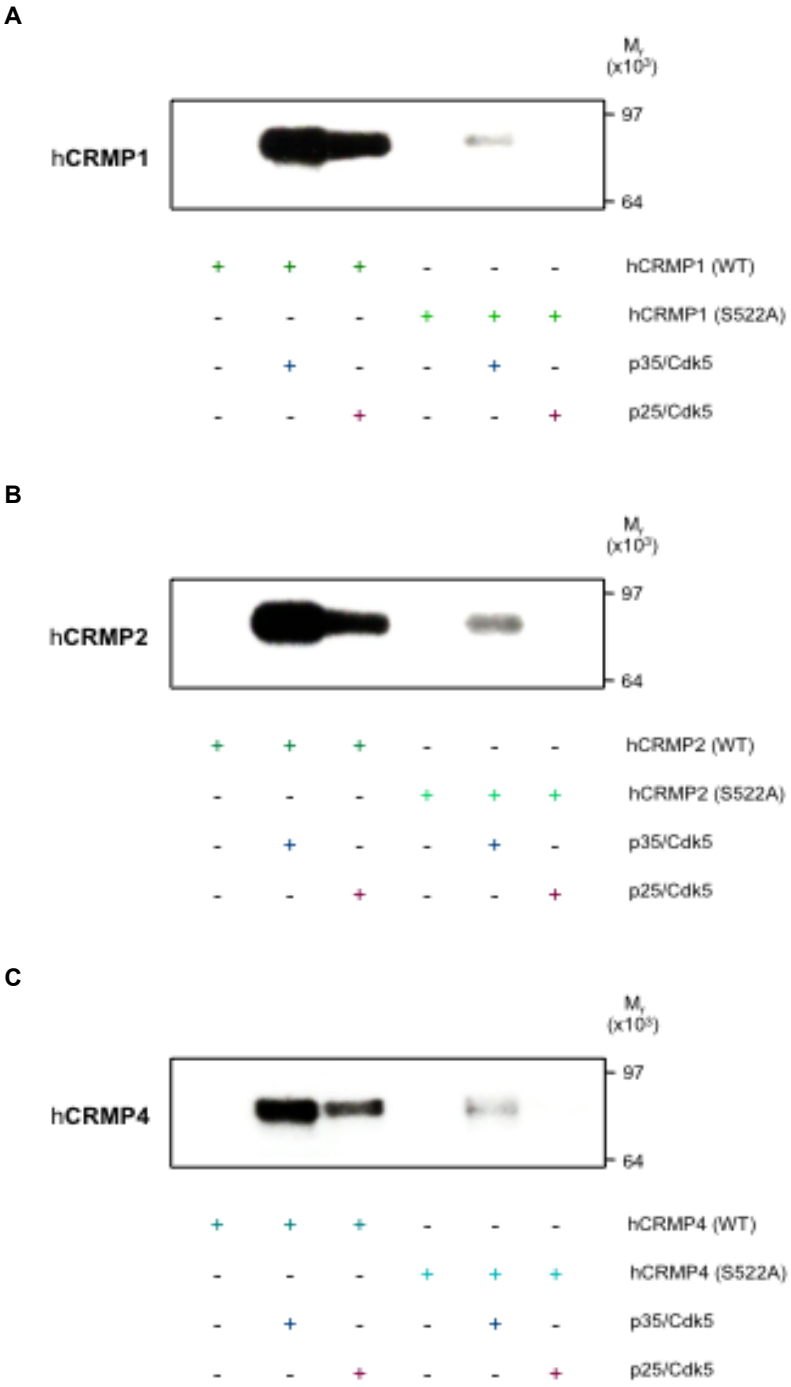


Figure 3.14. ***The major Cdk5 phosphorylation site is Ser522.*** Recombinant A) GST-hCRMP1 (WT) or GST-hCRMP1 (S522A) (0.5 μM final concentration), B) GST-hCRMP2 (WT) or GST-hCRMP2 (S522A), and C) GST-hCRMP4 (WT) or GST-hCRMP4 (S522A) was incubated with 2 mU of either p35/Cdk5 or p25/Cdk5 in the presence of radiolabelled ATP for 30 min before reactions were subject to SDS-PAGE and autoradiography.

3.2.2.1.2 p35/Cdk5 phosphorylates CRMP isoforms to a greater extent than p25/Cdk5 *in vitro*

To establish the optimal assay parameters for kinetic analysis, initial stoichiometric values were determined by incubating either p35/Cdk5 or p25/Cdk5, at specific activities of 0.2 mU and 2 mU, with each isoform (0.5 μM final concentration) in the presence of MgATP [γ^{32}-P] for the following time points: 5, 10, 20, 30, and 60 min. Following the assay, samples were subjected to SDS-PAGE and then autoradiography (***Figure 3.15 – Figure 3.17***). Stoichiometry was calculated as described in ***section 2.2.9.3***. The phosphorylation kinetics, K_m (half the active sites possessing substrate) and V_{max} (the maximal velocity that the reaction can reach when all the enzyme catalytic sites are occupied) were calculated as described in ***section 2.2.9.4***.

3.2.2.1.2.1 Collapsin response mediator protein 1 (CRMP1)

The maximum stoichiometry achieved following 1 hr incubation with p35/Cdk5 and CRMP1 was 0.75 mol/mol (***Figure 3.15B***). In comparison, a maximum stoichiometry of 0.58 mol/mol was achieved following incubation with p25/Cdk5 during this time period (***Figure 3.15D***). Phosphorylation kinetic parameters were determined for each complex by incubating 0.2 mU of either p35/Cdk5 or p25/Cdk5 with CRMP1 in the presence of radiolabelled ATP for 10 min. The calculated V_{max} for the p35/Cdk5 complex (0.7349 pmol/min) was dramatically higher than the V_{max} calculated for p25/Cdk5 (0.2 pmol/min). In contrast, the K_m values obtained for each of the complexes did not differ dramatically, with p35/Cdk5 having a K_m value of 1.3 and p25/Cdk5 a K_m value of 1.5. This suggests that the affinity of the Cdk5-CRMP1 interaction does not differ between p35 and p25 but that the p35/Cdk5 complex can phosphorylate CRMP1 to a faster rate (which may be related to product off rate rather than substrate on-rate), at least when compared to the generic MBP substrate.

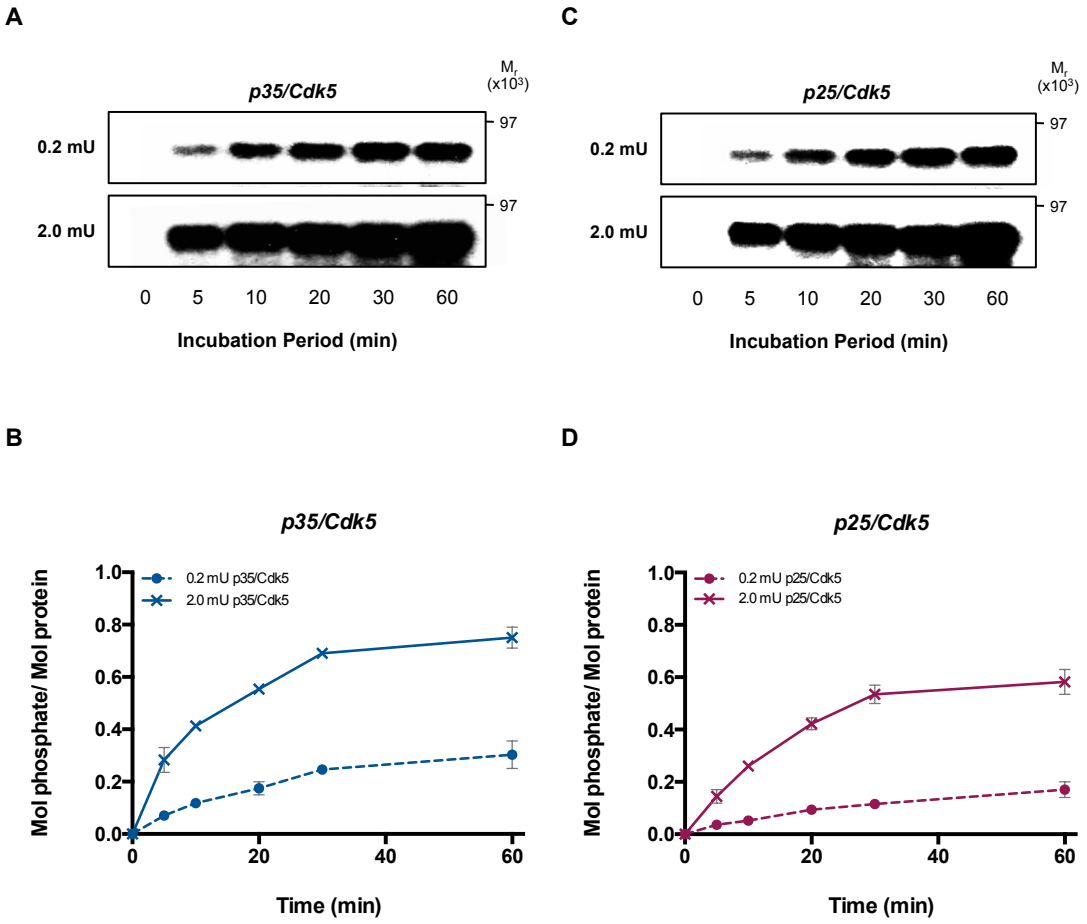


Figure 3.15. ***Kinetic analysis of CRMP1 phosphorylation with the major Cdk5 complexes.*** Recombinant CRMP1 (0.5 μM final concentration) was incubated with 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n = 3.

3.2.2.1.2.2 Collapsin response mediator protein 2 (CRMP2)

CRMP2 was phosphorylated to a greater extent by p35/Cdk5 compared to p25/Cdk5, reaching a maximal stoichiometry of 0.698 mol/mol after 1 hr incubation with p35/Cdk5 (***Figure 3.16B***). A maximal stoichiometry of 0.31 mol/mol was achieved for CRMP2 following 1 hr incubated with p25/Cdk5 (***Figure 3.16D***). Phosphorylation kinetic parameters were established by incubating 0.2 mU of either p35/Cdk5 or p25/Cdk5 with CRMP2 in the presence of radiolabelled ATP for 10 min. As with CRMP1, the calculated V_{max} values were higher for p35/Cdk5 compared to p25/Cdk5. The p35/Cdk5 complex had a calculated V_{max} value of 0.72 pmol/min whereas the p25/Cdk5 complex had a lower value of 0.18 pmol/min. However, the calculated K_m values were the same for the p35/Cdk5 (1.65 μM) and p25/Cdk5 complex (1.66 μM) suggesting that the affinity of the Cdk5-CRMP2 interaction does not differ when Cdk5 is complexed to p35 or p25.

3.2.2.1.2.3 Collapsin response mediator protein 4 (CRMP4)

As with both CRMP1 and CRMP2, p35/Cdk5 phosphorylated CRMP4 to a greater stoichiometry than p25/Cdk5 following 1 hr incubation. The p35/Cdk5 complex achieved a maximal stoichiometry of 1.035 mol/mol (***Figure 3.17B***), much greater in comparison to the 0.68 mol/mol stoichiometry achieved for the p25/Cdk5 complex (***Figure 3.17D***). Once again, there is a large difference between the calculated V_{max} values calculated for each complex. Calculated V_{max} for the p35/Cdk5 complex was 0.665 pmol/min compared to a lower V_{max} of 0.155 pmol/min for p25/Cdk5. However, as with the other isoforms, the K_m values do not differ dramatically between p35/Cdk5 (0.4 μM) and p25/Cdk5 (0.3 μM) suggesting that the affinity of the Cdk5-CRMP4 interaction does not differ between p35 and p25.

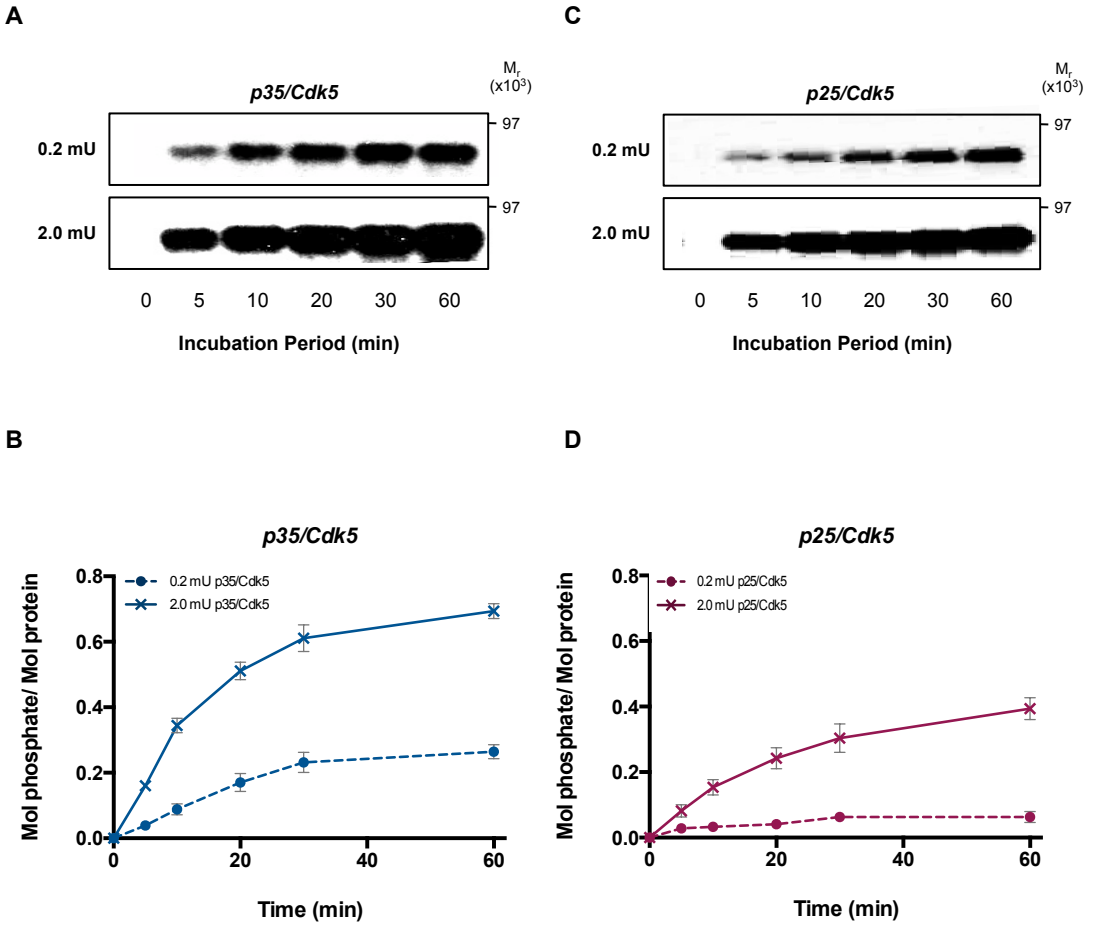


Figure 3.16. ***Kinetic analysis of CRMP2 phosphorylation with the major Cdk5 complexes.*** Recombinant CRMP2 (0.5 μM final concentration) was incubated with 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n = 3.

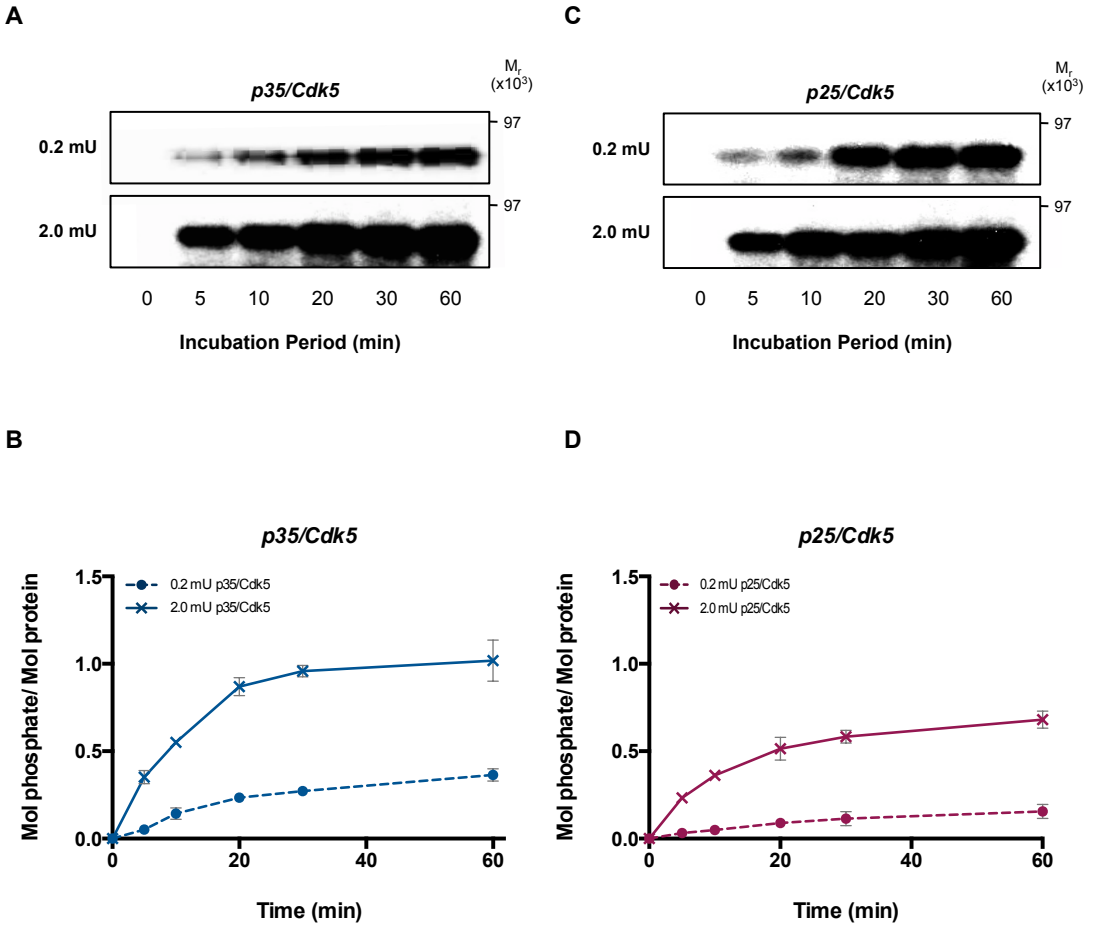


Figure 3.17. ***Kinetic analysis of CRMP4 phosphorylation with the major Cdk5 complexes.*** Recombinant CRMP4 (0.5 μM final concentration) was incubated with 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n =3.

3.2.3.2 Cdk5 *in vitro* tau phosphorylation site is a class 1 substrate

Multiple phosphorylation sites on tau are proposed as possible targets for Cdk5: Thr153, Thr181, Ser195, Ser199, Ser202, Thr205, Thr212, Ser214, Thr231, Ser235, Ser396, Ser404 (Hanger, D. *Tau phosphorylation sites*. Retrieved October 24, 2012, from http://cnr.iop.kcl.ac.uk/hangerlab/tautable). In order to identify the primary Cdk5 *in vitro* phosphorylation sites on tau, recombinant WT human tau (0.5 μM final concentration) was incubated with each Cdk5 complex in the presence of MgATP [γ32-P] for the times indicated and the phospho-tau subjected to phosphopeptide mapping (***Figure 3.18A***). To affirm that any phosphopeptides detected during mass spectrometry (MS) analysis were attributed to Cdk5 phosphorylation, p35/Cdk5 and p25/Cdk5 were also pre-incubated with Cdk inhibitors, purvalanol A and roscovitine, prior to incubation with tau. Incubation of recombinant WT human tau in the absence of either complex served as a negative control.

Following in-gel digestion with trypsin and MS analysis of Cdk5 phosphorylated tau; two phosphopeptides were identified for both Cdk5 complexes corresponding to sites Ser202 and Thr205, while no phosphopeptides were detected in the tau alone sample or those that had been treated with Cdk inhibitors. However, there was poor sequence coverage following the tryptic digestion of all samples and thus the analysis was repeated using lys-C instead of trypsin. Once again, no phosphopeptides were detected in tau alone samples or the samples in which Cdk5 was pre-incubated with either purvalanol A or roscovitine. This time, a single phosphopeptide, VAVVRTPPKpSPSSAK, corresponding to a single phosphorylation site (Ser235) was detected in the samples corresponding to both p35/Cdk5 and p25/Cdk5 incubation (***Figure 3.18B***). Ser202 and Thr205 failed to be detected suggesting that these may be minor sites compared to Ser235.

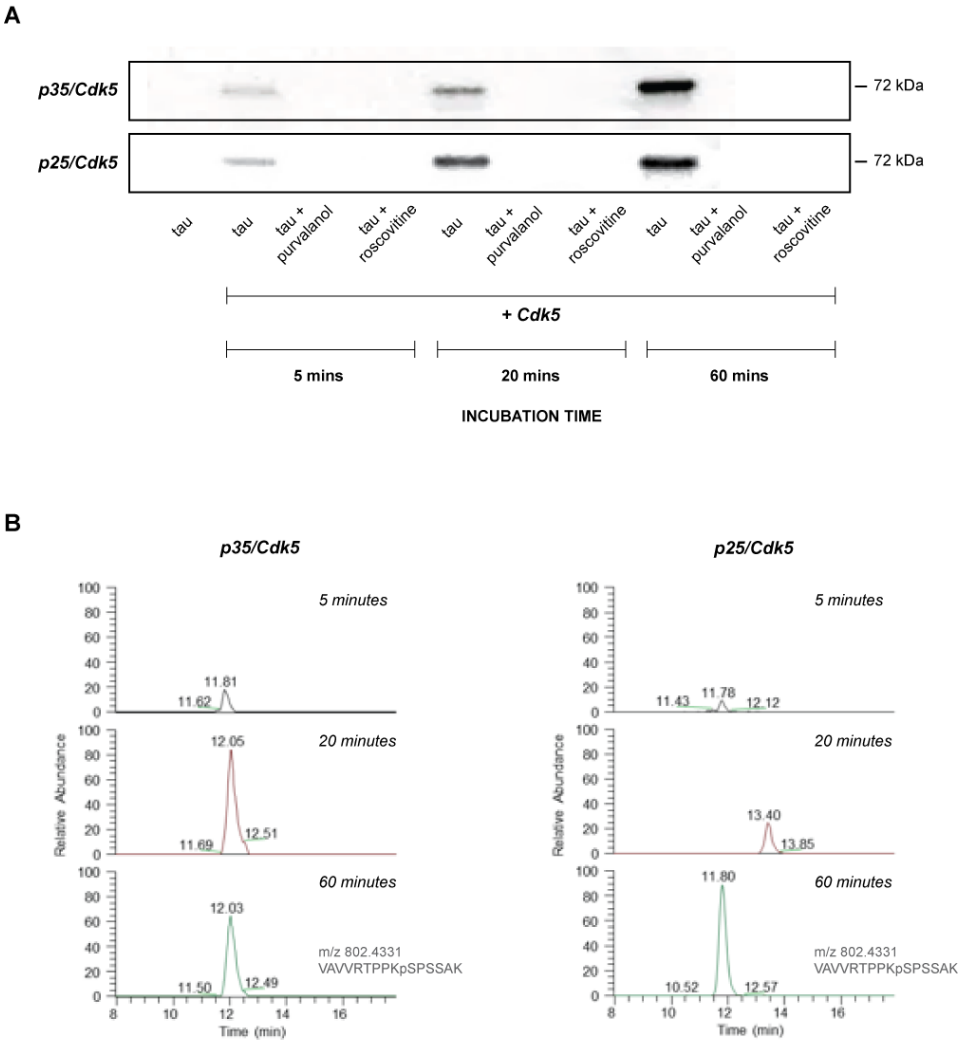


Figure 3.18. ***Both Cdk5 complexes phosphorylate tau.*** A) Recombinant GST-tau (0.5 µM final concentration) was incubated with either p35/Cdk5 or p25/Cdk5, with or without purvalanol A or roscovitine pre-incubation, in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated then subjected to SDS-PAGE followed by Colloidal Coomassie blue staining and autoradiography. B) Following the aforementioned methods, tau bands were digested with Lys-C and phosphopeptides isolated and identified as described in ***Chapter 2.2.11.1***.

3.2.3.2.1 Both Cdk5 complexes phosphorylate tau to the same extent

The degree of tau phosphorylation by p35/Cdk5 and p25/Cdk5 was directly compared by incubating recombinant WT human tau (0.5 μM final concentration) with either 0.2 mU or 2.0 mU of each complex in the presence of MgATP [γ32-P] for the times indicated (***Figure 3.19***).

Over 1 hr incubation period, tau was phosphorylated to the same extent by both Cdk5 complexes. A maximal stoichiometry of 0.55 mol/mol was reached following 60 min incubation with p35/Cdk5 (***Figure 3.19A***), while a similar maximal stoichiometry of 0.58 mol/mol was reached for p25/Cdk5 in the same time period (***Figure 3.19B***).

More detailed phosphorylation kinetic parameters were established for both of the Cdk5 complexes by incubating p35/Cdk5 or p25/Cdk5 with varying concentrations of tau in the presence of radiolabelled ATP for 10 min. The calculated V_{max} for p35/Cdk5 was 0.63 pmol/min while the V_{max} for p25/Cdk5 was 0.26 pmol/min, suggesting that p35/Cdk5 can phosphorylate tau to a greater velocity than p25/Cdk5. There was a higher K_m value calculated for p35/Cdk5 (1.89 μM) compared to p25/Cdk5 (1.419 μM). Overall, the V_{max}/K_m ratios were 0.3 and 0.2, respectively.

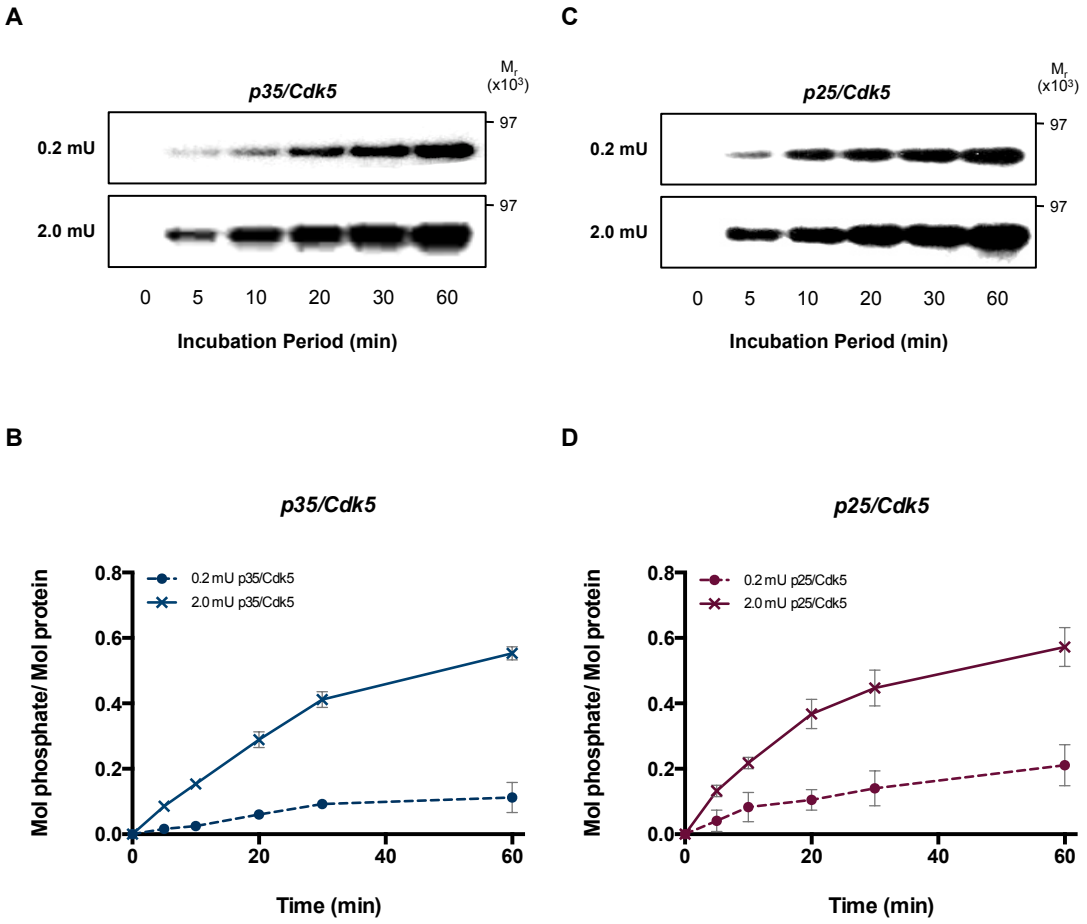


Figure 3.19. ***Kinetic analysis of tau phosphorylation with the major Cdk5 complexes***. Recombinant tau (0.5 μM final concentration) was incubated with 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n = 3.

3.2.3.2.2 Cdk5 primes tau for phosphorylation at Ser231 by GSK3

Phospho-mapping of Cdk5 phosphorylated tau identified Ser235 as the major *in vitro* Cdk5 phosphorylation site. It has been previously proposed that GSK3 requires phosphorylation at this residue for subsequent phosphorylation of tau at residue Thr231 (Li *et al*., 2006).

To confirm that Cdk5 can prime tau for subsequent GSK3 phosphorylation, a linked assay was performed with Cdk5 and GSK3. Cdk5 (1 mU) was incubated with recombinant WT human tau (0.5 μM final concentration) in the presence of non-radiolabelled ATP for 30 min prior to the addition of GSK3 (1 mU) for an additional 30 min. Following termination of the reaction, each reaction was subjected to SDS-PAGE and immunoblotted with phospho-specific antibodies to the Ser235 and Thr231 phosphorylation sites on tau (***Figure 3.20***).

Phosphorylation of Thr231 on tau was only observed following incubation with both Cdk5 and GSK3 confirming Cdk5 as a priming kinase and providing additional evidence that Cdk5 phosphorylates tau at Ser235. As Ser202/Thr205 had been identified as minor targets for Cdk5 in the phosphopeptide mapping, phosphorylation of these sites by Cdk5 was also investigated using phosphospecific antibodies to anti-Tau Ser202 and anti-Tau Thr205 (***Figure 3.20***). Interestingly, Cdk5 did induce some phosphorylation at both Ser202 and Thr205 and the phosphorylation of Thr205 may prime for additional phosphorylation at Ser202 by GSK3.

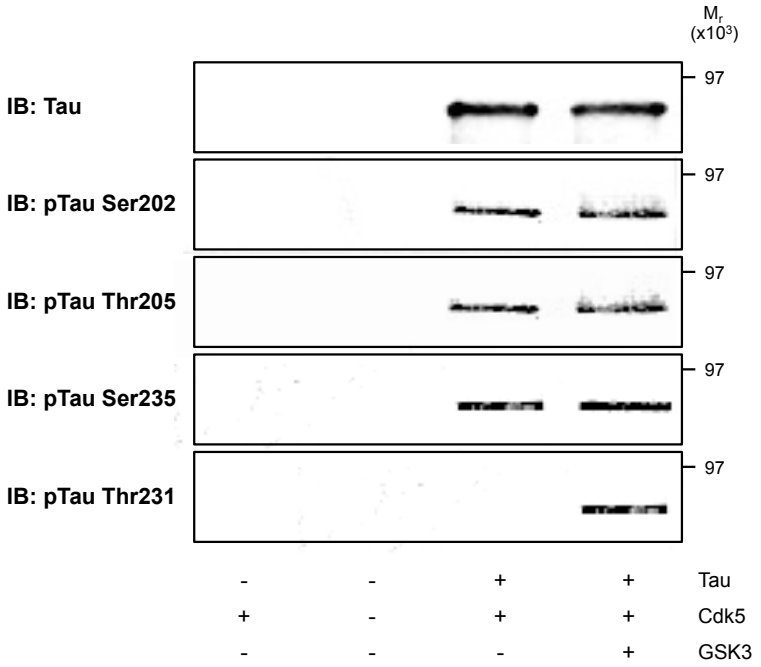


Figure 3.20. ***Validation of phospho-tau antibodies.*** Recombinant GST-hTau (WT) (0.5 μM final concentration) was incubated with 1 mU of p25/Cdk5 in the presence of non-radiolabelled ATP for 30 min. Subsequently, 1 mU GSK3 was added to the reaction and incubated for a further 30 min period. Following termination of the reaction, each reaction was subject to SDS-PAGE and immunoblotted with antibodies phospho-specific to Ser202, Thr205, Ser235 and Thr231. Blots are representative of two independent experiments.

3.2.3.3 Cdk5 phosphorylation of dynamin 1

GSK3 phosphorylation of dynamin 1 at Ser774 occurs following prior phosphorylation of Ser778 by Cdk5, modulating activity-dependent bulk endocytosis (ADBE) (Clayton *et al*., 2010). The Ser778 site of this substrate is flanked by both N- and C-terminal basic residues (RSPTSp**SP**SPQRR) but does not conform to the optimal sequence identified by peptide analysis above (no basic residue at +2/3).

To investigate dynamin 1 phosphorylation by Cdk5, 0.2 mU and 2.0 mU of either p35/Cdk5 or p25/Cdk5 were incubated with dynamin 1 (0.5 μM final concentration) and radiolabelled ATP as described in ***section 2.2.9.3***. Dynamin 1 was well phosphorylated by Cdk5, approaching a stoichiometry of 0.76 mol/mol after 60 min incubation with p35/Cdk5 (***Figure 3.21A).*** The stoichiometry obtained for p25/Cdk5 was of a similar magnitude, reaching a stoichiometry of 0.69 mol/mol after 60 min incubation with p25/Cdk5 (***Figure 3.21B)***.

Subsequently, 0.5 mU of either p35/Cdk5 or p25/Cdk5 was incubated with varying concentrations of dynamin 1 in the presence of MgATP [γ32-P] for 10 min. The V_{max} value of 0.309 pmol/min obtained for p35/Cdk5 was slightly greater than the V_{max} value of 0.187 pmol/min obtained for p25/Cdk5 however p25/Cdk5 had a lower K_m value (2.04 μM) than p35/Cdk5 (2.78 μM). Thus, the V_{max}/K_m ratio did not differ between the two complexes with both complexes having a V_{max}/K_m ratio of 0.1.

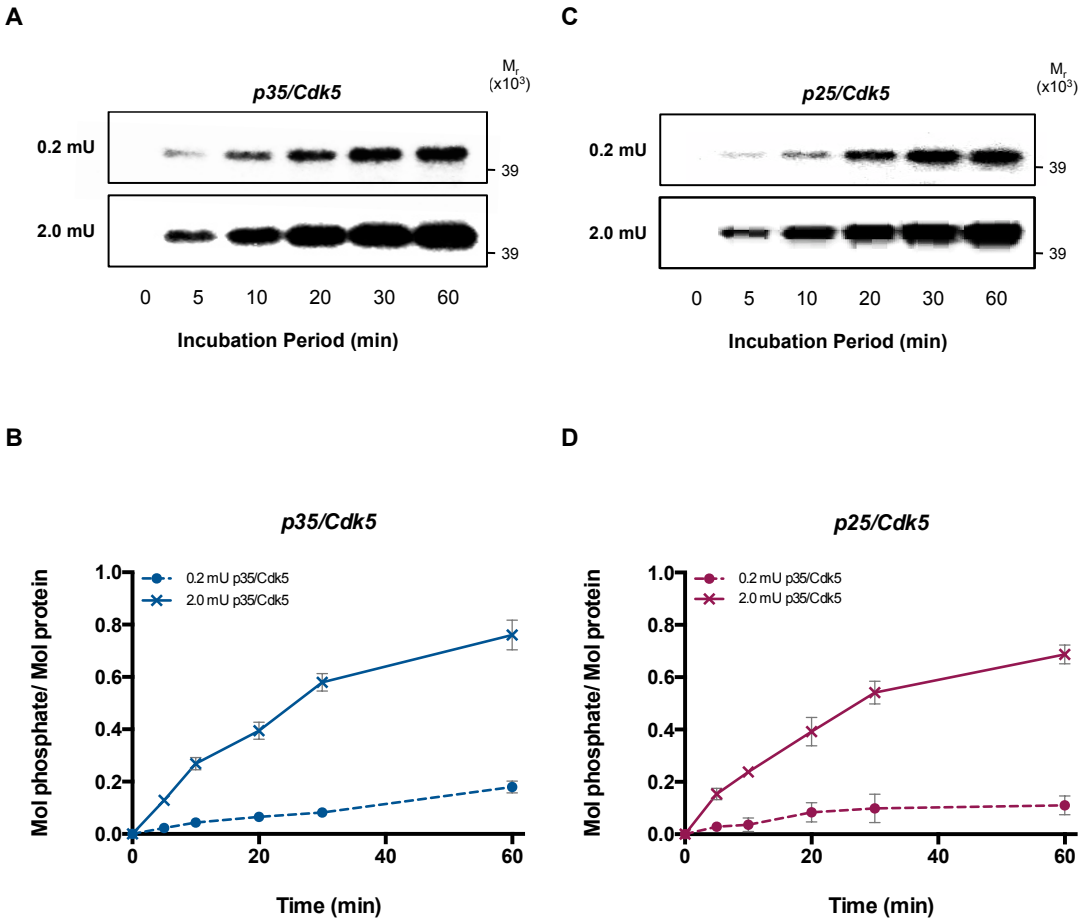


Figure 3.21. ***Kinetic analysis of dynamin 1 phosphorylation by the major Cdk5 complexes***. Recombinant human WT dynamin 1 (0.5 μM final concentration) was incubated with either 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n 3.

3.2.3.4 Phosphorylation of full-length substrates lacking basic residues

3.2.3.4.1 tumour suppressor protein, p53

Cdk5 reportedly phosphorylates p53 at two different SP motifs: Ser315 (NTSSp**SP**QPKKK) (Class 2) and Ser33 (NNVLp**SP**LPSQA) (Class 4)(Zhang *et al*., 2002). The previous peptide work had suggested Class 2 and Class 4 sequences were poor substrates for Cdk5 due to the lack of basic N-terminal residues therefore implying p53 would be a relatively weak substrate of Cdk5.

To investigate p53 phosphorylation by Cdk5, both major complexes of Cdk5 were incubated with recombinant WT human p53 (0.5 μM final concentration) and radiolabelled ATP as described in ***section 2.2.9.3***. The p35/Cdk5 complex phosphorylated p53 to a stoichiometry of 0.34 mol/mol after 1 hr incubation (***Figure 3.22A***), while p25/Cdk5 phosphorylated p53 to around 0.16 mol/mol in this time period (***Figure 3.22B***). Of course, it is not possible to say whether this phosphorylation occurs at more than one site in these experiments but this substrate was relatively weak compared to CRMPs, tau, and dynamin 1.

In an attempt to determine V_{max} and K_m values for p53 for a more direct comparison with Class 1 substrates, 2.0 mU of either p35/Cdk5 or p25/Cdk5 was incubated with varying concentrations of p53, in the presence of MgATP [γ32-P] for 10 min. However, under these assay conditions (comparable to those used with the earlier substrates) phosphorylation failed to reach twice above background and therefore it was not possible to confidently establish the phosphorylation kinetic parameters.

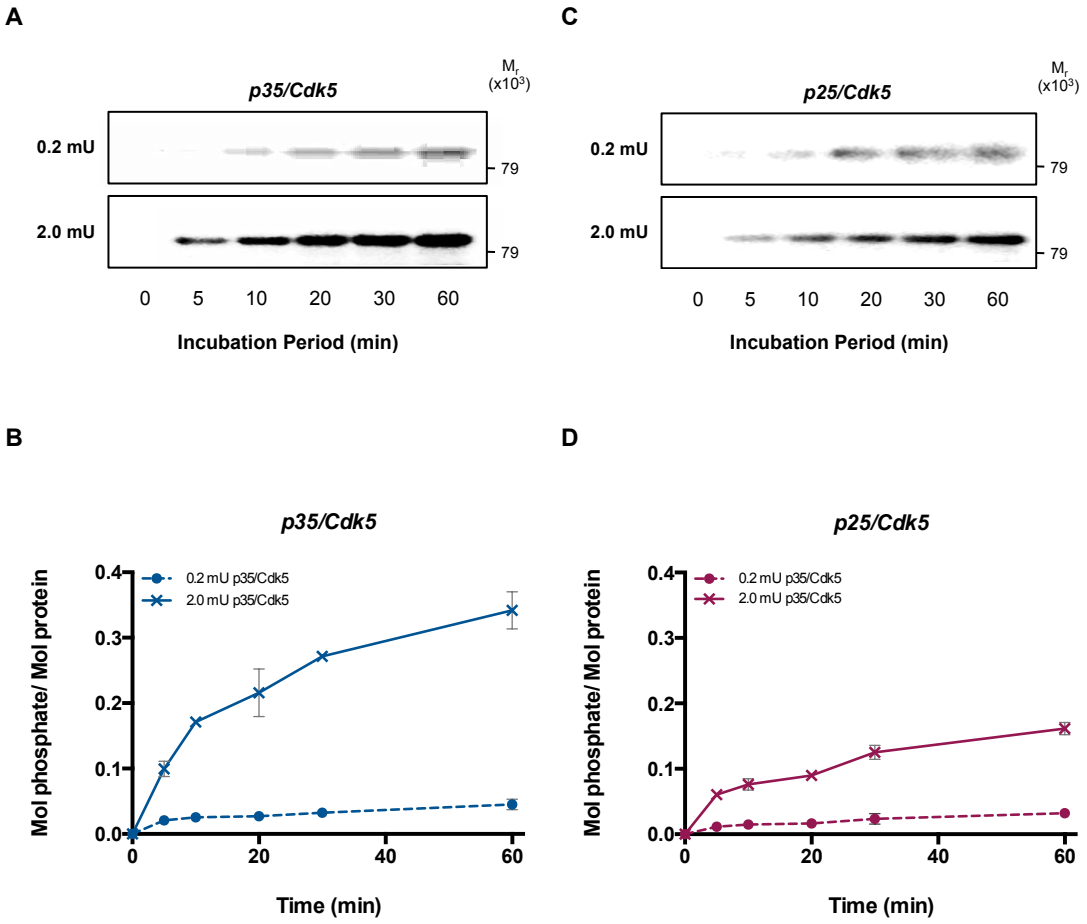


Figure 3.22. ***Kinetic analysis of p53 phosphorylation by the major Cdk5 complexes***. Recombinant human WT p53 (0.5 μM final concentration) was incubated with either 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n 3.

3.2.3.4.2 Cdk5 phosphorylation of PPARγ

Cdk5-mediated phosphorylation of PPARγ at serine 273 has been linked to obesity induced by high fat feeding in mice (Choi *et al*., 2010). The amino acid sequence surrounding Ser273 (TTDKp**SP**FVIY) contains an N-terminal lysine residue but no C-terminal basic residues therefore it is predicted that PPARγ would not be a substrate of Cdk5 phosphorylation based on the prior peptide assay.

In order to evaluate the validity of PPARγ as a substrate of Cdk5, both major complexes of Cdk5 were incubated with 0.5 μM of either PPARγ1 or PPARγ2 and radiolabelled ATP as described in ***Chapter 2.2.9.3***. Cdk5 phosphorylated PPARγ1 with relatively low stoichiometry, reaching a maximal stoichiometry of less than 0.15 mol/mol even after 1h incubation with 2 mU of p35/Cdk5 and only 0.034 mol/mol with 2 mU of p25/Cdk5 after 1h (***Figure 3.23***). The maximal stoichiometry values were slightly greater with PPARγ2; stoichiometry reached 0.2 mol/mol after 1h with 2 mU of p35/Cdk5 and 0.045 mol/mol with p25/Cdk5 (***Figure 3.24***).

Using the information obtained from the stoichiometric analysis, 2.0 mU of either p35/Cdk5 or p25/Cdk5 was incubated with varying concentrations of PPARγ1 and PPARγ2, independently, in the presence of MgATP [γ32-P] for 10 min. However, the activity in these assay conditions was too low to allow accurate calculation of the enzyme kinetics of these reactions. Additionally, work in our lab established that a PPARγ2 with Ser273 mutated to alanine was phosphorylated to the same level as WT PPARγ2, suggesting that this low level of phosphorylation by Cdk5 is not at Ser273 (Louise Saul, personal communication). As a result, this substrate was not included in further investigations.

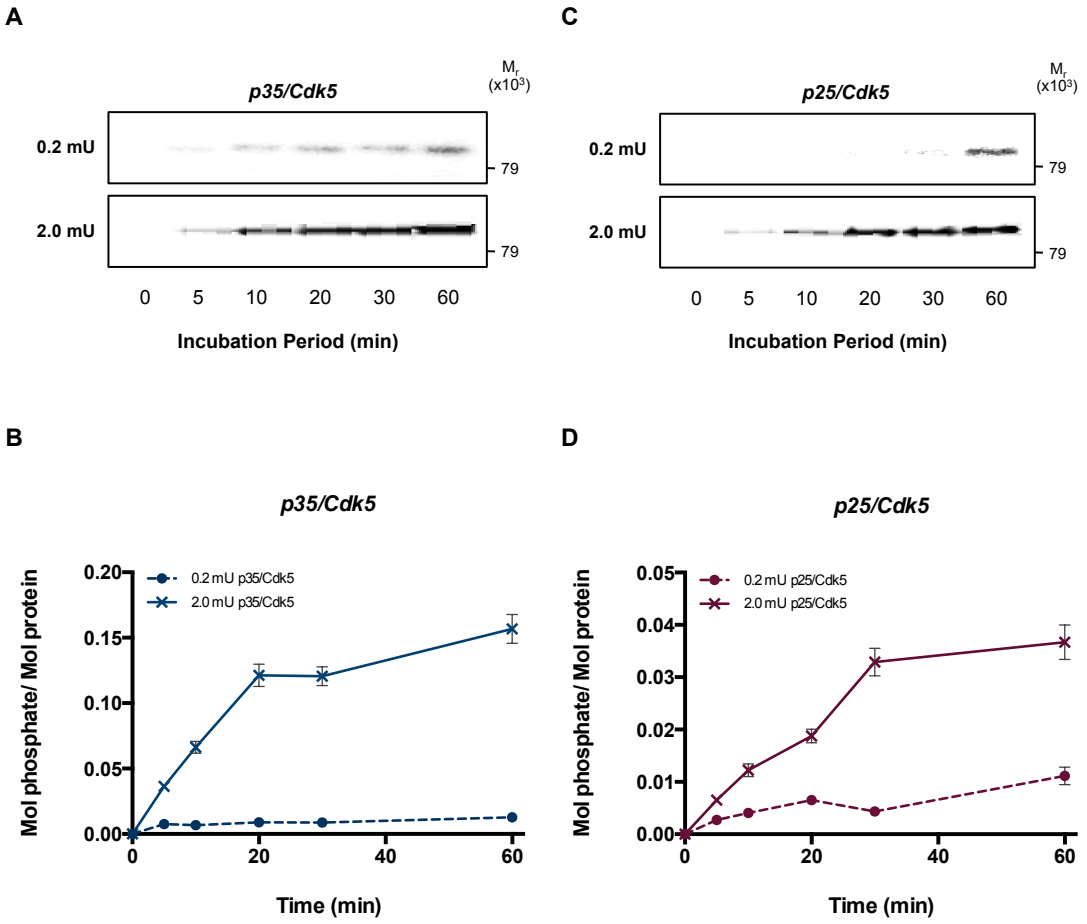


Figure 3.23. ***Kinetic analysis of PPARγ1 phosphorylation by Cdk5 complexes.*** Recombinant human WT PPARγ1 (0.5 μM final concentration) was incubated with either 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n 3.

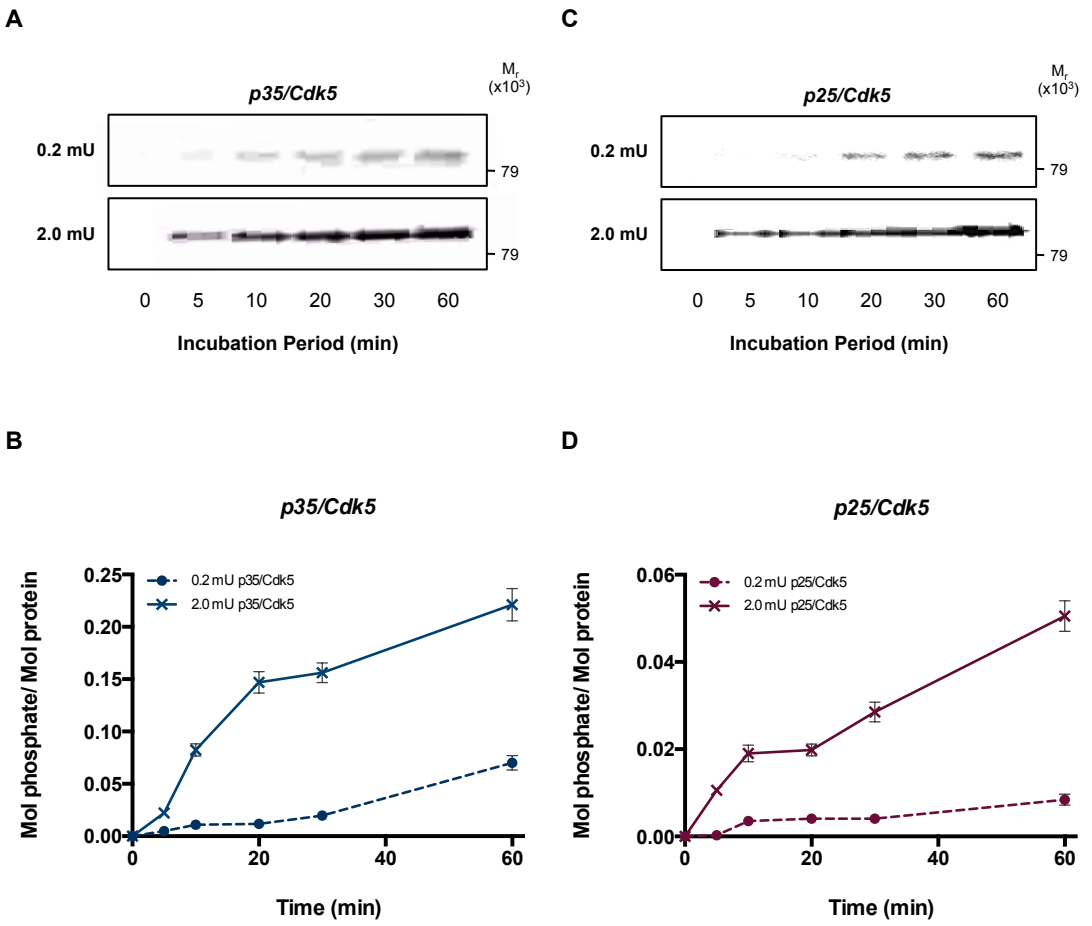


Figure 3.24. ***Kinetic analysis of PPARγ2 phosphorylation by Cdk5 complexes.*** Recombinant human WT PPARγ2 (0.5 μM final concentration) was incubated with either 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n 3.

3.2.3.5 The role of proline residues in Cdk5 recognition

It is well established that Cdk5 requires a proline residue at +1 relative to the phosphoacceptor residue to permit phosphorylation to occur. Interestingly, I found that Cdk5 phosphorylated peptide substrates that contained a proline residue located N-terminally (-2) from the phosphoacceptor residue but failed to phosphorylate such a peptide substrate when an additional proline residue was present at +2 (***Figure 3.6***). Such a sequence is contained within the transcription factor Mef2a surrounding Ser408 (SEPIp**SP**PRDR), a residue reportedly phosphorylated by Cdk5 which results in inhibition of Mef2a transcriptional activity leading to apoptosis of cortical neurons following induction by neurotoxicity (Gong *et al*., 2003). This site contains the proline (-2) beneficial to Cdk5 phosphorylation (as well as a basic residue at +3) however it also contains an additional proline (+2) and an asparate (+4), the residues found to be antagonistic to Cdk5 phosphorylation during the peptide analysis, bringing into question whether Ser408 of Mef2a is really a substrate for Cdk5.

To investigate Mef2a as a Cdk5 substrate, 0.2 mU and 2.0 mU of p35/Cdk5 or p25/Cdk5 was incubated with 0.5 μM Mef2a in the presence of MgATP [γ32-P] for the times indicated (***Figure 3.25***). At each time point, aliquots of the reactions were removed and subjected to gel electrophoresis to separate radiolabelled ATP from protein substrate, and the labelled substrate was visualised by autoradiography, prior to excision and quantification using liquid scintillation counting.

The stoichiometry of phosphorylation for Mef2a was relatively poor. Phosphorylation of Mef2a by p35/Cdk5 failed to achieve a stoichiometry greater than 0.1 mol/mol within 60 min (***Figure 3.25A***) while phosphorylation of Mef2a by p25/Cdk5 was even poorer, <0.05 mol/mol after 60 min (***Figure 3.25B***).

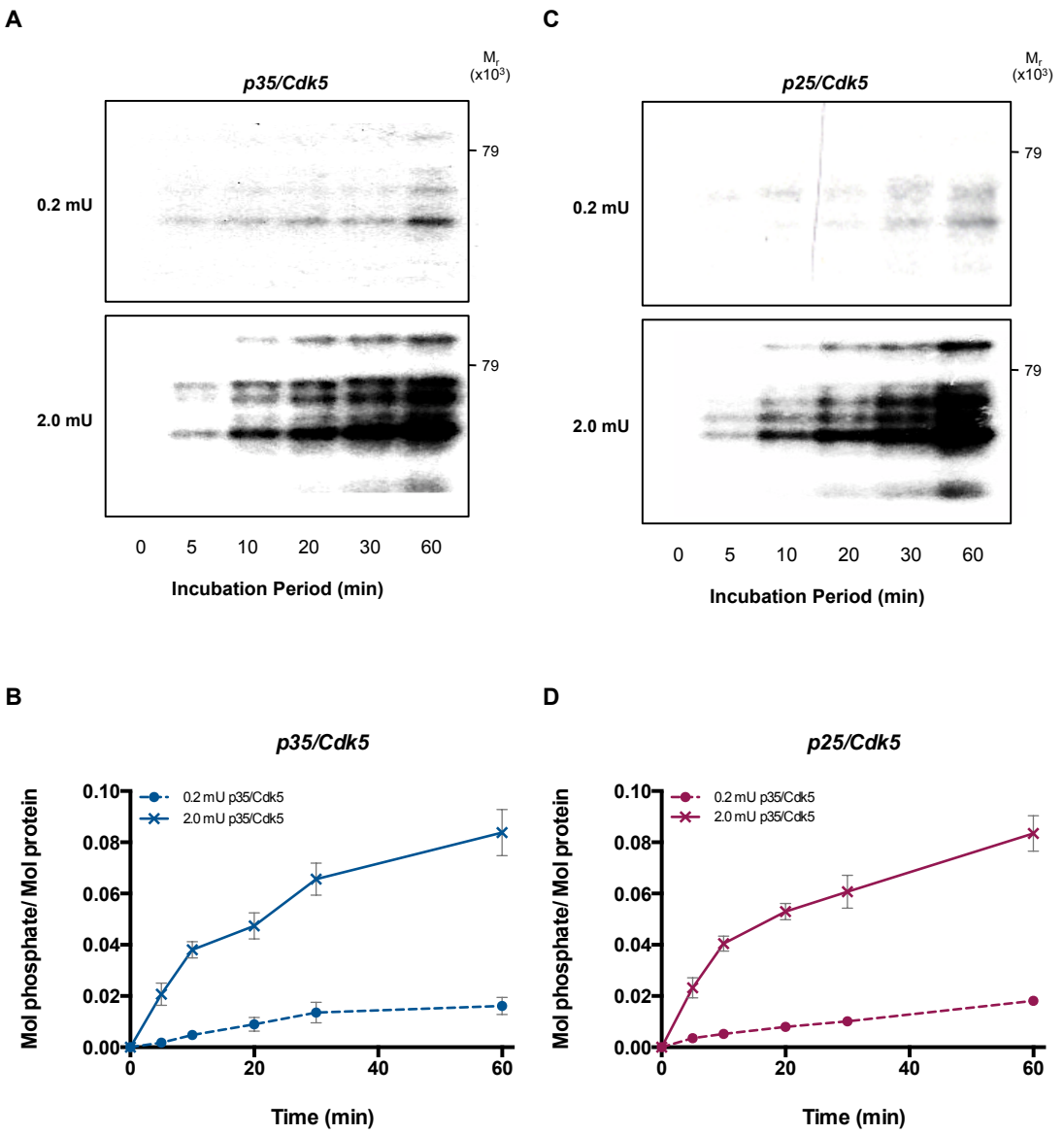


Figure 3.25. ***Kinetic analysis of Mef2a phosphorylation by the major Cdk5 complexes***. Recombinant Mef2a (0.5 μM final concentration) was incubated with either 0.2 mU or 2.0 mU of p35/Cdk5 (A and B) or p25/Cdk5 (C and D) in the presence of Mg [γ-32P] ATP (10 mM MgCl and 0.1 mM ATP) for the times indicated. At each time point, 5 pmol of protein was removed and added to SDS-PAGE loading buffer to terminate the reaction. Aliquots were then heated at 70°C before being subjected to SDS-PAGE. Subsequently, gels were stained with Coomassie Brilliant Blue (CBR-250), dried, and exposed to autoradiography film (A and C). The radiolabelled bands were excised and radioactivity incorporated measured by scintillation counting (B and D). Data expressed as mean ± SEM, n 3.

The data in ***Figure 3.25*** was obtained using the 82 kDa band. However, following gel electrophoresis and Coomassie staining of the gel, bands corresponding to varying molecular weights (82 kDa, 50 kDa, 42 kDa, 40 kDa, and 25kDa) were visible suggesting that the recombinant Mef2a may have been degraded and this could have contributed to the poor phosphorylation by Cdk5.

To quantify the level of protein degradation, and obtain a more accurate stoichiometry for full length Mef2a, a protein standard curve was generated using BSA (1.0 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml). The amount of each Mef2a product could then be estimated (***Figure 3.26***). Less than 20% of the protein in the recombinant Mef2a was in the 82 kDa band (expected full length) therefore it is difficult to be confident that the phosphorylation observed is representative of full length Mef2a. Thus, detailed phosphorylation kinetic parameters could not be determined on these protein preparations.

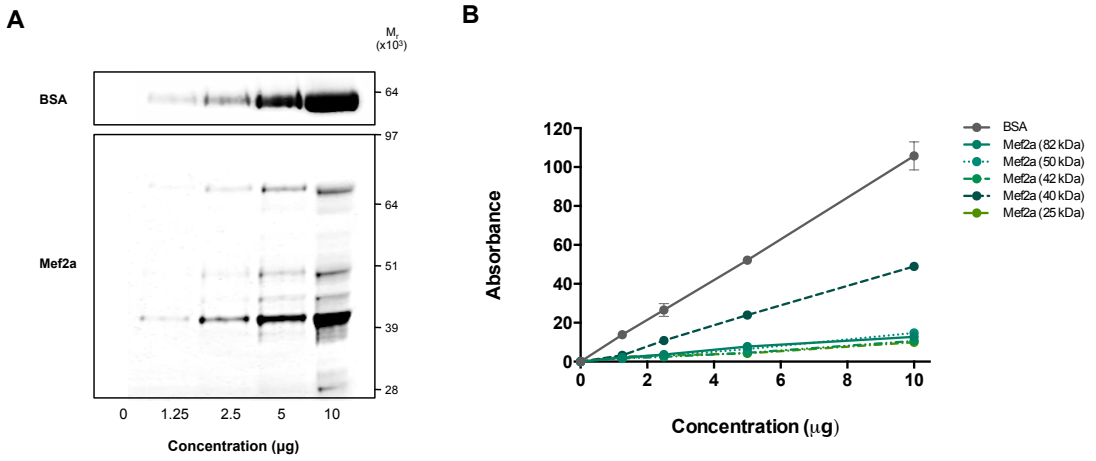


Figure 3.26. ***BSA standard curve for determining protein concentration of Mef2a preparation*****.** A standard curve was obtained for BSA by obtaining the absorbance readings of known concentrations of BSA. A) BSA at four concentrations (1.0 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml) was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (CBR-250) along with known amounts of Mef2A protein. B) Absorbance readings for the indicated concentrations were obtained using the Licor imaging system. Data expressed as mean $\pm$ SEM, n = 2.

3.3 DISCUSSION

Cdk5 is depicted as a highly promiscuous kinase actively targeting a vast array of substrates that are not confined by a specific consensus. As a result of the observation that levels of p25 are elevated in Alzheimer's disease, activation of Cdk5 by the truncated cofactor is believed to enhance Cdk5 activity and alter specificity thereby contributing to the pathogenesis of neurodegenerative disease however there has been little evidence to support either claim. Therefore, the aim of this chapter was to establish a consensus for Cdk5 substrate selection and compare the inherent activities of the p25/Cdk5 and p35/Cdk5 complexes.

3.3.1 Redefining the Cdk5 consensus

Most protein kinases that have been studied in detail have a minimum and preferred consensus peptide sequence that contributes to substrate specificity. However, the multitude of proposed Cdk5 substrates appear to only have a single common primary structural feature, a Pro residue at the +1 position relative to the phosphoacceptor site, a property shared by the majority of the CGMC family of protein kinases. On the contrary, biochemical and structural studies have suggested that the Cdk family members discriminate substrates through additional differences in the primary sequence surrounding the phosphoacceptor residue (Echalier *et al*., 2010; Brown *et al*., 2007) implying that a Cdk5 substrate consensus should exist. Thus, the initial aim of this chapter was to isolate residues that are important for Cdk5 phosphorylation and, ultimately, redefine the degenerate Cdk5 consensus.

3.3.1.1 Important residues that guide Cdk5 substrate selection

The current consensus sequence described for the Cdk family members suggests a preference for a basic residue towards the C-terminus of the phosphoacceptor residue

(Beuadette *et al*., 1993; Shetty *et al*., 1993) however many of the substrates proposed for Cdk5 do not possess this residue therefore questioning its importance for Cdk5 specificity. In the present study, *in vitro* investigation into the primary sequence requirements of Cdk5 uncovered an unexpected, highly selective, peptide substrate profile for Cdk5. Whilst basic residues towards the C-terminus were found to be favourable for Cdk5 phosphorylation, surprisingly, the N-terminal residue at -2 actually had more influence on the rate of peptide phosphorylation by Cdk5. Peptides with a proline residue at -2 had high affinity for Cdk5 phosphorylation whereas Cdk5 selectively failed to phosphorylate peptides that lacked a proline residue at this position. In correlation with this finding, previous work using an oriented degenerate peptide library with p35/Cdk5, along with other Ser/Thr protein kinases, determined that the optimal sequence for Cdk5 when complexed to p35 contained either a histidine (selectivity value = 2.5) or a proline (selectivity value = 2.1) at the -2 position (Songyang *et al*., 1996). Taken together, these results suggest that the amino acid in the -2 position contributes to Cdk5 substrate selection and the presence of a proline or basic residue increases the probability of this substrate being phosphorylated by Cdk5.

In addition to having a positive effect on Cdk5 phosphorylation, proline residues were also found to have a detrimental effect on substrate phosphorylation. The presence of a proline residue directly following the SP motif (+2) resulted in complete ablation of Cdk5 phosphotransferase activity, even in peptides containing an N-terminal proline residue (-2). The amino acid substitution work in this chapter provides evidence to suggest that the primary sequence in direct proximity of the phosphoacceptor residue is critical for Cdk5 substrate selection, and the sequence determinants could be used

to identify potential novel substrates while providing confidence (or, alternatively, questioning) that proposed substrates are Cdk5 targets.

3.3.1.2 Validation of consensus in full-length substrates

The second goal of this chapter was to investigate whether the primary sequence requirements identified in the peptide work were valid in full-length protein substrates. Interestingly, the major site on tau found in this study to be phosphorylated by Cdk5 *in vitro* possessed the N-terminal proline (at -2) and basic residues both N- and C-terminal within ±5 of the phosphoacceptor residue (TPPK**SP**SSAK). In addition, CRMP1 and CRMP2 were excellent substrates for Cdk5 *in vitro* and both contain basic residues N- and C-terminal to the phosphoacceptor site (lysine residues at +2 and -2). Interestingly, CRMP4, which contains arginine residues at +2 and -2, was found to be the best *in vitro* substrate of Cdk5 suggesting that even small changes in amino acid properties modulate Cdk5 phosphorylation rate.

Substrates that lacked the optimal residues were phosphorylated to a lesser extent. For example, PPARγ was a relatively poor substrate for Cdk5 *in vitro*. These results provide further confidence in the optimal Cdk5 consensus and question whether potential substrates that do not meet consensus requirements are *bona fide* Cdk5 substrates. However, it is important to note that kinases have devised several strategies in addition to primary sequence to specifically recognise their substrates. For example, additional docking sequences can increase the specificity of kinases towards their substrates. This is especially well characterised for the Cdks: an RXL motif that is present in many of their substrates binds to a hydrophobic patch on the catalytic subunit, increasing the affinity of the kinase for the substrate (Schulman *et al*., 1998). Furthermore, classical MAPKs directly bind to their substrates and do not

phosphorylate peptides very well implying that primary sequence motifs may be overridden by other mechanisms such as binding. Therefore, further work is required to understand Cdk5 substrate recognition and fully validate potential Cdk5 substrates, however this work provides evidence to suggest that Cdk5 substrate selection can at least be inferred or seriously questioned by studying the primary sequence.

3.3.1.3 Consensus sequence is specific to Cdk5

Phosphorylation of peptides by Cdk5 was compared with a panel of kinases representing different branches of the CMGC family to establish if the Cdk5 peptide consensus was conserved across CMGC members. Cdk5 expressed the highest phosphotransferase towards most of the peptides studied, with the other kinases showing relatively minimal phosphotransferase towards each of the peptides, suggesting that Cdk5 substrate selection has primary sequence determinants specific to the kinase. The exception was a higher phosphotransferase activity by PCTAIRE with a peptide that Cdk5 failed to phosphorylate (KKASAPK**SP**PRPRS). This result provided confidence that this peptide was soluble and that Cdk5 did not phosphorylate it, and suggests that the primary sequence around the phosphoacceptor site of substrates can provide strong selectivity bias toward even relatively homologous protein kinases. In this case, the proline at +2 reduces phosphorylation by Cdk5 to a much greater extent than for PCTAIRE.

In hindsight, it would have been interesting to compare Cdk2 with Cdk5 on these peptides as the high homology between these enzymes suggests there is likely to be some overlap in substrate recognition. It is quite possible that the known Cdk2 substrates could also be Cdk5 substrates, thereby providing regulation outside of the cell cycle (as Cdk5 is active in terminally differentiated cells).

3.3.1.4 Perspectives and future directions for a Cdk5 consensus sequence

The results described here provide evidence to suggest that Cdk5 substrate selection is defined, at least in part, at the primary sequence level. Fully elucidating the sequence requirements of Cdk5 would require peptide studies covering more sequence combinations, as there appears to be multiple contributions to the recognition parameters for Cdk5 phosphorylation. The CRMP family of proteins were good *in vitro* substrates of Cdk5 therefore these could serve as a useful substrate to use as a template in which to study the effect of substituting residues that we predict to be important (positively or negatively) which were determined through peptide analysis. For example, substituting lysine residues to arginine residues (CRMP1/CRMP2 to CRMP4) enhanced phosphotransferase activity *in vitro*, and it would be interesting to try all 20 amino acid substitutions within this context on each and both of these sites. Of course, our previous work found that Cdk5 deletion in neurons reduced CRMP1 and CRMP2 phosphorylation at Ser522, but did not alter CRMP4 phosphorylation (Cole *et al*., 2007). Thus, although Cdk5 may prefer arginine over lysine it would appear that there are other kinases in neurons with an absolute requirement for arginine over lysine which means CRMP4 can be regulated independently of Cdk5 and the other CRMPs. Therefore, it is important to keep in mind that primary consensus sequences for kinases have evolved not only to provide the best possible target sequence for efficient phosphorylation but also to provide (or not) specificity to a given kinase(s). In addition, recognition of the sequence by phosphatases will also dictate steady state phosphorylation levels. The pSer522 motif in CRMP2 is highly resistant to dephosphorylation *in vitro* (Cole *et al*., 2008) and this is related to the presence of the same basic residues N- and C-terminal to the phosphoacceptor site that make it a better Cdk5 substrate. These may form a structure with the PO_4^{2-} that reduces accessibility for phosphatases and results in relatively high stoichiometry in

cells (Cole *et al*., 2007). This model would predict that other Cdk5 substrates with basic charged residues either side of the phosphoacceptor would also be relatively highly phosphorylated due to low rates of dephosphorylation. One could then suggest that substrates with basic residues to only one side of the phosphorylation site may have slower rates of phosphorylation by Cdk5 but allow more access to a phosphatase and be more dynamically controlled.

In conclusion, although kinases have devised several strategies to recognise substrates and substrate sequences have evolved in an environment where specificity and dephosphorylation are also factors for sequence selection, this chapter provides evidence to suggest that the primary sequence surrounding the phosphoacceptor residue contributes key information to aid in the identification and validation of Cdk5 substrates. Importantly, substrates that do not meet the minimum requirements for phosphorylation *in vitro* should be heavily scrutinised, while classification of substrates by primary sequence could allow modelling of specificity and likely stoichiometry of phosphorylation in cells.

3.3.2 Cdk5 as a potential priming kinase for GSK3 on CRMP5

CRMP5 is highly expressed during brain development and plays an important role in the regulation of neuronal polarity by inhibiting dendrite outgrowth at early developmental stages (Brot *et al*., 2013). When expressed in adults, CRMP5 is associated with neurological disorders (Brot *et al*., 2013). Results obtained with CRMP5-deficient mice stress the role of CRMP5 in the development, and synaptic plasticity, of cerebellar Purkinje cells (Yamashita *et al*., 2011). However, few studies have investigated the regulation of CRMP5 and Cdk5 has not been considered as a

CRMP5 kinase due to the lack of conservation around the Ser522 region with the other CRMPs.

3.3.2.1 Cdk5 displayed phosphotransferase activity towards CRMP5

Previous work within the laboratory (Cole *et al*., 2006) established Cdk5 as the priming kinase that allowed subsequent GSK3 phosphorylation of CRMP2. Due to the conservation of the 509-522 motif within CRMP1, CRMP2, and CRMP4, only these three isoforms were investigated as Cdk5 targets. In the present study, CRMP3 and CRMP5 were included in the analysis and it was confirmed that Cdk5 does not phosphorylate CRMP3. In contrast, this work demonstrated for the first time that Cdk5 was able to phosphorylate CRMP5, albeit at a lower rate and to a lower stoichiometry than CRMP1, CRMP2, and CRMP4. Interestingly, the sequence surrounding the Thr514 residue on CRMP5 meets some of the requirements for Cdk5 phosphorylation elucidated in the peptide studies. Of particular note is that, if Thr514 were the Cdk5 target residue then this would prime CRMP5 for regulation by GSK3 at Thr509, in a similar fashion to the other CRMPs. This would be relatively straightforward to investigate through use of T514A and T509A mutants of CRMP5.

3.3.3 Differential inherent activities of the p25/Cdk5 and p35/Cdk5 complexes *in vitro* does not account for the role of p25/Cdk5 in the pathogenesis of neurodegenerative disease

Development of the mammalian central nervous system is dependent on the tight regulation of Cdk5 activity, an activity that results following association with its regulatory subunit, p35. At present, there is common belief that truncation of this cofactor results in an increase in Cdk5 kinase activity, prolonged activation, as well as a change in cellular localisation and alteration of its substrate specificity (Patrick *et*

al., 1999), which subsequently promotes pathogenesis of neurodegenerative diseases. A major goal of this chapter was to directly compare the p35/Cdk5 and p25/Cdk5 complexes and establish potential differences in rates of substrate phosphorylation that may result from proteolytic cleavage of the p35 regulatory subunit.

3.3.3.1 Substrate profile and inherent activity is similar between complexes

If proteolytic processing of p35 to p25 results in an increase in inherent catalytic activity or an altered substrate specificity profile then it should be possible to confirm this through direct comparison of substrate phosphorylation *in vitro*. However, the two Cdk5 complexes phosphorylated a range of peptides and proteins to almost identical levels and similar rates (when activities were first matched against a generic substrate) (***Table 3.3***). Additionally, phospho-mapping identified the same phosphorylation site on tau for both complexes indicating that both complexes target substrates using the same discrimination techniques and we could find no evidence for additional sites on tau being hit by p25/Cdk5. These findings indicate that the substrate profile of Cdk5 is unaltered by the cleavage of p35 and does not support the theory that a greater inherent activity of p25/Cdk5 contributes to its potential role in the pathogenesis of neurodegenerative disease.

	p35/Cdk5			*p25/Cdk5*		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
CRMP4	0.67	0.40	1.66	0.16	0.25	0.62
CRMP1	0.73	1.26	0.59	0.19	1.46	0.13
CRMP2	0.72	1.65	0.44	0.18	1.66	0.11
Tau	0.63	1.89	0.33	0.26	1.42	0.19
Dynamin 1	0.31	2.78	0.11	0.19	2.04	0.09

Table 3.3. ***Phosphorylation kinetic parameters of Cdk5 substrates and comparison between the major Cdk5 complexes.*** Phosphorylation kinetic parameters were established by incubating equal amounts of p35/Cdk5 and p35/Cdk5 with varying concentrations of the indicated substrates in the presence of Mg [γ-32P] ATP (10 mM Mg 0.1mM ATP), for 10 minutes at 30°C. V_{max} and K_m values were calculated by plotting the substrate-velocity curves and using the Lineweaver-Burk equation. V_{max} values are in pmol/min and K_m values are in μM. Values are representative of at least two independent experiments.

3.3.3.2 Perspectives and future directions to delineate the effect of p35 proteolytic cleavage

There are two major mechanisms that could not be investigated in the *in vitro* analysis performed in this study. Firstly, if clipping of p35 alters the stability of this subunit then it is possible that p25 would accumulate to higher levels than p35. There is some evidence that p25 is more stable than p35 (Patrick *et al*., 1999). Secondly, the cleavage of p35 removes a membrane targeting sequence meaning that p25 could relocate Cdk5 to a different cellular compartment and thus target an alternative set of substrates. This seems an important aspect to investigate and a direct comparison of the global phosphoproteome of cells expressing each complex may provide evidence as to how great a change occurs when p25 is produced. This latter point may influence the interpretation of the *in vitro* system used in the present study, which compared p25/Cdk5 and p35/Cdk5 catalytic activities in a soluble assay. A myristolyation site is present on p35, tethering p35/Cdk5 to the membrane, however proteolytic cleavage of p35 by calpain results in loss of the myristolyation site and a complex that is no longer retained by the membrane. If membrane association alters Cdk5 complex structure then this may affect the rate at which it phosphorylates specific substrates. The development of a micelle/vesicle assay system to study p35/Cdk5 substrate phosphorylation may be required to fully address this issue.

In conclusion, the inherent catalytic affinity of Cdk5 and substrate specificity profile of Cdk5 is not dramatically altered upon cleavage of p35 when measured using purified components *in vitro*. However, intracellular protein-protein, protein-lipid interactions, or post-translational modifications may differentially affect catalytic specificity *in vivo*. Otherwise, the toxicity of the p25/Cdk5 complex may be attributed to its specifically altered subcellular compartmentalisation and accessibility to

different substrates. In addition, the prolonged activation of Cdk5 may contribute to pathophysiology, if the half-life of p25/Cdk5 is substantially greater than that of p35/Cdk5, and the mis-localisation and lapse in the tight regulation of Cdk5 activity need further investigation. However, this chapter concludes that the potential role of Cdk5 in the pathogenesis of neurodegenerative disease can not be attributed to "hyperactivation" of the specific activity of the Cdk5 complex, as measured *in vitro*.

3.4 SUMMARY

In summary, *in vitro* peptide analysis studies were performed to evaluate the importance of primary sequence requirements in Cdk5 substrate selection and concluded that Cdk5 substrate selection is mediated, to some extent, at the primary sequence level. Proline residues at the -2 position relative to the phosphoacceptor residue appeared important for guiding Cdk5 substrate selection whereas proline residues at the +2 position were antagonist for Cdk5 phosphorylation. The optimal consensus sequence was subsequently defined as P-K-**S**-**P**-R-K. Further *in vitro* analysis using full-length proteins confirmed that proteins adhering to the consensus were phosphorylated to a greater extent, for example tau was phosphorylated to a greater extent than PPARγ *in vitro*. Substrate profile and inherent activity was also similar between complexes suggesting that the specific activity of Cdk5 is not affected by the cleavage of p35.

CHAPTER 4

Validating the tools to assess Cdk5 substrate phosphorylation in cancer

4.1 INTRODUCTION

The work described in the previous chapter examined Cdk5 substrate phosphorylation through *in vitro* analysis. While significant *in vitro* phosphorylation of a proposed substrate is essential in identifying potential substrates, there are additional criteria that are required to confidently validate a proposed substrate as a true physiological target. Namely, manipulation of kinase activity in cells and in *vivo* through genetic, pharmacological, and physiological methods should alter the phosphorylation of specific residues on substrates targeted *in vitro*. Finally, alteration of phosphorylation should result in a change in the function of the substrate. Full validation of kinase substrates is important as it aids in understanding the physiological significance of the kinase and enables examination of kinase activity in a pathophysiological context. Moreover, it allows subsequent assessment of the benefits of manipulating kinase activity as a therapeutic option.

In this context, this chapter attempts to further validate the substrate consensus sequence and specific substrates identified in the previous chapter by manipulating Cdk5 kinase activity in cells and intact tissue, through overexpression and inhibition of Cdk5 activity, and subsequently assessing the effect on substrate phosphorylation at the sites targeted *in vitro*. Substrates confirmed as direct targets of Cdk5 by these means will then be investigated in human tumours to provide insight into the status of Cdk5 activity in cancer.

4.1.2 Chapter Aims

The major goal of this chapter was to establish whether specific proposed substrates of Cdk5 examined in the previous chapter were indeed physiological substrates and to subsequently use these to monitor Cdk5 activity in cancer.

4.1.2.1 Sequence determinants

Having investigated the phosphorylation of potential Cdk5 substrates *in vitro* in the previous chapter, this chapter sets out to investigate the relationship between the degree of substrate phosphorylation observed within a mammalian cell line and the preferred substrate sequence determinants identified in the previous chapter. Proposed substrates would be confirmed as Cdk5 targets if phosphorylation of these substrates was altered following manipulation of Cdk5 activity in cell lines and intact tissue.

4.1.2.2 Tools to study Cdk5 in cells and tissue

Following the validation of Cdk5 substrates through modulation of Cdk5 activity in cell lines and intact tissue, the second aim of the chapter was to establish a phospho-specific antibody panel that would enable the measurement of phosphorylation of these validated Cdk5 substrates, and subsequently validate these tools for use in immunohistochemistry.

4.1.2.3 Cdk5 and cancer

The final aim of this chapter was to screen human tumours for abnormal Cdk5 substrate phosphorylation to determine if changes in Cdk5 activity may contribute to the initiation, progression, or prognosis of human cancer.

4.2 RESULTS

4.2.1 Establishing an appropriate cell model system in which to study Cdk5 substrate phosphorylation

Cells provide a physiological system in which to validate and characterise protein phosphorylation events identified *in vitro*. The most appropriate system for characterisation of substrate phosphorylation is generally a cell system where both the kinase and substrate are known to be endogenously expressed however one of the aims of this chapter was to compare the action of each Cdk5 complex on given protein substrates, therefore cell systems were developed where each of the Cdk5 complexes were not endogenously expressed and the p25 and p35 isoforms could be ectopically introduced.

4.2.1.1 Investigating Cdk5 substrate phosphorylation within HEK293 cells

The human embryonic kidney (HEK) cell line has been extensively used for the study of recombinant proteins as it is of human origin and is also relatively easy to transfect. This cell line expresses relatively little, if any, p35/p25 or Cdk5 and therefore provides the opportunity to study the specific effect of each cofactor on Cdk5 activity with regard to substrate phosphorylation.

4.2.1.1.1 Transfection of Cdk5 and cofactors results in an active complex in HEK293 cells

As the purpose of transfection was to study the effect of Cdk5 phosphorylation on potential substrates it was therefore important to establish if overexpression of Cdk5 along with either cofactor (p35 or p25) would result in the production of an active Cdk5 complex.

Equal amounts of Cdk5 and either p35 or p25 expression vectors were co-transfected into HEK293 cells using the calcium phosphate precipitation method (***refer to methods, section 2.2.4.1***). Successful overexpression of Cdk5 and p35/p25 protein was confirmed by Western blot analysis (***Figure 4.1A***). In order to isolate complexes of Cdk5, immunoprecipitation (IP) was performed using the p35 antibody, which recognises both the p35 and p25 cofactors. IP using the Cdk5 antibody was not performed as this could isolate recombinant Cdk5 not complexed to a cofactor and, furthermore, this antibody can inhibit the activity of the Cdk5 complex (Dr. M. Nikolic, personal communication). The efficiency of the p35 IP was assessed by immunoblotting (***Figure 4.1B***). Following successful pull-down, Cdk5 activity was examined by *in vitro* kinase assay using MBP as the standard substrate.

Both p35/Cdk5 and p25/Cdk5 isolated from HEK293 cells showed a substantial increase in phosphotransferase activity, compared with control, confirming that overexpression of Cdk5 with either cofactor results in generation of an active Cdk5 complex within HEK293 cells (***Figure 4.1C***). The phosphotransferase activity of the p25/Cdk5 complex was slightly greater in comparison to the p35/Cdk5 complex, which may be attributed to a slight difference in expression levels or, alternatively, the p25/Cdk5 complex phosphorylating MBP with a slightly greater specific activity. The immunoblots did not indicate a major difference in expression levels and thus it seems possible that the inherent activity of the recombinant p25/Cdk5 complex against MBP is slightly higher than that of p35/Cdk5. However, this theory was not explored further.

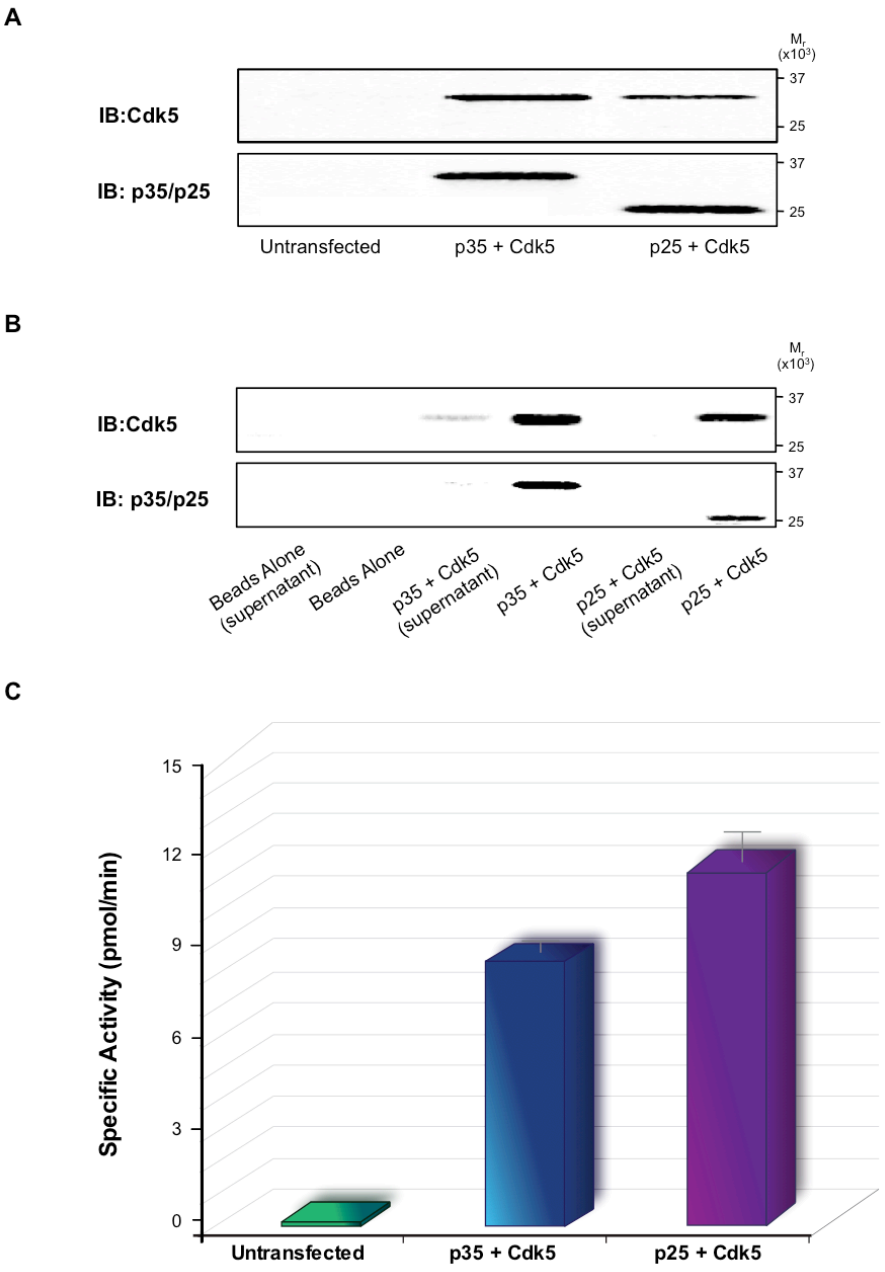


Figure 4.1. ***Generation of an active Cdk5 complex within a mammalian cell line.*** Mammalian constructs containing cDNA for Cdk5 and either p35 or p25 were co-transfected into HEK293 cells using calcium phosphate. A) Successful overexpression was confirmed by Western blot analysis. B) Cdk5 complexes were immunoprecipitated using an antibody to anti-p35/25 and pull-down efficiency determined by immunoblotting. C) Specific activity of each complex was determined by means of *in vitro* kinase assay using MBP as substrate. The degree of phosphate transfer was measured by scintillation counting and used to calculate the specific activity (pmol/min). Data expressed as mean ± S.E.M, n = 2.

4.2.1.1.2 Co-transfection of Cdk5, cofactor and substrate in HEK293 cells

Having confirmed that overexpression of Cdk5 and either p35 or p25 in HEK293 cells resulted in the formation of an active Cdk5 complex, this system was then expanded to investigate substrate phosphorylation by Cdk5 following triple transfection of kinase, cofactor, and substrate in the cell line.

Consistency of total DNA content in transfections was achieved by including appropriate amounts of parent vector plasmid when comparing a single plasmid to multiple plasmid transfections. Cells were plated around 24 hrs prior to transfection to allow the cells to reach around 60% confluency (***Figure 4.2A***). Triple transfection of constructs was performed using the calcium phosphate precipitation method (***refer to methods, section 2.2.4.2***). Transfection reactions were repeated at least 3 times. In every case, Western blot analysis was performed to quantify successful protein overexpression (***Figure 4.2B***).

Although double transfection of both Cdk5 and cofactor consistently produced active Cdk5 complexes, triple transfection experiments (to include a substrate) were more problematic. Single transfection of substrate expression vectors confirmed the validity of these constructs (***Figure 4.2B***), however all substrates failed to overexpress when co-transfected with two other plasmids with expression cassettes (i.e. Cdk5 and either p35 or p25). Transfction with PPARγ is given as an example where transfection of constructs results in PPARγ overexpressing only in single transfections and not during triple transfection (***Figure 4.2B***).

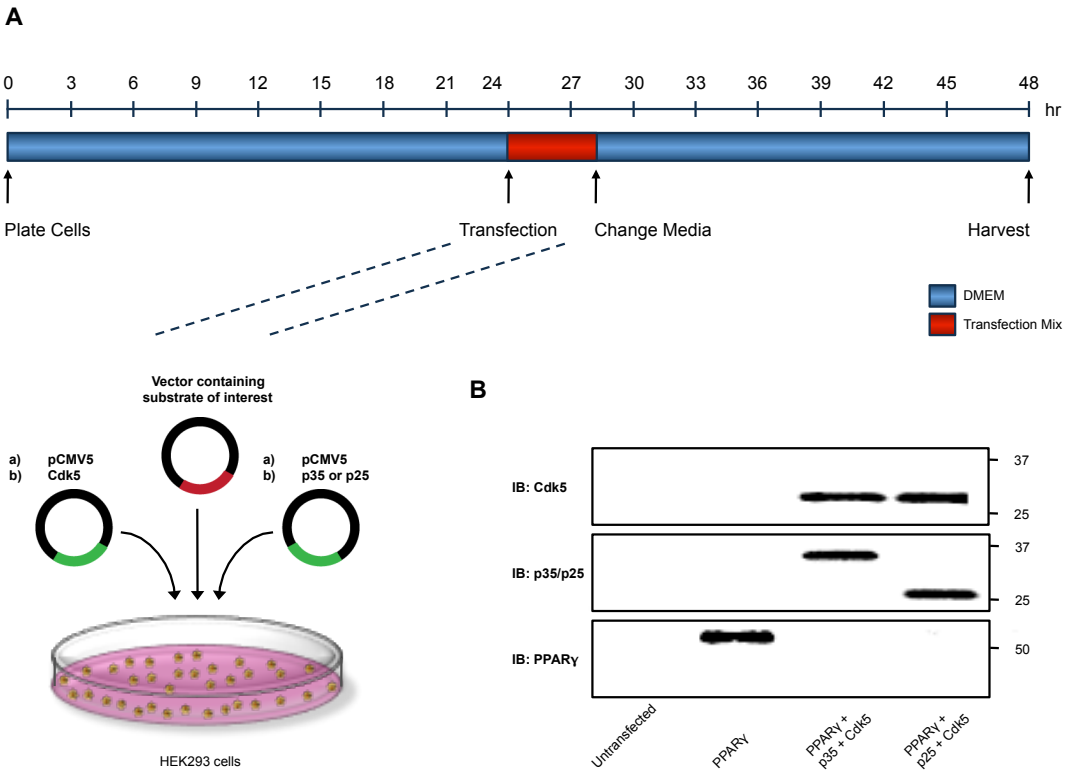


Figure 4.2. ***Triple transfection in HEK293 cells successfully overexpresses Cdk5 and cofactor but fails to overexpress substrate.*** A) Schematic representation of transfection protocol. HEK293 cells were plated 24 hrs prior to transfection to allow the cells to reach the appropriate level of confluency. The different number of plasmids transfected was controlled for by including empty vector plasmids (a) in transfections with only a single construct to ensure each transfection received the same amount of plasmid. Cells were triple transfected with constructs containing the substrate of interest, Cdk5 and cofactor (b). Transfection mix was removed after 4 hrs and cells were incubated in fresh DMEM for 24 hrs to allow protein overexpression. B) Western-blot analysis was performed to confirm overexpression.

Extensive optimisation of the triple transfection protocol was performed in order to overcome the technical issues described above. For example, the ratio of constructs was altered per transfection reaction in an attempt to find the minimal amount of each plasmid DNA required to generate robust protein expression. Concentrations were tested for each plasmid in the range from 1 ng/µl, in intervals of 1, up to 10 ng/µl in different ratios of Cdk5 to cofactor to substrate. Unfortunately, no conditions could be found that produced robust and consistent expression of all three proteins and hence an alternative cell system was approached.

4.2.1.2 Investigating Cdk5 substrate phosphorylation within HeLa cells

HeLa cells are a human cervix adenocarcinoma used extensively in cell biology research since the 1950s. Interestingly, previous work in this cell line suggested that HeLa cells contain endogenous Cdk5 but lack either cofactor (Sun *et al*., 2008). In theory, this provides an opportunity to study Cdk5 substrate phosphorylation following transfection of either cofactor along with substrate, negating the requirement for a triple transfection.

4.2.1.2.1 Cdk5 activity isolated from HeLa cells increases more dramatically following overexpression of Cdk5 and cofactor compared to cofactor alone

To ensure that overexpression of a Cdk5 cofactor resulted in generation of an active Cdk5 complex beyond endogenous activity in the HeLa cells, they were transfected with either p35 or p25, with or without co-transfection of Cdk5, and a subsequent IP kinase assay was performed on the resultant cell lysates. IP reactions were performed using the p35/p25 antibody, with untransfected lysate as a control, and successful pull-down confirmed by Western blot. The specific activity of each isolated complex was determined by performing an *in vitro* kinase assay with MBP as substrate.

No activity was found in beads alone control IP and only a small amount of activity was observed from pull-down using lysates from untransfected cells suggesting a low level of p35 and Cdk5 endogenous expression (***Figure 4.3***). Activity isolated from lysates generated from cells transfected with either cofactor was greater than endogenous activity suggesting Cdk5 is present in the HeLa cells, but by far the greatest Cdk5 activity was obtained in lysates from cells transfected with both kinase and cofactor (***Figure 4.3***). This suggests that the endogenous level of Cdk5 is much lower than that following transfection of the Cdk5 expression vector. Unfortunately, when potential Cdk5 substrates were co-transfected with only p35 or p25, there was no enhancement of substrate phosphorylation detected (*data not shown*). This could be due to the very low level of kinase activity in cells transfected with p35 and p25 in the absence of Cdk5 or, alternatively, it could mean that these are not truly Cdk5 substrates. Therefore, triple-transfections were attempted, this time using the HeLa cell system in an attempt to distinguish between these two possibilities.

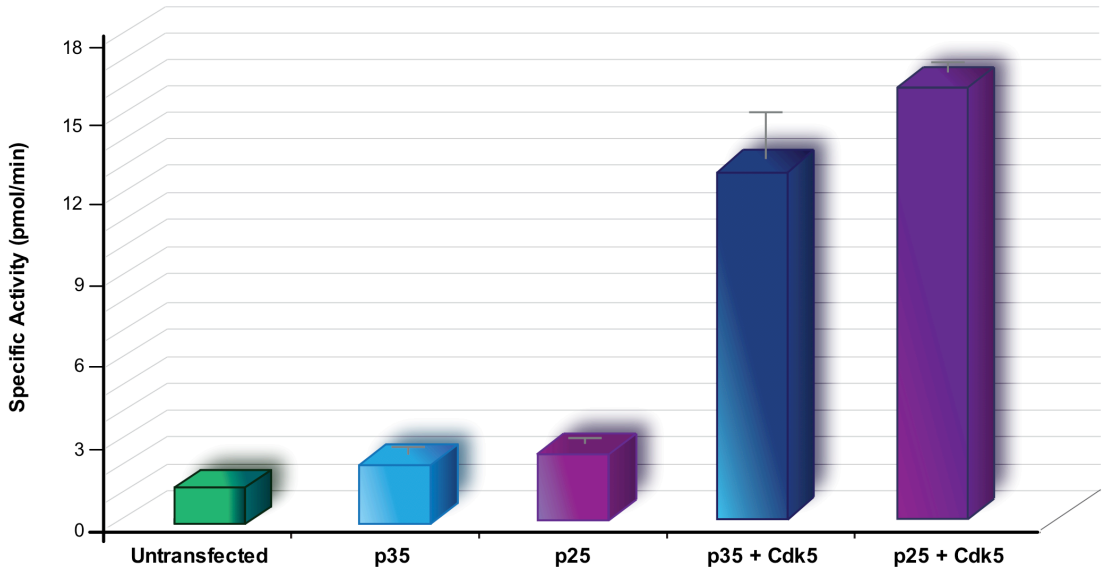


Figure 4.3. ***Kinase activity is dramatically greater following overexpression of Cdk5 and cofactors compared to cofactors alone.*** Mammalian constructs containing cDNA for Cdk5 and either p35 or p25 were co-transfected into HeLa cells using calcium phosphate. Successful overexpression was confirmed by Western blot analysis before Cdk5 complexes were immunoprecipitated using an antibody to anti-p35/25 and pull-down efficiency determined by immunoblotting. Specific activity of each complex was determined by means of *in vitro* kinase assay using MBP as substrate. The degree of phosphate transfer was measured by scintillation counting and used to calculate the specific activity (pmol/min). Data expressed as mean $\pm$ S.E.M, n = 2.

4.2.1.2.2 Overexpression of Cdk5, cofactor, and substrate in HeLa cells

Previously, triple transfections in HEK293 cells using calcium phosphate proved problematic therefore in an attempt to increase the efficiency of triple transfections in HeLa cells, the method of transfection was altered from calcium phosphate to Lipofectamine (***refer to methods, section 2.2.4.2***).

HeLa cells were plated around 24 hrs prior to transfection to enable the cells to reach around 60% confluency. Subsequently, expression vectors for multiple constructs (***refer to methods, section 2.2.4.3***) were transfected into HeLa cells along with Cdk5 and either activator using Lipofectamine. Total DNA in transfections was equalised by including empty vector plasmids where appropriate.

As with the HEK293 system transfection of three plasmids interfered with the overexpression of at least one of the proteins of interest. This mostly affected overexpression of the substrate where overexpression was much greater in the substrate alone transfections and minimal in the triple transfections, as was the case with Mef2a and PAK1 (***Figure 4.4A-B***), or where overexpression either failed completely, as was the case with dynamin 1 and STAT3 (*data not shown*, the constructs were both FLAG-tagged though not detected by the FLAG antibody therefore these blots are not shown in ***Figure 4.4C-D***). Additionally, overexpression of p25 protein appeared to be dramatically greater compared to p35 overexpression.

Of all the potential Cdk5 substrates investigated by triple transfection in HeLa cells, only CRMP2 and tau overexpressed to a robust level when co-expressed with Cdk5 and a cofactor therefore these are described in greater detail in the next section.

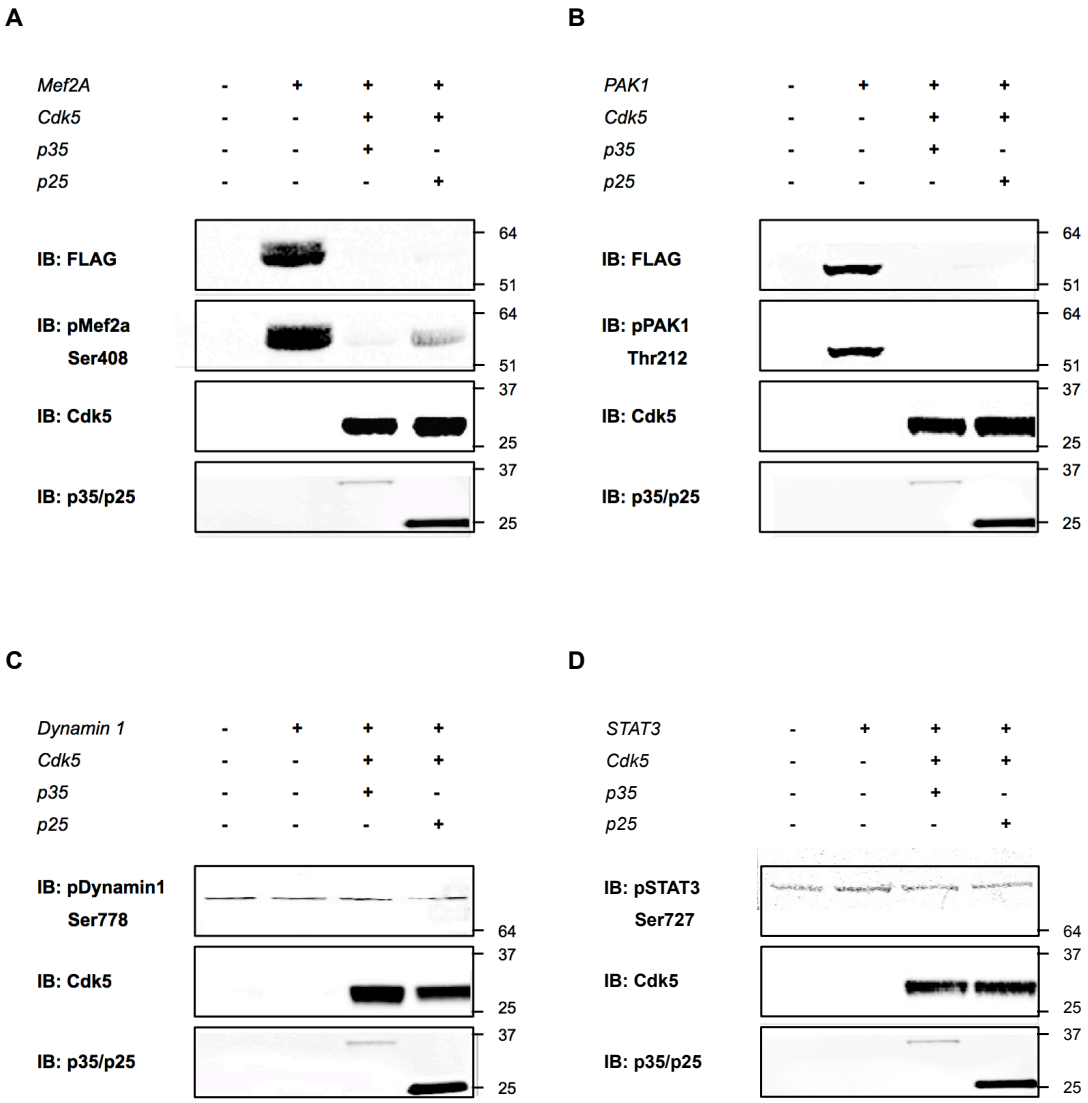


Figure 4.4. ***Triple transfections in HeLa cells results in Cdk5 and cofactor overexpression but overexpression of substrate is affected.*** HeLa cells were transfected with equal amounts of expression constructs for Cdk5, p35 or p25, and substrate of interest to assess Cdk5 phosphorylation of A) Mef2a, B) PAK1, C) Dynamin 1, and D) STAT3. Subsequently, cells were lysed, subjected to SDS-PAGE, and successful overexpression was determined by Western blot analysis.

4.2.1.2.2.1 Co-expression of CRMP2, Cdk5, and cofactors results in increased phosphorylation of Ser 522

The major Cdk5 phosphorylation site on both CRMP2 and CRMP4 was previously reported as Ser522 following *in vitro* analysis experiments (Cole *et al*., 2004). In order to assess these substrates in intact cells and additionally investigate potential differences between p35 and p25 regulation of this target, HeLa cells were transfected with Cdk5, either cofactor, and CRMP2 or CRMP4.

Transfection reactions were performed at around 60% confluency using Lipofectamine (***refer to methods, section 2.2.4.2***). Cells were harvested after 24 hrs and protein expression was confirmed using Western blot analysis (***Figure 4.5A, D***). In the single transfection reaction, expression of either CRMP2 or CRMP4 was slightly greater compared to triple transfected cells. In cells where Cdk5 was overexpressed with CRMP2, an increase in pCRMP2 Ser522 was observed compared to the CRMP2 alone control, confirming that overexpressed Cdk5 results in increased phosphorylation of this site (***Figure 4.5B***). Conversely, Ser522 of CRMP4 was phosphorylated when expressed in HeLa cells, even though there is little Cdk5 activity, and co-expressing Cdk5/cofactor did not increase CRMP4 Ser522 phosphorylation suggesting that this site is either not an *in vivo* target of Cdk5 or is fully phosphorylated by endogenous Cdk5 kinase (***Figure 4.5C***). This observation is consistent with previous work in a Cdk5 knockout mouse from our lab indicating that Cdk5 is required for regulation of CRMP2 but not CRMP4 *in vivo* (Cole *et al*., 2006). In general, the degree of phosphorylation of CRMP2 was greater with p25 expression however expression of p25 was greater than that of p35 in these experiments. This may account for differences in CRMP2 phosphorylation between complexes.

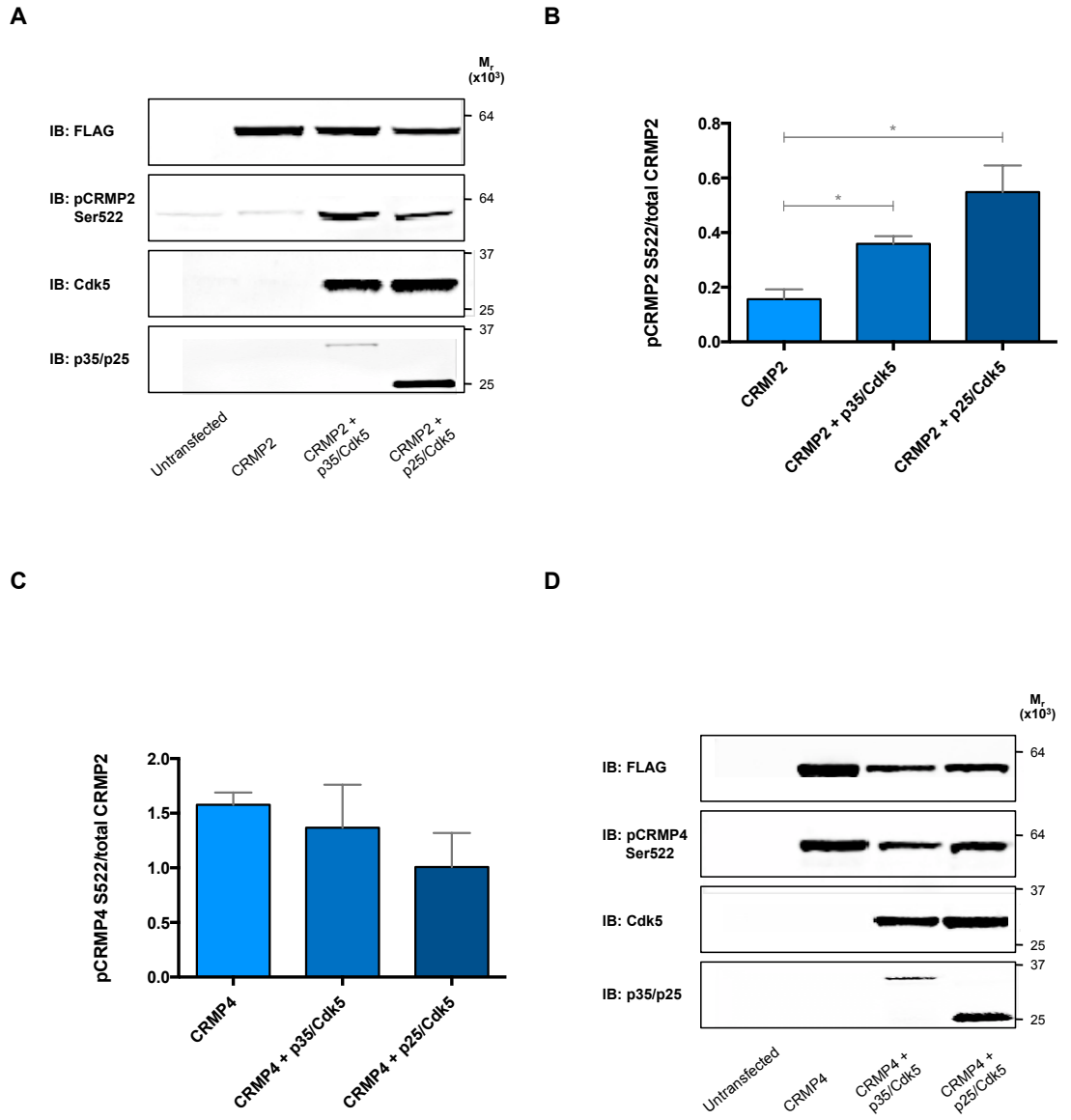


Figure 4.5. ***Overexpression of Cdk5 complexes results in increased Ser522 phosphorylation in CRMP2 but not CRMP4.*** HeLa cells were transfected with equal amounts of expression constructs for Cdk5, p35 or p25, and CRMP2 or CRMP4. Cells were lysed, subjected to SDS-PAGE, and A) Western blot analysis establishing successful overexpression. B-D) Quantification was performed using Licor software. Data expressed as mean ± SEM, n = 6. Data was analysed by one-way ANOVA with Tukey's post hoc test, *p<0.05.

4.2.1.2.2.2 Overexpression of Cdk5, cofactor and tau resulted in increased phosphorylation of tau at Ser 202, Thr 205, and Ser 235

In the previous chapter, phospho-mapping of phosphorylated tau identified Ser235 as the major Cdk5 phosphorylation site on tau, with potential minor sites at Ser202 and Thr205 (***Chapter 3, Figure 3.18***). To confirm that Cdk5 phosphorylates these sites in a cell system and to investigate the contributions of cofactors, an expression vector for tau was co-transfected along with Cdk5 and cofactor expression constructs in HeLa cells, as with CRMPs above.

Transfection reactions were performed at around 60% confluency using Lipofectamine (***refer to methods, section 2.2.4.2***) and cells harvested after 24 hrs. Protein overexpression of tau, Cdk5 and cofactors was confirmed by Western blot analysis, as was the effect of Cdk5 phosphorylation of tau at residues Ser202, Thr205, and Ser235 using phospho-specific antibodies to each of these sites (***Figure 4.6A***).

In the single transfection reaction, overexpression of tau was slightly greater compared to the triple transfected cells. In cells where Cdk5 was overexpressed with tau, an increase in phosphorylation of tau was observed at sites Ser202, Thr205, and Ser235 confirming these are potential Cdk5 phosphorylation sites in intact cells. There was not a significant difference in tau phosphorylation at any of the sites when p25 expression was compared to p35 expression data suggesting each complex was equally able to induce phosphorylation of this substrate.

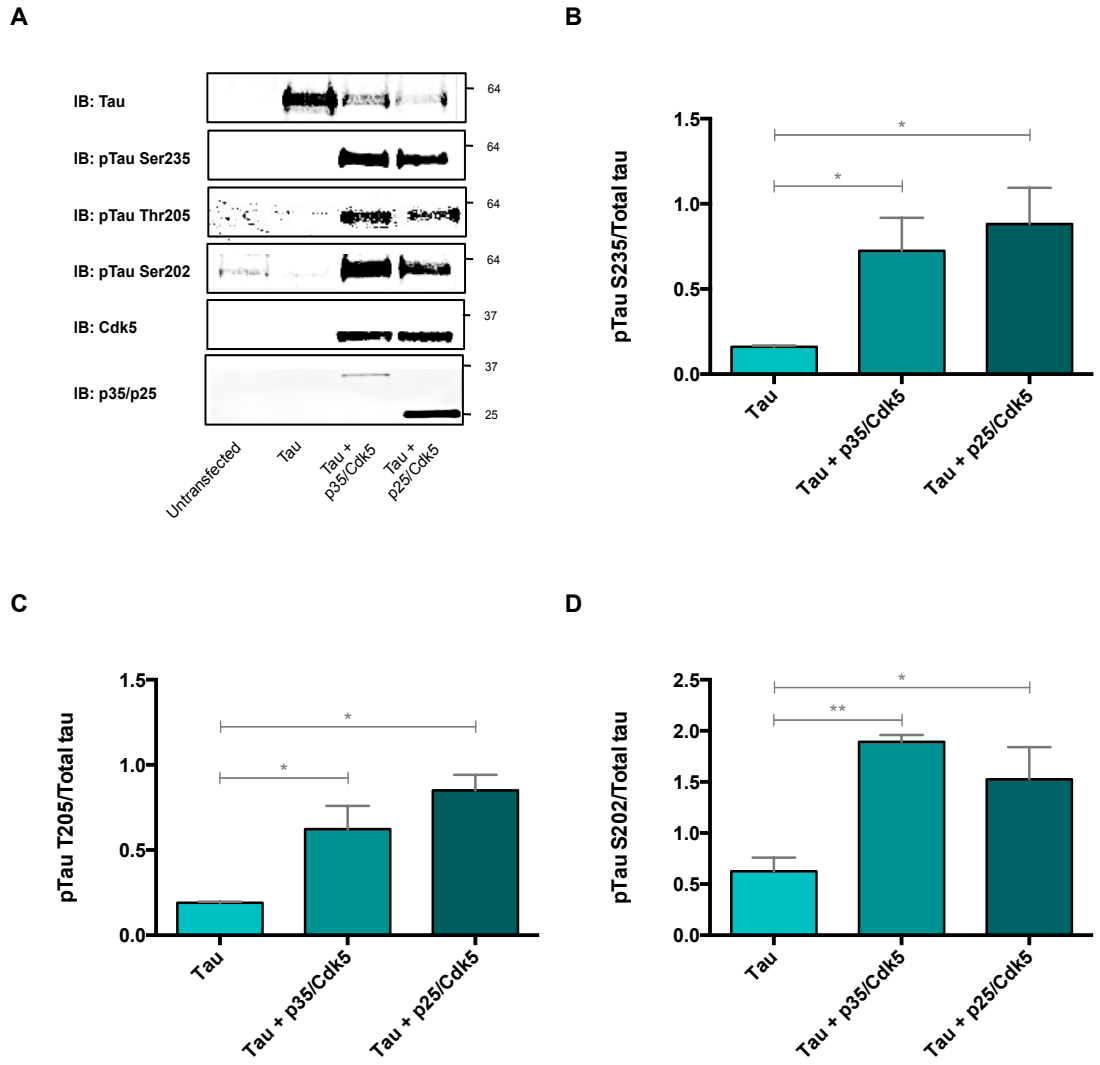


Figure 4.6. ***Overexpression of Cdk5 complexes results in increased phosphorylation of Tau.*** HeLa cells were transfected with equal amounts of expression constructs for Cdk5, p35 or p25, and tau. Cells were lysed, subjected to SDS-PAGE, and A) Western blot analysis which confirmed successful overexpression. B-D) Quantification was performed using Licor software and the ratio between phospho-tau: total tau calculated for each phospho-tau antibody. Data expressed as mean ± SEM, n = 6. Data was analysed by one-way ANOVA with Tukey's post hoc test, *p<0.05, **p<0.01.

4.2.1.2.3 Cdk5 inhibitors, purvalanol A and roscovitine, are toxic to HeLa cells after transfection using Lipofectamine

Following overexpression of Cdk5 activity, the effect of inhibition of endogenous Cdk5 activity on substrate phosphorylation in HeLa cells was subsequently investigated. HeLa cells were transfected with substrates, as described in the previous sections, before transfection media was removed and cells incubated for 24 hrs to allow protein overexpression. Subsequently, serum-containing media was removed from the transfected cells and replaced with media containing either purvalanol A or roscovitine (both at 10 μM final concentration) to inhibit endogenous Cdk5 activity. However, cell death was apparent within 1 hr of inhibitor treatment on transfected cells, therefore the effect of Cdk5 inhibition on overexpressed substrate phosphorylation could not be assessed. Inhibitor concentration was lowered to 1 μM however this still proved toxic to transfected cells.

4.2.1.3 Examining substrate phosphorylation following Cdk5 inhibition in primary neuronal cells

Primary neuronal cultures derived from rodents are commonly used to study basic physiological properties of neurons and, as such, present a useful tool in which to study the effect of kinase inhibition on protein phosphorylation. Cdk5 is primarily active in post-mitotic neurons therefore primary cortical neurons were used to study the effect of inhibition of Cdk5 activity on the phosphorylation of substrates identified *in vitro* in the previous chapter.

The primary cortical neuron cultures used in the following experiments were cultured and donated by Dr. Ritchie Williamson, University of Bradford, as described in ***Chapter 2, section 2.2.3.4***.

4.2.1.3.1 Phosphorylation of CRMP2 at Ser522 but not CRMP4 is reduced in primary neurons following Cdk5 inhibition

Both CRMP2 and CRMP4 are phosphorylated by Cdk5 *in vitro* (***Section 3.2.3.1***) however only CRMP2 was phosphorylated at Ser522 in cells overexpressing each CRMP isoform and active Cdk5 complex (***Figure 4.5***). Therefore, this site was examined in primary neurons following inhibition of endogenous Cdk5 activity.

Following cell suspension preparation (***refer to methods, 2.2.3.4***), cells were maintained in DMEM/F12 culture medium to enable maturation and differentiation. Cultures following 6 days *in vitro* (DIV) were treated with purvalanol A (10 μM final concentration) for 3 hrs to inhibit Cdk5 activity, with 0.1% DMSO as vehicle. After 3 hrs treatment with purvalanol A, cells were fixed for a duration of 3 min with 4% (w/v) formaldehyde solution, washed three times with 500 μL PBS then subjected to phospho-specific antibody staining to determine the effect of Cdk5 inhibition on CRMP2 and CRMP4 phosphorylation. Cells were initially incubated with either an anti-pCRMP2 Ser522 or anti-CRMP4 Ser522 antibody followed by incubation with a fluorophore-conjugated antibody to mouse IgG (Cys3, absorption 543 nm = red) and counter-stained with the DNA-binding dye DAPI (absorption 350 nm = blue) to label nuclei. Cells were then mounted and viewed with an automated confocal microscope (***Figure 4.7***).

In the vehicle-treated controls, phosphorylation of CRMP2 and CRMP4 at Ser522 was apparent throughout the cell body and axons. Treatment with purvalanol A resulted in a complete loss of phosphorylated CRMP2 (***Figure 4.7A***). Conversely, pCRMP4 Ser522 immunopositive staining was still apparent in the cell body and axons of primary neurons treated with purvalanol A (***Figure 4.7B***).

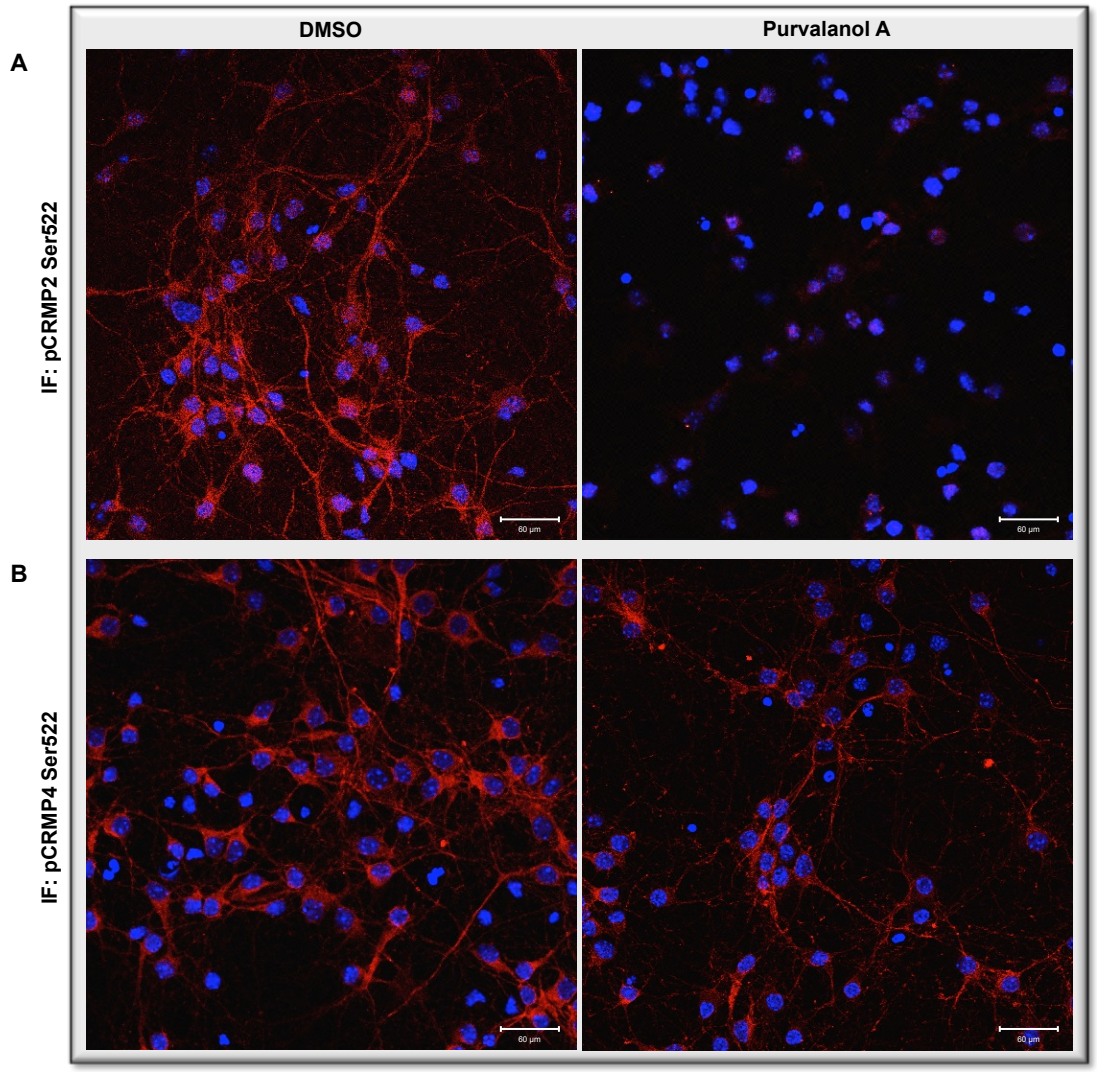


Figure 4.7. ***Purvalanol A treatment reduces Cdk5 phosphorylation of CRMP2 but not CRMP4 at Ser522.*** Primary cortical neurons were cultured for 6 days *in vitro* (6 DIV) then treated with 10 µM purvalanol A or vehicle (0.1% DMSO) for 3 hrs prior to fixation, permeabolisation, and staining with phospho-specific antibodies to A) pCRMP2 Ser522 and B) pCRMP4 Ser522. Phospho-specific antibodies were detected by Cy-3 bound secondary antibodies (red) and nuclei were counter-stained with the DNA-binding dye DAPI (blue). *Scale bar* = 60 µm.

4.2.1.3.2 Phosphorylation of Tau is reduced following Cdk5 inhibition

Phospho-mapping of tau phosphorylated by Cdk5 *in vitro* identified Ser235 as the major Cdk5 phosphorylation site on tau with minor sites at Ser202 and Thr205 (***Section 3.2.3.1***). Phosphorylation of each of these sites was increased following overexpression of tau protein and active Cdk5 complexes (***Figure 4.6***). Therefore, these sites were investigated in primary neurons following Cdk5 inhibition.

Primary neurons were treated with purvalanol A as described previously for both CRMP2 and CRMP4. Subsequent to washing in PBS, cells were incubated with phospho-antibodies specific to either anti-pTau Ser202, anti-pTau Thr205 or anti-pTau Ser235 followed by incubation with a fluorophore-conjugated antibody to rabbit IgG (Cys3, absorption 543 nm = red) and then counter-stained with DAPI to label nuclei (absorption 350 nm = blue). Cells were then mounted and viewed with an automated confocal microscope (***Figure 4.8***).

Staining was apparent throughout the cell body and axons of primary neurons treated with vehicle (***Figure 4.8***). Following inhibitor treatment, a slight reduction in the number of pTau Ser202 immunopositive cells was observed (***Figure 4.8A***). The number of pTau Thr205 immunopositive cells was reduced to a greater extent following purvalanol A treatment, with a weaker signal observed in the cell body and axons of the primary neurons compared to vehicle treatment (***Figure 4.8B***). Staining of primary neurons with pTau Ser235 appeared weaker when treated with inhibitor however staining using this antibody was weak in vehicle cells (***Figure 4.8C***). This was also the case with the HeLa cells were Ser235 only became phosphorylated when Cdk5 was co-expressed (***Figure 4.6***).

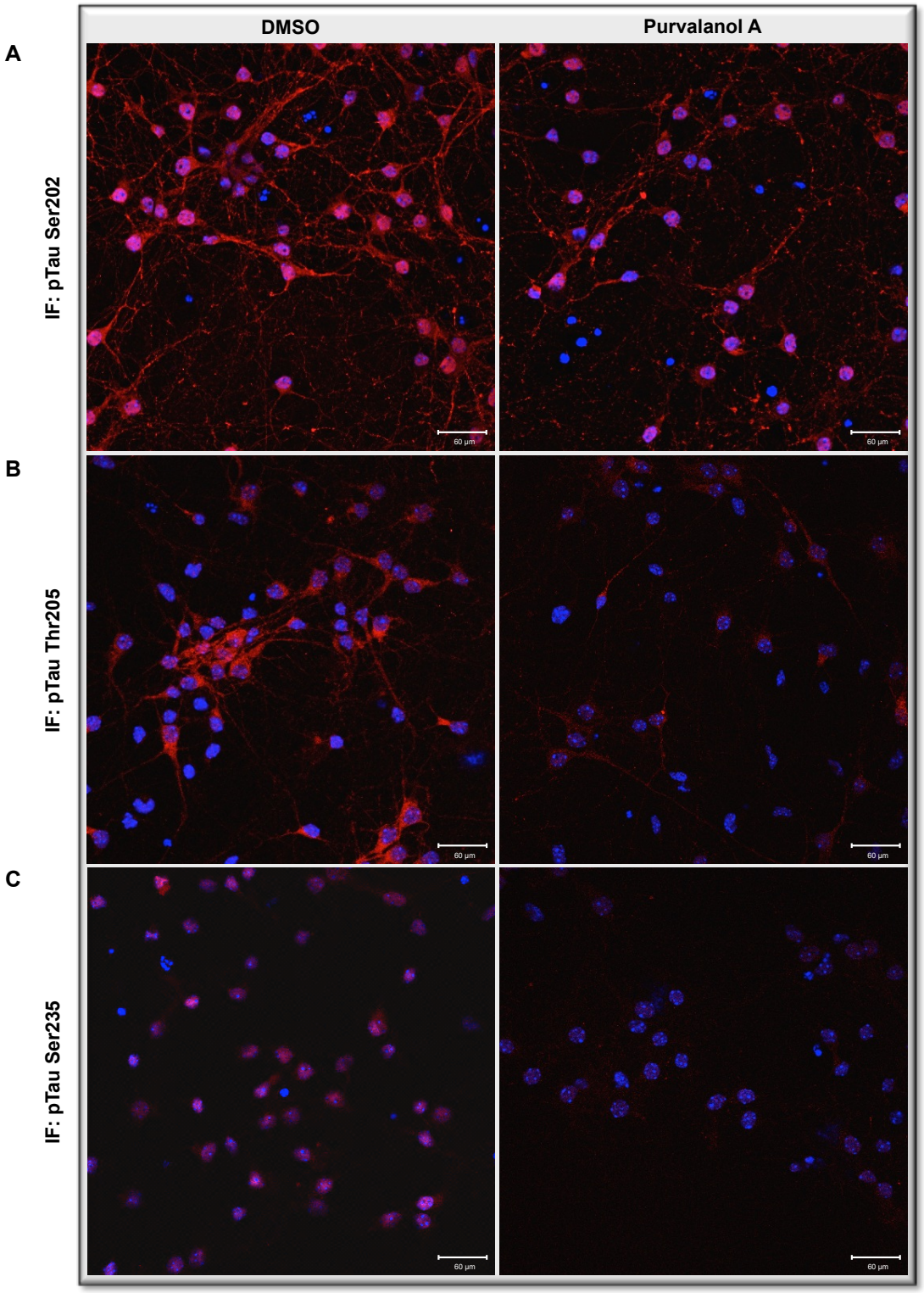


Figure 4.8. ***Purvalanol A treatment reduces Cdk5 phosphorylation of tau.*** Primary cortical neurons were cultured for 6 days *in vitro* (6 DIV) and then treated with 10 µM purvalanol A or vehicle (0.1% DMSO) for 3 hrs prior to fixation, permeabolisation, and staining with phospho-specific antibodies to A) pTau Ser202, B) pTau Thr205, and C) pTau Ser235. Phospho-specific were detected by Cy-3 bound secondary antibodies (red) and nuclei were counter-stained with the DNA-binding dye DAPI (blue). *Scale bar* = 60 µm.

4.2.2 Assessing Cdk5 substrate phosphorylation in human tumours

Treatment of primary neurons with purvalanol A gave promising results that both CRMP2 and tau were physiological substrates of Cdk5 that could be monitored by immunohistochemistry (IHC). This suggested that these antibodies might be suitable to screen human tumours to examine Cdk5 substrate phosphorylation during tumourigenesis therefore they underwent preliminary antibody optimisation for IHC.

4.2.2.1 Antibody Optimisation for Immunohistochemistry

To ensure accurate interpretation of immunohistochemistry results, the following controls were performed to demonstrate that the specific antigens were localised to the correct specialised tissues, cell types, or subcellular localisation. Optimisation of fixation, blocking, antibody incubation, and antigen retrieval steps improves the chances of a strong and specific signal. IHC characterisation requires appropriate positive and negative controls to support the validity of staining observed and identify experimental artefacts. ***Figure 4.9*** shows a schematic representation of the antibody validation process.

As substrate phosphorylation could be reduced following Cdk5 inhibition with purvalanol A (***section 4.2.1.3***), primary neurons were used for the initial antibody validation to establish compatibility with the IHC protocol. Primary neuron cultures (6 DIV) were embedded in paraffin and sectioned as described in ***Chapter 2, sections 2.2.10.1 – 2.2.10.2***. The antibody panel (including total CRMP2, pCRMP2 Ser522, total tau, pTau Ser235, pTau Ser202 and Ser205) to be used for assessing Cdk5 substrate phosphorylation in human tumours underwent stepwise rounds of optimisation and validation, as depicted in ***Figure 4.9***.

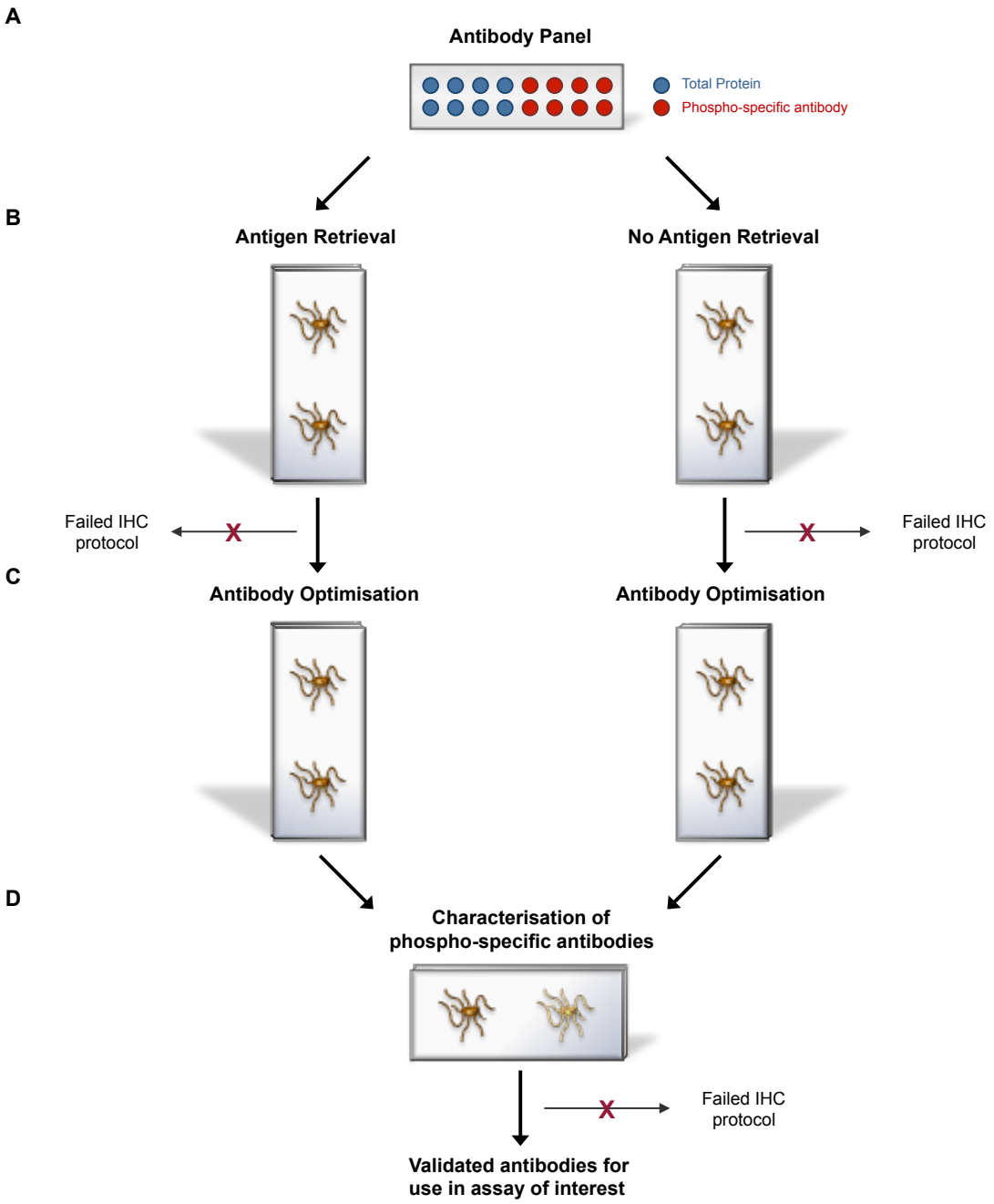


Figure 4.9. ***Schematic representation of stepwise antibody validation.*** A) Antibody panel composed of phospho-specific and total protein antibodies for validation. B) Initially, antibodies undergo either no antigen retrieval step (see text for definition) or antigen retrieval using citrate buffer. Antibodies that failed the IHC protocol would be eliminated at this point. C) Several dilutions would be performed to optimise optimal antibody concentration, evident by optimal antibody staining. D) Phospho-specific antibodies would then be characterised by observing the effect of Cdk5 inhibitor treatment on immunostaining of primary cortical neurons.

4.2.2.2 Antigen Retrieval Optimisation

For preservation of tissue morphology it is essential that fixation of the tissue is performed however this process can have a negative impact on IHC detection. The fixation process can alter protein biochemistry and, as a result, the epitope of interest may become masked thereby preventing binding of the primary antibody. This may be due to cross-linking of amino acids within the epitope, cross-linking of unrelated peptides at or near the epitope, alteration of the electrostatic charge of the antigen, or alteration of the conformation of the epitope. Antigen retrieval refers to any technique in which masking of an epitope is reversed and epitope-antibody binding is restored. The requirement for antigen retrieval is dependent on multiple variables including, but not limited to, tissue type, method and duration of fixation, antibody, and the target antigen therefore this technique must be investigated for each individual antibody prior to staining of tissue.

During the initial round of antibody screening, all antibodies in the antibody panel (***Table 4.1***) went through either the antigen retrieval step or the IHC protocol omitting the antigen retrieval step to establish the requirement for antigen retrieval for each antibody. Briefly, during the antigen retrieval step, sections were placed in boiling citrate buffer (pH 6.0) and heated in a microwavable oven for 15 min before being allowed to cool at room temperature for 30 min and subsequently incubated with each antibody in the antibody panel. Following this initial round of screening, it was apparent that all of the antibodies tested favoured the antigen retrieval step (***Table 4.1***), expressing better staining after antigen retrieval. Following this, all antibodies used in the subsequent experiments were subjected to the antigen retrieval step prior to antibody incubations during immunohistochemical staining of tissue.

Protein	Residues Surrounding	Source	Antibody	Detects	Antigen Retrieval by HIER
CRMP2	total protein	Sheep	Polyclonal	Recognises phosphorylated and unphosphorylated human, mouse and rat CRMP2 protein	Yes
pCRMP2 Ser522	Ser 522	Sheep	Polyclonal	Recognises human, mouse, and rat CRMP2 protein phosphorylated at Ser 522	Yes
Tau	total protein	Rabbit	Polyclonal	Reacts with phosphorylated and non-phosphorylated forms of human, mouse and rat tau	Yes
pTau Ser235	Ser 235	Rabbit	Monoclonal	Detects human, mouse and rat tau phosphorylated at threonine residue 235	Yes
pTau Thr205	Thr 205	Rabbit	Polyclonal	Detects human, mouse and rat tau phosphorylated at threonine residue 205	Yes
Cdk5	total protein	Rabbit	Polyclonal	Antibody detects Cdk5 protein of mouse, rat and human origin	Yes
p35/p25	total protein	Rabbit	Polyclonal	Detection of p25 and p35 regulatory subunits of Cdk5 of mouse, rat and human origin	Yes

Table 4.1. ***Antibodies to assess Cdk5 substrate phosphorylation by IHC require antigen retrieval by heat induced epitope retrieval (HIER).*** Optimised antigen retrieval conditions with antibody specificity information.

4.2.2.3 Optimal Antibody Concentrations

In the previous validation studies, all antibodies tested displayed compatibility with IHC on primary neurons and were shown to favour antigen retrieval. Subsequently, the optimal antibody concentration was determined for each of these antibodies prior to screening of human tissue. Heat induced epitope retrieval (HIER) was performed before each antibody was incubated on sections of primary neurons in stepwise increases to determine the optimal antibody dilution for IHC. Initially, antibody dilutions were preformed at 1:333 followed by increases in the dilutions from 1:1000 to 1:3000 to 1:9000.

Following the initial dilution series, sections were examined under a light microscope to assess the level of staining and the requirement of additional dilutions based on the level of staining observed. The following antibodies: pCRMP2 Ser522, total tau, and pTau Thr205 showed strong staining at all concentrations above 1:3000 however the staining became weaker at a dilution of 1:9000, therefore several dilutions were preformed between this range to determine the optimal antibody concentration. Intense staining was observed for pTau Ser235 at all antibody dilutions therefore further dilutions of up to 1:20,000 were preformed with this antibody to establish optimal conditions. Good staining was seen for the p35/p25 antibody for dilutions of 1:1000 and 1:3000 therefore a dilution of 1:2000 was performed to dictate the antibody dilution used for IHC. The final two antibodies to be optimised, Cdk5 and total CRMP2, both showed weak staining at all antibodies dilutions with the most intense staining observed at a dilution of 1:333. The final antibody dilutions that were selected for IHC can be found in ***Table 4.2***.

Antibody	Dilution Series 1	Dilution Series 2	Final Concentration for IHC
Total CRMP2	1:333 1:1000 1:3000 1:9000	1:100 1:250	1:250
pCRMP2 Ser522	1:333 1:1000 1:3000 1:9000	1:4000 1:6000	1:5000
Total Tau	1:333 1:1000 1:3000 1:9000	1:4000 1:6000	1:5000
pTau Ser235	1:333 1:1000 1:3000 1:9000	1:15 000 1:20 000	1:20 000
pTau Thr205	1:333 1:1000 1:3000 1:9000	1:4000 1:6000	1:5000
Total Cdk5	1:333 1:1000 1:3000 1:9000	1:100 1:250	1:250
Total p35/p25	1:333 1:1000 1:3000 1:9000	1:2000 1:2500	1:2000

Table 4.2. ***Optimised antibody concentrations selected for IHC*****.** Dilution series for antibodies used to assess Cdk5 substrate phosphorylation using IHC. Initial dilutions were performed at 1:333, 1:1000, 1:3000, and 1:9000, followed by a second round of dilutions, before reaching an optimal antibody concentration.

4.2.2.4 Validation of Phospho-Specific antibodies

The three phospho-specific antibodies (pCRMP2 Ser522, pTau Ser235 and pTau Thr205) that showed Cdk5 inhibitor sensitive staining in fixed primary neurons were used to interrogate the embedded neurons. To assess their ability to detect the phosphorylated peptide and validate each antibody as phospho-specific for use in IHC, primary neurons were treated at 6 DIV with either 10 μM purvalanol A, 10 μM roscovitine, or DMSO vehicle for 3 hrs to inhibit Cdk5 activity. After treatment, the cultures were fixed in formaldehyde solution and then embedded in paraffin (***refer to methods, section 2.2.10.1***). Subsequently, sections were taken from the paraffin block and incubated with each of the phospho-specific antibodies. Negative controls were also performed in which the sections were incubated with antibody diluent, without the primary antibody, followed by secondary antibody incubation.

Subsequent to examination of immunopositive staining under a light microscope, antibodies were classified into two groups: antibodies for which the staining was reduced or lost upon inhibitor treatment (passed); or, antibodies that did not show reduction of staining (failed) (***Figure 4.10***). There was not an apparent loss or reduction in staining with the pTau Thr205 antibody between control and inhibitor treated neurons therefore this antibody was not a good measure of Cdk5 activity and was not taken further. There was a slight reduction in the number of primary neurons stained with the pTau Ser235 antibody when treated with inhibitors however the intensity of the stain was not reduced and therefore this antibody did not pass the validation step. Staining of inhibitor treated neurons with the pCRMP2 Ser522 antibody was less intense and a reduction in the number of immunopositive cells was apparent, compared to vehicle treated neurons, therefore this antibody was passed for IHC use with human tumour samples.

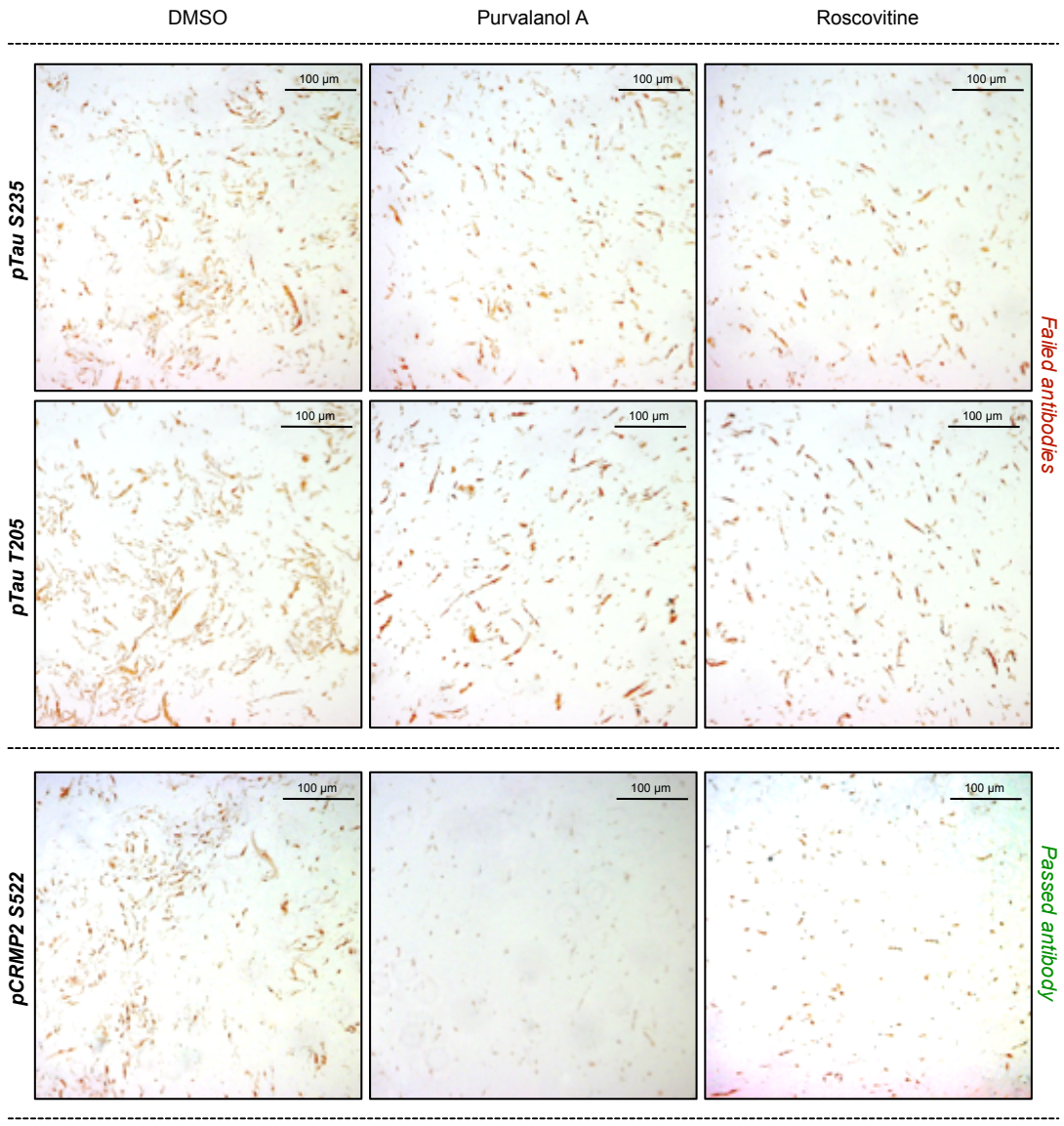


Figure 4.10. ***Cdk5 inhibition reduces pCRMP2 Ser522 immunostaining in primary cortical neurons.*** Primary cortical neuron cultures (6 DIV) were treated with either 10 µM purvalanol A, 10 µM roscovitine, or vehicle (0.1% DMSO) for 3 hrs. Subsequently, cultures were fixed in formaldehyde solution and then embedded in paraffin. Sections were taken from each paraffin block and incubated with phospho-specific antibodies to anti-pTau Thr205, pTau Ser235, and pCRMP2 Ser522 followed by biotinylated secondary antibodies and then streptavidin complexed with biotinylated peroxidases which were visualised using DAB chromagen. Cell nuclei were counterstained with hematoxylin and mounted in DPX. Antibodies were then classified as passed or failed dependent on the observed staining pattern. Images were taken at a magnification of x 20. *Scale bar* = 100 µm.

4.2.2.5 Cdk5 substrate phosphorylation in lung carcinomas

As described in ***Chapter 1***, downregulation of a CRMP isoform was associated with poor clinical outcome in lung cancer (Shih *et al*., 2001), therefore investigation of Cdk5 substrate phosphorylation was initiated in lung carcinoma as CRMP2 was the major substrate under investigation. Lung tumour biopsies used in the following experiments were sectioned and provided by Dr. Phil Coates, Tayside Tissue Bank, University of Dundee.

Lung biopsies from 21 different NSCLC patients were obtained and immunostained using antibodies to anti-Cdk5 (total) and anti-p35/p25 (total) to confirm the presence of both Cdk5 and its cofactors in lung tissue (***Figure 4.11)***. Additionally, tissue sections were stained with an antibody to anti-CRMP2 (total) to establish expression of CRMP2 protein in lung tissue and, also, a phospho-specific antibody to anti-pCRMP Ser522 in order to compare the expression and degree of CRMP2 phosphorylation between normal and tumour tissue (***Figure 4.11)***. Tissue section staining was performed as described in ***Chapter 2.2.10.3***.

Tau was previously confirmed as a Cdk5 substrate therefore lung biopsies was also stained using an antibody to anti-tau (total). Although the pTau Ser235 antibody did not fully pass the previous phospho-specific antibody IHC validation, it was included in this investigation with the total tau antibody as a potential read-out of Cdk5 activity in lung tumour cells as there was a slight reduction in pTau Ser235 immunopositive staining with inhibitor. Once again, these tissue sections were stained as described in ***Chapter 2.2.10.3***.

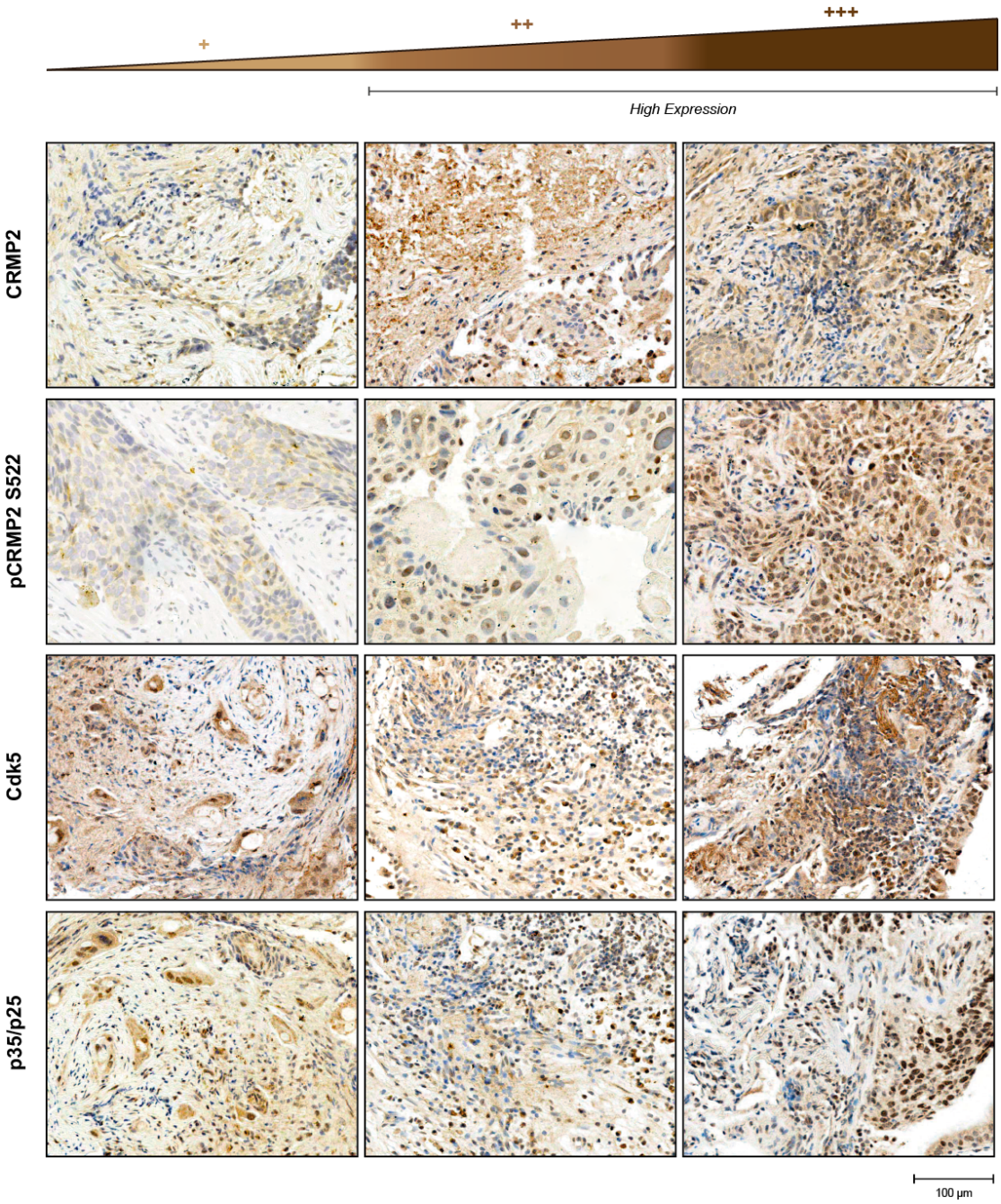


Figure 4.11. ***Representative images of phosphorylated CRMP2 protein expression in lung tumour biopsies.*** Tissue sections were immunostained with antibodies to anti-CRMP2 (total), anti-pCRMP2 Ser522, anti-Cdk5, and anti-p35/p25. Degree of staining was graded using a semi-quantitative scale from 0-3, with 0 representing no staining, 1 representing light brown staining, 2 representing moderate brown staining and 3 representing dark brown staining. Scores of 2 or 3 were interpreted as high expression. Images were taken at a magnification of x 20. *Scale bar* = 100 µm.

The immunostained tissue sections were independently assessed by two investigators (Professor Frank Carey and Dr Yvonne Woods, Department of Pathology, Ninewells Hospital) and the degree of staining was graded using a semi-quantitative scale from 0-3, with 0 representing no staining, 1 representing light brown staining, 2 representing moderate brown staining, and 3 representing dark brown staining (***Table 4.3***). Those with scores of 2 or 3 were interpreted as high expression.

Unfortunately, staining was not robust enough with the Cdk5, p35/p25, and total CRMP2 antibodies to make significant conclusions on the data with these antibodies (***Figure 4.11***). Interestingly, however, immunopositive staining with the pCRMP2 Ser522 antibody was consistently enriched in the nuclei of tumour cells while no staining was detected in healthy tissue from the same biopsies (***Figure 4.11***).

Despite detectable immunopositive staining, the pattern of staining with the tau antibody was unclear which made interpretation of results difficult with this antibody. Additionally, interpretation of results with the pTau Ser235 phospho-specific antibody was difficult as staining was relatively low using this antibody therefore both antibodies were not included in further analyses.

Of potential clinical interest, pCRMP2 Ser522 immunopositive staining appeared more robust in squamous cell carcinoma (8/11 with a score of 2 or 3, average score = 1.91) than within the adenocarcinoma tumour biopsies (5 biopsies, none with score 3, average score = 1.8) in this preliminary analysis (***Figure 4.12***). Nonetheless, the numbers of adenocarcinoma biopsies were relatively low in comparison to squamous cell carcinoma biopsies and therefore this result is not conclusive.

	Antibody/Staining					
	CRMP2	**pCRMP2 Ser522**	**p35/p25**	**Cdk5**	**Tau**	**pTau Ser235**
Squamous Cell Carcinoma	1	2 (n/t)	2 (n>c) t	2 (n>c) t	2 (n>c)	1
	1	2 (n) t	2 (n) t	3 (n) t	3 (n>c) t	1
	1	1	2 (n) t	2 (n) t	2 (n>c) t	1
	1	2 (n) t	1	1		
	1	2 (n) t	1	3 (n) t	2 (n>c) t	1
	2 (c)	2 (n) t			2 (n/c) t	1
	1	3 (n) t			2 (n>c) t	2 (n) t
	3	3 (n) t				
	1	1				
	1	2 (n) t	1	1	1	1
	1	1	2 (n/c) t	2 (n/c) t		
Adenocarcinoma	1	1	1	1		
	1	2 (n) t				
	1	1	2 (n/c) t	2 (n/c) t	2 (n>c) t	1
	2 (c)	2 (n) t	2 (n/c) t	3 (n/c) t		
	2 (n)	2 (n) t				
Other Cancer Types	1	2 (n/t)	1	1	2 (c) t	1
	2 (n/c) t	3 (c) t	2 (n/c) t	3 (n/c) t	3 (n) t	1
	n/a	3 (n)				
	2 (n) t	2 (n) t				
	1	2 (n) t	2 (n>c) t	2 (n>c) t	2 (n/c)	1

Table 4.3. ***Evaluation of immunohistochemistry.*** Immunostained biopsies were graded using a semi-quantitative scale from 0-3, with 0 representing no staining, 1 representing light brown staining, 2 representing moderate brown staining and 3 representing dark brown staining. Scores of 2 or 3 were interpreted as high expression, n = nuclear, c = cytoplasmic and t = more in tumour than healthy tissue.

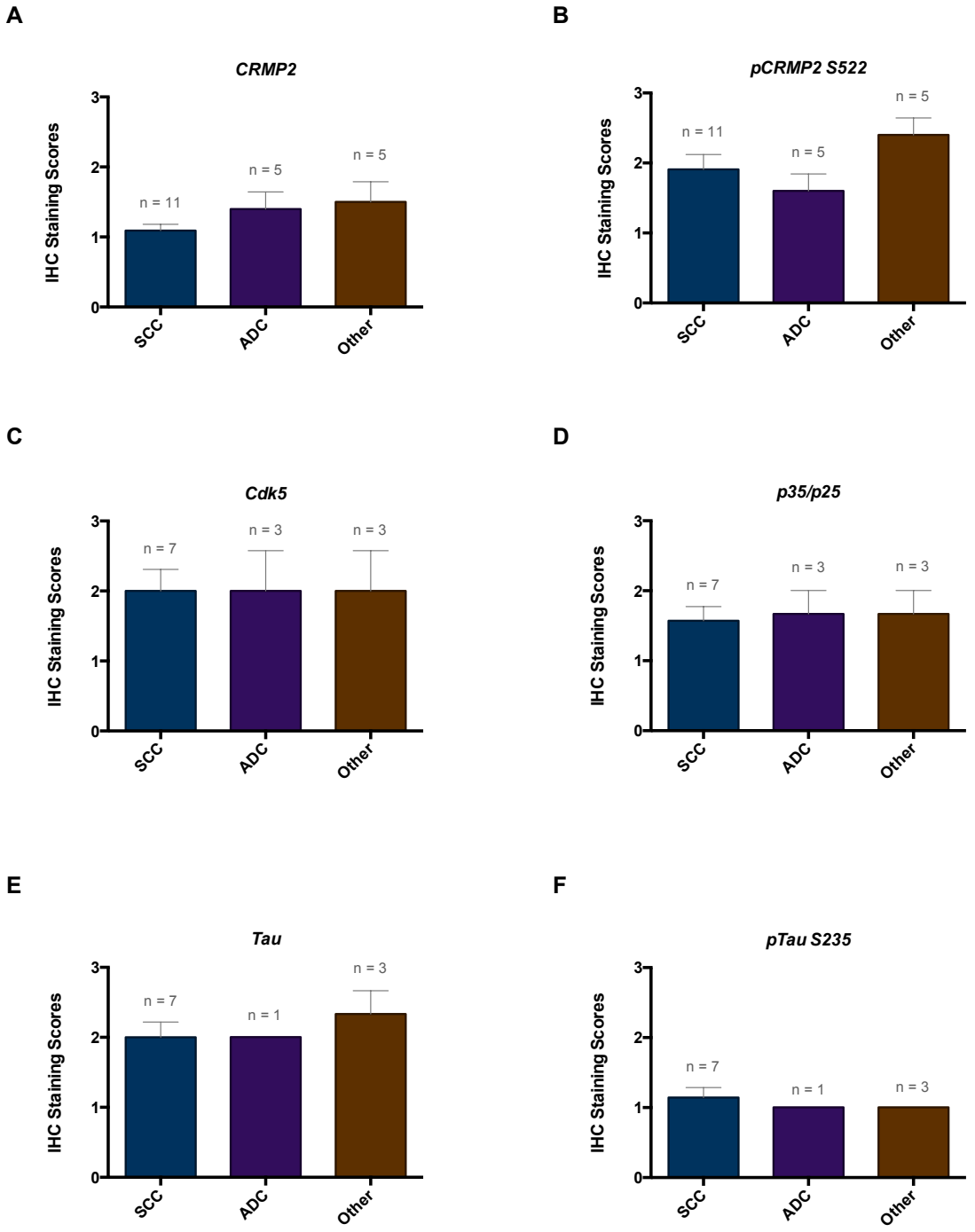


Figure 4.12. ***Immunohistochemistry staining scores of lung tumour biopsies.*** Immunohistochemical staining of squamous cell carcinoma (SCC), adenocarcinoma (ADC), and other lung tumour biopsies was graded using a semi-quantitative scale from 0-3, with 0 representing no staining, 1 representing light brown staining, 2 representing moderate brown staining and 3 representing dark brown staining. Scores of 2 or 3 were interpreted as high expression. Data expressed as ± SEM and the number of patient biopsies (n) is presented on the graph.

4.2.2.6 Further analysis of pCRMP2 staining in squamous cell carcinoma and adenocarcinoma

Following the observation of a potential variation in pCRMP2 Ser522 expression between squamous cell carcinoma and adenocarcinoma, an additional 18 lung tumour biopsies were obtained, containing roughly equal numbers of both tumour types, in order to compare histologically graded staining between adenocarcinoma and squamous cell carcinoma. In addition, a squamous carcinoma from skin was included in this analysis as squamous lung carcinoma is histologically different from squamous carcinoma sourced from other areas of the body.

Tissue sections were obtained from Dr. Phil Coates, Tayside Tissue Bank, University of Dundee. As described previously, the biopsies were stained using the phospho-specific pCRMP2 Ser522 antibody and evaluated independently by two investigators (Professor Frank Carey and Dr Yvonne Woods, Department of Pathology, Ninewells Hospital). The degree of staining was graded using a semi-quantitative scale as described previously (***refer to section 4.2.2.5)***.

Consistent with the preliminary observation, phosphorylated CRMP2 was predominantly localised in the nuclei of lung cancer cells and, importantly, absent in healthy tissue thereby confirming the unusual nuclear localisation and association with tumourigenesis (***Figure 4.13A***). Once more, immunostaining was detected in both cancer types however CRMP2 phosphorylation was of a significantly higher intensity in squamous cell carcinoma compared to adenocarcinoma immunostaining (***Figure 4.13B-C***).

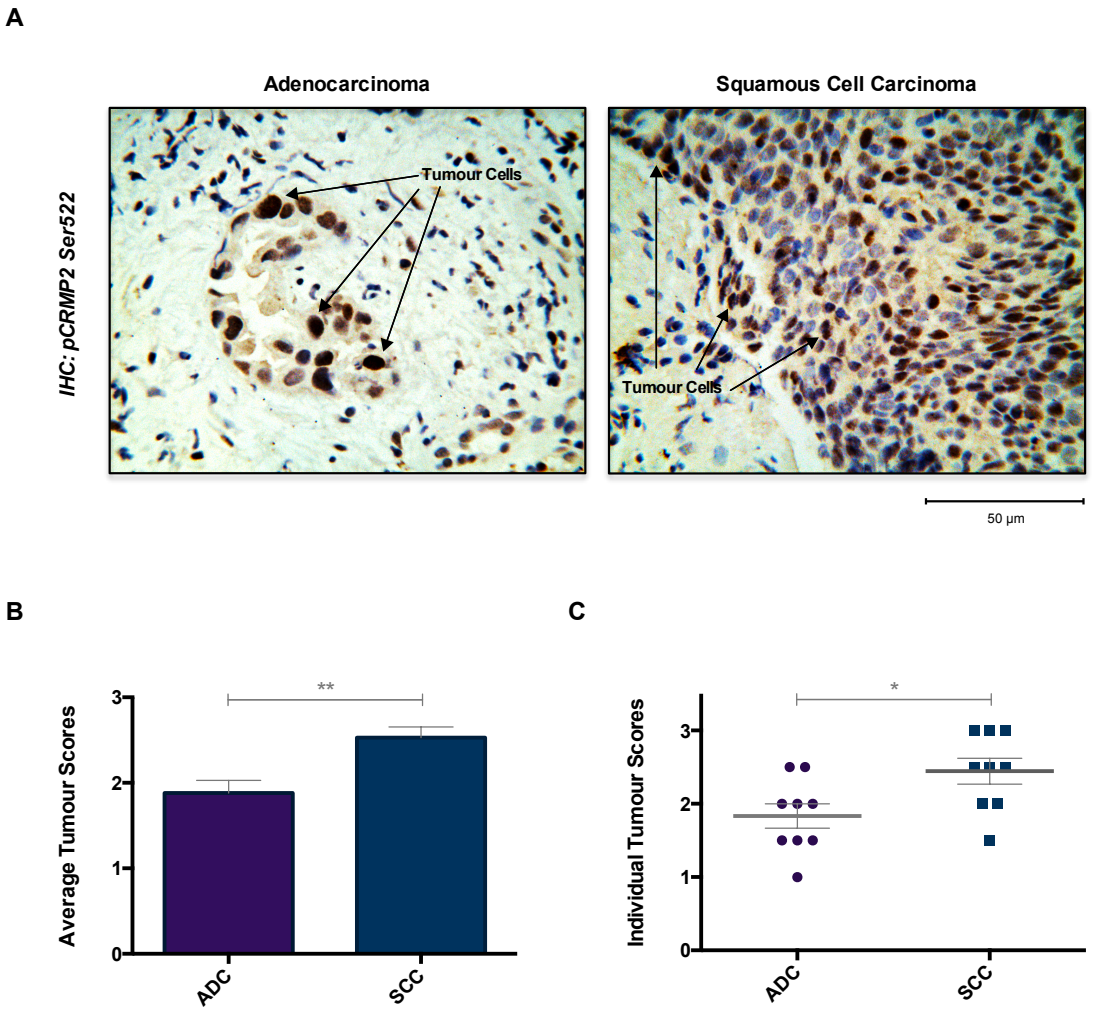


Figure 4.13. ***Phosphorylation of CRMP2 by Cdk5 is stained more intensity in squamous cell carcinoma compared to adenocarcinoma.*** A) Representative histology for adenocarcinoma and squamous cell carcinoma biopsies immunostained with pCRMP2 Ser522 antibody. *Scale bar* = 50 µm. B) Quantification of the average scores (from two independent pathologists) for pCRMP2 Ser522 immunostaining of adenocarcinoma (ADC) and squamous cell carcinoma (SCC) tumours. Data shown as mean ± S.E.M, n = 9 (ADC), 9 (SCC), *t*-test, $^{**}p<0.01$. C) Quantification of individual scores for pCRMP2 Ser522 staining of adenocarcinoma (ADC) and squamous cell carcinoma (SCC) tumours. Data shown as mean ± S.E.M, n = 9 (ADC), 9 (SCC), *t*-test, $^{*}p<0.05$.

4.2.2.7 How widespread is the abnormal pCRMP2 Ser522 in cancer?

The strong positive staining of lung tumour tissue with the pCRMP2 Ser522 antibody prompted a more general assessment of the reactivity of this antibody in different types of cancer using tissue microarrays (TMA) of human tumours, generated by Dr. Phil Coates in the Tayside Tissue Bank. Further optimisation and validation of antibodies was performed for use on the TMA platform.

4.2.2.7.1 Antibody staining in whole embryo

Prior to investigating which tumour types may exhibit pCRMP2 Ser522 staining, the tissue expression profile of CRMP2 and pCRMP2 Ser522 was examined in embryos from healthy animals. Unfortunately, as shown earlier, in contrast to work with rat primary neurons, the total CRMP2 antibody exhibited high background staining in human lung tissue. As it would be informative to have data on total CRMP2 expression, an alternative CRMP2 antibody was sourced (*Cell Signaling*) and subjected to the antibody optimisation protocol as described previously (***refer to sections 4.2.2.1 – 4.2.2.3***). This antibody required antigen retrieval and was used at a concentration of 1:1000. Both CRMP2 antibodies were screened against whole sagittal sections of a rat fetus (for further optimisation and to establish antibody specificity prior to screening human tumours).

Once more, the original total CRMP2 antibody (*DSTT*) exhibited high levels of background and the staining pattern was therefore not clear (***Figure 4.14A***). Negligible staining was observed with the second CRMP2 antibody (*Cell Signaling*) other than around the brain tissue (***Figure 4.14C-D***), which may question the specificity of the original CRMP2 antibody. This does suggest that the CRMP2 whole protein antibodies may only be of use in brain sections.

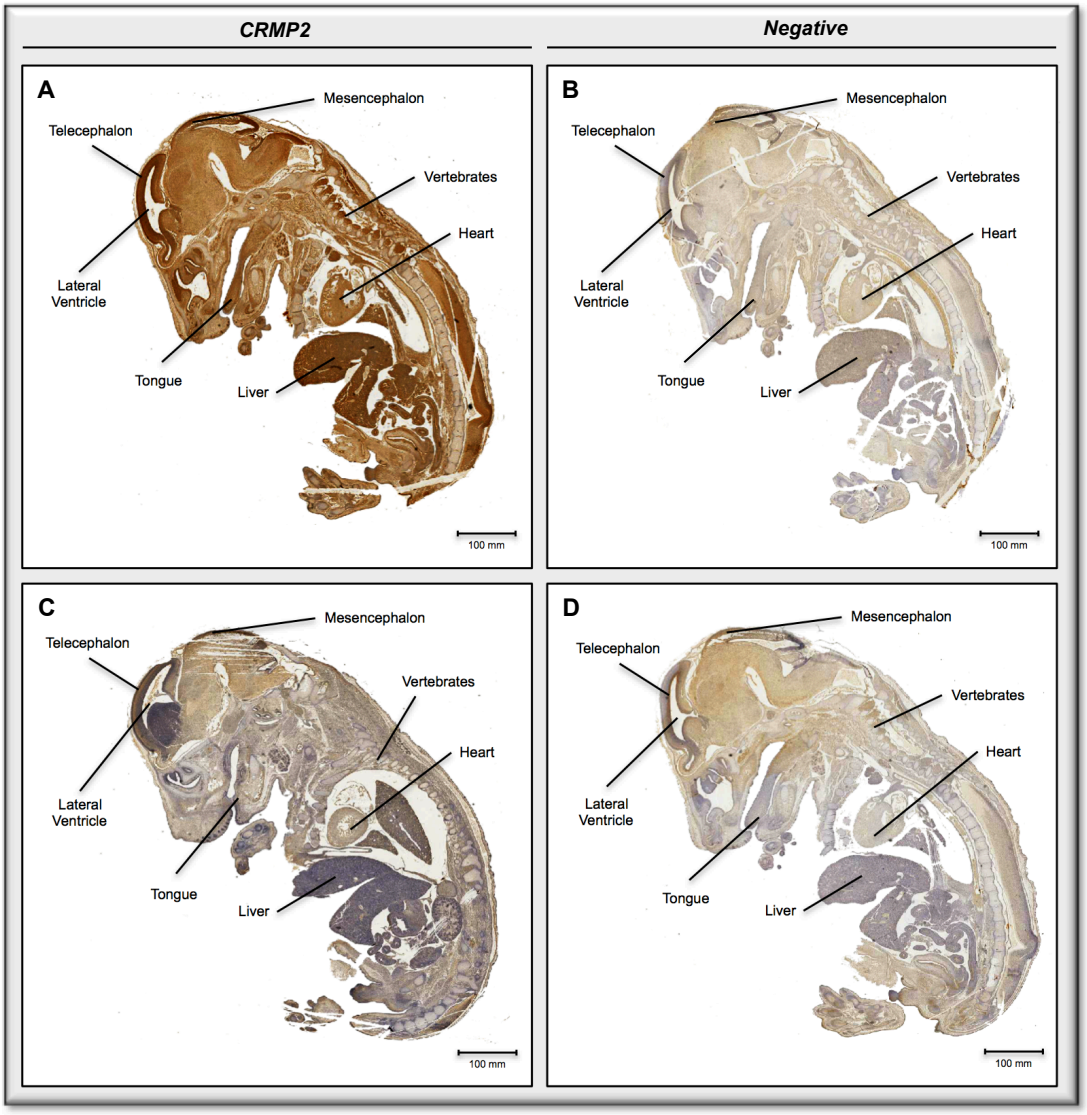


Figure 4.14. ***Total CRMP2 antibodies show a lack of tissue specificity.*** Rat fetus sections were incubated with antibodies to A) anti-CRMP2 (DSTT) and B) anti-CRMP2 (Cell Signaling), and C-D) corresponding negative controls. Subsequently, sections were incubated with biotinylated secondary antibodies followed by streptavidin complexed with biotinylated peroxidases that were visualised using brown DAB chromagen. *Scale bar* = 100 mm.

4.2.2.5.2 *Tissue specificity of pCRMP2 S522 antibody*

To further investigate tissue specificity of the pCRMP2 Ser522 antibody, staining was examined in healthy rat tissue from regions that would be analysed during the planned tumour TMA screen. Tissue sections were provided by Dr. Phil Coates, Tayside Tissue Bank, University of Dundee.

Sections consisting of ovary, prostate, lung, skin, thymus, and stomach tissue from healthy adult rats were immunostained using the pCRMP2 Ser522 phospho-specific antibody as described in ***Chapter 2, section 2.2.10.3***.

The pattern of pCRMP2 Ser522 antibody immunostaining appeared to be sporadic (***Figure 4.15***). Immunopositive staining was observed in the follicular cells of the ovary and strong staining was also apparent in the corpus luteum. The prostate displayed a high level, and possibly artefactual staining, around the periphery of the tissue, which made interpretation of results difficult. However, a degree of immunopositive staining was observed in some neurons. Within lung tissue, immunopositive staining appeared scattered throughout alveolar cells and positive staining was also observed in the occasional bronchus cell, the cells in which lung cancer is believed to originate. Staining with the pCRMP2 Ser522 antibody was negative in lymphocytes in the white pulp of the spleen however immunopositive staining was seen in myeloid cells present in the red pulp. Skin was mainly negative for pCRMP2 Ser522 expression however immunopositive staining was observed in some proliferative cells, including the basal cells of the epithelium and hair follicles. Finally, high intensity staining was observed in epithelial cells that line the stomach.

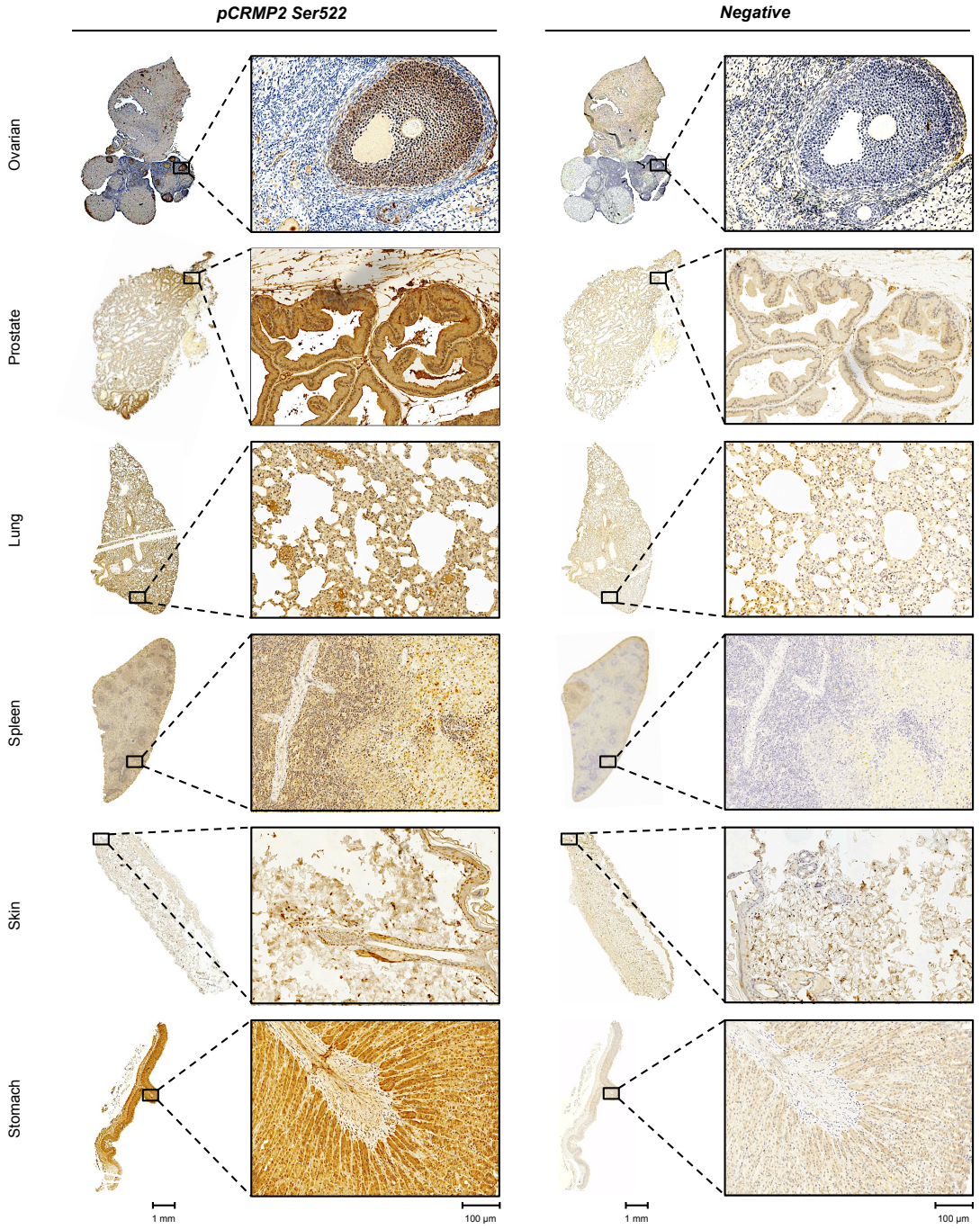


Figure 4.15. ***Immunohistochemical staining of rat tissue with a phospho-specific pCRMP2 Ser522 antibody.*** Following immunostaining of ovary, prostate, lung, spleen, skin, and stomach sections, bound primary pCRMP2 Ser522 was detected using VECTASTAIN® ABC system (*Vector Laboratories*) and visualised using DAB chromagen. Nuclei were counterstained with hematoxylin. Images were taken with Olympus x20/0.75NA objectives on an Aperio ImageScope (*Aperio Technologies, California, USA*), with a magnified image (at x20 zoom) of the same. *Scale bar* = 1 mm and 100 µm (inset).

4.2.2.8 Assessing phosphorylation of CRMP2 in human tumours by means of Tumour Microarray (TMA)

Tissue microarrays enable the simultaneous analysis of multiple tissue specimens for a large number of markers. Similarly, the TMA technique can be applied to multiple selected cancers (Tumour Microarray), where these are gathered into a single paraffin block prior to sectioning, mounting, and screening using IHC. TMAs available for analysis of CRMP2 phosphorylation included a range of human tumours, such as colorectal adenoma, prostate, renal, ovarian cancer, and follicular lymphoma.

TMA sections were stained using an antibody to anti-pCRMP2 Ser522 and visualised using DAB chromagen, as described in ***Chapter 2, sections 2.2.10.3 – 2.2.10.5***. The immunohistochemical stained sections were graded by Dr. Phil Coates, Tayside Tissue Bank, University of Dundee. The degree of immunohistochemical staining was assessed using quick score categories that were based on both the intensity and the proportion of brown staining: the proportion of malignant cells staining positively throughout the section was assigned scores from 1-6 (1 = >0-5%; 2 = 6-20%; 3 = 21-40%; 4 = 41-60%; 5 = 61-80%; 6 = 81-100%) and termed Category A; the average intensity in malignant cells was scored as 0, 1, 2, or 3, corresponding to the presence of negative, weak, moderate, and strong brown staining, respectively, and termed Category B. Negative cores were scored zero whereas positive cores were allocated Category A and Category B scores to form an additive quick score result. The observed immunohistochemical expression is depicted in TMA tissue cores punched from different locations on the tumour (four sample cores from each patient) (***Figure 4.16***, as an example of DLBCL scores). In the event that sample cores did not contain tumour cells, this was excluded from the analysis as an unrepresentative sample. A tumour sample was considered negative when all sample cores were negative,

whereas ≥ 2 positive cores was considered a positive result. The results of pCRMP2 Ser522 immunohistochemical staining are presented in ***Figure 4.17***.

There was only a single positive score for the control tissue TMAs (and that was the lowest quick score possible) therefore control tissue was deemed negative for pCRMP2 Ser522 staining. Likewise, ovarian, renal, prostate and colorectal cancers were almost completely negative for pCRMP2 Ser522 staining, with less than 1.7% of TMAs expressing immunopositive staining. Positive staining was observed in breast TMAs, however positive staining constituted only 8% of total patient samples and these were all only a quick score of 3 (4/48).

Interestingly, a greater number of sample cores taken from lymphomas were positive for pCRMP2 Ser522 staining. One in every ten of follicular lymphoma cores was positive for phosphorylated CRMP2, while a higher proportion of immunopositive cells were observed in samples cores from diffuse large B-cell lymphoma (DLBCL), with almost half (48.1%) of patient cases scoring positive and half of those with a quick score >3 (***Figure 4.16***). This data suggests that pCRMP2 Ser522 is present in more than just lung carcinoma.

Consistent with the data in individual sections of lung tumours (***Section 4.2.2.6***), positive staining was predominantly localised within the nuclei of tumour cells (***Figure 4.18***). Furthermore, positive staining of tumour cells was more apparent in squamous cancers and less consistent in adenocarcinoma across these TMAs (Personal communication, Dr. Phil Coates).

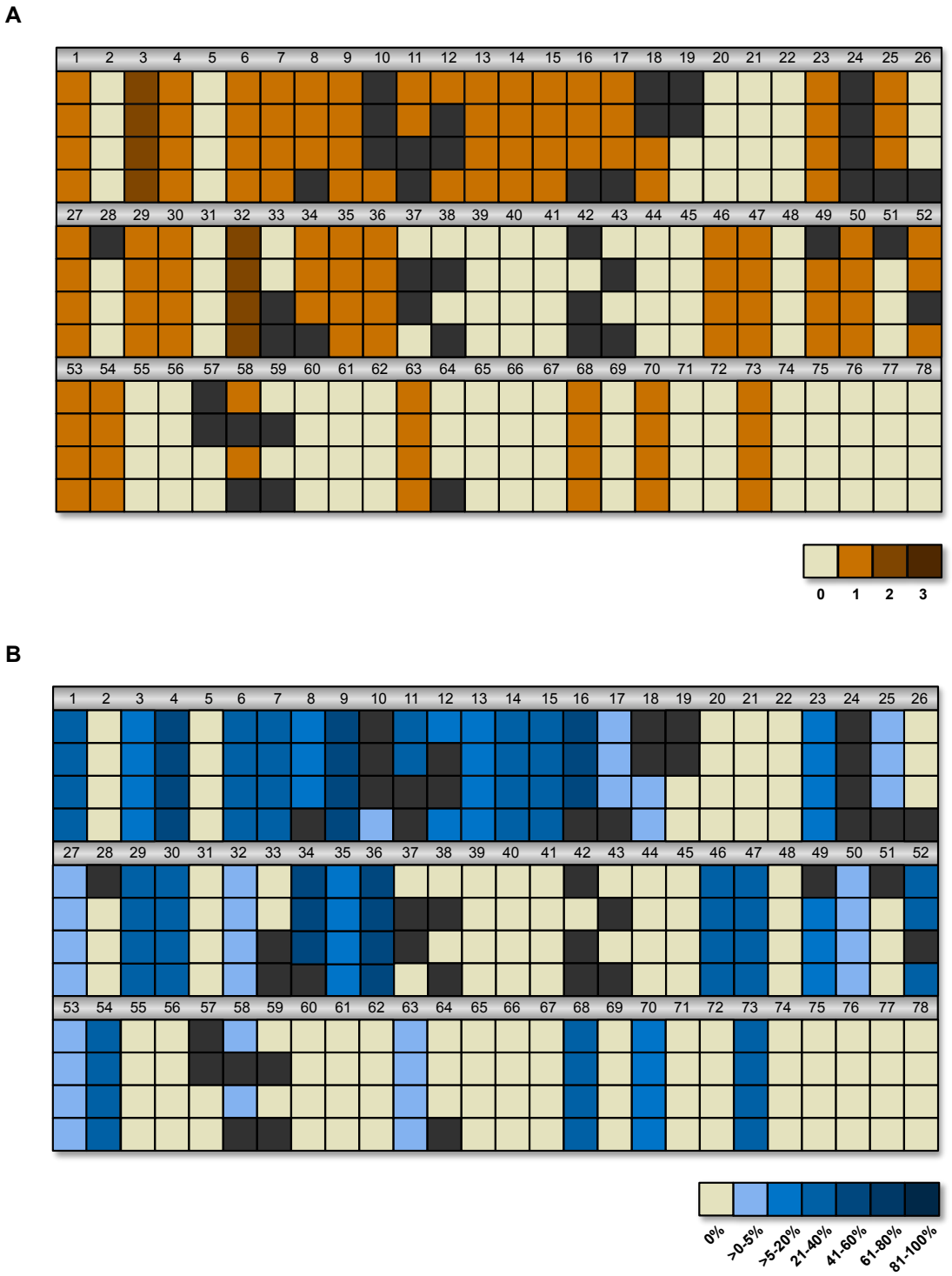


Figure 4.16. ***Diffuse Large B-Cell Lymphoma (DLBCL).*** Heat-map of TMA scoring for DLBCL cases. Each panel illustrates the scoring, observed for different cases of follicular lymphoma following immunohistochemistry with the pCRMP2 Ser522 phospho-specific antibody, in terms of A) staining intensity (scale of 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) and B) percentage of stained cells (scale: >0-5%; >5-20%; 21-40%; 41-60%; 61-80%; 81-100%, represented by increasingly darker shades of blue). Patient cases are represented numerically. Dark grey shading refers to the absence of a core for that patient case.

A

Quick Score

Category A (score for proportion staining)	Category B (score for intensity staining)
0 = no staining 1 = >0 – 5% 2 = 6 – 20% 3 = 21 – 40% 4 = 41 – 60% 5 = 61 – 80% 6 = 81 – 100%	0 = no staining 1 = weak staining 2 = moderate staining 3 = strong staining
Total Score = A+B = 0 to 9	

B

	Quick Score (A+B)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Cases**
Controls	0	1	0	0	0	0	0	0	0	14
Ovarian	0	0	0	1	0	0	0	0	0	79
Kidney	0	0	1	2	0	0	0	0	0	181
Prostate	0	1	1	0	0	0	0	0	0	25
Breast	0	0	4	0	0	0	0	0	0	48
Colorectal Adenoma	0	0	0	2	0	0	0	0	0	127
Follicular Lymphoma	0	2	1	2	0	0	0	0	0	46
Diffuse Large B-Cell Lymphoma	0	9	10	13	5	0	0	0	0	77

Figure 4.17. ***Frequency of additive quick scores in TMA result subsets.*** TMAs immunostained with pCRMP2 Ser522 antibody were scored using the quick score grading system. A) Quick score was determined by adding the scores for the proportion of cells stained (Category A) and the intensity of staining (Category B). Quick scores ranged from 0 to 9. B) Number of positively stained TMA quick scores for ovarian, kidney, prostate, breast, colorectal adenoma, follicular lymphoma, and diffuse large B-Cell lymphoma. The numbers in the table represent the total number of positive patient cases for the tumour specified while the case value represents the total number of patient samples for each TMA screen.

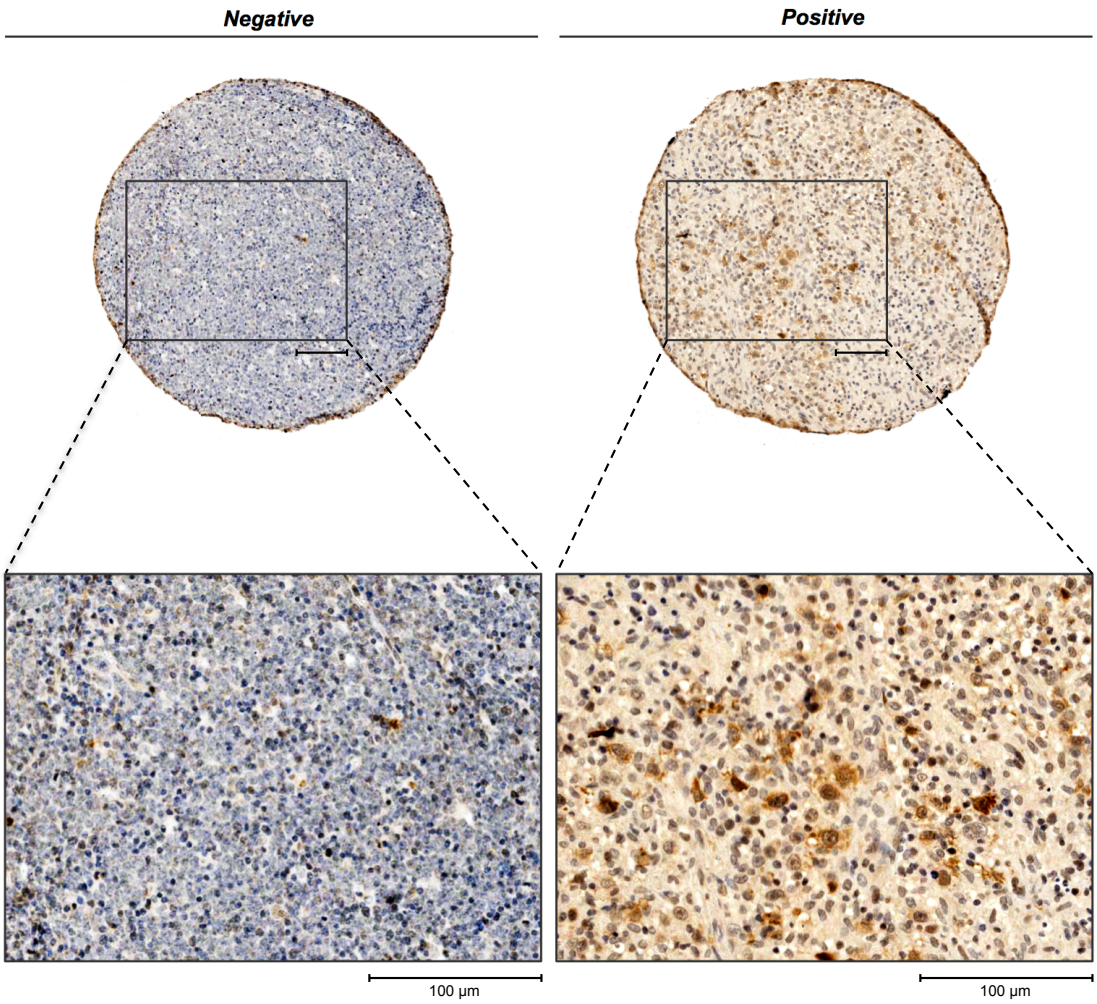


Figure 4.18. ***Immunohistochemical evaluation of pCRMP2 S522 protein expression in tissue microarrays of Diffuse Large B-Cell Lymphoma (DLBCL).*** Representative images of Diffuse Large B-Cell Lymphoma cores stained using pCRMP2 Ser522 illustrating A) negative expression of pCRMP2 Ser522 and B) positive (scored 2x2) expression of pCRMP2 Ser522. Images were taken with Olympus x20/0.75NA objectives on an Aperio ImageScope (*Aperio Technologies, California, USA*), with a magnified image (at x20 zoom) of the same. *Scale bar* = 100 µm.

4.2.3 Does phosphorylation of CRMP2 at Ser522 occur in the nucleus of tumour cells in culture?

The immunostaining of the nucleus of lung tumour cells with the anti-pCRMP2 Ser522 antibody was an unexpected result as there is very limited evidence that CRMP2 enters the nucleus of cells (at least in neurons where CRMP2 has mostly been studied). Therefore it was worthwhile to investigate whether CRMP2 protein expression could be detected in the nucleus of lung cancer cell lines.

4.2.3.1 Cdk5 and its cofactor are expressed in human lung cancer cell lines

Three human lung cancer cell lines representing each class of NSCLC were obtained to investigate CRMP2 phosphorylation and localisation; namely, the EBC-1 cell line derived from human lung squamous cell carcinoma, the A549 which is used as a cell-based model of adenocarcinoma, and, finally, the NCI-H460 cell line which serves as a model of large cell carcinoma.

Protein lysates of the three cell lines were subjected to SDS-PAGE and immunoblotted with antibodies to anti-CRMP2 (*DSTT*) and anti-pCRMP2 Ser522. The SHSY5Y human neuroblastoma cell line was included as a positive control. CRMP2 positive protein bands corresponding to the molecular weight of CRMP2 (62 kDa) were detected in all lines and the pSer522 phosphospecific antibody also detected a band at this same molecular mass (***Figure 4.19***). Indeed, the ratio of pCRMP2 Ser522 to total CRMP2 appeared in the same range to that found in SHSY5Y cells. Similarly, all cell lines contained Cdk5 and p35, as judged by IB using selective antibodies, however the lung lines appeared to contain less p25 than the SHSY5Y cells (***Figure 4.19***). This data confirms that these cell models of lung carcinoma express CRMP2 and p35/Cdk5 and CRMP2 is phosphorylated at Ser522.

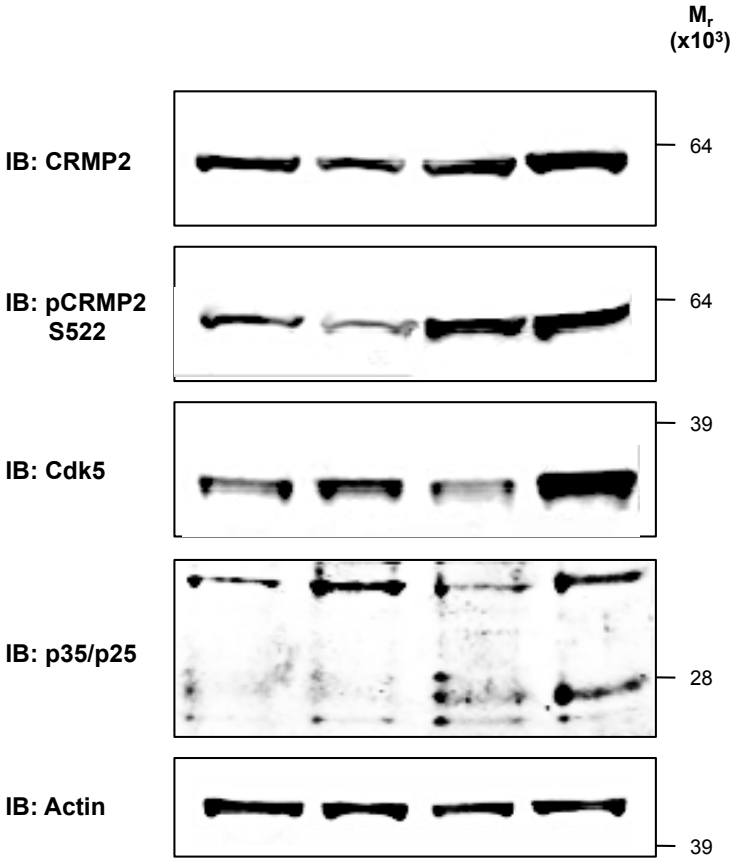


Figure 4.19. ***CRMP2 phosphorylation at Ser522 is recapitulated in NSCLC cell lines positive for CRMP2 and Cdk5 protein expression.*** Western blot analysis was performed to confirm CRMP2 and Cdk5 expression in three human immortal lung cancer cell lines representing each class of NSCLC. A549, EBC-1, and H460 cell lines were subject to SDS-PAGE and Western immunoblots probed with the following antibodies: anti-CRMP2, anti-pCRMP2 Ser522, anti-Cdk5, and anti-p35/p25. The SHSY5Y cell line was used as a positive control known for endogenous CRMP2, Cdk5 and cofactor protein expression. Western blots are representative of three independent experiments.

4.2.3.2 Fractionation of NSCLC cell lines reveals nuclear localisation of a potential CRMP2 isoform

CRMP proteins are reported to be predominantly cytosolic protein however investigation of lung tumour biopsies identified a nuclear epitope recognised by the phosphospecific pCRMP2 Ser522 antibody (***section 4.2.2.5***). This raises the intriguing possibility that the presence of phosphorylated CRMP2 in the nucleus is a cause or consequence of tumourigenesis and could provide diagnostic or mechanistic insight into lung carcinoma.

To obtain additional experimental evidence that CRMP2 truly exists in the nucleus, subcellular fractionation of the cancer cell lines that expressed phosphorylated CRMP2 protein was performed (***Figure 4.20***), and the resultant nuclear and cytoplasmic protein lysate fractions immunoblotted using total CRMP2 (*DSTT*) and pCRMP2 Ser522 antibodies. Cell lines were fractionated (as described in ***Chapter 2***, ***section 2.2.7.4***.) to generate cytoplasmic, membrane, nuclear (soluble), and nuclear (chromatin-bound) protein lysates. Fractionation efficiency was assessed by immunoblot using an antibody to anti-GAPDH (cytoplasm) and anti-histone H4 (nuclear (chromatin-bound)) (***Figure 4.20***).

CRMP2 protein expression (62 kDa) was found only in the cytoplasmic fraction for all cell lines (***Figure 4.20A-D***), however an apparent larger form of CRMP2 was detected in the soluble nuclear fraction. This band corresponded to the molecular mass of a known isoform of CRMP2 (CRMP2A) (75 kDa) that has an N-terminal extension due to alternative splicing (***Figure 4.20A-D***). This same band of apparent mass 75 kDa was also detected using the anti-pCRMP2 Ser522 antibody giving greater confidence that this is truly the CRMP2A isoform and indicating that

phospho-CRMP2 does exist in the nucleus. Interestingly, the shorter cytoplasmic form of CRMP2 (CRMP2B) was detected by the pCRMP2 Ser522 antibody, and this may be responsible for the weak cytoplasmic signal seen in the IHC studies above. This data suggests that alternative splicing of CRMP2 may contribute to its ability to localise to the nucleus and both isoforms of CRMP2 are regulated by phosphorylation at Ser522.

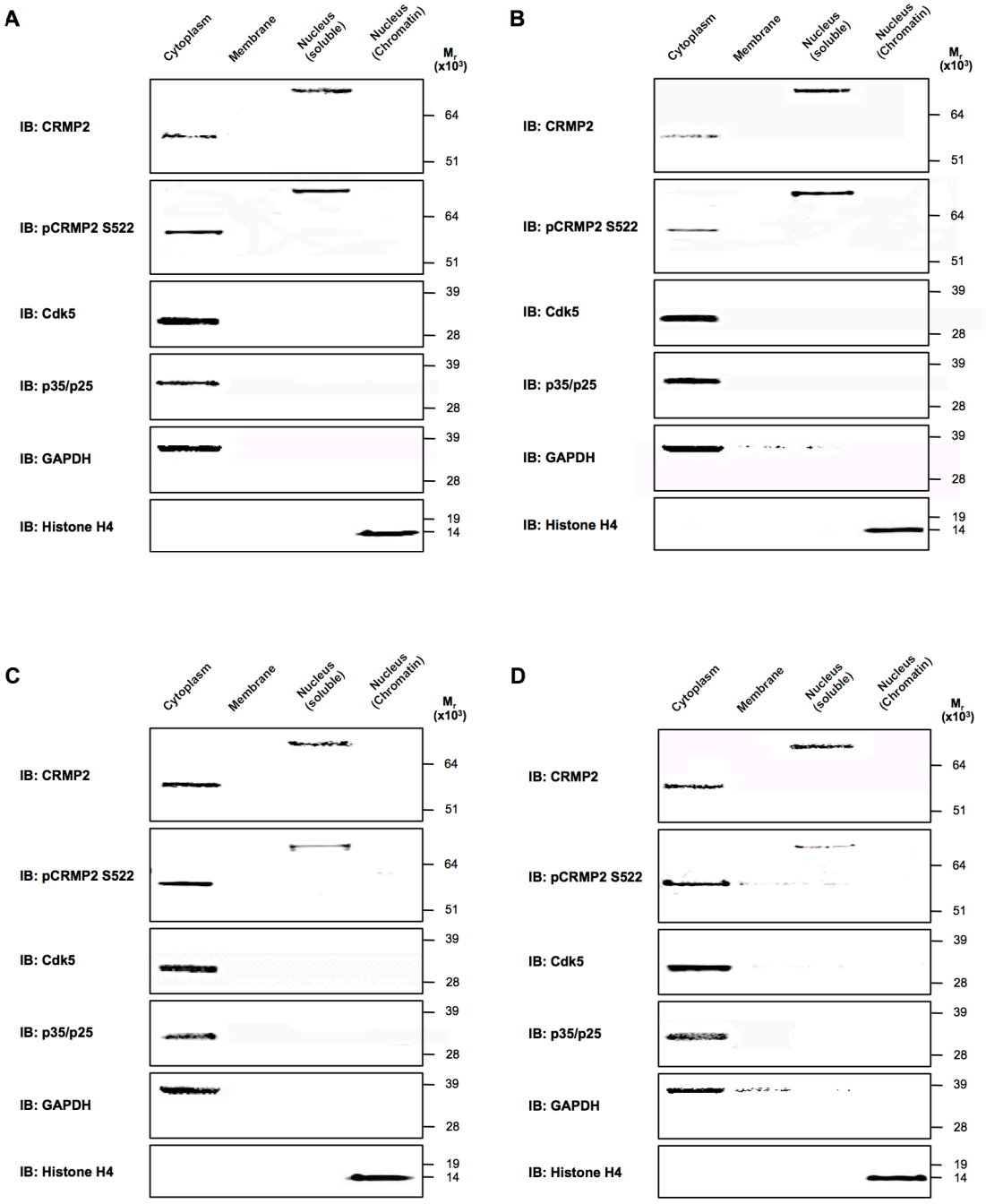


Figure 4.20. ***Identification of phosphorylated CRMP2 in nuclear fraction.*** Subcellular fractionation of A) A549, B) EBC-1, C) H460, and D) SHSY5Y cell lines. Cells were fractionated into cytoplasmic, membrane, soluble nuclear and chromatin-bound nuclear fractions, subjected to SDS-PAGE and immunoblots probed with the following antibodies: anti-CRMP2, anti-pCRMP2 Ser522, anti-Cdk5 and anti-p35/p25. Antibodies to anti-GAPDH and anti-histone H4 were used as subcellular markers for the cytoplasm and chromatin-bound nuclear fraction, respectively. Western blots are representative of three independent experiments.

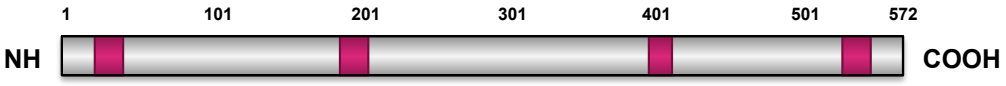
4.2.3.3 Nuclear expression of CRMP2 isoform confirmed by Mass Spectroscopic fingerprint analysis

It remained possible that the signal detected by the pCRMP2 Ser522 antibody in the nuclear fraction and IHC was unrelated to CRMP2 therefore Mass Spectroscopic (MS) analysis was performed to obtain additional evidence supporting nuclear CRMP2A localisation as this technique is independent of antibody specificity.

The EBC-1, HeLa, and HEK293 cell lines were subjected to subcellular fractionation to isolate the cellular nuclear fraction. Duplicate samples of these protein lysates were separated by SDS-PAGE before the gel was subsequently split into two, each half containing one sample from each cell line. One half of the gel was subjected to immunoblotting to confirm the presence and molecular mass of an anti-P-Ser522 CRMP2 reactive protein; whereas, the second half of the gel was Coomassie stained and each band equivalent to pCRMP2 Ser522 staining (*approx.* 75 kDa) was excised, digested by trypsin, and sent to the Dundee Proteomic Service for MS fingerprint analysis, searching solely for tryptic peptides from human CRMP2 isoforms.

MS analysis of the HEK293 nuclear fraction positively identified three peptides that corresponded to either isoform of CRMP2 (***Figure 4.21***). Additionally, a fourth peptide that corresponded to the N-terminal extension region only found on CRMP2A was identified, providing compelling evidence that CRMP2A is present within the nucleus, at least in HEK293 cells. Unfortunately, CRMP2 peptides were not detected to a significant level in HeLa and EBC-1 nuclear lysates suggesting that expression is relatively low in these cell lines, therefore it would be worth repeating this analysis starting from a larger number of cells and possibly including analysis of additional proteins with known nuclear expression levels as an internal positive control.

A



B

	1	11	21	31	41	51	61	71
1	MSYQGKKNIP	RITSDRLLIK	GGKIVNDDQS	FYADIYMEDG	LIKQIGENLI	VPGGVKTIEA	HSRMVIPGGI	DVHTRFQMPD
91	QGMTSADDFF	QGTKAALAGG	TTMIIDHVVP	EPGTSLLAAF	DQWREWADSK	SCCDYSLHVD	ISEWHKGIQE	EMEALVKDHG
161	VNSFLVYMAF	KDRFQLTDCQ	IYEVLSVIRD	IGAIAQVHAE	NGDIIAEEQQ	RILDLGITGP	EGHVLSRPEE	VEAEAVNRAI
241	TIANQTNCPL	YITKVMSKSS	AEVIAQARKK	GTVVYGEPLT	ASLGTDGSHY	WSKNWAKAAA	FVTSPPLSPD	PTTPDFLNSL
321	LSCGDLQVTG	SAHCTFNTAQ	KAVGKDNFTL	IPEGTNGTEE	RMSVIWDKAV	VTGKMDENQF	VAVTSTNAAK	VFNLYPRKGR
401	IAVGSDADLV	IWDPDSVKTI	SAKTHNSSLE	YNIFEGMECR	GSPLVVISQG	KIVLEDGTLH	VTEGSGRYIP	RKPFPDFVYK
481	RIKARSRLAE	LRGVPRGLYD	GPVCEVSVTP	KTVTPASSAK	TSPAKQQAPP	VRNLHQSGFS	LSGAQIDDNI	PRRTTQRIVA
561	PPGGRANITS	LG						

Figure 4.21. ***Identification of peptide corresponding to CRMP2A identified in HEK293 nuclear fraction.*** Schematic representation of MS analysis results following isolation of HEK293 nuclear component, SDS-PAGE, and excision of band of interest to depict A) the position of identified peptides within CRMP2A and B) the amino acid sequence of each peptide (highlighted) within the full protein sequence.

4.3 DISCUSSION

Recent studies have linked abnormal expression of Cdk5 in many malignancies, including prostate cancer, lung cancer, and glioblastoma (Strock *et al*., 2006; Hsu *et al*., 2011; Feldmann et *al*., 2010; Eggers *et al*., 2011; Demelash *et al*., 2012; Liu *et al*., 2011); however, the association of Cdk5 activity with tumourigenesis still remains controversial. Therefore, the work described in this chapter attempted to develop protocols to quantify the degree of Cdk5 substrate phosphorylation in human tumours as a surrogate for Cdk5 activity. Ideally a panel of protein sites that are very specifically regulated by Cdk5, with optimised and highly selective phosphospecific antibodies, would provide the most useful assay for Cdk5 action in tumourigenesis. This chapter presented characterisation of two potential substrates as initial members of such a panel, namely CRMP2 and tau. In addition, evidence for the first time was provided that abnormal phosphorylation of CRMP2, at a site that is sensitive to Cdk5 inhibition or deletion (Ser522), is enriched in human tumour cells. Uncharacteristic for CRMP2, this abnormal phosphorylation is present in the nucleus of tumour cells and may represent a specific isoform of CRMP2. Hence, this work implicates abnormal Cdk5 activity as a cause or consequence of specific cancers and further investigation of the mechanism that leads to this abnormal epitope may provide diagnostic or therapeutic value.

4.3.1 Cdk5 substrate phosphorylation is apparent in human tumours

The biological function of CRMP2 is modulated by phosphorylation at residues Ser509 through Ser522, with phosphorylation of Ser522 acting as the priming mechanism that leads to the other modifications (Fukata *et al*., 2002, Brown *et al*., 2004, Uchida *et al*., 2005, Yashimura *et al*., 2005, Cole *et al*., 2007). Consistent with previous findings inthe laboratory (Cole *et al*., 2006), evidence presented in this study

confirms that Cdk5 is both necessary and sufficient for phosphorylation of CRMP2 at Ser522. Furthermore, this work shows for the first time that Ser522 phosphorylated CRMP2 is present in lung tumour cells, with no detection in healthy lung tissue in the same sections. Phosphorylated CRMP2 was predominantly localised in the nuclei of lung tumour cells and may therefore be a distinctive characteristic of highly proliferative cells. If so, this would support the notion that abnormal CRMP2 phosphorylation by Cdk5 may be associated with lung cancer development. Of course, it remains possible that an alternative Ser522 kinase is enhanced in tumours, or that the phosphatase responsible for dephosphorylation of Ser522 is lost. The identification of additional Cdk5 substrates for inclusion in these analyses would help dissect these possibilities. What appears likely, however, is that CRMP2 function is altered in lung tumours compared with surrounding healthy tissue.

Until very recently, a role for the CRMP family in cancer biology had not been proposed however during this study of CRMP2 phosphorylation in tumours, two groups reported abnormal CRMP2 phosphorylation in human tumours (Oliemuller *et al*., 2013; Shimada *et al*., 2013). Firstly, total CRMP2 protein expression was increased in NSCLC tissue compared to healthy tissue, and phosphorylated CRMP2 was predominantly detected in tumours cells and absent in healthy tissue (Oliemuller *et al*., 2013). The second group reported phosphorylated CRMP2 predominantly in the nuclei of breast cancer cells, and not in healthy mammary tissue, despite a significant decrease of CRMP2 mRNA and protein expression in the breast cancer tissue (Shimada *et al*., 2013). Although the role of CRMP2 protein expression remains unclear (increased CRMP2 expression in lung; decreased CRMP2 expression in breast cancer), possibly due to the poor quality of total CRMP2 antibodies, both groups report increased expression of phosphorylated CRMP2 predominantly localised in the

nuclei of tumour tissue. Of importance, these groups used a different phosphospecific CRMP2 antibody to the current work, although the epitope was overlapping with Ser522. They used an antibody that recognises a multiply phosphorylated peptide (pThr509, pSer518, and pSer522) and suggest an association of GSK3 to cancer progression as our lab have reported Thr509 and Ser518 as targets for this protein kinase. However this clearly provides further confidence that it is truly CRMP2 that is being detected in these different studies. Hence, the results described in this chapter add to a growing body of evidence recognising abnormal CRMP2 in tumour cells and implicating the modulation of CRMP2, potentially through Cdk5 activation, as an important aspect of tumourigenesis. It is worth noting that there are three completely novel aspects to the findings presented in the current study: firstly, the link with Cdk5 rather than GSK3; secondly, the identification of the long isoform of CRMP2 as the nuclear form; and thirdly, the potential difference between cancer types in intensity of staining of pCRMP2 Ser522.

4.3.2 CRMP2 phosphorylation is greater in squamous cell carcinoma compared to adenocarcinoma

Two separate studies of lung tumour cells with the pCRMP2 Ser522 antibody identified both adenocarcinoma and squamous cell carcinoma, and both suggested that staining was significantly more intense ($p = 0.03$, student's t-test) in squamous cell carcinoma, exhibiting a higher histological grade in comparison to adenocarcinoma. This data may indicate increased CRMP2 phosphorylation in squamous cell carcinoma as a result of increased Cdk5 activity however an assessment of Cdk5 activity would be required to confirm this.

While immunohistochemistry is emerging as a useful resource in the subtyping of

non-small cell carcinoma, it is important to emphasise that the majority of adenocarcinoma and squamous cell carcinoma can already be distinguished based on standard morphological criteria, with immunostains required in only a subset of cases (Rehktman *et al*., 2011; Righi *et al*., 2011; Lou *et al*., 2010; Edwards *et al*., 2010). Therefore, immunostaining with pCRMP2 Ser522 antibodies may not provide added diagnostic value as a marker for differentiating between adenocarcinoma and squamous cell carcinoma however an immunopositive pCRMP2 Ser522 stain might be able to distinguish a metastatic squamous cell carcinoma of primary lung origin from an origin in alternative sites. Of course, this would need to be confirmed by future more directed studies. Additionally, dissecting the role of Cdk5 and/or CRMP2 in the mechanism of tumourigenesis in the tumour subtypes may provide novel targeted cancer therapeutic strategies. Interestingly, in the study by Oliemuller and colleagues, a significant correlation between phosphorylated CRMP2 and poor outcome in NSCLC patients was proposed (Oliemuller *et al*., 2013). Such an analysis was not possible in the present work but would be worth investigating using our more specific antibody. It is worth noting that adenocarcinoma and squamous cell carcinoma have variable rates of growth, therefore the staining intensity may potentially be linked to the rate of progression of the disease.

4.3.3 Phosphorylated CRMP2 in other cancers

Phosphorylated CRMP2 was not a common feature of all cancers, suggesting that the phospho-specific CRMP2 antibody is not simply identifying a general upregulation of proliferation, rather a more specialised property of a few cancer types (e.g. lung, lymphoma, and breast). In all cases, immunopositive staining was localised predominantly in the nuclei and not found in surrounding healthy tissue. Furthermore, immunopositive staining was not detected in all tumour cells providing confidence

that the nuclear staining was not background noise but rather indicative of some selective mechanism that could provide diagnostic or therapeutic value.

Oliemuller *et al*., proposed that CRMP2 participates in cell division in a phosphorylation-dependent manner (Oliemuller *et al*., 2013). It has been known for some time that CRMP2 phosphorylation impairs its ability to bind tubulin structures therefore, these researchers extrapolate that knowledge to suggest phosphorylated CRMP2 could delay spindle assembly and entry into the metaphase of the cell cycle; conversely, non-phosphorylated CRMP2 would increase microtubule stabilisation and interfere with cell cycle progression into anaphase. During cell division, the mitotic spindle, a highly specialised and dynamic structure that mediates the alignment of replicated chromosomes to the equatorial plane and their subsequent transmission to daughter cells, is formed by microtubules (Rovini *et al*., 2011). Microtubules are dynamic polymers formed by tubulin heterodimers, in continuous equilibrium between growth and destruction (Downing *et al*., 1998) and the concurrent activity of microtubule-associated proteins (MAPs), including the CRMP family, are required to achieve microtubule formation. MAPs contribute to the transport, localisation, and regulation of microtubule components (Bhat & Setaluri, 2007). CRMP2 has been detected in the mitotic spindle of transformed mouse and human cells (Tahimic *et al*., 2006) and, recently, it was demonstrated that CRMP2 binds to tubulin during mitosis, whereas its depletion leads to destabilised anaphase microtubules and altered spindle position (Lin *et al*., 2011). This suggests that CRMP2 plays an important role in mitosis and CRMP2 phosphorylation defects may result in important defects during cell cycle progression, which is often present in cancer cells enabling uncontrolled cell proliferation. However, this would not explain the selective pCRMP2 staining across different tumour types and requires further study.

4.3.4 CRMP2 nuclear localisation in cancer cell lines

The CRMP family have always been considered cytosolic proteins as they are predominantly expressed in the neurites and cell bodies of neurons, and when the predominant isoform (CRMP2B) is transfected into COS cells, the recombinant protein localises to the cytoplasm ((Goshima *et al*., 2009), *data not shown*). Therefore it was surprising to observe pCRMP2 Ser522 expression in the nuclei of tumour cells.

4.3.4.1 CRMP2A is observed in the nucleus of cancer cell lines

CRMP2 is alternatively spliced to generate two subtypes (A and B; long- and short-form, respectively) (Bretin *et al*., 2005; Minturn *et al*., 1995; Goshima *et al*., 1995; Inagaki *et al*., 2001; Fukata *et al*., 2002), derived through an N-terminal extension (Yuasa-Kawada *et al*., 2003). When total cell lysates are analysed by IB for CRMP2 expression both isoforms are usually detected, the major isoform (CRMP2B) with apparent molecular mass of 62 kDa and a second long-form at 75 kDa (CRMP2A). Interestingly, subcellular fractionation of multiple immortalised cell lines revealed CRMP2B protein expression only in the cytoplasmic fraction, while CRMP2A was detected solely in the nuclear fraction. This isoform specific localisation has never been previously reported and additional mass fingerprinting data from HEK293 cells supported this finding.

There is evidence to suggest that post-translational modifications of CRMP2 can alter its cellular localisation. For example, post-translational processing of CRMP2 into a 58 kDa isoform has been reported to alter the spatio-temporal localisation (Hao *et al*., 2006). CRMP2 is thought to contain a nuclear localisation signal (NLS) within residues Arg^{171} – Lys^{472} that becomes unmasked after C-terminal clipping. It is possible that the N-terminal extension in CRMP2A either unmasks this NLS or

contains an alternative NLS. Clearly, mutation of the central NLS within CRMP2A or addition of the N-terminal extension to fluorescent proteins could uncover the relative importance of each region to the nuclear localisation of CRMP2A. It would also be worth investigating whether polymorphisms in the N-terminal region of the CRMP2A gene are associated with cancer genesis, progression, or prognosis. Of note, the N-terminal extension of CRMP2A contains an additional proline-directed phosphorylation site with some aspects of a Cdk5 consensus sequence.

4.3.4.2 A novel role for the long form of CRMP1 in tumourigenesis

There are some lines of evidence implicating the long-form of a related CRMP protein in cancer (Pan *et al*., 2010, Pan *et al*., 2011, Wang *et al*., 2012). Pan and colleagues reported that the long-form of CRMP1 (LCRMP1), which undergoes a similar N-terminal exon 1 splice event as CRMP2A, has a positive correlation with lung cancer cell invasiveness using both *in vitro* and *in vivo* invasion assays (Pan *et al*., 2010). Furthermore, high LCRMP1 mRNA expression was associated with poor overall and disease-free survival in NSCLC patients.

4.3.5 Developing additional readouts of Cdk5 activity *in vivo*

The original aim of this chapter was to develop a panel of validated Cdk5 substrates that could simultaneously provide quantitative information on overall Cdk5 activity in human tissue. A major hurdle in this quest was the development of a suitable cell model system in which to validate Cdk5 substrates and further investigate the relationship between preferred substrate sequence determinants and degree of substrate phosphorylation.

4.3.5.1 Inefficiency of the triple transfection system

Despite successful overexpression and generation of active Cdk5 complexes in HEK293 cells, triple transient transfection in these cells proved unreliable. It is known that co-transfection of large copy numbers of multiple different gene promoters can 'squelch' key transcription factors, resulting in reduced activity of one or more of the gene promoters. However, substantial experiments comparing different ratios of expression constructs did not overcome the problems. HeLa cells presented a reasonable alternative as they were reported to express endogenous Cdk5 and would, therefore, only require a double transfection (cofactor and substrate), while allowing direct comparison of p35 with p25 (as neither was thought to be present in these cells). Unfortunately, Cdk5 expression was not sufficient to allow induction of endogenous activity by transfection with p35 or p25. Future development of a suitable cell system may require the use of targeted stable insertion of inducible Cdk5 and cofactor genes, permitting subsequent single transient or stable transfection with substrates of interest.

4.3.6 Conclusions

The results described in this chapter present four major advances: firstly, they provide additional evidence that abnormal CRMP2 phosphorylation occurs in the nuclei of multiple tumour types; secondly, they implicate abnormal Cdk5 activity in lung cancer, breast cancer, and lymphoma; thirdly, they identify the long isoform of CRMP2 (CRMP2A) for the first time as the nuclear form of this protein and the one linked to tumourigenesis; and finally, they provide preliminary evidence that the intensity of staining of pCRMP2 Ser522 varies between adenocarcinoma and squamous cell carcinoma.

Overall, the data will serve as a platform to improve mechanistic insight into tumourigenesis in specific forms of cancer that will hopefully guide additional diagnostic and therapeutic strategies for cancer in the near future.

4.4 SUMMARY

In summary, cell-based and immunohistochemical techniques were utilised in order to validate substrates and develop tools to investigate Cdk5 substrate phosphorylation in human cancer. Work in this chapter generated evidence that CRMP2 phosphorylation at Ser522 reduced when Cdk5 was inhibited in primary cells, and increased when Cdk5 was overexpressed. This gave confidence that phosphorylation of Ser522 on CRMP2 was a reasonable surrogate for Cdk5 activity in intact cells and tissue. In NSCLC and DLBCL, pCRMP2 Ser522 was predominantly observed in the nuclei of tumour cells but absent in normal cells, thereby implicating Cdk5 activity and modulation of CRMP2 as an important aspect of tumourigenesis. Other groups recently published related findings, which gave confidence that this phosphorylation event is a potential marker of an aspect of tumourigenesis.

CHAPTER 5

Final Discussion

5.1 RESEARCH QUESTIONS SURROUNDING CDK5

The initial discovery of Cdk5 was made due to its strong sequence homology to Cdk1 and Cdk2 and added a new member to the Cyclin-dependent kinase family, albeit an apparently uncharacteristic member with no association with known cyclins. The majority of studies have since focused on the functions of Cdk5 in the development of the nervous system and in differentiated neurons where it appears enriched. More recently, however, the physiological functions of Cdk5 are being reassessed due to an increasing number of proposed substrates not involved in neuronal functions. Collectively, these substrates do not appear to have a specific consensus sequence differentiating them from substrates of other Cdks, making it even more vital to validate physiological and pathophysiological targets. Cdk5 has previously been associated with malignancy however this remains extremely controversial. The role of Cdk5 in disease is further complicated by the proposal that proteolytic cleavage of the Cdk5 activator, p35 results in altered subcellular localisation, dysregulation of kinase activity and altered substrate profile, with this process contributing to the pathophysiology of multiple diseases including neurodegenerative disease and cancer. In order to provide evidence for or against the proposed role of Cdk5 in disease, the work in this thesis aimed to further characterise Cdk5 substrate selection at the primary sequence level, determine whether or not proteolytic cleavage of p35 to p25 alters the specificity of the Cdk5 complex to specific substrates, and, finally, investigate whether abnormal phosphorylation of Cdk5 substrates was present in tumour cells.

5.2 MAIN CONTRIBUTIONS OF THE THESIS

The present study systematically defined a consensus sequence for Cdk5 substrate recognition, validated Cdk5 substrates using specific assessment criteria to establish whether they could act as a read-out of Cdk5 activity in cells and tissues, and examined substrate phosphorylation in human tumours. A major outcome of this work was the identification of an abnormally phosphorylated Cdk5 substrate in lung tumours suggesting that abnormal Cdk5 substrate phosphorylation may contribute to an aspect of the disease process or serve as a clinical diagnostic. There were two unusual and novel aspects to this abnormal tumour associated phosphorylation event: firstly, that the substrate, CRMP2 was phosphorylated at Ser522 only in the nucleus of the tumour cells; and secondly, that it appeared to be a minor isoform of this family of proteins.

5.2.1 Abnormal Cdk5 phosphorylation in cancer

CRMP2 was characterised as a substrate specifically regulated by Cdk5 in neurons therefore abnormal phosphorylation of this site was investigated as a read-out of altered Cdk5 activity in tumour cells. As a result, this is the first study to associate Cdk5 regulation of CRMP2 in tumourigenesis. Interestingly, studies published towards the end of the current work reported abnormal CRMP2 phosphorylation at Thr509, Ser518, and Ser522 in both breast and lung cancer (Shimada *et al*., 2013; Oliemuller *et al*., 2013). However, they did not link Cdk5 to this event, rather the downstream regulator GSK3, therefore they may have missed the key regulatory pathway in this process as Cdk5 phosphorylation of Ser522 is a rate-limiting step for GSK regulation of CRMP2. Both of these studies identified abnormal CRMP2 phosphorylation in the nuclei of tumour cells. Detection in breast tissue suggests that abnormal Cdk5 phosphorylation is more widespread than lung tumours and this work

found immunopositive staining in a few cancer types but not all, suggesting this is not just a marker of general upregulation of proliferation. Furthermore, the current work provides preliminary evidence that CRMP2 phosphorylation appeared to be more intense in squamous cell carcinoma compared to adenocarcinoma.

The abnormal CRMP2 phosphorylation was enriched in the nuclei of tumour cells in direct contrast to neuronal CRMP2 where it only exists as a cytosolic protein. This work suggests that it is the long-form of CRMP2, which was not investigated in the recent reports of CRMP2, GSK and tumourigenesis.

In summary, the work presented in this thesis implicates abnormal Cdk5 regulation of CRMP2 as a cause or consequence of specific cancers, and presents a potential new pathway to serve as a platform to enhance mechanistic insight into tumourigenesis in specific forms of cancer. Additionally, further investigation of the mechanism that leads to abnormal phosphorylation of CRMP2 may present new opportunities for diagnostic and therapeutic strategies.

5.2.2 Towards a Cdk5 consensus sequence and the role of p35 cleavage

In the present study, *in vitro* peptide analysis revealed an unanticipated, extremely selective peptide substrate profile for Cdk5 leading to the conclusion that Cdk5 substrate selection is defined, at least in part, at the primary sequence level. Subsequently, P-K-**S-P**-R-K was proposed as the optimal sequence consensus for Cdk5 substrate phosphorylation from the examined peptide combinations. In support of this consensus, a previous publication determined the consensus sequence of optimal substrates by sequencing the mixture of products generated during a brief reaction with a kinase of interest and established the optimal consensus for p35/Cdk5

as H/P-K-**S-P**-K/R-H/R/K (Songyang *et al*., 1996). Expanding on these findings, the current work showed that the N-terminal -2 residue appears to be crucial for peptide substrate phosphorylation and residues surrounding the phosphoacceptor residues affect the rate of substrate phosphorylation, signifying the importance of primary sequence for Cdk5 substrate selection. Unfortunately, an optimal consensus sequence for substrate recognition is not sufficient to identify all *bona fide* substrates, as substrate phosphorylation *in vivo* does not rely solely on primary sequence for recognising substrates. However, this information provides an evidence base to examine proteins for this motif and investigate whether Cdk5 phosphorylates these proteins.

It is often reported that the catalytic ability of Cdk5 is significantly increased when complexed to p25, in comparison to p35; therefore conversion of p35 to p25 is believed to induce Cdk5 'hyperactivity' contributing to pathophysiology (Tsai & Cruz, 2004) however very few studies have rigorously demonstrated relative hyperactivity of the p25/Cdk5 complex. In the present study, there were no clear differences in substrate phosphorylation rates for the two major complexes *in vitro* using peptide or protein substrates, leading to the conclusion that inherent activity was similar for both complexes. Consequently, cellular toxicity of p25/Cdk5 *in vivo* cannot be attributed to simple differences in the catalytic parameters between the two complexes. Although a previous publication proposed that truncation of p35 resulted in increased phosphorylation of tau, the difference was at a single point in the rate curves and was not overly convincing (Hashiguchi *et al*., 2002). Other publications have also failed to detect differences in specific activity of the two complexes (Peterson *et al*., 2002; Kusakawa *et al*., 2000; Sukaue *et al*., 2005). Therefore, truncation of p35 does not alter the substrate specificity of Cdk5 nor the catalytic

efficiency measured *in vitro*.

This finding is perhaps not that surprising as the Cdk5 activation domain on p35 lies between residues 138 – 291, consisting of three domains essential for interaction and activation (Pant *et al*., 2003), and cleavage of p35 by calpain at residues 98 and 99 does not interfere with the activation domain structure. However it is important to note that both Cdk5 complexes were compared using a soluble assay system and as cleavage removes the membrane localisation domain of p35 this may affect the inherent structure of the Cdk5 interaction domains. Although it is perhaps more likely that the cleaved p25 cofactor exposes Cdk5 to a distinct substrate pool or alters its stability and this promotes altered Cdk5 activity in intact cells. Further studies are required to distinguish between these possibilities and could help develop strategies to selectively inhibit the p25/Cdk5 complex as a potential therapeutic target.

5.3 FUTURE DIRECTIONS

The work described in this thesis has presented a consensus sequence motif specific to Cdk5 that may aid in the identification of novel Cdk5 substrates and provided evidence to implicate abnormal Cdk5 phosphorylation of CRMP2 in tumourigenesis. However, the mechanisms underlying the nuclear localisation and isoform selective actions of CRMP2 and Cdk5 in tumours are still to be uncovered. Additional functional and mechanistic studies to further elucidate the role of Cdk5 in tumourigenesis would extend the work described here and are necessary to pave the way to more clinically relevant findings.

5.3.1 Identification of novel Cdk5 substrates

The consensus sequence proposed for Cdk5 in this study could be used in future studies to search for and identify novel substrates of Cdk5, extending understanding of the physiological relevance of Cdk5. The predicted Cdk5 consensus sequence extends the minimal SP motif and introduces specificity-determining residues that may be utilised *in silico* to identify potential *in vivo* substrates of Cdk5. Computational analysis using the proposed consensus substrate phosphorylation motif could be used to identify novel substrates that harbour the P-K-**S**-**P**-R-K consensus phosphorylation motif by employing short peptide sequences as specificity determinants specific to Cdk5 and searching protein and phosphorylation-site databases for proteins containing the specificity-determining consensus sequence motif. The use of short peptide motifs are large enough to confer specificity however they are also small enough to cover a broad spectrum of proteins, therefore introducing the use of physiologically relevant filters such as protein structure and accessibility of the catalytic site as well as subcellular localisation could be applied to refine the substrates identified as potential substrates of Cdk5. Potential substrates would then be analysed further to substantiate the screening results through substrate validation experiments, such as immunoprecipation kinase assay of full-length proteins and site-directed mutagenesis of the target motifs. Alternatively, the proposed Cdk5 consensus sequence could be used in conjunction with Stable Isotope Labelling of cultured Cells (SILAC)-type experiments coupled with mass spectrometry to identify novel substrates of Cdk5.

5.3.2 Investigating the role of Cdk5 in Cancer

The present study presented evidence that implicated altered Cdk5 activity as an important aspect of tumourigenesis, presenting Cdk5 as a potential therapeutic target,

therefore future studies should focus on confirming altered Cdk5 activity in tumours. Expanding the current research by examining a greater panel of confirmed *in vivo* substrates of Cdk5 for enhanced phosphorylation in tumours at sites specifically regulated by Cdk5 using optimised and highly selective phosphospeciifc antibodies would provide further evidence of potentially altered Cdk5 activity in tumour cells. Additionally, the use of murine models could be used to examine the effect of altered Cdk5 activity in tumourigenesis. For example, strategies to transfer tumour cells or injecting mice with viruses that can induce lymphomas could be used to investigate the impact on tumourigenesis in Cdk5 knockout models and examine the role of Cdk5 in tumour formation. Furthermore, future studies are required to fully elucidate the role of Cdk5 in tumourigenesis and determine if altered Cdk5 activity is associated with invasion, migration, and metastasis of cancer cells and determine if inhibiting Cdk5 activity can reduce metastasis and offer Cdk5 as a potential therapeutic target. Therefore, work evaluating the role of Cdk5 in tumours should focus on migration regulation and the potential for changes in invasion and metastasis in cancer. The use of knockdown or overexpression vectors, or small molecule Cdk5 inhibitors coupled with migration and invasion assays may help establish the role of Cdk5 in metastasis.

5.3.3 Mechanistic Studies

Further work with CRMP2-receptor fusion proteins would help evaluate the role of the isoform specific N-terminus of CRMP2 in its localisation and phosphorylation by Cdk5, both in healthy cells and tumours. Similarly, development of cell systems with strong pCRMP2 Ser522 nuclear staining would enable the dissection of upstream regulation (for example, Cdk5 dependence) and investigation of the role of cell cycle regulation in its appearance. Furthermore, the use of CRMP2 mutants in such systems

(for example, S522A or tubulin binding deficient) would help establish the importance of CRMP2 regulation in the survival and proliferation of the tumour cells.

5.3.4 Clinical Utility

If altered Cdk5 regulation of CRMP2 is associated with initiation, invasion, migration, and/or metastasis of specific cancers then this antibody may provide some diagnostic value on prognosis, while Cdk5 would become a potential therapeutic target. The tumour profile of pCRMP2 Ser522 and differential intensity in squamous cancer compared to adenocarcinoma requires further investigation as this may provide additional clinical utility for this marker. A greater proportion of immunopositive cells with higher intensity was observed for the faster growing, more aggressive form of non-Hodgkins lymphoma (double large B-cell lymphoma) compared to the indolent form of non-Hodgkins lymphoma (follicular lymphoma) raising the hypothesis that staining intensity of the pCRMP2 Ser522 antibody may be related to the proliferation rate and/or aggressiveness of the tumour. The two types of NSCLC have variable rates of growth however differences in biological aggressiveness between squamous cell carcinoma and adenocarcinoma of the lung are not well understood and the staining intensity may potentially be linked to the rate of progression and/or aggressiveness of the disease. Further investigation may uncover the use of this antibody as a biomarker to distinguish between aggressive versus non-aggressive cancer. Furthermore, this antibody may be useful in providing information about the origins of secondary tumours. Cancers of epithelial origin invade local tissues and metastasise to distal sites forming secondary tumours therefore a phospho-CRMP2 stain might be able to distinguish a metastatic squamous carcinoma of primary lung origin from an origin in other sites however this would require further investigation.

5.4 CONCLUSION

These studies have provided novel insight into Cdk5 activity and one of its substrates in cancer and will hopefully provide greater impetus to investigate Cdk5 activity as a therapeutic or diagnostic target in cancer.

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