PROTEIN PHOSPHATASE 2A HOLOENZYME STRUCTURE AND FUNCTION

Specific Insights into the PR70 Holoenzyme

By Nathan Joseph Wlodarchak

A dissertation submitted in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

(Cancer Biology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2014

Date of final oral examination: 6-27-14

The dissertation is approved by the following members of the Final Oral Committee:

Yongna Xing, Associate Professor, Oncology

Hazel M. Holden, Professor, Biochemistry

Robert F. Kalejta, Professor, Oncology

Janet E. Mertz, Professor, Oncology

Wei Xu, Professor, Oncology

Acknowledgments

First, cliché as it may be, I would like to thank God for all of the wonderful things He has given me in life. All that I am started from Him and He seems to want to keep me alive for some purpose, and I believe it is to use my gifts to help others.

Secondly, I would like to thank my advisor, Dr. Yongna Xing. For the last half a decade, she has mentored and guided me in my development as a scientist, encouraged me to think in new ways, and done all she can to position me for a productive career. I am very grateful for all of her help. I would also like to thank my committee, Dr. Hazel Holden, Dr. Robert Kalejta, Dr. Janet Mertz, and Dr. Wei Xu. They have all been extraordinarily helpful in developing my critical thinking and offering many suggestions, and were instrumental in giving advice and recommendations on the next step in my career. They also have been genuinely interested and supportive of my research, and I am glad to have had such positive encouragement.

I would also like to thank the McArdle (and Cancer Biology) community as a whole. The many great professors that comprise this department have been some of the most pleasant and curious people I have had the pleasure with whom to work, and all of our interactions have always been so pleasant. To the unsung heroes of the department…administrative staff and support staff…I am eternally indebted. I have had the pleasure to work with all of you much more than the average graduate student, and I am astonished at the lengths you will go to accommodate both myself and all those on whose behalf I have spoken. Everyone here has such wonderful personalities and has made working here an immensely pleasurable experience. I can tell that you all are fervently dedicated to the cause, and I am privileged to call many of you my friends. Speaking of friends, I am overwhelmed as to the strong connections I have made with my peers in the program. There are many friends—too numerous to count—that have made my after-work life so joyous. We have all

gone through so many ups and downs in life and our research careers, and I thank God for every one of you. I have developed strong friendships here that I expect to last a lifetime, and I look forward to working with you all as colleagues for the rest of our careers.

I could not have done any of what I did without the fantastic support from everyone in my lab, past and present. Many people have come and gone in the lab, and I have enjoyed working with all of you. I will single out a few to whom I am specifically indebted. Patrick Menden trained me during my rotation and early months in the lab. Dr. Li Jiang was always helpful and did me so many small favors I can't possibly recount them. Dr. Vitali Stanevitch was always willing to let me bounce ideas off of him and was always a lot of fun. Dave Smith and Keith Brister were of tremendous help with data collection at LS-CAT (APS). Sunah Shin helped assemble information for the peptide assay (chapter IV) and helped me with various other projects. Dr. Ken Satyshur has the patience of a saint and spent so much time teaching me a great deal about molecular modeling. I hope one day to have the breadth of knowledge he has. Finally, and most importantly, is Dr. Feng Guo. He is the man with whom I worked most closely for most of my time here. I cannot possibly begin to recount all that he taught me, all of the help he gave me, and all of the joys and frustrations we shared. He is kind and easy going, and is someone to whom I truly look up. I learned a great deal from him and hope that I will bring some of his personality to my mentoring style during my career. To all of those in the lab who were with us shortly or recently, I am sorry I couldn't mention all of you specifically, but know I have enjoyed working with each and every one of you.

I have been fortunate to have many people in my life who have inspired me to become the person I am. I have a great many teachers and mentors who helped me along my journey. In particular, I want to mention my 7th and 8th grade science teacher, Mrs. Mary Archer, who taught me that the search for truth is the common thread between science and religion. My high school biology teacher, Mrs. Angelique Depenbrock, mentored me and inspired me to learn all that I could. Words

cannot describe my appreciation for my undergraduate advisors, Dr. Robert Skrivin and Dr. Margaret Norton. Dr. Skirvin truly inspired me to be a scientist with his amazing personality and childlike inquisitiveness. He also has the best teaching style I have ever witnessed, and I hope to bring as much of that as I can to my students in the future. Dr. Norton has the warmest and most loving personality of anyone with whom I have ever worked. She always lent an ear to any problems I was having and gave sage advice (sometimes with real sage). Her cheesecakes also are of legend and lore and deserve a published shout-out. I don't know where I would be without all of these great people.

Finally, and most importantly, I need to thank all of those people in my life who have provided their love, support, and guidance in all areas of my life. To all of my friends—too numerous to count—I am nothing without the good people with whom I surround myself. To all of my old neighbors, in particular Marge and Bob Kapke and Lois and Joe Narog, who taught me so much about life, thank you and rest in peace. To all of my family…you guys know what unconditional love is and how to stick together. I have not seen many families as close as we are. To Grandma Sophie, thank you for all you did for me growing up, and rest in peace. To my sisters Carrie and Kelly, and brothers-in-law Chris and Nick, you have all been so kind to me and close, and I don't know what I would do without you. To my nieces Addie and Anna and my Nephew Cooper, I never knew what love was until I knew you. I can't wait to see what life has in store for each of you and I am so proud of you already. To my partner, Shawn, I never knew I was deserving of such love, affection, and devotion as you give to me. You make me incredibly joyful. Finally, to my Mom and Dad: no words can describe all of the love I have for you, the appreciation I have for all that you have done and sacrificed for me, and the inspiration you have given me to become the person I am. Few are as blessed as I am for having you both.

Abstract

Cancer, cardiovascular disease, and neurological disease are currently three top public health concerns in much of the world. One protein found deregulated in many of these diseases is Protein Phosphatase 2A (PP2A), a group of diverse heterotrimeric holoenzymes containing regulatory subunits which are responsible for substrate specificity. There is limited structural and biochemical information on PP2A holoenzyme-substrate interactions and no PP2A substrate binding consensus sequences are yet identified.

The study here has an important focus on one family of PP2A regulatory subunits, the B"/PR72 family, which is involved in several cellular processes and uniquely regulated by calcium binding. The PR70 subunit in this family interacts with cell division control 6 (Cdc6), a cell cycle regulator important for control of DNA replication. Here I report the crystal structure of the trimeric PR70 holoenzyme at a resolution of 2.4 Å and *in vitro* characterization of the PP2A-PR70 holoenzyme and its interaction with Cdc6. The holoenzyme structure reveals that one of the PR70 calcium-binding motifs directly contacts the scaffold subunit, resulting in the most compact scaffold subunit conformation among all known PP2A holoenzymes. *In vitro* biochemical analysis demonstrated that PR70 can enhance PP2A-mediated dephosphorylation of pCdc6; in contrast, the B' family of holoenzymes barely have any phosphatase activity on pCdc6, likely due to steric hindrance by a B' loop near the active site. PR70 binds distinctively to the catalytic subunit near the active site, and this interaction is required for PR70 to enhance phosphatase activity toward Cdc6. These studies provide a structural basis for unique regulation of B"/PR72 holoenzymes by calcium ions, and suggest mechanisms for precise control of substrate specificity among PP2A holoenzymes.

The study above suggests that local structural features and global conformational changes regulate substrate binding. As such, PP2A substrate recognition is dictated by three-dimensional architecture of holoenzymes, and PP2A substrates might not have consensus recognition sequences for holoenzyme binding. The study above also demonstrates how peptide-based assays help characterize PP2A substrate interactions. Given the vast amount of processes and pathways in which PP2A is involved, novel high-throughput methods are discussed for efficient characterization of PP2A substrates *in vitro*.

Table of Contents

List of Abbreviations

4E-BP	4E binding protein
AC8	adenylyl cyclase 8
AES	amino-terminal enhancer of split
AKT	protein kinase B
ALB	cell growth inhibiting protein 42
AMPK	AMP activated protein kinase
AP1 & AP2	clatherin coat associated protein 1 & 2
APC	anaphase promoting complex
APC	adenomotous polyposis coli
APP	amyloid beta (A4) precursor protein
AR	androgen receptor
ARL2	ADP-ribosylation factor
ARS	autonomously replicating sequences
ATG3	autophagy related 3 homolog
ATM	ataxia-telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related protein
ATRA	all trans-retinoic acid
ATXN7L2	ataxin-7-like protein 2
BAD	bcl-XL/Bcl-2-associated death promoter
BAF	barrier to autointegration factor
BARD1	BRCA1 associated RING domain 1 isoform ep
Bax	BCL2-associated X protein
Baz	bazooka
BCL2	protein phosphatase 1, regulatory subunit 50
BLNK	Src homology [SH2] domain-containing leukocyte protein
B-myb	v-myb myeloblastosis viral oncogene homolog (avian)-like 2
BRCA	breast and ovarian cancer sususceptibility protein
BUB	Budding Uninhibited By Benzimidazoles
BUBR1	budding uninhibited by benzimidazoles 1 homolog beta (yeast)
CACNA1S	calcium channel, L type, alpha 1 polypeptide, isoform 3
Camk	cam kinase
CAS	breast cancer anti-estrogen resistance protein 1
Cav1.2	calcium voltage channel 1.2
CBX1	chromobox homolog 1
CCT2	haperonin containing t-complex polypeptide 1, beta subunit
CDC	cell division control
CDK16	cyclin dependent kinase 16
CFP	cyan fluorescent protein
CFTR	cystic fibrosis transmembrane conductance regulator
CG-NAP	centrosome- and Golgi-localized PKN-associated protein

Chk1	check 1
CIP2A	cancerous inhibitor of PP2A
CK1	casein kinase 1
CK2	casein kinase 2
c-MET	met proto-oncogene
CRIP2	cystein-rich intestinal protein
CSNK1E	casein kinase I isoform epsilon
CSNK1G1	casein kinase I isoform gamma-1
CSNK1G2	casein kinase 1 isoform gamma-2
CTLA4	ligand and transmembrane spliced cytotoxic T lymphocyte associated antigen 4
CUL3	cullin 3
CXCR2	high affinity interleukin-8 receptor B
Dab2	disabled homolog 2, mitogen-responsive phosphoprotein
DARPP32	protein phosphatase 1, regulatory subunit 1B
D-box	destruction box
Dlg4	discs, large homolog 4
DP	dimerization partner
Drc1	53 kDa squamous epithelial-induced stress protein
DSP	aspartate-dependent phosphatase
E4orf3	E4 openn reading frame 3
ECT2	pithelial cell transforming sequence 2 oncogene
EDTA	ethylenediaminetetraacetic acid
Eef2	eukaryotic translation elongation factor 2
EGFR	epidermal growth factor
EIF1AK2	eukaryotic translation initiation factor 2alpha
eIF4B	eukaryotic translation initiation factor 4b
eIF4e	eukaryotic translation initiation factor 4e
EIF4EBP1	eukaryotic initiation factor 4E-binding protein-1
ELAVL1	embryonic lethal, abnormal vision-like 1
Emi2	endogenous meiotic inhibitor
Era	estrogen receptor alpha
Erf1	sup45 homolog-like 1
ERK	extracellular signal-related kinase
ERK1/2	developmentally-regulated eph-related tyrosine kinase
FAM107A	family with sequence similarity 107 member A transcript
FANCG	Fanconi Anemia complementation group G
FGF	fibroblast growth factor
FMRP	fragile X mental retardation 1 protein
FOX	forkhead box transcription factor
FRET	fluorescent resonance energy transfer

GM130	Golgi autoantigen, golgin subfamily a, 2
Gp130	glycoprotein 130
GRB2	epidermal growth factor receptor-binding protein GRB2
GRK5	g protein-coupled receptor kinase
GSK3β	glycogen synthetase kinase 3 beta
GST	glutathione S-transferase
H2AX	histone family, member X
HCN1	Na/K hyperpolarization-activated cyclic nucleotide-gated channel 1
HCP-6	non-SMC condensin II complex, subunit D3
HDAC	histone deacetylase
HDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein
HEAT	Huntington-elongation-A subunit-TOR
hHR6A	ubiquitin-conjugating enzyme E2A (RAD6 homolog)
HIRA	HIR histone cell cycle regulation defective homolog A (S. cerevisiae)
HMGB2	high-mobility group (nonhistone chromosomal) protein 2
HOX11	Homeo box-11 (T-cell leukemia-3 associated breakpoint, homologous to Drosophila Notch)
HPV E1 protein	human papalloma virus E1
HRX	Hyperreflexia
HSC40)	Heat Shock Cognate 40
HSF2	heat shock transcription factor 2
IEX-1	radiation-inducible immediate-early gene IEX-1
IKK	inhibitor of kappa kinase
IQGAP1	IQ motif containing GTPase activating protein 1
IRAK	interleukin-1 receptor-associated kinase
IRS1	insulin receptor substrate 1
JAK2	Janus kinase 2 (a protein tyrosine kinase)
JNK	mitogen-activated protein kinase 8 isoform JNK1 alpha1
JNK	c-jun N-terminal kinase
KCNQ2	potassium voltage-gated channel, KQT-like subfamily, member 2
KID	gap junction protein, beta 2, 26kDa
KIF11	thyroid receptor interacting protein 5
KSR1	kinase suppressor of ras 1
LATS2	large tumor suppressor, homolog 2 (Drosophila)
LCMT1	leucine carboxy methyltransferase 1
LEM4	LEM domain containing 4
LIMD1	LIM domain-containing protein 1
LNX1	multi-PDZ-domain-containing protein, E3 ubiquitin-protein ligase LNX
LRP	low-density lipoprotein receptor-related protein
LS-CAT	life sciences collaborative acess team
Mad2	mitotic arrest deficient-like 2

MAP4	microtubule-associated protein 4
MAPK	mitogen activated protein kinase
MARCKS	myristoylated alanine-rich protein kinase C substrate
MCLR	microcystin LR
MCM2	minichromosome maintenance
Mdm	3T3 cell double minute
MEF	murine embryonic fibroblast
MEK3	dual specificity mitogen-activated protein kinase kinase 3
MEKK3	mitogen-activated protein kinase kinase kinase 3
Mib-CK	creatine kinase, sarcomeric mitochondrial
Mid-1	neuromancer 2
MKK4	dual specificity mitogen-activated protein kinase kinase 4
MKP	map kinase phosphatase
MLH1	mutL homolog 1
MLLT3	myeloid/lymphoid or mixed-lineage leukemia
Mps1 p	40S ribosomal protein S27
Mst3	serine/threonine kinase 24
MTOR	mechanistic target of rapamycin
MyoD	class C basic helix-loop-helix protein 1
NDRG1	reducing agents and tunicamycin-responsive protein
NEK1	never in mitosis-related kinase 1
Nemo	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
Net1	guanine nucleotide regulatory protein
NF-Y	nuclear factor gamma
NHE3	solute carrier family 9 (sodium/hydrogen exchanger), member 3
Nir2	phosphatidylinositol transfer protein, membrane-associated 1
NKCC1	solute carrier family 12 (sodium/potassium/chloride transporters), member 2
NLS	nuclear localization signal
NM23H2	non-metastatic cells 1
NMDA receptor	N-methyl-D-aspartate receptor
NME1v2	non-metastatic cells 1
NMEv1	non-metastatic cells 1
NOD2	nucleotide-binding oligomerization domain
NOTCH1	ranslocation-associated notch protein TAN-1
NPAT	nuclear protein of the ataxia telangiectasia mutated locus
NR3A	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A
NRF1	alpha palindromic-binding protein
NUCKS	nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate
OA	okadaic acid
ORC	origin recognition complex
OSBP	oxysterol-binding protein

P27kip	interferon alpha-inducible protein 27
PACS1	phosphofurin acidic cluster sorting protein 1
PAK1	p21/Cdc42/Rac1-activated kinase 1
PDE4D3	Phosphdiesterase 4D3
PEA-15	15 kDa phosphoprotein enriched in astrocytes
PI3K	phosphoinositide-3 kinase
PIM1	proto-oncogene serine/threonine-protein kinase pim-1
Pin1	protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1
PKA	protein kinase A
PKB	protein kinase b
PKR	protein kinase, interferon-inducible double stranded RNA dependent
PME1	PP2A methylesterase 1
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B
PP4	protein phosphatase 4
PP5	protein phosphatase 5
PP6	protein phosphatase 6
PP7	protein phosphatase 7
PPFIA1	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 1
PPM	metal dependent phosphatase
PPP	phospho protein phosphatase
pras40	proline rich AKT substrate 1
Prkar1a	protein kinase, cAMP-dependent, regulatory, type I, alpha
PSP	phospho-serine phosphatase
PTPA	PP2A tyrosine phosphatase activator
PTPN14	cytoskeletal-associated protein tyrosine phosphatase
Rab4	member RAS oncogene family
Rack1	protein homologous to chicken B complex protein, guanine nucleotide binding
Raf1	raf proto-oncogene serine/threonine protein kinase
RAG	ras related GTP binding
RalA	v-ral simian leukemia viral oncogene homolog A (ras related)
Rb	Retinoblastoma protein
RCC1	regulator of chromosome condensation 1
Rec8	meiotic recombination and sister chromatid cohesion phosphoprotein of the rad21p family
RelA	nuclear factor of kappa light polypeptide gene enhancer in B-cells 3
REPOman	Recruits PP1 Onto Mitotic Chromatin At Anaphase Protein
RHEB	Ras homolog enriched in brain 2
RPL14	60S ribosomal protein L14

RPS6KA5	nuclear mitogen- and stress-activated protein kinase
R-RAS	related ras oncogene viral protein
RRN3	polymerase I transcription factor homolog
Runx2	polyomavirus enhancer-binding protein 2 alpha A subunit
SAD	single anomalous dispersion
SDS-PAGE	sodium dodecyl sulphate polyacrylimide gel electrophoresis
SEM	standard error of the mean
SET	template-activating factor-I, chromatin remodelling factor
SG2NA	striatin, calmodulin binding protein 3
SGK1	serum and glucocorticoid regulated kinase 1
SHC	(Src homology 2 domain containing) transforming protein 1
Sk1	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1
Smad3	mothers against decapentaplegic homolog 3
SMURF1	specific E3 ubiquitin protein ligase 1
SOS	son of sevenless
SOX2	SRY (sex determining region Y)-box 2
Sp1	Sp1 transcription factor
SRC	Schmidt-Ruppin A-2
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SRPK2v2	serine/arginine-rich protein-specific kinase 2
STAT5	signal transducer and activator of transcription 5A
STE20	serine/threonine kinase 24
STK3	serine/threonine kinase 3
SUMO1	MT3 suppressor of mif two 3 homolog 1
Sv40	simian vacuolating virus 40
Swi5	recombination repair homolog
TADA2L	transcriptional adaptor 2 alpha
TAP42	alpha 4 yeast homologue
TAX	transiently-expressed axonal glycoprotein
TBC1D3	Rab GTPase-activating protein PRC17
TC	tetracysteine
TCEAL1	transcription elongation factor S-II protein-like 1
TCF	transcription factor
tel2	telomere maintainence 2
TEV	tobacco etch virus
TGFBR1	transforming growth factor beta receptor 1
TH	tyrosine hydroxylase
TIP	ntegrin-alpha FG-GAP repeat-containing protein 1
TIPRL	tor signaling pathway regulator-like
TOM22	translocase of outer mitochondrial membrane 22 homolog

TOP1	type I DNA topoisomerase
TOPK	lymphokine-activated killer T-cell-originated protein kinase
TRAF2	tumor necrosis factor type 2 receptor associated protein 3
β-TRCP	β-transducin repeat containing protein
TSC2	tuberous sclerosis 2 protein
TSC2	tuberous sclerosis complex 2
tti1	TELO2 interacting protein
TTP	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 13
UBD	ubiquitin-like protein FAT10
UBR5	ubiquitin protein ligase
ULK1	unc51-like autophagy activating kinase 1
UPF1	regulator of nonsense transcripts homolog
ZNF519	zinc finger protein 519
ZRANB1	zinc finger Ran-binding domain-containing protein 1

Chapter I

Introduction and Background:

PP2A and the Cell Cycle

A review article is in preparation which will include information presented in this chapter.

Introduction

Cell division is the most important aspect of life, allowing life to continuously grow and evolve. This process itself evolved from a relatively simple copying of a few macromolecules to a beautiful and intricate process that the very minds created by it are now only beginning to understand. Unfortunately, the more complex this process becomes, the more severe the consequences are when errors occur. These errors can lead to cancer, one of the most devastating diseases that afflict mankind. To conquer or suppress cancer, it is important for us to have a deep understanding of the cell cycle and know what we can do to correct or prevent these errors. There are many varied and complex proteins involved in the cell division, including kinases and phosphatases, the primary regulators of the cell cycle. Protein Phosphatase 2A, the focus of this thesis, is a phosphatase with many links to the cell cycle and has been implicated in tumor suppression. Important advances have been made in understanding the cell cycle, and the role of PP2A in regulating this process is beginning to be elucidated. These begin with the understanding of cell signaling pathways involved in the cell cycle and the history of those discoveries.

Historical Background of Cell Division

Shortly after cells were discovered, it was observed that they grow and divide. "Mitosis" was coined by Flemming in the late 1800's and has been extensively studied since then[1]. Theodor Boveri, a zoologist at Würzburg, extensively studied cell division at the end of the 1800's and beginning of the 1900's and gave extensive insight as to the importance of chromosomes for the transmission of heritable traits and the necessity for these to be divided properly for daughter cells to behave as the parent cells[2]. Boveri also gave remarkable insight into the relatedness of cell division and carcinogenesis, a connection well supported and agreed upon in the modern age. He argued that malignancy resulted from chromosomal abnormalities, most often resulting in clonal outgrowths, and that tumors were the result of some loss of function which allowed cells to proliferate without restraint[3].

Research into cell division continued throughout the early 1900's, investigating mostly morphological mitotic and meiotic events, but scientists began investigating in the 50's the role of biological macromolecules in the cell cycle, most notably DNA. In 1950, Hewson Swift determined that the amount of DNA in the cells is consistent with and is a characteristic of a particular species[4]. In 1953, Howard & Pelc discovered that DNA synthesis occurred in a specific, narrow time period during the middle of interphase (now known as S phase)[5]. The same year, Watson & Crick would publish their landmark paper on the structure of DNA[6]. By 1970, it was understood that the cell cycle had different phases, and Rao and Johnson demonstrated that the cell cycle phases occur in an ordered, unidirectional manner, and that the progression is controlled by the exchange of chemicals between the cytoplasm and nucleus[7]. Still, the manner of cell cycle initiation was unresolved in the early 70's. To address this, Arthur Pardee performed a group of experiments demonstrating that cells must pass a "restriction point"

to enter the cell cycle in G1, and the cell is committed after this point. He also suggested that this control point does not function normally in malagnancies[8]. Together, these studies established the stages, directionality, and restriction of the cell cycle, as well as the importance in properly copying and segregating the cell's genomic material. This discovery would set the stage for the molecular biology era.

The last third of the 20th century would be heavily focused on understanding the molecular mechanisms that regulate the cell cycle and gene expression. A key discovery in cell division control was the discovery of regulatory kinases and phosphatases to which the Nurse group made several key contributions. They first discovered several mutations that allowed yeast cells to divide at a smaller size[9, 10]. Eventually, these genes were defined as the kinase wee1 and the phosphatase cdc25, and they determined that cdc2 (CDK1 in humans), a critical substrate of wee1 and cdc25, is phosphorylated and dephosphorylated on Y15, and this phosphorylation regulates the entry of cells into mitosis[11-13]. Concurrent with these discoveries, the Hunt group discovered proteins that were created and destroyed with each cell division. They termed these proteins "cyclins" and postulated that they are intimately involved in the cell cycle[14]. Shortly thereafter, the Kirschner group demonstrated that these proteins were required for embryonic cell divisions and that cyclin B is required for the activation of maturation promoting factor (CDK1) kinase activity[15, 16].

While many scientists in the 80's were hot on the trail of cyclins and CDKs, another group of scientists began looking into S phase and were able to shed some light on how such a large amount of DNA is efficiently and accurately replicated via origins of replication. By the early 80's, autonomously replicating sequences (ARS) of DNA were isolated from yeast and were identified as the origins of replication in DNA synthesis[17, 18]. It was not until 1992 that

Diffley & Cocker demonstrated that a large protein complex is targeted to the origins of replication in G1 phase[19], and the same year, Bell & Stillman identified and characterized the six origin recognition complex proteins[20]. These proteins were demonstrated to be necessary for DNA synthesis, but questions remained as to what other proteins were required for initiation. The cdc6 gene was isolated and characterized in 1988, and it was also determined to be necessary for DNA synthesis[21]. Eight years later, it was shown to bind directly to the ORC proteins, which in turn bound to the ARS. This complex would be termed the pre-replication complex[22], and the other components of this complex, mini-chromosome maintenance 2-7 and cdt1, were discovered a few years later[23, 24].

The discovery of the pre-replication complex was a milestone in the molecular biology of DNA synthesis, but open questions remained regarding how the complex was regulated and how synthesis (S phase) was initiated. Two discoveries were critical in answering these questions: G1 cyclins/CDKs and the retinoblastoma tumor suppressor protein (pRb or Rb).

In 1991, a number of groups identified a new group of cyclins, cyclin C, D, and E, the expression of which peaked in the G1 phase of the cell cycle[25-27]. The authors all suggested that these cyclins play an important role in ushering the cell from G1 to S phase, as they all peak sometime in G1 phase and then fall. Previous work demonstrated that cyclin A and B were important for CDK1 activity in human cells, and it was clear that cyclin A and B levels correlated with the level of CDK1 activity, which both peaked in the G2 phase[28,29]. The levels of the new cyclins peaked in G1 phase, prompting researchers to search for new cyclin-CDK interactions. CDK2 was discovered in 1991[30], and it was found to interact with cyclin E and, later, cyclin D which was also demonstrated to interact with several other CDKs[31,32].

In the early 80's, scientists were searching for a causal agent of retinoblastoma, a very rapidly developing cancer affecting the eyes in a small population of people, primarily children. A genetic link, the retinoblastoma gene, was soon found, and the gene was cloned and characterized in 1987, becoming the first discovered tumor suppressor gene[33, 34]. The protein product of this gene (pRb or Rb) was characterized as a phosphoprotein which has the ability to bind DNA[35]. Shortly thereafter, numerous viral proteins were found to interact with Rb and mediate transformation through this interaction[36-38]. Non-phosphorylated Rb was discovered to bind to the E2F transcription factor, with SV40 large T antigen and adenovirus E1A proteins disrupting this interaction, an important requirement for viral transformation[39-41]. These data indicated the critical importance of Rb in cell cycle progression, and its function as the G1-S checkpoint and the role of phosphorylation by cyclin/CDKs in this regulation would soon be elucidated[42, 43].

By the early 90's, researchers suspected there were other checkpoints important in cell cycle regulation and began screening for candidate genes. A screen of yeast mutants led to the discovery of a protein, Mad2, which is necessary for microtubule spindle assembly[44]. The same year, more microtubule regulating genes would be discovered: the bub loci[45]. The Mad and Bub genes would eventually become known for their role in the spindle checkpoint. The role of one final and incredibly important cell cycle protein would be discovered in the early 90's: p53. The Old group had discovered p53 in 1979 and found that it was highly expressed in a number of cancers[46]. After finding this protein highly expressed in transformed tissues, it was thought that p53 was an oncogene[47]. Ten years later, the Vogelstein group found that the p53 gene is mutated in a large subset of colon cancers and expression of wild-type p53 could suppress the growth of a large subset of these cancer cells[48, 49]. This was the first evidence that p53 was not an oncogene

but, instead, a tumor suppressor gene. The role of p53 in stopping DNA synthesis by responding to DNA damage was discovered shortly thereafter, which established the role of p53 in the DNA damage checkpoint, one of the most crucial cell cycle checkpoints.

By the end of the 20th century, most of the major proteins involved in the cell cycle were discovered. The roles of major checkpoint players such as Rb and p53 were known, the coordinated synthesis of DNA was seen, and the role of cyclins and kinases in regulating most of the cell cycle proteins by phosphorylation was uncovered. The 21st century would dive deeper into the details surrounding all of these proteins—the larger assemblies of which they were part, the substrates on which they acted, and the existence of feedback loops. Cell cycle initiating pathways such as mTOR, Wnt, and MAPK would also be well studied due to their role in proliferation and carcinogenesis. Another area of interest would be the role of phosphatases—once thought to be housekeeping enzymes, but now known to have a great deal to do with cell cycle regulation. These enzymes would turn out to be far more diverse and their actions more complex than previously thought and shift a kinase-focused paradigm of the cell cycle to one of exquisitely coordinated antagonism between phosphorylation and dephosphorylation.

Protein phosphatase 2A: A Complex and Diverse Family of Phosphatases

Background: Eukaryotic phosphatases are encoded by a small number of genes and can be divided into three super families: the serine/threonine phosphatases (PSPs), the tyrosine phosphatases (PTPs) and the dual specificity phosphatases (DSPs) (reviewed in 50-52). The number of PTPs is approximately equivalent to the amount of tyrosine kinases in the genome, but the number of serine/threonine kinases exceeds the PSPs by fourteen fold. This difference is due to the complexity of PSP oligomerization. The PSPs are further divided into three families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases (Figure 1.1). The PPP family contains many of the phosphatases involved in cell cycle regulation such as PP1, PP2B, PP4, PP5, PP6, PP7, and of course, PP2A[50-52]. The PPP family all have a similar active site configuration, containing two catalytic metal ions, 6 conserved residues (D, N, and H's), and a catalytic water molecule. The dephosphorylation reaction proceeds via an S_N2 mechanism with the activated water serving as a nucleophile to attack the phosphorus, hydrolyzing the phosphate bond[50]. One of the PPPs, PP2A, functions in the regulation of many processes such as neuronal stabilization, cardiac muscle function, and as discussed below, the cell cycle. As such, it is implicated in a vast array of diseases such as Alzheimer's disease, cardiac disease, and cancer[53-56]. It can affect all of these processes due to its complex regulation and diverse holoenzyme formation.

Regulation and Activation of PP2A: *A diagrammatic summary of this section is given in figure 1.2*. The PP2A holoenzyme is formed by a combination of three subunits: a catalytic (C) subunit containing the active site, a regulatory (B) subunit which confers substrate specificity, and a scaffolding (A) subunit that interacts with B and C[57]. There are two isoforms, α and β, for both A and C that share high sequence homology. The α subunit for each is

expressed at a much higher level and is the predominant isoform studied in PP2A research. In addition to A and B subunits, cellular PP2A C subunit (PP2Ac) is found associated with α4 protein and TOR Signaling Pathway Regulator-like (TIPRL)[58]. The function of α4 on PP2Ac has been controversial in the past; however, now it is known that α4 can bind and partially unfold PP2Ac and inactivate it[59]. α4 can also protect PP2Ac from ubiquitination by Midline 1 (MID1) and subsequent degradation. This binding of stable inactive PP2Ac provides the cell with a pool of available PP2Ac while simultaneously protecting the cell from non-targeted dephosphorylation by free PP2Ac. Before assembly into active holoenzymes, PP2A must be activated. The phosphotyrosyl phosphatase activator (PTPA) is a critical protein in PP2A activation[60]. PTPA is necessary for folding of the active site and loading of catalytic metal ions. PP2A together with PTPA can form an enzyme with ATPase activity, and this association is necessary for loading the catalytic metal ions and activating PP2A. Evidence suggests there is a Zn^{2+} ion in the active site, and ATP is required to load a Mg^{2+} ion into the second position and activate PP2A[60]. PP2Ac also undergoes post-translational modification on its unstructured carboxy-terminal tail. Phosphorylation occurs on T304 and Y307, and carboxymethylation occurs on L309. L309 is methylated by a PP2A specific enzyme: leucine carboxy methyltransferase (LCMT1) and this methylation is reversed by another specific enzyme, PP2A methylesterase 1 (PME1)[61, 62]. PP2A methylation is essential for cellular function, and cells will undergo apoptosis in the absence of LCMT-1[63]. LCMT-1 is also required for the anti-tumor effect of PP2A. Methylated PP2Ac has an increased affinity for some B subunits and has been shown to fluctuate in the cell cycle, making it a likely regulator of holoenzyme assembly[64].

Structural Diversity of Holoenzymes: PP2A can act on a large array of substrates because of the diverse holoenzymes it can form. The B subunits contribute to the largest

variation on holoenzyme formation and are divided into four families: the B (PR55), B' (PR56), B'' (PR72) and B''' (Striatin). Currently, the identified regulatory subunits are encoded by 15 genes which can be alternatively spliced to yield 26 different B subunits[56]. These subunits share sequence homology within each family, but have little-to-no sequence homology between the families. As such, there is not a specific "recognition motif" that PP2A substrates contain, and the recognition is likely due to structural elements inherent in each subunit. The structure of the core dimer of PP2A revealed important insights on how holoenzyme assembly and activity is regulated[65]. The C subunit consists of a core domain with two β-sheets flanked by α-helicies, with connecting loops of β-sheets forming the active sites. These loops contain the 6 conserved residues which bind the active site metal ions. The outside of the loops are likely dynamic as every holoenzyme (and core enzyme) structure solved to date required the addition of a selective inhibitor such as microcystin LR (MCLR) or okadaic acid (OA) to enable crystallization[57, 65-67]. The A-subunit consists of 15 Huntington-elongation-A subunit-TOR (HEAT) repeats arranged in a horseshoe shape. The C subunit binds to repeats 10-15 and the regulatory subunits bind to the N-terminal repeats. The A subunit can exhibit a large degree of conformational flexibility, explaining how so many structurally diverse B subunits can form active holoenzymes with the same AC dimer. The B'γ1 holoenzyme was the first holoenzyme structure solved[57]. The B'γ1 subunit structure is similar to the A subunit, as it is a linear arrangement of HEAT repeats. There is also an ordered loop near the active site which may be involved in restricting substrate access (Chapter II). The structure of the Bα holoenzyme demonstrated a much wider conformation for the A subunit than the core enzyme or B' holoenzyme, with little interaction between the B and C subunits. The Bα subunit is a 7-bladed β-propeller with a hairpin that extends to interact with the side face of the N-terminal HEAT repeats of the A subunit[66]. Until recently, these were the

only two holoenzyme structures known, with no structural information on the B'' or B''' family. Chapter III is devoted to the elucidation of the high-resolution structure of the PR70 holoenzyme, a member from the B" family.

Given the diversity of holoenzyme structures, it is no surprise that PP2A has been suggested or confirmed to dephosphorylate over 300 substrates (Chapter IV). The majority of these substrates are involved in cell cycle regulation, and although some of PP2A-mediated dephosphorylation cause positive regulation of proliferation pathways, the majority of these reactions function as negative regulators, so the net effect is to retard cell division and promote apoptosis. It is not a surprise that PP2A has been implicated in such a wide array of human diseases and is well studied due to its effects as a tumor suppressor.

The Cell Cycle Initiation: Signaling Pathways

The initiation of the cell cycle is controlled by many diverse and complex signaling pathways. There is a large and incredibly detailed body of information on each of these signaling pathways, and they all continue to be thoroughly researched and reviewed. Presented here is a brief review on three critical signaling pathways, Wnt, mTOR, and MAPK, with a focus on the role of PP2A in their regulation.

Wnt Signaling Pathway: *A diagrammatic representation of the wnt pathway is shown in figure 1.3*. The Wnt pathway is involved in the regulation of cell proliferation and polarity as well as embryonic development. Under baseline cell conditions, the protein β-catenin is degraded by the action of a complex composed of axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (CK1) (reviewed in 68, 69). GSK3β and CK1 phosphorylate β-catenin, targeting it for ubiquitination and proteasomal degradation[70, 71]. APC and axin have unique domains that bind to CK1 and GSK3β to serve as scaffolds to increase β-catenin phosphorylation, and these scaffolds are often found mutated in cancers[72-74]. To activate Wnt signaling, extracellular Wnt binds to the receptor frizzled and co-receptor LRP 5/6. An intracellular complex is then formed with the receptors, disheveled, axin, CK1, and GSK3β, which then prevents the phosphorylation and subsequent degradation of β-catenin[75]. β-catenin can then accumulate in the nucleus and bind to TCF family transcription factors and activate Wnt responsive genes[76]. Many of these genes are critical promoters of cell division such as cyclin D1 and c-Myc[77, 78].

β-catenin is the central substrate in Wnt signaling, and its regulation is highly dependent on phosphorylation and dephosphorylation. The phosphorylation events are sequential, with CK1 phosphorylating S45 followed by GSK3β phosphorylating T41, S37, and S33[70, 71].

Phosphorylation at S37 and S33 allows the ubiquitin ligase β-transducin repeat containing protein (β-TRCP) to bind β-catenin and target it for degradation[79]. In addition to phosphorylating β-catenin, CK1 and GSK3β can phosphorylate APC and axin which increases the affinity of β-catenin for these scaffolds[80-82]. These phosphorylation events are disrupted when the complex is perturbed by Wnt signaling. β-catenin, APC, and axin can be dephosphorylated by phosphatases such as PP2A and PP1, and this event can also increase β-catenin levels. PP1 increases β-catenin levels by dephosphorylating axin which reduces its affinity for GSK3β[83]. Unlike PP1, PP2A has a dual and opposing role in β-catenin regulation (Figure 1.3). The PP2A-Bα holoenzyme has been shown to directly interact with and dephosphorylate β-catenin to enhance Wnt signaling. This holoenzyme can not only dephosphorylate the residues relevant to destruction, but it can also dephosphorylate residues S552 and S675, the functionality of which has yet to be elucidated. The B55α holoenzyme can also directly bind axin, likely through a different domain than the one that PP2Ac can bind[84]. In contrast, the PP2A B'α holoenzyme has been implicated in negative regulation of Wnt signaling. B'α can bind to the destruction complex through APC, and overexpression of B'α results in decreased β-catenin levels, with the amino terminus of β-catenin being required for this effect[85]. PP2A negatively regulates Wnt signaling through GSK3β both directly and indirectly. GSK3β is inhibited by phosphorylation on S9, and DNAJB6 with Heat Shock Cognate 40 (HSC40) can recruit PP2A to GSK3β where it can directly dephosphorylate S9 and activate GSK3β[86]. PP2A also indirectly regulates GSK3β by inhibiting Protein Kinase B (AKT or PKB). AKT phosphorylates GSK3β on S9 to inactivate it, but if AKT is inhibited, there is increased active GSK3β which targets more β-catenin for destruction[87]. This pathway provides an important intersection with the mTOR pathway, another critical cell cycle initiating pathway.

Mechanistic Target of Rapamycin (mTOR): *A diagrammatic summary of the mTOR pathway is shown in figure 1.4*. The mTOR pathway is involved in many diverse cellular processes. It is stimulated by amino acids, cellular metabolism, and growth factors, and results in increased growth, metabolism, biomolecule synthesis, and proliferation[88]. Because of the multifaceted role of mTOR in cell regulation, it is an intensely studied pathway with major implications in cancer, heart disease, and even some neurological diseases such as autism[88-91]. Rapamycin was known to have toxic effects on yeast, and the responsible genes (DDR1&2/TOR1&2) were discovered in 1993, with the protein being discovered one year later[92-94]. Two complexes are formed with the mTOR catalytic protein: mTORC1 and mTORC2. Both complexes contain some shared as well as some unique components. The shared components are the tti1 and tel2 scaffolds, deptor, and mLST8. mTORC1 contains the unique proteins raptor and pras40, whereas mTORC2 contains rictor, mSin1, and protor1/2[88]. Deptor is an inhibitor of both mTOR complexes and suppresses the function of S6 kinase 1 (S6K1), AKT, and Serum and Glucocorticoid regulated kinase 1 (SGK1). Deptor is highly overexpressed in some multiple myelomas, and this overexpression can induce AKT function due to loss of feedback inhibition of phosphoinositide-3 kinase (PI3K) from mTORC1[95]. Raptor and rictor help regulate substrate specificity to mTORC1 and mTORC2, respectively. Raptor binds with mTOR in the mTORC1 complex and is necessary for binding and phosphorylation of S6K1 and 4E-BP1, which in turn induce protein synthesis and proliferation[96, 97]. GβL (also known as mLST8) is found in both mTORC2 and mTORC1 and is essential in stabilizing the interaction of mTOR with raptor[88, 98].

Both complexes are regulated by highly diverse processes. Wnt signaling can activate both mTOR complexes, as can stimulation by insulin[99, 100]. Wnt can stimulate mTORC2 directly

through the GTPase RAC1 and stimulates mTORC1 indirectly by inactivating glycogen synthase kinase 3β, which is necessary to activate an mTORC1 inhibitor, tuberous sclerosis complex 2 (TSC2)[99, 100]. Insulin, long known to stimulate protein synthesis, activates mTOR through a central molecule, phosphatydilinositol-3,4,5-triphosphate, produced by PI3K. This molecule can directly stimulate mTORC2 and can indirectly stimulate mTORC1 by activation of AKT which inhibits TSC2[100, 101]. Free amino acids activate mTORC1 via binding to RAS-related GTP binding protein (RAG) heterodimers, causing a global conformational change. The RAG complex associated with amino acids can recruit mTORC1 to the lysosome where it is activated by binding to RAS homologue enriched in brain (RHEB)[100, 102].

There is generally a good body of knowledge on how mTOR is regulated, with some details still being worked out. Conversely, the substrates of mTOR are not very well known. Mass spectrometric studies have identified 93 potential substrates in human embryonic kidney cells and 174 potential substrates in mouse embryonic fibroblasts[103]. Very few of these substrates have been studied in detail. The best known substrates of mTOR are the ribosomal S6 kinase (S6K) and eIF4e binding protein 1 (4E-BP)[100]. Many proteins that are involved in growth and proliferation are encoded by mRNAs that have secondary structures in their UTR that inhibit scanning by the 40S ribosomal subunit (reviewed in 104). Phosphorylation of 4E-BP by mTORC1 can inhibit its binding to eIF4E which is necessary to recruit the pre-initiation complex[104, 105]. An important component of the pre-initiation complex is eIF4B, which allows for more efficient unwinding of secondary mRNA structures. The binding of eIF4B is enhanced by phosphorylation by S6K, which is phosphorylated and activated by mTORC1[104, 105]. These mTOR substrates both work in concert to translate these structured mRNAs to enhance growth and proliferation. mTOR regulates autophagy by the phosphorylation of Unc 51-like kinase 1

(ULK1). Autophagy is promoted by ULK1 when activated by phosphorylation on S317 and S777 by AMP activated protein kinase (AMPK). mTOR phosphorylates ULK1 on S757, which prevents AMPK phosphorylation and subsequent activation[106]. Recently, LIPIN1, a protein that helps promote lipid biosynthesis, was identified as a potential mTOR substrate, but more work is needed to further characterize this substrate[107].

There is certainly a lack of information on mTOR substrates, and even more lacking is the role of phosphatases in regulating mTOR activity and activation of its substrates. There is evidence that PP2A can associate and dephosphorylate S6K, and the same report indicated that mTOR can inactivate PP2A, providing two modes of S6K activation[108] (Figure 1.4). PP2A has also been shown to affect mTOR activity by regulating AKT. AKT, which inhibits TSC2, requires phosphorylation on T308 and S473 for activation, and the PP2A B'α holoenzyme has been shown to dephosphorylate AKT on T308, thereby inactivating it[109] (Figure 1.4). Upstream of AKT, driven by insulin signaling, is insulin receptor substrate 1 (IRS1) which is necessary to transduce insulin receptor signaling to PI3K. PP2A can dephosphorylate IRS1, leading to its stabilization, and mTOR can inhibit PP2A activity toward IRS1 potentially directly phosphorylating IRS1 at S307, leading to IRS1 degradation[110, 111] (Figure 1.4). Multiple reports have indicated that mTOR can negatively regulate PP2A activity, and most of this negative regulation supports mTOR activation through insulin signaling and PI3K. In contrast, PP2A can negatively regulate mTOR when amino acids are not present. MAP4K3 can signal to activate mTOR when amino acids are present. Autophosphorylation on S170 is necessary for this activation. When amino acids are withdrawn, PP2A dephosphorylates S170 and prevents mTOR activation by this pathway[112] (Figure 1.4). To make matters more complex, TIPRL can overcome amino acid withdrawal and stimulate mTOR activation by binding with the C subunit

of PP2A and inhibiting its activity; however, the yeast homologue, TIP41, has a negative effect on mTOR activation by binding to TAP42, which would bind PP2Ac and inhibit its function[58]. The mammalian homologue of TAP42, α4, does bind PP2Ac, but unlike in yeast, this association is not dependent on mTOR, indicating that the functions of TIPRL and α4 are not conserved[113].

The mTOR pathway is a critical pathway to activate growth and proliferation, and the regulation of this pathway is exceedingly complex, involving many feedback loops and antagonistic partnerships, especially with PP2A. There is a large amount of information known about mTOR, and it is a frequently targeted pathway for potential treatments of disease such as cancer. Despite this knowledge, there are gaps in our understanding of mTOR substrates, as well as its activation, regulation, and crosstalk with other signaling pathways. Further study will deepen our understanding of growth signaling and possibly lead to significant drug development.

Mitogen Activated Protein (MAP) Kinase Signaling Pathway: *A diagrammatic overview of the MAPK pathway is shown in figure 1.5*. MAP kinase pathways help regulate many cellular functions such as proliferation, differentiation, and apoptosis. There are 4 families of MAP kinases: ERK1/2, ERK5, JNK, and p38 (reviewed in 114-117). When activated, MAP kinases phosphorylate downstream substrates to induce cellular responses. MAPKs are activated by upstream kinases (MAPK kinases), and those MAPKKs are activated by further upstream kinases, MAPKK kinases (MAPKKKs). These kinases are activated by cellular growth signals, cytokines, or stress signals. ERK5, JNK, and p38 generally have pro-apoptotic functions and are activated by stresses, whereas ERK1/2 promotes proliferation and transformation. The ERK1/2 pathway was the first MAPK pathway discovered and is the best studied. Growth factors such as epidermal growth factor (EGF) or fibroblast growth factor (FGF) bind to their respective receptors and recruit a complex of SRC homology-2-containing protein (SHC), growth factor

receptor bound protein 2 (GRB2), and son of sevenless (SOS)[118]. This complex changes Ras conformation, disrupting GDP interaction and promoting GTP association which activates Ras and recruits Raf (MAPKKK) to the membrane bound complex, where Raf is activated by dimerization and phosphorylation[119, 120]. Raf then phosphorylates and activates MEK1, which subsequently phosphorylates and activates ERK1/2[121, 122]. ERK1/2 phosphorylates transcription factors Jun and Fos which can then translocate to the nucleus and bind DNA to initiate transcription of genes involved in cell cycle regulation such as AP-1, which in turn can promote cyclin D1 expression[123, 124]. ERK1/2 can also phosphorylate and stabilize c-Myc, which can then enhance its transcriptional activity toward cell cycle promoting genes such as cyclin D1 and CDC25A[117, 125].

The ERK1/2 MAPK pathway and its downstream substrates are also regulated by the action of phosphatases. There are at least 11 MAPK phosphatases (MKPs), which are split into three families based on cellular localization. There is significant cross-activity between the MKPs and all four of the MAPK pathways, and many of the MKPs, such as MKP-1 and MKP-3, have an overall transforming effect and are implicated in many cancers (reviewed in 126). MKP-1 has been the best studied of the MKPs, and is implicated in a variety of cancers. One of the mechanisms implicated in maintaining cell survival is its ability to prevent stress-induced apoptosis by preferentially dephosphorylating p38 and JNK, inactivating two critical stress-induced apoptotic pathways in the cell[127, 128].

PP2A appears to have a role in both positive and negative regulation of the ERK MAPK pathway (Figure 1.5). SHC is an important member of the complex that binds growth receptors and activates Ras. PP2A can bind to the phospho-tyrosine binding domain of SHC and negatively regulate Ras activation. After growth factor stimulation, T317 phosphorylation of

SHC can dissociate PP2A and allow downstream activation. It is currently unknown whether PP2A actively dephosphorylates SHC or which regulatory subunit is responsible for PP2A's inhibitory effect[129]. PP2A can also directly inactivate ERK by dephosphorylation (Figure 1.5). This is mediated by the B'β and B'γ subunits, which can also be phosphorylated by ERK if IEX-1 is expressed, thus reversing the PP2A mediated inactivation[130]. Sprouty2 is an inhibitor of FGF stimulated ERK activation. Sprouty2 is normally phosphorylated and cannot bind Grb2, and phosphorylation on T55 allows c-Cbl to bind and target sprouty2 for degradation by the proteasome. On FGF stimulation, sprouty2 is dephosphorylated by PP2A, which exposes a Grb2 binding motif on the C-terminus (Figure 1.5). When bound to Grb2, ras is unable to be recruited to the complex and be activated, thus downregulating ERK activation by FGF. PP2A binds to sprouty2 between residues 50-60, competing with c-Cbl binding to activate and protect sprouty2[131]. PP2A appears to have diverse effects negatively regulating the assembly of the ras activating complex in addition to working downstream on ERK itself. Conversely, PP2A can also positively regulate ERK MAPK signaling. The kinase repressor of ras (KSR1) is a critical positive regulator of ras signaling. It is a necessary scaffold to transduce the activation signal from ras-1 to MEK to ERK. PP2A Bα holoenzyme is associated with the KSR1 complex and is required for MEK activation. When phosphorylated, S392 of KSR1 associates with 14-3-3 protein and remains cytoplasmic. PP2A Bα directly dephosphorylates KSR1 at S392 which is then freed from 14-3-3 and can translocate to the membrane, an event required for MEK activation[132] (Figure 1.5). Similarly, PP2A and PP1 have been shown to positively regulate Raf-1 activity by dephosphorylating S259, allowing 14-3-3 release from Raf-1 and membrane translocation[133] (Figure 1.5). In addition to working downstream of growth receptors, PP2A can exert a positive regulation of ERK MAPK signaling. EGF receptors are targeted for

ubiquitination and degradation by c-Cbl, which requires phosphorylation on various residues. This interaction is disrupted upon recruitment of SRC homology 2 domain containing inositol polyphosphate phosphatase, SHIP2. PR130, a PP2A regulatory subunit from the B'' family, forms a holoenzyme which can form a complex with SHIP2 and is required for SHIP2-mediated stabilization of EGFR. Mapping studies indicate that the catalytic domain of SHIP2 interacts with the EF hands of PR130, and mutation in this region disrupts the stabilizing effect of PR130 holoenzyme on EGFR[134]. It is currently unknown whether catalytic activity is required for this effect and whether PP2A-PR130 dephosphorylates EGFR, SHIP2, or other associated targets.

Taken together, much of the phosphatase activity of MKPs has a net overall proliferative effect, and most of the activity of PP2A has a net overall anti-proliferative effect; however, this regulation is especially complex and may have other as-yet-unidentified regulatory proteins involved. There is also crosstalk with many of these pathways as well as other cell cycle promoting pathways, highlighting the importance of phosphatases in regulating the initiation of the cell cycle and the complexity by which they do so.

Cell Cycle Progression: Rb and the G1-S Transition

The initiation of the cell cycle can occur by a variety of processes such as those mentioned above. In G1 phase, these pathways initiate growth and transcription of factors, such as cyclin D1, that control cell cycle progression. Before the cell can enter into synthesis phase, it must pass through a critical cell checkpoint to ensure it is ready for DNA synthesis. The master regulator of this checkpoint, and first discovered tumor suppressor protein, is the retinoblastoma tumor suppressor protein (Rb).

Rb Phosphorylation: Rb is an approximately 105 kDa protein consisting of three functional domains: an N-terminal structured region, a two-part central pocket region, and a C-terminal unstructured region. There are two other proteins structurally and functionally related to Rb: p107 and p130. Together, these proteins make up the pocket protein family and have all been implicated in diverse cellular processes such as cell cycle progression, apoptosis, senescence, differentiation, and angiogenesis (reviewed in 135). These proteins bind and inactivate E2F transcription factors, with Rb binding E2F 1-3, and p107/p130 binding E2F4/5 (Figure 1.6). E2F 1-3 are transcriptional activators and mostly express cell cycle genes such as cyclins E and A, and CDC25 (reviewed in 135-137). E2F4 & 5 are transcriptional repressors and are involved in maintaining genomic stability and redundant functions with Rb (reviewed in 138, 139). The pocket proteins bind to E2F transcription factors along with their dimerization partners (DPs), preventing their translocation to the nucleus and transcriptional activation. The pocket proteins are phosphorylated by cyclin/CDK holoenzymes on numerous residues, weakening the interaction between them and the E2Fs, causing dissociation and E2F transcriptional activation (reviewed in 140, 141) (Figure 1.6). Rb is phosphorylated by cyclin D/CDK4 and cyclin E/CDK2 in G1, and numerous phosphorylation events gradually lead to the

release of E2F transcription factors[142]. One possible mechanism for this gradual release is due to the association of E2F with multiple domains of Rb[143]. The pocket domain alone is not sufficient for E2F dimerization, as the C-terminal region of Rb adopts a stable conformation upon association with E2F1-DP and increases the binding of the complex more than 36 fold. Loss of this interaction predisposes Rb-E2F to dissociate, and this region is phosphorylated by cyclin D/CDK4/6 early in G1, thus providing a model for how sequential phosphorylation events dissociate Rb-E2F. Rb levels do not change throughout the cell cycle, indicating that phosphorylation events need to be reversed to reset Rb after cell division[140].

Phosphatases in Rb Regulation: Although the role of phosphorylation in Rb regulation was discovered early on and has been extensively studied, the role of phosphatases in reversing these phosphorylation events has not been as thoroughly studied. Protein phosphatase 1 and PP2A are the primary phosphatases that regulate Rb (and p107/130) function. PP1 is responsible for complete dephosphorylation of Rb after mitosis, whereas PP2A functions throughout the cell cycle, dephosphorylating Rb and p107/130 in response to various stimuli (reviewed in 140, 144).

PP1 and PP2A are both known to dephosphorylate Rb, and PP1 appears to compete for the same CDK docking sites, possibly explaining why it is only effective after mitosis when CDK activity drops[145, 146]. This sort of competitive interaction has also been suggested between CDKs and PP2A for all three pocket proteins, and during the cell cycle when CDKs are elevated, the net effect is toward hyperphosphorylation[147]. Indeed, specific overexpression of Bα induces p107 dephosphorylation. Bα can directly associate with p107 but has little affinity for pRb, therefore, additional holoenzymes may be required to mediate cell cycle arrest by pocket protein activation[148].

In contrast to constitutive competitive interactions, extracellular signaling or stress factors can induce PP2A-modulated dephosphorylation of the pocket proteins with no significant changes in CDK activity or PP2A expression[149]. Sustained FGF signaling can arrest cell growth in chondrocytes, which is the opposite effect in most other cell types. FGF can dephosphorylate Bα, increasing its affinity for p107, and chondrocytes have a large constitutively expressed Bα population. Phosphorylation of Bα allows increased association of PP2A-Bα holoenzymes with p107 and subsequent dephosphorylation and cell cycle arrest[150]. The extracellular factor all-*trans*-retinoic acid (ATRA) appears to induce PP2A specific dephosphorylation of p130. Upon ATRA treatment, PP2A can bind to and dephosphorylate p130, protecting it from ubiquitination and degradation. PP2A can also mediate p130's translocation to the nucleus due to dephosphorylation of S1080 and T1097, exposing the NLS and allowing binding by importins α and β[151, 152]. Under oxidative stress conditions, the PP2A-PR70 holoenzyme can dephosphorylate Rb, and this activity is dependent on Ca^{2+} stimulation[153] (Figure 1.6). One potential underlying mechanism is that oxidative stress induces an influx of Ca^{2+}, which stimulates PR70 holoenzyme formation and results in specific Rb dephosphorylation by PP2A-PR70.

The regulation of pocket proteins by dephosphorylation is complex and results from the interplay between competition from CDKs and specific mitogenic or stress stimuli. There may also be crosstalk between various signaling pathways in this process, as pocket proteins are central effectors through which many pathways funnel. S phase induction by Rb represents a commitment by the cell to DNA synthesis and phosphatases continue to be important in regulating this process.

DNA Synthesis and Regulation of the Origin Recognition Complex

Once the cell passes the G1-S checkpoint, it is committed to the process of synthesizing DNA. The genome in eukaryotes is far too large for synthesis to proceed in a linear fashion from one end to another, so synthesis proceeds from discrete origins of replication. In yeast, these origins are defined by specific DNA sequences; however, human origins are likely defined by DNA structural features (reviewed in 154).

ORC Assembly and Regulation: The origin recognition complex (ORC) is a large protein complex that binds to DNA at the origins of synthesis and recruits all of the proteins required to unzip and polymerize DNA (reviewed in 155, 156). There are 6 ORC proteins (ORC1-6) that bind to DNA at the origins (referred to as autonomously replicating sequences (ARS) in yeast). These proteins all bind and hydrolyze ATP, and ATPase activity is required for their assembly and recruitment of other complex members[20]. In late G1/early S, cell division control 6 protein (Cdc6) binds to the ORC proteins and is the critical component for further ORC assembly[22]. Cdc6 facilitates the loading of Cdt1 and ORC6 to ORC1-5 which then facilitate the loading of the mini-chromosome maintenance proteins (MCM2-7)[24]. Cdc6 also has ATPase activity, and hydrolysis of ATP leads to conformational changes which increase the binding affinity of the MCM proteins for the complex[157]. The MCM proteins have helicase activity, and can begin to unwind DNA for replication. The MCM proteins are then phosphorylated by Dbf4/Cdc7 which allows the recruitment of RPA and Cdc45 to the unzipped origin, which facilitates the loading of DNA polymerase and synthesis of DNA[158, 159].

Regulation of Cdc6 by Phosphorylation: *A diagrammatic illustration of Cdc6 regulation is shown in figure 1.7.* For error free cell division, DNA must be synthesized once and only once. One of the chief ways the cell regulates this process is by only allowing the

origins to fire one time. This restriction is accomplished by the tight regulation of Cdc6. In the absence of phosphorylation near its N-terminal destruction motifs, the anaphase-promoting complex/cyclosome (APC/C) targets Cdc6 for ubiquitination and proteasomal degradation. In G1, Cdc6 is phosphorylated by cyclin E/CDK2 on S54 and S74, protecting it from degradation and allowing it to be transported into the nucleus and to bind to the ORC[160, 161] (Figure 1.7). After origin firing in late S phase, Cdc6 is acetylated by general control nonderepressible 5 (GCN5) and phosphorylated by cyclin A/CDK2 on S106[162, 163]. These modifications tag Cdc6 for nuclear export where it is degraded in the cytoplasm. Cdc6 degradation can only happen when the protective residues are dephosphorylated, and PP2A-PR70 has been shown to dephosphorylate Cdc6, although this interaction has not been fully characterized (Figure 1.7). Chapter II will focus on the interaction of PP2A-PR70 and Cdc6 with insights on its interaction and dephosphorylation.

Concluding Remarks

The cell cycle is a complex and intricate process and may be the most studied aspect in all of biology. This highlights its importance in understanding the origins of most of our diseases and what we can do to intervene and provide our species with an unprecedented quality of life and longevity. We have made great strides in understanding the complex players of the cell cycle, and the importance of regulation by reversible phosphorylation cannot be underestimated.

The role of PP2A in regulating the cell cycle is only beginning to be investigated. PP2A is a complex group of enzymes and is highly regulated, making it difficult to study. There are no known PP2A-substrate binding consensus sequences, indicating that structural and biochemical information is required to understand the mechanisms by which PP2A regulates substrate dephosphorylation. The knowledge on the B'' family of PP2A holoenzymes is particularly lacking, despite the importance of its member in regulating key cell cycle proteins such as Rb and Cdc6. Structural information on a PP2A-B'' holoenzyme will fill in an important gap in knowledge of PP2A function. This information will lead to better characterization of PP2A function and identify predictive markers and possible targets for therapeutic intervention.

In the modern era, we are beginning to understand the numerous variations in the population, which requires us to study cell division both as a general process and on an individual-specific basis. The "omics" revolution gives us new understanding in both of these aspects, and we can begin to use these tools to better prevent, diagnose, and treat disease. High-throughput assays can identify substrates and characterize protein-protein interactions significantly faster than traditional methods while still having high specificity. These large scale assays combined with structural and biochemical characterization will provide an unprecedented

amount of information to the PP2A field and possibly identify new targets of cell cycle regulation. These new targets may be incredibly useful in developing drugs or biomarkers for preventing, diagnosing, or treating human disease, and more refined knowledge on existing PP2A-substrate interactions may help improve current treatments.

Figures

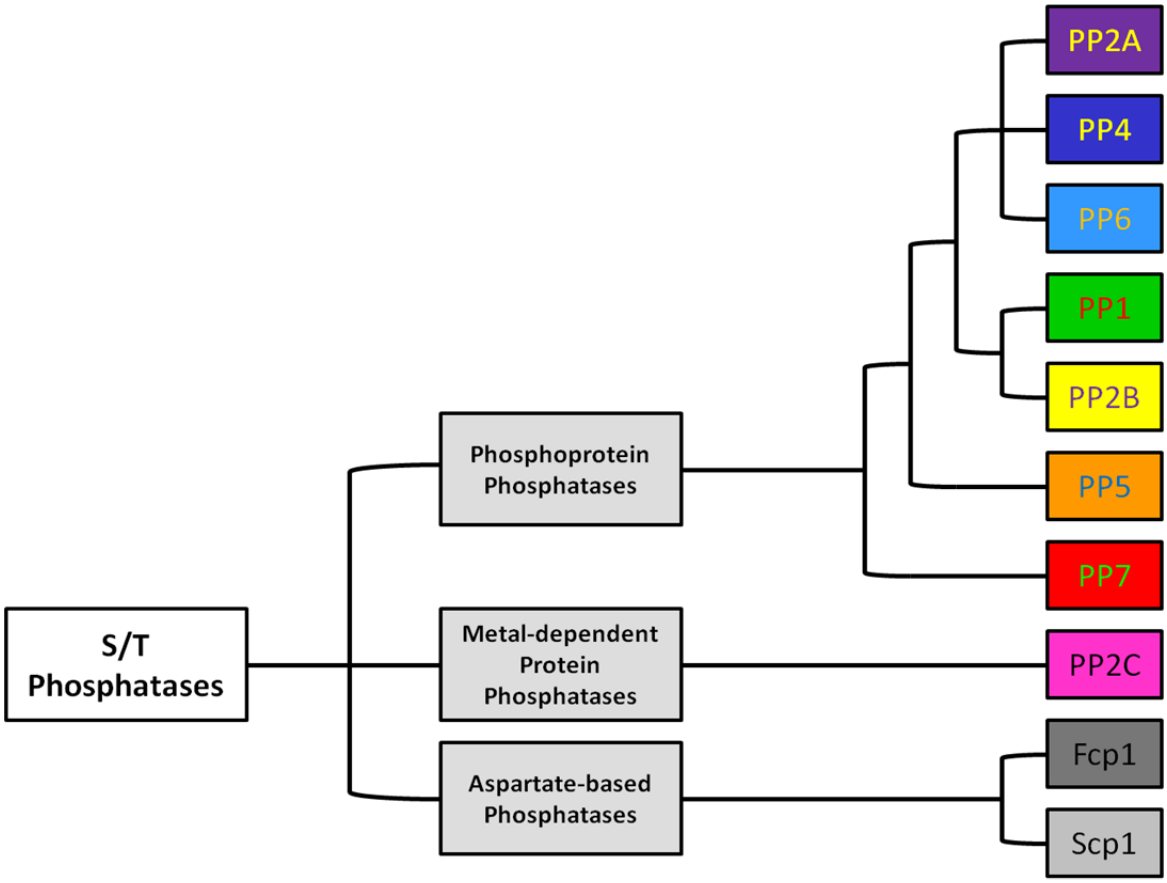


Figure 1.1. Serine/threonine phosphatases are classified based on biochemical mechanism. They are divided into three families, the aspartate-based phosphatases, the metal dependent protein phosphatases, and the phosphoprotein phosphatases. The phosphoprotein phosphatases have similar active site configurations and require catalytic metal ions in the active site. PP2A is a member of this family. Adapted from Stanevitch (2013).

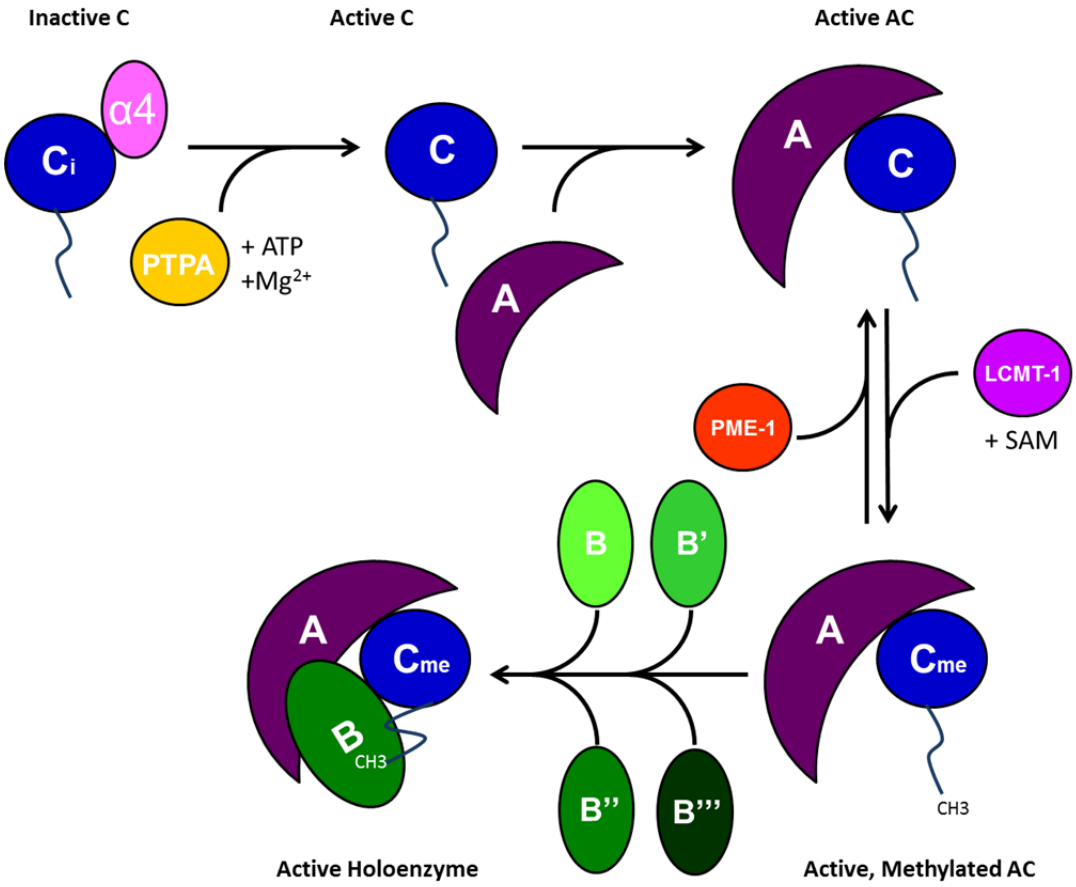
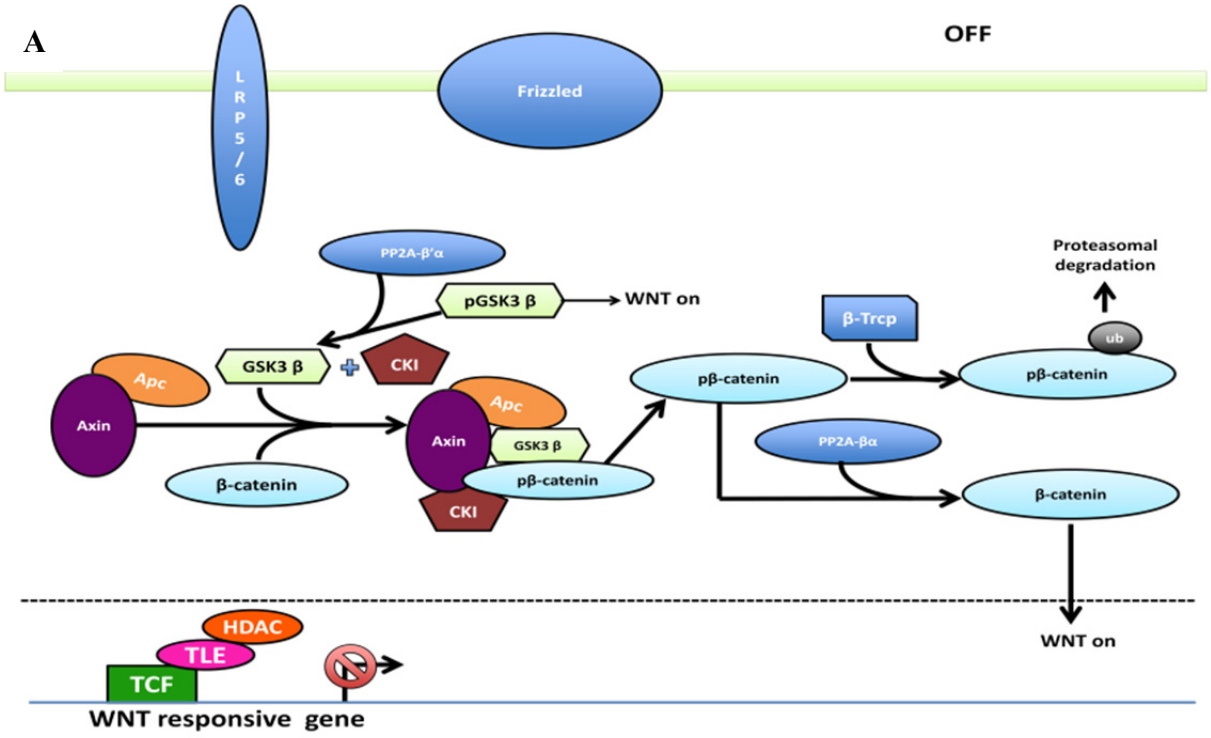


Figure 1.2. Biogenesis and assembly of PP2A holoenzymes. α4 protects inactive PP2Ac from ubiquitination by MID1. Activating metal ions are loaded by PTPA, and active PP2Ac binds to the scaffold subunit (A). The C-terminal tail of PP2Ac can be methylated by LCMT-1 and reversed by PME-1. Active, methylated PP2A-AC can then form holoenzymes with various B subunits which are divided into 4 families: B, B', B'', and B'''.


A
OFF
LRP5/6
Frizzled
PP2A-β'α
pGSK3 β
WNT on
Proteasomal degradation
β-Trcp
ub
GSK3 β
CKI
Apc
Axin
β-catenin
pβ-catenin
PP2A-βα
HDAC
TLE
TCF
WNT responsive gene

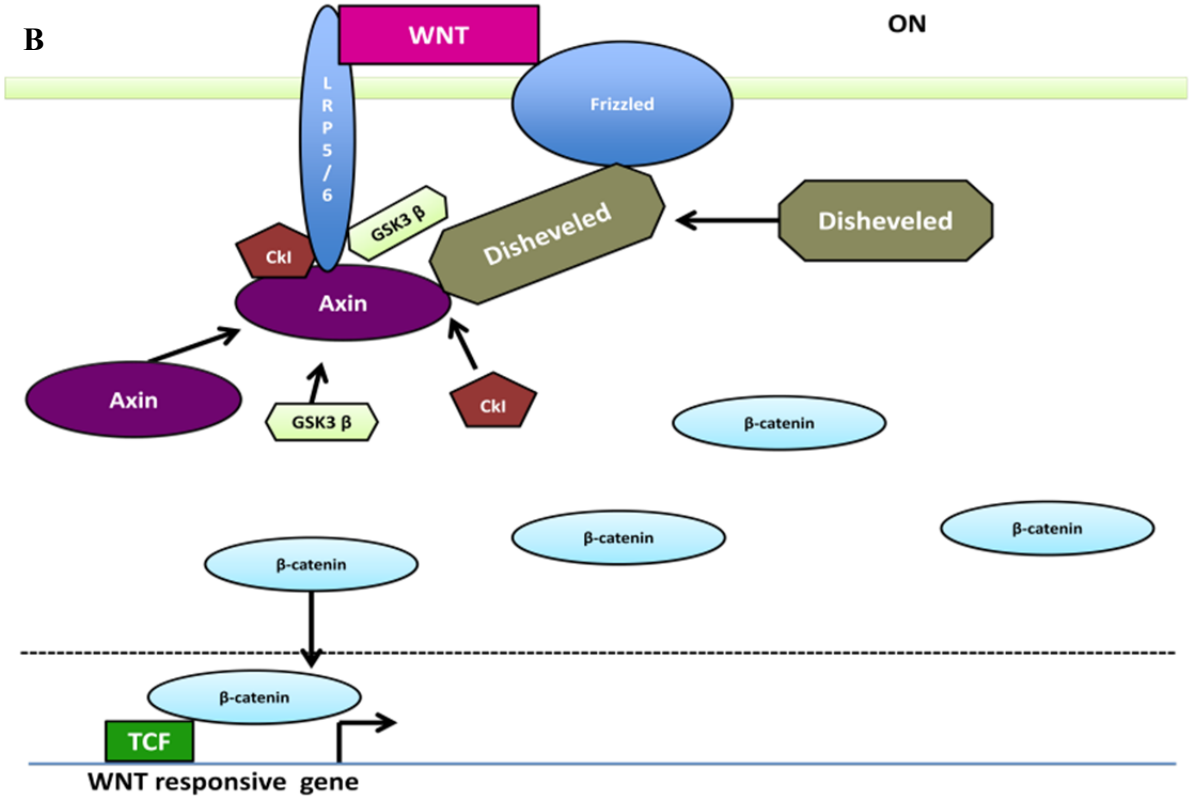

B
ON
WNT
LRP5/6
Frizzled
GSK3 β
CkI
Disheveled
Axin
β-catenin
TCF
WNT responsive gene

Figure 1.3. The Wnt signaling pathway. **A**. Wnt OFF. In the absence of Wnt signaling, a complex of Axin, Apc, GSK3β, and CK1 phosphorylate β-catenin, targeting it for proteasomal degradation. PP2A-B'α promotes β-catenin degradation by removing an inhibitory phosphorylation on GSK3β. PP2A-Bα can directly dephosphorylate β-catenin, promoting the activation of wnt responsive genes. **B**. Wnt signaling ON. In the presence of Wnt ligand, Wnt receptors LRP5/6 and frizzled sequester the Axin, GSK3β, and CK1, preventing the phosphorylation of β-catenin. β-catenin accumulates and translocates to the nucleus, promoting the transcription of Wnt responsive genes. Figure adapted from MacDonald et. al. (2009).

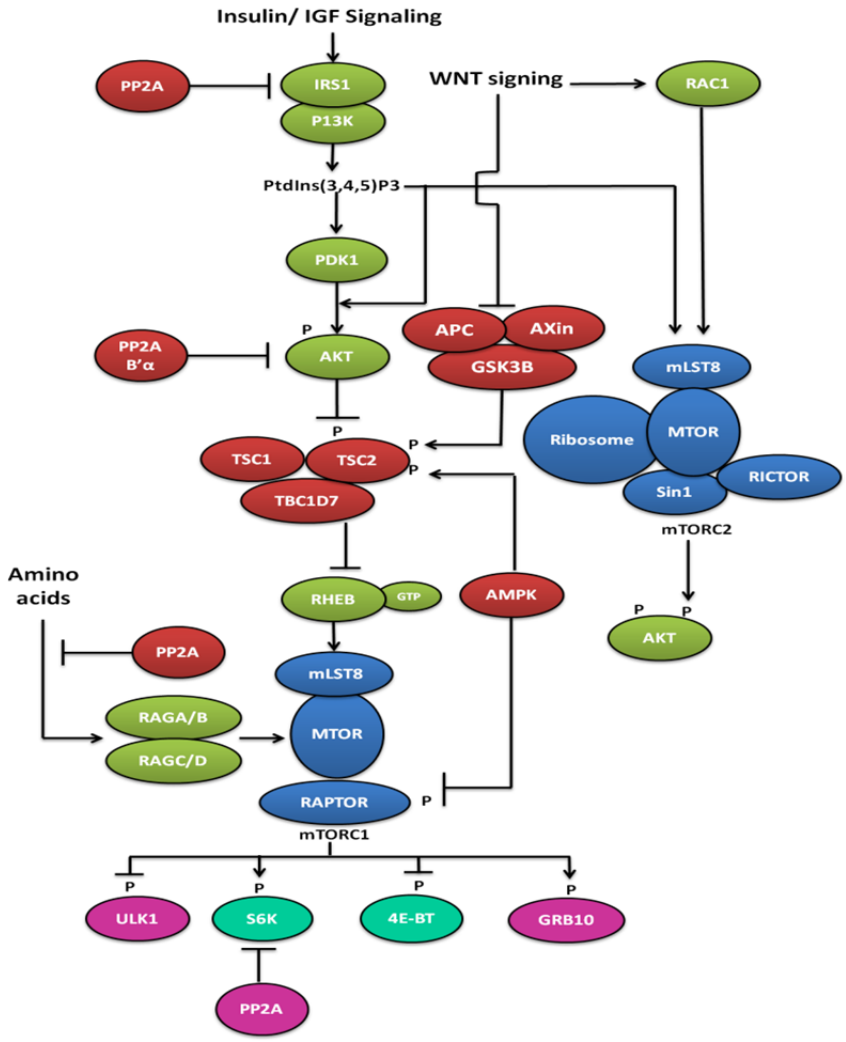


Figure 1.4. The mTOR signaling pathway. The mTOR complexes are colored blue. Proteins involved in mTOR inhibition and activation are colored red and green, respectively. Downstream factors inhibiting and stimulating growth are colored magenta and teal, respectively. Growth factors stimulate the mTOR pathway via inhibiting the function of the TSC complex that inhibits mTOR activation. Wnt signaling can inhibit the TSC complex or directly stimulate mTORC2. Amino acids can also stimulate mTOR activity. PP2A inhibits the mTOR pathway by inhibiting IRS1 in the insulin signaling pathway or MAP4K3 in the amino acid pathway, or by inhibiting AKT function. PP2A can also reverse mTOR phosphorylation of S6K. Figure adapted from Shimobayshi & Hall (2014).

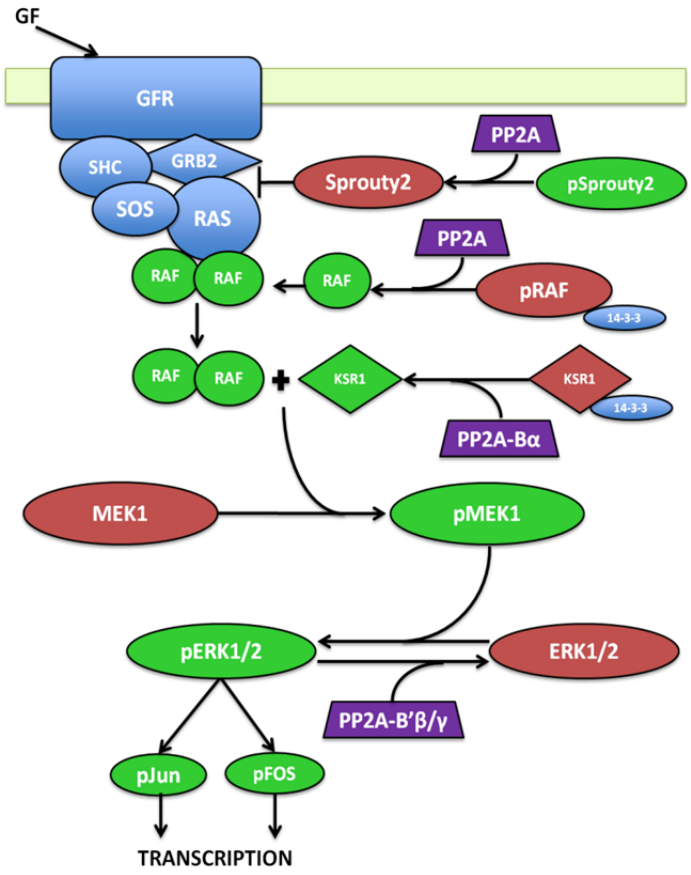


Figure 1.5. The MAPK signaling pathway. Growth factors stimulate a complex of proteins: SHC, GRB2, and SOS to a growth factor receptor. This complex activates Ras which starts a signal cascade from activation of Raf, to activation of MEK, ERK, and eventually the transcription factors that activate the transcription of growth related genes. PP2A has both positive and negative regulatory functions in this pathway. PP2A can activate Raf by dephosphorylating S259 and causing 14-3-3 release. PP2A-Bα dephosphorylates S392 of KSR1, which leads to dissociation of 14-3-3 from KSR1, essential for MEK1 activation. PP2A negatively regulates MAPK upstream by activating Sprouty2, which inhibits GRB2 and subsequent RAS complex formation. PP2A-B'β/B'γ can directly dephosphorylate ERK1/2 downstream of the signaling cascade, thereby inactivating it. Figure loosely adapted from McCubrey et. al. (2009).

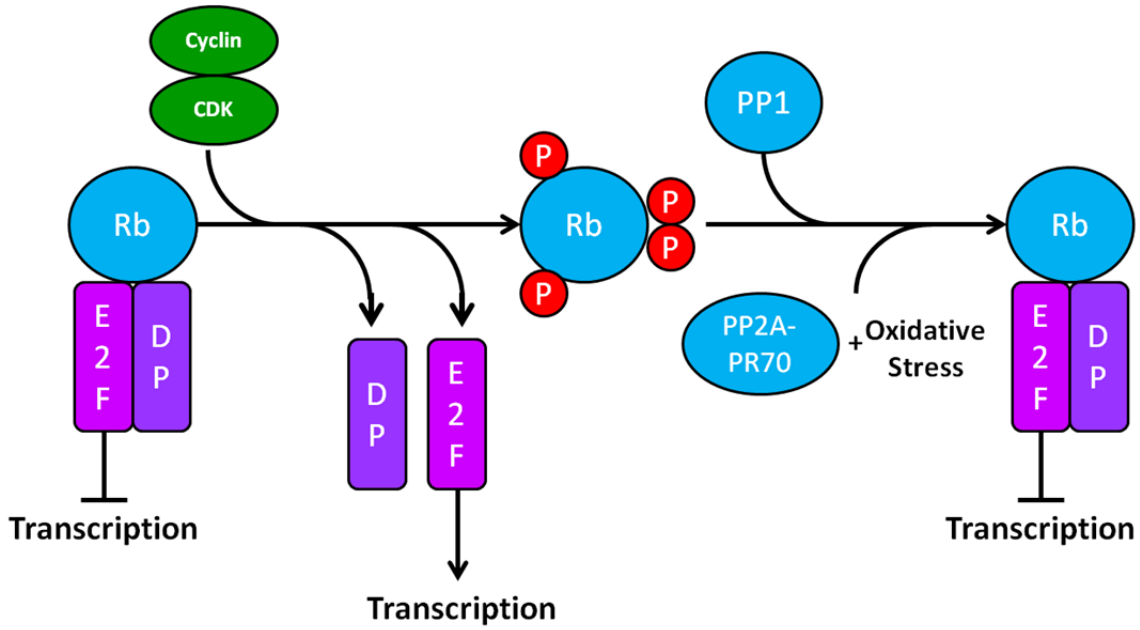


Figure 1.6. Rb phosphorylation promotes transcription of E2F responsive genes. Rb normally binds E2F transcription factors and their dimerization partners. When phosphorylated by cyclin/CDK heterodimers, Rb loses affinity for E2F and free E2F is allowed to promote transcription. Rb is dephosphorylated at the end of mitosis, allowing re-association with E2F. PP2A-PR70 can dephosphorylate Rb under oxidative stress conditions.

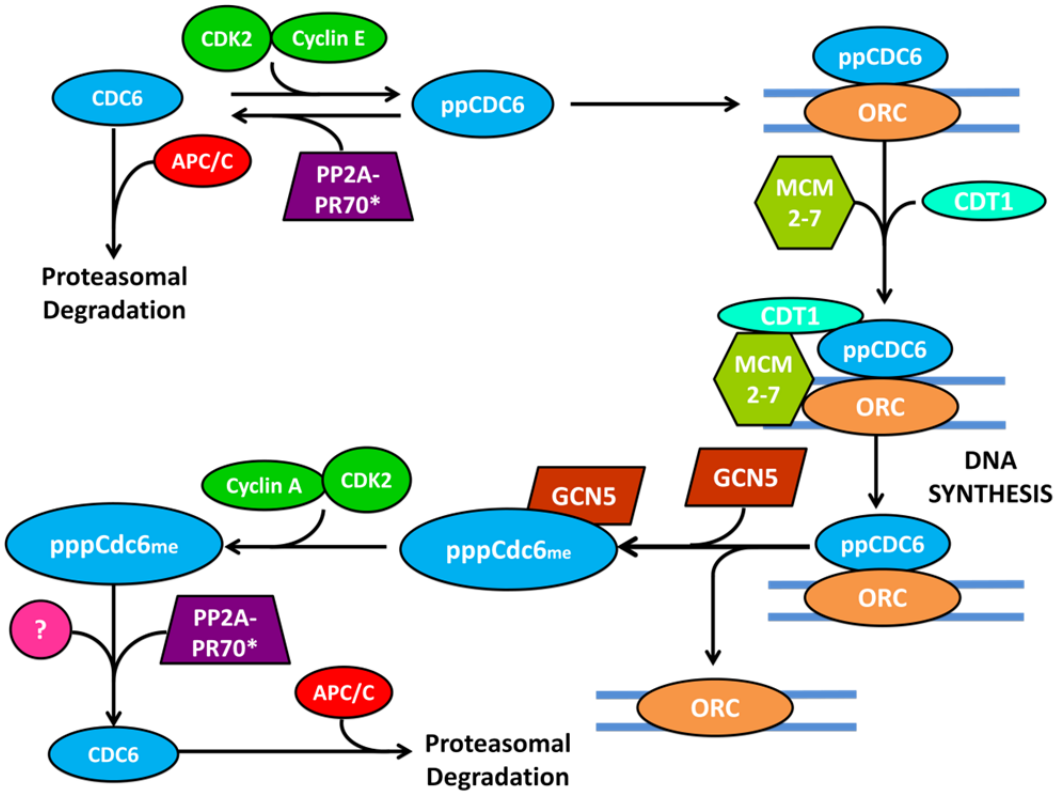


Figure 1.7. Cdc6 is necessary for assembly of the pre-replication complex and subsequent DNA synthesis. In G0, Cdc6 is ubiquinated by the anaphase promoting complex/cyclosome (APC/C) and degraded by the proteasome. In G1, Cyclin E/CDK2 phosphorylates Cdc6 on S54 and S74, protecting it from degradation. Cdc6 is translocated into the nucleus where it binds the origin recognition complex and is required to recruit Cdt1 and MCM2-7 and form the pre-replication complex. After firing of the origins, Cdc6 is methylated by GCN5 causing its dissociation from the ORC. Cdc6 is then phosphorylated on S106 by Cyclin A/CDK2 and translocated to the cytoplasm. PP2A-PR70 is thought to dephosphorylate Cdc6 either at this point in G2 and/or in G1, ensuring Cdc6 destruction and regulating DNA synthesis. Figure loosely adapted from Mumby (2009).

References

1 Mitchison TJ, Salmon ED. Mitosis: a history of division. *Nat Cell Biol* 2001; **3**:E17-21.

2 Surridge C. milestone 1 Chromosomes to the fore. Available from: http://www.nature.com/celldivision/milestones/full/milestone01.html.

3 Boveri T. Concerning the origin of malignant tumours by Theodor Boveri. Translated and annotated by Henry Harris. *J Cell Sci* 2008; **121 Suppl 1**:1-84.

4 Swift H. The constancy of desoxyribose nucleic acid in plant nuclei. *Proc Natl Acad Sci U S A* 1950; **36**:643-654.

5 Voorhees JJ, Duell EA, Chambers DA, Marcelo CL. Regulation of cell cycles. *J Invest Dermatol* 1976; **67**:15-19.

6 Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* 1953; **171**:737-738.

7 Rao PN, Johnson RT. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature* 1970; **225**:159-164.

8 Pardee AB. A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci U S A* 1974; **71**:1286-1290.

9 Nurse P. Genetic control of cell size at cell division in yeast. *Nature* 1975; **256**:547-551.

10 Nurse P, Thuriaux P. Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe. *Genetics* 1980; **96**:627-637.

11 Russell P, Nurse P. Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. *Cell* 1987; **49**:559-567.

12 Russell P, Nurse P. cdc25+ functions as an inducer in the mitotic control of fission yeast. *Cell* 1986; **45**:145-153.

13 Gould KL, Nurse P. Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. *Nature* 1989; **342**:39-45.

14 Evans T, Rosenthal ET, Youngblom J, Distel D, Hunt T. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* 1983; **33**:389-396.

15 Murray AW, Kirschner MW. Cyclin synthesis drives the early embryonic cell cycle. *Nature* 1989; **339**:275-280.

16 Murray AW, Solomon MJ, Kirschner MW. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* 1989; **339**:280-286.

17 Stinchcomb DT, Struhl K, Davis RW. Isolation and characterisation of a yeast chromosomal replicator. *Nature* 1979; **282**:39-43.

18 Brewer BJ, Fangman WL. The localization of replication origins on ARS plasmids in S. cerevisiae. *Cell* 1987; **51**:463-471.

19 Diffley JF, Cocker JH. Protein-DNA interactions at a yeast replication origin. *Nature* 1992; **357**:169-172.

20 Bell SP, Stillman B. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* 1992; **357**:128-134.

21 Lisziewicz J, Godany A, Agoston DV, Kuntzel H. Cloning and characterization of the Saccharomyces cerevisiae CDC6 gene. *Nucleic Acids Res* 1988; **16**:11507-11520.

22 Liang C, Weinreich M, Stillman B. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* 1995; **81**:667-676.

23 Chong JP, Mahbubani HM, Khoo CY, Blow JJ. Purification of an MCM-containing complex as a component of the DNA replication licensing system. *Nature* 1995; **375**:418-421.

24 Nishitani H, Lygerou Z, Nishimoto T, Nurse P. The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature* 2000; **404**:625-628.

25 Lew DJ, Dulic V, Reed SI. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell* 1991; **66**:1197-1206.

26 Matsushime H, Roussel MF, Ashmun RA, Sherr CJ. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 1991; **65**:701-713.

27 Xiong Y, Connolly T, Futcher B, Beach D. Human D-type cyclin. *Cell* 1991; **65**:691-699.

28 Draetta G, Luca F, Westendorf J, Brizuela L, Ruderman J, Beach D. Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell* 1989; **56**:829-838.

29 Minshull J, Golsteyn R, Hill CS, Hunt T. The A- and B-type cyclin associated cdc2 kinases in Xenopus turn on and off at different times in the cell cycle. *Embo j* 1990; **9**:2865-2875.

30 Elledge SJ, Spottswood MR. A new human p34 protein kinase, CDK2, identified by complementation of a cdc28 mutation in Saccharomyces cerevisiae, is a homolog of Xenopus Eg1. *Embo j* 1991; **10**:2653-2659.

31 Koff A, Cross F, Fisher A *et al.* Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell* 1991; **66**:1217-1228.

32 Xiong Y, Zhang H, Beach D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* 1992; **71**:505-514.

33 Benedict WF, Murphree AL, Banerjee A, Spina CA, Sparkes MC, Sparkes RS. Patient with 13 chromosome deletion: evidence that the retinoblastoma gene is a recessive cancer gene. *Science* 1983; **219**:973-975.

34 Lee WH, Bookstein R, Hong F, Young LJ, Shew JY, Lee EY. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* 1987; **235**:1394-1399.

35 Lee WH, Shew JY, Hong FD *et al.* The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. *Nature* 1987; **329**:642-645.

36 DeCaprio JA, Ludlow JW, Figge J *et al.* SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* 1988; **54**:275-283.

37 Whyte P, Buchkovich KJ, Horowitz JM *et al.* Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* 1988; **334**:124-129.

38 Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989; **243**:934-937.

39 Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR. The E2F transcription factor is a cellular target for the RB protein. *Cell* 1991; **65**:1053-1061.

40 Ludlow JW, DeCaprio JA, Huang CM, Lee WH, Paucha E, Livingston DM. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell* 1989; **56**:57-65.

41 Bagchi S, Raychaudhuri P, Nevins JR. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. *Cell* 1990; **62**:659-669.

42 Goodrich DW, Wang NP, Qian YW, Lee EY, Lee WH. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* 1991; **67**:293-302.

43 Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev* 1993; **7**:331-342.

44 Li R, Murray AW. Feedback control of mitosis in budding yeast. *Cell* 1991; **66**:519-531.

45 Hoyt MA, Totis L, Roberts BT. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell* 1991; **66**:507-517.

46 DeLeo AB, Jay G, Appella E, Dubois GC, Law LW, Old LJ. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc Natl Acad Sci U S A* 1979; **76**:2420-2424.

47 Dippold WG, Jay G, DeLeo AB, Khoury G, Old LJ. p53 transformation-related protein: detection by monoclonal antibody in mouse and human cells. *Proc Natl Acad Sci U S A* 1981; **78**:1695-1699.

48 Baker SJ, Fearon ER, Nigro JM *et al.* Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* 1989; **244**:217-221.

49 Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 1990; **249**:912-915.

50 Shi Y. Serine/threonine phosphatases: mechanism through structure. *Cell* 2009; **139**:468-484.

51 Hunter T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 1995; **80**:225-236.

52 Virshup DM, Shenolikar S. From promiscuity to precision: protein phosphatases get a makeover. *Molecular cell* 2009; **33**:537-545.

53 Martin L, Latypova X, Wilson CM, Magnaudeix A, Perrin ML, Terro F. Tau protein phosphatases in Alzheimer's disease: the leading role of PP2A. *Ageing Res Rev* 2013; **12**:39-49.

54 Heijman J, Dewenter M, El-Armouche A, Dobrev D. Function and regulation of serine/threonine phosphatases in the healthy and diseased heart. *J Mol Cell Cardiol* 2013; **64**:90-98.

55 Kotlo K, Johnson KR, Grillon JM, Geenen DL, deTombe P, Danziger RS. Phosphoprotein abundance changes in hypertensive cardiac remodeling. *J Proteomics* 2012; **77**:1-13.

56 Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochimica et biophysica acta* 2009; **1795**:1-15.

57 Xu Y, Xing Y, Chen Y *et al.* Structure of the protein phosphatase 2A holoenzyme. *Cell* 2006; **127**:1239-1251.

58 Nakashima A, Tanimura-Ito K, Oshiro N *et al.* A positive role of mammalian Tip41-like protein, TIPRL, in the amino-acid dependent mTORC1-signaling pathway through interaction with PP2A. *FEBS Lett* 2013; **587**:2924-2929.

59 Jiang L, Stanevich V, Satyshur KA *et al.* Structural basis of protein phosphatase 2A stable latency. *Nat Commun* 2013; **4**:1699.

60 Guo F, Stanevich V, Wlodarchak N *et al.* Structural basis of PP2A activation by PTPA, an ATP-dependent activation chaperone. *Cell Res* 2014; **24**:190-203.

61 Stanevich V, Jiang L, Satyshur KA *et al.* The structural basis for tight control of PP2A methylation and function by LCMT-1. *Molecular cell* 2011; **41**:331-342.

62 Xing Y, Li Z, Chen Y, Stock JB, Jeffrey PD, Shi Y. Structural mechanism of demethylation and inactivation of protein phosphatase 2A. *Cell* 2008; **133**:154-163.

63 Lee JA, Pallas DC. Leucine carboxyl methyltransferase-1 is necessary for normal progression through mitosis in mammalian cells. *J Biol Chem* 2007; **282**:30974-30984.

64 Yu XX, Du X, Moreno CS *et al.* Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen. *Mol Biol Cell* 2001; **12**:185-199.

65 Xing Y, Xu Y, Chen Y *et al.* Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell* 2006; **127**:341-353.

66 Xu Y, Chen Y, Zhang P, Jeffrey PD, Shi Y. Structure of a protein phosphatase 2A holoenzyme: insights into B55-mediated Tau dephosphorylation. *Molecular cell* 2008; **31**:873-885.

67 Xu Z, Cetin B, Anger M *et al.* Structure and Function of the PP2A-Shugoshin Interaction. *Molecular cell* 2009; **35**:426-441.

68 MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 2009; **17**:9-26.

69 Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell* 2012; **149**:1192-1205.

70 van Noort M, Meeldijk J, van der Zee R, Destree O, Clevers H. Wnt signaling controls the phosphorylation status of beta-catenin. *J Biol Chem* 2002; **277**:17901-17905.

71 Amit S, Hatzubai A, Birman Y *et al.* Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes Dev* 2002; **16**:1066-1076.

72 Dajani R, Fraser E, Roe SM *et al.* Structural basis for recruitment of glycogen synthase kinase 3beta to the axin-APC scaffold complex. *Embo j* 2003; **22**:494-501.

73 Kinzler KW, Nilbert MC, Su LK *et al.* Identification of FAP locus genes from chromosome 5q21. *Science* 1991; **253**:661-665.

74 Nishisho I, Nakamura Y, Miyoshi Y *et al.* Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science* 1991; **253**:665-669.

75 Julius MA, Schelbert B, Hsu W *et al.* Domains of axin and disheveled required for interaction and function in wnt signaling. *Biochem Biophys Res Commun* 2000; **276**:1162-1169.

76 Behrens J, von Kries JP, Kuhl M *et al.* Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 1996; **382**:638-642.

77 Rimerman RA, Gellert-Randleman A, Diehl JA. Wnt1 and MEK1 cooperate to promote cyclin D1 accumulation and cellular transformation. *J Biol Chem* 2000; **275**:14736-14742.

78 He TC, Sparks AB, Rago C *et al.* Identification of c-MYC as a target of the APC pathway. *Science* 1998; **281**:1509-1512.

79 Latres E, Chiaur DS, Pagano M. The human F box protein beta-Trcp associates with the Cul1/Skp1 complex and regulates the stability of beta-catenin. *Oncogene* 1999; **18**:849-854.

80 Ferrarese A, Marin O, Bustos VH *et al.* Chemical dissection of the APC Repeat 3 multistep phosphorylation by the concerted action of protein kinases CK1 and GSK3. *Biochemistry* 2007; **46**:11902-11910.

81 Ha NC, Tonozuka T, Stamos JL, Choi HJ, Weis WI. Mechanism of phosphorylation-dependent binding of APC to beta-catenin and its role in beta-catenin degradation. *Mol Cell* 2004; **15**:511-521.

82 Jho E, Lomvardas S, Costantini F. A GSK3beta phosphorylation site in axin modulates interaction with beta-catenin and Tcf-mediated gene expression. *Biochem Biophys Res Commun* 1999; **266**:28-35.

83 Luo W, Peterson A, Garcia BA *et al.* Protein phosphatase 1 regulates assembly and function of the beta-catenin degradation complex. *Embo j* 2007; **26**:1511-1521.

84 Zhang W, Yang J, Liu Y *et al.* PR55 alpha, a regulatory subunit of PP2A, specifically regulates PP2A-mediated beta-catenin dephosphorylation. *J Biol Chem* 2009; **284**:22649-22656.

85 Seeling JM, Miller JR, Gil R, Moon RT, White R, Virshup DM. Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. *Science* 1999; **283**:2089-2091.

86 Mitra A, Menezes ME, Pannell LK *et al.* DNAJB6 chaperones PP2A mediated dephosphorylation of GSK3beta to downregulate beta-catenin transcription target, osteopontin. *Oncogene* 2012; **31**:4472-4483.

87 Kumar A, Pandurangan AK, Lu F *et al.* Chemopreventive sphingadienes downregulate Wnt signaling via a PP2A/Akt/GSK3beta pathway in colon cancer. *Carcinogenesis* 2012; **33**:1726-1735.

88 Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* 2012; **149**:274-293.

89 Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. *Nat Rev Drug Discov* 2014; **13**:140-156.

90 Sciarretta S, Volpe M, Sadoshima J. Mammalian target of rapamycin signaling in cardiac physiology and disease. *Circ Res* 2014; **114**:549-564.

91 Chen J, Alberts I, Li X. Dysregulation of the IGF-I/PI3K/AKT/mTOR signaling pathway in autism spectrum disorders. *Int J Dev Neurosci* 2014; **35c**:35-41.

92 Cafferkey R, Young PR, McLaughlin MM *et al.* Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. *Mol Cell Biol* 1993; **13**:6012-6023.

93 Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, Hall MN. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell* 1993; **73**:585-596.

94 Brown EJ, Albers MW, Shin TB *et al.* A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* 1994; **369**:756-758.

95 Peterson TR, Laplante M, Thoreen CC *et al.* DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* 2009; **137**:873-886.

96 Kim DH, Sarbassov DD, Ali SM *et al.* mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 2002; **110**:163-175.

97 Nojima H, Tokunaga C, Eguchi S *et al.* The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J Biol Chem* 2003; **278**:15461-15464.

98 Kim DH, Sarbassov DD, Ali SM *et al.* GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell* 2003; **11**:895-904.

99 Inoki K, Ouyang H, Zhu T *et al.* TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* 2006; **126**:955-968.

100 Shimobayashi M, Hall MN. Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol* 2014; **15**:155-162.

101 Klippel A, Kavanaugh WM, Pot D, Williams LT. A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol Cell Biol* 1997; **17**:338-344.

102 Sancak Y, Peterson TR, Shaul YD *et al.* The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 2008; **320**:1496-1501.

103 Hsu PP, Kang SA, Rameseder J *et al.* The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science* 2011; **332**:1317-1322.

104 Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 2009; **10**:307-318.

105 Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A* 1998; **95**:1432-1437.

106 Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* 2011; **13**:132-141.

107 Yuan M, Pino E, Wu L, Kacergis M, Soukas AA. Identification of Akt-independent regulation of hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2. *J Biol Chem* 2012; **287**:29579-29588.

108 Peterson RT, Desai BN, Hardwick JS, Schreiber SL. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. *Proc Natl Acad Sci U S A* 1999; **96**:4438-4442.

109 Kuo YC, Huang KY, Yang CH, Yang YS, Lee WY, Chiang CW. Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. *J Biol Chem* 2008; **283**:1882-1892.

110 Hartley D, Cooper GM. Role of mTOR in the degradation of IRS-1: regulation of PP2A activity. *J Cell Biochem* 2002; **85**:304-314.

111 Carlson CJ, White MF, Rondinone CM. Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation. *Biochem Biophys Res Commun* 2004; **316**:533-539.

112 Yan L, Mieulet V, Burgess D *et al.* PP2A T61 epsilon is an inhibitor of MAP4K3 in nutrient signaling to mTOR. *Mol Cell* 2010; **37**:633-642.

113 Yoo SJ, Jimenez RH, Sanders JA, Boylan JM, Brautigan DL, Gruppuso PA. The alpha4-containing form of protein phosphatase 2A in liver and hepatic cells. *J Cell Biochem* 2008; **105**:290-300.

114 Imajo M, Tsuchiya Y, Nishida E. Regulatory mechanisms and functions of MAP kinase signaling pathways. *IUBMB Life* 2006; **58**:312-317.

115 Hommes DW, Peppelenbosch MP, Deventer SJHv. Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. 2003.

116 McCubrey JA, Steelman LS, Chappell WH *et al.* Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta* 2007; **1773**:1263-1284.

117 Meloche S, Pouyssegur J. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene* 2007; **26**:3227-3239.

118 Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* 1993; **363**:45-51.

119 Milburn MV, Tong L, deVos AM *et al.* Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. *Science* 1990; **247**:939-945.

120 Freedman TS, Sondermann H, Friedland GD *et al.* A Ras-induced conformational switch in the Ras activator Son of sevenless. *Proc Natl Acad Sci U S A* 2006; **103**:16692-16697.

121 Wu X, Noh SJ, Zhou G, Dixon JE, Guan KL. Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells. *J Biol Chem* 1996; **271**:3265-3271.

122 Crews CM, Alessandrini A, Erikson RL. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* 1992; **258**:478-480.

123 Weber JD, Raben DM, Phillips PJ, Baldassare JJ. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem J* 1997; **326 (Pt 1)**:61-68.

124 Monje P, Hernandez-Losa J, Lyons RJ, Castellone MD, Gutkind JS. Regulation of the transcriptional activity of c-Fos by ERK. A novel role for the prolyl isomerase PIN1. *J Biol Chem* 2005; **280**:35081-35084.

125 Mathiasen DP, Egebjerg C, Andersen SH *et al.* Identification of a c-Jun N-terminal kinase-2-dependent signal amplification cascade that regulates c-Myc levels in ras transformation. *Oncogene* 2012; **31**:390-401.

126 Wu GS. Role of mitogen-activated protein kinase phosphatases (MKPs) in cancer. *Cancer Metastasis Rev* 2007; **26**:579-585.

127 Franklin CC, Kraft AS. Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. *J Biol Chem* 1997; **272**:16917-16923.

128 Franklin CC, Srikanth S, Kraft AS. Conditional expression of mitogen-activated protein kinase phosphatase-1, MKP-1, is cytoprotective against UV-induced apoptosis. *Proc Natl Acad Sci U S A* 1998; **95**:3014-3019.

129 Ugi S, Imamura T, Ricketts W, Olefsky JM. Protein phosphatase 2A forms a molecular complex with Shc and regulates Shc tyrosine phosphorylation and downstream mitogenic signaling. *Mol Cell Biol* 2002; **22**:2375-2387.

130 Letourneux C, Rocher G, Porteu F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *Embo j* 2006; **25**:727-738.

131 Lao DH, Yusoff P, Chandramouli S *et al.* Direct binding of PP2A to Sprouty2 and phosphorylation changes are a prerequisite for ERK inhibition downstream of fibroblast growth factor receptor stimulation. *J Biol Chem* 2007; **282**:9117-9126.

132 Ory S, Zhou M, Conrads TP, Veenstra TD, Morrison DK. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr Biol* 2003; **13**:1356-1364.

133 Jaumot M, Hancock JF. Protein phosphatases 1 and 2A promote Raf-1 activation by regulating 14-3-3 interactions. *Oncogene* 2001; **20**:3949-3958.

134 Zwaenepoel K, Goris J, Erneux C, Parker PJ, Janssens V. Protein phosphatase 2A PR130/B"alpha1 subunit binds to the SH2 domain-containing inositol polyphosphate 5-phosphatase 2 and prevents epidermal growth factor (EGF)-induced EGF receptor degradation sustaining EGF-mediated signaling. *Faseb j* 2010; **24**:538-547.

135 Indovina P, Marcelli E, Casini N, Rizzo V, Giordano A. Emerging roles of RB family: new defense mechanisms against tumor progression. *J Cell Physiol* 2013; **228**:525-535.

136 Harbour JW, Dean DC. Rb function in cell-cycle regulation and apoptosis. *Nature cell biology* 2000; **2**:E65-67.

137 Nevins JR. The Rb/E2F pathway and cancer. *Human molecular genetics* 2001; **10**:699-703.

138 Plesca D, Crosby ME, Gupta D, Almasan A. E2F4 function in G2: maintaining G2-arrest to prevent mitotic entry with damaged DNA. *Cell Cycle* 2007; **6**:1147-1152.

139 Dominguez-Brauer C, Brauer PM, Chen YJ, Pimkina J, Raychaudhuri P. Tumor suppression by ARF: gatekeeper and caretaker. *Cell Cycle* 2010; **9**:86-89.

140 Kolupaeva V, Janssens V. PP1 and PP2A phosphatases--cooperating partners in modulating retinoblastoma protein activation. *Febs j* 2013; **280**:627-643.

141 Rubin SM. Deciphering the retinoblastoma protein phosphorylation code. *Trends Biochem Sci* 2013; **38**:12-19.

142 Brown VD, Phillips RA, Gallie BL. Cumulative effect of phosphorylation of pRB on regulation of E2F activity. *Mol Cell Biol* 1999; **19**:3246-3256.

143 Rubin SM, Gall AL, Zheng N, Pavletich NP. Structure of the Rb C-terminal domain bound to E2F1-DP1: a mechanism for phosphorylation-induced E2F release. *Cell* 2005; **123**:1093-1106.

144 Kurimchak A, Grana X. PP2A holoenzymes negatively and positively regulate cell cycle progression by dephosphorylating pocket proteins and multiple CDK substrates. *Gene* 2012; **499**:1-7.

145 Alberts AS, Thorburn AM, Shenolikar S, Mumby MC, Feramisco JR. Regulation of cell cycle progression and nuclear affinity of the retinoblastoma protein by protein phosphatases. *Proceedings of the National Academy of Sciences of the United States of America* 1993; **90**:388-392.

146 Hirschi A, Cecchini M, Steinhardt RC, Schamber MR, Dick FA, Rubin SM. An overlapping kinase and phosphatase docking site regulates activity of the retinoblastoma protein. *Nature structural & molecular biology* 2010; **17**:1051-1057.

147 Garriga J, Jayaraman AL, Limon A *et al.* A dynamic equilibrium between CDKs and PP2A modulates phosphorylation of pRB, p107 and p130. *Cell cycle (Georgetown, Tex)* 2004; **3**:1320-1330.

148 Jayadeva G, Kurimchak A, Garriga J *et al.* B55alpha PP2A holoenzymes modulate the phosphorylation status of the retinoblastoma-related protein p107 and its activation. *The Journal of biological chemistry* 2010; **285**:29863-29873.

149 Cicchillitti L, Fasanaro P, Biglioli P, Capogrossi MC, Martelli F. Oxidative stress induces protein phosphatase 2A-dependent dephosphorylation of the pocket proteins pRb, p107, and p130. *The Journal of biological chemistry* 2003; **278**:19509-19517.

150 Kolupaeva V, Daempfling L, Basilico C. The B55alpha regulatory subunit of protein phosphatase 2A mediates fibroblast growth factor-induced p107 dephosphorylation and growth arrest in chondrocytes. *Mol Cell Biol* 2013; **33**:2865-2878.

151 Soprano KJ, Purev E, Vuocolo S, Soprano DR. Rb2/p130 and protein phosphatase 2A: key mediators of ovarian carcinoma cell growth suppression by all-trans retinoic acid. *Oncogene* 2006; **25**:5315-5325.

152 Purev E, Soprano DR, Soprano KJ. PP2A interaction with Rb2/p130 mediates translocation of Rb2/p130 into the nucleus in all-trans retinoic acid-treated ovarian carcinoma cells. *Journal of cellular physiology* 2011; **226**:1027-1034.

153 Magenta A, Fasanaro P, Romani S, Di Stefano V, Capogrossi MC, Martelli F. Protein phosphatase 2A subunit PR70 interacts with pRb and mediates its dephosphorylation. *Molecular and cellular biology* 2008; **28**:873-882.

154 Hyrien O, Rappailles A, Guilbaud G *et al.* From simple bacterial and archaeal replicons to replication N/U-domains. *J Mol Biol* 2013; **425**:4673-4689.

155 Duncker BP, Chesnokov IN, McConkey BJ. The origin recognition complex protein family. *Genome biology* 2009; **10**:214.

156 Bell SP. The origin recognition complex: from simple origins to complex functions. *Genes & development* 2002; **16**:659-672.

157 Shin JH, Grabowski B, Kasiviswanathan R, Bell SD, Kelman Z. Regulation of minichromosome maintenance helicase activity by Cdc6. *J Biol Chem* 2003; **278**:38059-38067.

158 Tanaka T, Nasmyth K. Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. *Embo j* 1998; **17**:5182-5191.

159 Sheu YJ, Stillman B. Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. *Mol Cell* 2006; **24**:101-113.

160 Jallepalli PV, Brown GW, Muzi-Falconi M, Tien D, Kelly TJ. Regulation of the replication initiator protein p65cdc18 by CDK phosphorylation. *Genes & development* 1997; **11**:2767-2779.

161 Mailand N, Diffley JF. CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. *Cell* 2005; **122**:915-926.

162 Petersen BO, Lukas J, Sorensen CS, Bartek J, Helin K. Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *The EMBO journal* 1999; **18**:396-410.

163 Paolinelli R, Mendoza-Maldonado R, Cereseto A, Giacca M. Acetylation by GCN5 regulates CDC6 phosphorylation in the S phase of the cell cycle. *Nat Struct Mol Biol* 2009; **16**:412-420.

Chapter II

The Biochemical Characterization of

PP2A and its Interaction with Cdc6

The data discussed here is published in Cell Research (Wlodarchak et. al. 2013) with the following exceptions: the data in figure 2.5 has been submitted to the New England Journal of Medicine (Kinsler et. al 2014) as part of a collaboration, and the data in figure 2.9 is not published.

The data in figure 2.1 C and 2.2 C & D was obtained in collaboration with Dr. Feng Guo. Dr. Guo also performed some replications for the data in figures 2.7 & 2.8 and assisted with calculations.

Abstract

The B"/PR72 family of protein phosphatase 2A (PP2A) is an important PP2A family involved in diverse cellular processes and uniquely regulated by calcium-binding to the regulatory subunit. The PR70 subunit in this family interacts with cell division control 6 (Cdc6), a cell cycle regulator important for control of DNA replication. This chapter focuses on the *in vitro* characterization of the PP2A PR70 holoenzyme and its interaction with Cdc6. PR70 interacts with Cdc6 around the N-terminal phosphorylation sites of Cdc6 and can facilitate dephosphorylation of residue(s) S54 and/or S74. PR70 holoenzyme assembly is facilitated by Ca^{2+}-binding and results in the most compact scaffold subunit conformation seen to date. PR70 can enhance PP2A-mediated dephosphorylation of pCdc6; in contrast, the B' family of holoenzymes have no phosphatase activity on pCdc6, likely due to steric hindrance by a B' loop near the active site. This study provides unique insights on PR70 holoenzyme assembly and its regulation of Cdc6 dephosphorylation.

Introduction

The tight control of reversible protein phosphorylation and dephosphorylation is crucial for the regulation of cellular function[1, 2]. Protein phosphatase 2A (PP2A) is a major Ser/Thr phosphatase in all eukaryotic cells that participates in many cellular processes via formation of diverse trimeric holoenzymes. Each holoenzyme is comprised of a common core enzyme formed by the scaffold (A) and catalytic (C) subunits (two isoforms each) that recruits a variable regulatory subunit derived from four different families (B/B55/PR55, B'/B56/PR61, B"/PR72, and B"'/striatin)[3, 4]. The regulatory subunits control substrate specificity and intracellular distribution of PP2A holoenzymes. Collectively, PP2A holoenzymes target a broad array of cellular phosphoproteins[3, 4]. The mechanisms PP2A uses to regulate substrate dephosphorylation are not well understood, and it is unclear how PP2A regulatory subunits restrict the access to the PP2A active site, allowing only selected substrates to be dephosphorylated.

Members of B"/PR72 family of PP2A regulatory subunits are involved in multiple cellular processes, including modulation of neuronal signaling[5], Wnt signaling[6, 7], regulation of calcium-channel phosphorylation[8, 9], and tumor suppression[10, 11]. The PR72 and PR70 members of this family have been proposed to play a role in cell cycle progression by regulating the retinoblastoma (Rb) and cell division control 6 (Cdc6) proteins, which are both crucial for the G1/S transition[12-14]. The precise control of Cdc6 levels is important for the correct timing of DNA replication and helps to ensure that only one copy of the genome is synthesized during each cell cycle [15, 16]. The N-terminal domain of Cdc6 harbors R*XX*L (D-box) and KEN (KEN box) destruction motifs, which targets Cdc6 for ubiquitination and degradation [17]. Ubiquitination is negatively regulated by phosphorylation of serine residues at 54, 74, and 106 of Cdc6 by cyclin-dependent kinases during G1[18]. Evidence suggests that the PR70 regulatory subunit binds

to Cdc6 and plays a role in controlling its cellular level [14]. How the phosphatase activity toward Cdc6 is regulated, however, remains largely unclear.

The results below describe the *in vitro* characterization of the PR70 holoenzyme-mediated dephosphorylation of Cdc6. PR70 binds to the N-terminal region of Cdc6 overlapping the destruction motifs and phosphorylation sites. PR70 holoenzyme formation requires calcium ions, and PR70 can also out-compete B' family subunits. Assembly also forces the A subunit to adopt a highly compact conformation. PR70 enhances PP2A phosphatase activity toward pCdc6; in contrast, the B' regulatory subunits markedly hinder Cdc6 dephosphorylation, likely by steric hindrance of Cdc6 entry route to the active site on the catalytic subunit. These studies provide important insight into tight control of Cdc6 dephosphorylation.

Materials and Methods

Protein Preparation. All constructs and point mutations were generated using a standard PCR-based cloning strategy. Expression and purification of PP2A Aα (9-589), Cα (1-309), and assembly of the PP2A core enzyme (Aα-Cα heterodimer) followed procedures described previously[19]. Expression and purification of CFP-Aα (9-589)-TC fusion protein and the core enzyme containing this protein were similar to Aα (9-589). Expression of Cyclin A and CDK2 followed procedures described previously[20]. Cdc6 and PR70 with different boundaries were cloned in pQlink vector (Addgene) harboring a GST-tag and a His_8-tag, respectively, and a TEV cleavage site between the affinity tag and the protein. The proteins were overexpressed at 23°C in *E. coli* strain DH5α. The soluble fraction of the *E. coli* cell lysate was purified over GS4B resin (GE Healthcare) or Ni-NTA resin (Qiagen), and further fractionated by anion exchange chromatography (Source 15Q, GE Healthcare) and gel filtration chromatography (Superdex 200, GE Healthcare). PR70 and PR70 holoenzymes were purified in the presence of 1-2mM $CaCl_2$. Free Cdc6 peptides were purified by gel filtration after TEV digestion of the GST tag.

Pull-Down Assays. Approximately 10 μg of GST-AC (core enzyme) or GST-Cdc6 was bound to 10 μl of glutathione resin via GST tag. The resin was washed with 200 μl assay buffer three times to remove the excess unbound protein. Then, 10 μg or the indicated amount of PR70 constructs, wild type PR70 (108-575 or 108-519) was added to the resin in a 200 μl volume suspended in the assay buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, and 3 mM DTT. The mixture was washed three times with the assay buffer. The proteins remained bound to resin were examined by SDS-PAGE, and visualized by Coomassie blue staining. His-PR70 pull-down assays were performed similarly, with His-PR70 being passed over Ni-NTA resin and Cdc6 peptides being passed over the bound protein. For binding of PR70 vacant of calcium, 1mM $CaCl_2$ was replaced with 0.1 mM EDTA as indicated. For determination of PR70

competition with B'γ1, a titration of PR70 (108-519) or B'γ1 (0-12.8 μM) was added to fixed 2 μM B'γ1 or PR70, and the resulting mixture was added to 2 μM GST-AC. The GST-AC-B was pulled-down with glutathione resin and were examined by SDS-PAGE, and visualized by Coomassie blue staining. The level of binding was quantified using Image J, and results were fitted in GraphPad Prism (GraphPad Software, Inc.). All experiments were repeated three times and standard error of the mean (SEM) were shown where quantified.

FRET Assay. The donor fluorescent signal of CFP of 100μg/ml of PP2A core enzyme containing CFP-Aα (9-589)-TC fusion protein was measured in the presence and absence of FlAsH-EDC_2 compound (Invitrogen) using a Victor V 1420 Multilabel HTS counter (Wallac) with excitation at 450 nm and emission at 490 nm. Addition of FlAsH-EDC_2 compound at 1:1.1 molar ratio creates the highly fluorescent TC-FLASH that serves as the acceptor in the FRET assay. The rate of energy transfer was calculated based on loss of donor fluorescence using the following equation: $E=1-(F_{DA}/F_D)$, where F_{DA} and F_D are the fluorescence of CFP in the presence and absence of TC-FLASH, respectively. The effect of regulatory subunits on the rate of energy transfer was determined similarly in the presence of five time molar concentration of B' subunits or wild type or mutant PR70 (108-575).

Phosphatase Assay. The purified GST-Cdc6 (49-90) was phosphorylated *in vitro* by Cyclin A/CDK2 (1/20 w/w) with 10 mM $MgCl_2$ and 10x molar concentration of ATP for 1 hour at 30^{o}C. The phosphorylated protein was purified by gel filtration chromatography (Superdex 200, GE Healthcare) to remove free ATP, followed by overnight cleavage of the GST-tag with TEV protease (1/20 w/w). The pCdc6 peptide was then separated from GST or uncleaved peptide using untrafiltration membrane (Millipore) with a 10 kDa cut-off. The measurement of phosphatase activity followed a procedure of a commercially available kit (Upstate) modified as

follows. The phosphatase activity of 50nM - 1μM of PP2A core enzyme or holoenzyme containing B' or PR70 (108-519 or 108-575) was measured using 60 μM pCdc6 peptide in a buffer containing 25 mM Tris pH 8.0, 150 mM NaCl, 3mM DTT, 50 μM $MnCl_2$ and 1 mM $CaCl_2$. The reaction was performed in 50μl assay volume at room temperature for 15 minutes and stopped by the addition of a malachite green solution (100μL) which binds to free phosphate and undergoes a colorimetric absorbance shift. The absorbance of the mixture was measured at 620 nm after 10 min incubation at room temperature. For steady state kinetics, the assays were performed using 0.16 μM of the indicated PP2A complexes and titration of pCdc6 peptide (10-380 μM). The data were fitted using GraphPad Prism (GraphPad Software, Inc.) to calculate K_m and K_{cat}.

The phosphatase activity of PP2A samples (0.4-20 nM) toward a universal phosphopeptide substrate (K-R-pT-I-R-R) was measured similarly as previous described [21]. For steady state kinetics, the assays were performed using 0.4 nM of the indicated PP2A complexes and titration of pThr peptide (75 μM-1.2 mM). All experiments were performed in triplicate and repeated three times. Mean ± SEM were calculated.

Results

PR70 binds to Cdc6 near the N-terminal phosphorylation sites and destruction motifs. Phosphorylation sites on the N-terminus of Cdc6 (S54 and S74) have been implicated in protection from degradation, and PP2A was shown to mediate this degradation[22, 23]. GST pull-down assays were employed to probe the PR70 binding region on Cdc6. Full-length Cdc6 (1-550) was not able to be purified, but a region from 18-550 was soluble and was used for pull-down assays. Previous reports indicated Cdc6 bound to PR70 from 442-575[22], and PR70 constructs were chosen for pull-down with various C-terminal truncations, with PR72 used as a negative control. PR70 108-575 bound Cdc6 18-550 (not shown) as did PR70 108-519 and 108-490 (Figure 2.1A). Neither construct bound well to Cdc6 140-550, indicating that the binding region was in the unstructured N-terminal region (Figure 2.1B). The binding region was then narrowed to a region encompassing both S54 and S74 (Cdc6 49-90) (Figure 2.1C). The GST tag may cause some steric interference when assaying smaller pieces of Cdc6, so a His-tagged PR70 was used to pull-down Cdc6 peptides. This assay revealed that Cdc6 70-90 is necessary and sufficient for binding to PR70 (Figure 2.1D).

PR70 interacts weakly with Cdc6. Cdc6 and PR70 did not co-migrate over anion exchange chromatography, but did over size exclusion chromatography. On a size exclusion column, the Cdc6 70-90 fragment alone eluted around 20 mL; in the presence of PR70, it co-eluted with PR70 in the peak near 15 mL (Figure 2.2A&B). GST-70-90 did not interact with the PP2A-PR70 holoenzyme, likely due to steric interference by the N-terminal GST-tag. Additional Cdc6 sequence following the GST-tag allowed GST-Cdc6 49-90 to interact with AC-PR70 108-519, and the two co-migrated on a size exclusion column (Figure 2.2C&D). These data further support the conclusion that PP2A-PR70 interacts with Cdc6 near its N-terminal

phosphorylation sites. The interaction is weak, however, since it survives size exclusion chromatography but not anion exchange chromatography.

PP2A holoenzyme assembly is regulated by diverse mechanisms. It is still unclear how holoenzyme assembly is coordinated. One proposed mechanism is through the methylation of the C-terminal tail of PP2Ac, and some B subunits such as Bα require this methylation for assembly[24, 25]. To test this hypothesis, PR70 holoenzymes were prepared with methylated and unmethylated PP2Ac. The level of holoenzyme assembly exhibited no difference between methylated and unmethylated PP2Ac *in vitro* (Figure 2.3). One unique feature of the PR72 family of B subunits is the presence of two EF hands in each subunit. Previous studies show that mutations in these regions that disrupt Ca^{2+} binding do not allow PR70 holoenzyme formation[22]. PR70 (108-575) treated with EDTA to chelate Ca^{2+} exhibited the same results, even at twice the molar amount of PR70 relative to AC (Figure 2.4A). Assembly of the B'γ1 holoenzyme was not affected by EDTA and bound to AC in the presence of PR70 without Ca^{2+}, but bound less when Ca^{2+} is present (Figure 2.4B). Furthermore, PR70 appeared to have higher affinity than B'γ1 for AC and fully out-competed B'γ1 for holoenzyme assembly at 6x the molar ratio of PR70:AC (Figure 2.5A). B'γ1 barely displaced PR70 across the same concentration range (Figure 2.5B).

The PR70 holoenzyme has a more compact conformation than B' holoenzymes or AC alone. The known structures involving PP2A holoenzymes or AC dimer all show differences in A-subunit conformation[19, 26, 27]. A fluorescence resonance energy transfer (FRET) assay was developed as a tool to measure the conformation of the A subunit when assembled into different holoenzymes. The A subunit exists as a horseshoe shape forming 15 HEAT (Huntingtin-elongation- A subunit-TOR) repeats with considerable flexibility. To examine the conformation of the A-subunit in different holoenzymes, cyan fluorescent protein (CFP) was

fused to the N-terminus of A and a tetracysteine (TC) peptide was fused to its C-terminus. CFP served as a fluorescence acceptor, and the TC peptide bound to a fluorescein derivative, the FlAsH compound, resulting in a fluorophore that acted as a FRET acceptor (Figure 2.6A). Purified AC dimer with this FRET pair was assayed with various B subunits to measure FRET efficiency by donor loss. All proteins were normalized and added in a 5x molar excess to ensure full saturation of the fluorescent AC dimer (Figure 2.6C). Free B subunits did not have any effect on fluorescence for this assay. The assay revealed that the PR70 holoenzyme had a significant increase in FRET as compared to B'γ1, B'ε, and AC alone (Figure 2.6B). This result indicated that the A-subunit is in a tighter conformation in the PR70 holoenzyme than that of free AC or holoenzymes comprised of B' family regulatory subunits.

PR70 can dephosphorylate Cdc6 49-90 *in vitro*. Cdc6 49-90 contains two known CDK phosphorylation sites: S54 and S74. *In vitro* phosphorylation of the PR70 binding motif of Cdc6 (70-90) by CDK2 and subsequent dephosphorylation by PP2A-PR70 revealed low levels of dephosphorylation; therefore, Cdc6 49-90 was used for all subsequent *in vitro* dephosphorylation assays and prepared as described in the methods. The steady-state kinetics for Cdc6 dephosphorylation by PP2A-PR70 were determined using the phosphatase assay described in the methods. Little difference was seen between AC and two PR70 holoenzymes on dephosphorylation of a small universal pT substrate [KR(pT)IRR] (Figure 2.7A). The PR70 holoenzymes exhibited enhanced dephosphorylation of pCdc6 as compared to AC (Figure 2.7B). Although the K_{cat} and K_m values were slightly different between the two truncations of PR70 used in the assay, their K_{cat}/K_m ratio were identical (0.12 $\mu M^{-1}min^{-1}$) and over twice the value of AC alone (0.05 $\mu M^{-1}min^{-1}$). These results indicated that PR70 enhances PP2A-mediated dephosphorylation of Cdc6.

B' holoenzymes cannot dephosphorylate Cdc6. B subunits are thought to regulate substrate specificity. B'γ1 and B'ε holoenzymes were tested for their ability to dephosphorylate pCdc6 49-90. While PR70 enhanced pCdc6 dephosphorylation relative to AC, similar to that shown in Figure 2.7, the B'γ1 and B'ε holoenzymes were hardly able to dephosphorylate pCdc6, even at high enzyme concentration (Figure 2.8A). The same holoenzymes were used to dephosphorylate the artificial pT peptide as a control, and none had significantly different activity (Figure 2.8B). An examination of the B'γ1 holoenzyme structure (2NPP) revealed an extended loop (~99-110) near the PP2A active site (Figure 2.9A). To test whether this loop restricted the access of PP2A substrates to the exclusion of Cdc6, a B'γ1 construct with residues 99-110 removed (Δ99-110) was created and used for the *in vitro* dephosphorylation assay. A holoenzyme containing B'γ1 Δ99-110 showed a restoration of non-specific activity similar to that of AC, indicating that this loop prevents dephosphorylation of pCdc6 by B'γ1 holoenzymes (Figure 2.9B).

Discussion

PP2A is intimately involved in human disease, in large part due to its regulation of the cell cycle. Despite this critical importance and increased study, much remains to be known about the interaction of PP2A with its substrates. It is known that the PR70-PP2A holoenzyme is responsible for Cdc6 dephosphorylation and overexpression leads to increased Cdc6 degradation[22]. These results provide detailed biochemical characterization of PP2A with Cdc6. The smallest PR70-binding region of Cdc6 is from residues 70-90; however, for most of the experiments, 49-90 was used since it contains both phospho-sites and binds PR70 similar to full-length protein (Figure 2.1 & 2.2). This region also overlaps with the CDK2-binding region (71-77), but not the cyclin A-binding region (89-100)[28], suggesting that a competitive mechanism may be involved in controlling the phosphorylation status of Cdc6. Preliminary data indicated that pCdc6 49-90 was more efficiently dephosphorylated by PP2A than pCdc6 70-90 (data not shown); however, differences in *in vitro* phosphorylation by CDK2 cannot be ruled out. In addition, preliminary phosphatase assays with S54A, S54D, S74A, and S74D pCdc6 mutants indicated that more phosphate is released from S74A and S74D than S54A and S54D, suggesting that S54 may be the target site of PP2A, but more experiments are needed to confirm this result.

Little is known about what regulates holoenzyme assembly and if holoenzymes change during the cell cycle. As discussed in chapter I, PP2A holoenzymes are involved in many diverse processes regulating the cell cycle, some positively and most negatively. Holoenzymes must be tightly regulated to ensure these processes proceed in the proper fashion. One possible mechanism discovered here is through the competition of B subunits for PP2A-AC. PR70 outcompetes B'γ1 for holoenzyme assembly *in vitro* (Figure 2.5), but in the absence of Ca^{2+}, PR70 can hardly bind, giving B'γ1 a clear advantage (Figure 2.4). Ca^{2+} levels rise during the cell

cycle, and there are numerous spikes at various points where Ca^{2+} is needed[29]. Changes in Ca^{2+} levels may be one avenue by which holoenzyme assembly is regulated, temporally regulating holoenzyme formation and substrate dephosphorylation.

It has been widely accepted in the PP2A field that the B subunits control substrate specificity[30, 31]. Although many studies on PP2A have demonstrated binding preferences between B subunits, very few have characterized the phosphatase activity on those substrates. PP2A-PR70 holoenzyme enhances the dephosphorylation of pCdc6 49-90 relative to AC alone, and holoenzymes containing the B'γ1 and B'ε subunits cannot dephosphorylate pCdc6 at all (Figure 2.7 & 2.8). This finding appears to be the first quantified evidence that a specific holoenzyme enhances the dephosphorylation of one of its substrates, and that holoenzymes from another family cannot dephosphorylate the substrate at all. The structure of the B'γ1 holoenzyme reveals an extended loop that may be the mechanism for substrate restriction, and phosphatase assays with the loop removed support this idea (Figure 2.9). Other structural features may be involved in this recognition, and those will be discussed in the next chapter.

The PP2A-PR70 holoenzyme is only beginning to be studied despite its critical importance in the regulation of cell cycle proteins. This study mapped the PR70-binding region on Cdc6, revealed a tight interaction of PR70 in the holoenzyme and the higher binding affinity than other B' subunits, and showed specific phosphatase activity on pCdc6 by PP2A-PR70. The evidence presented suggests that the activity of PP2A on its substrates may be due to competition between various B subunits, or the specific structural architecture of each holoenzyme. Chapter III will present further insights based on the structure of the PP2A-PR70 holoenzyme.

Figures

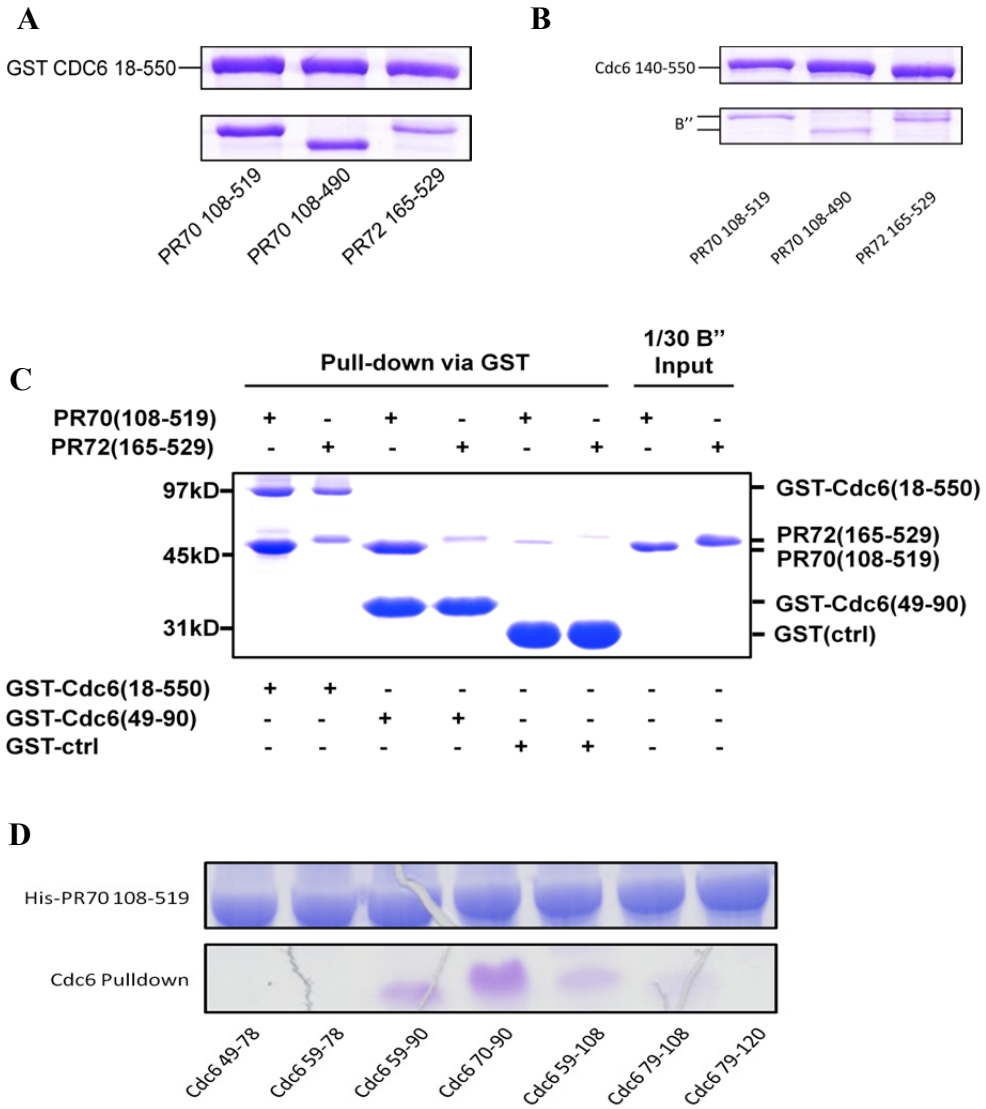


Figure 2.1. PR70 directly binds to the N-terminus of Cdc6. For A-C, various GST-tagged Cdc6 fragments were used to pull-down various PR70 truncations. **A**. The PR70 core domain (108-490) binds to Cdc6 (18-550). **B**. The PR70 core domain binds weakly to Cdc6 140-550. **C**. PR70 binds near known N-terminal phosphorylation sites (49-90). PR72 does not associate in this region and only weakly associates with FL Cdc6. **D**. PR70 binds Cdc6 70-90. Peptides of Cdc6 were produced as GST-tagged proteins and cleaved before being pulled-down by His-tagged PR70 108-519.

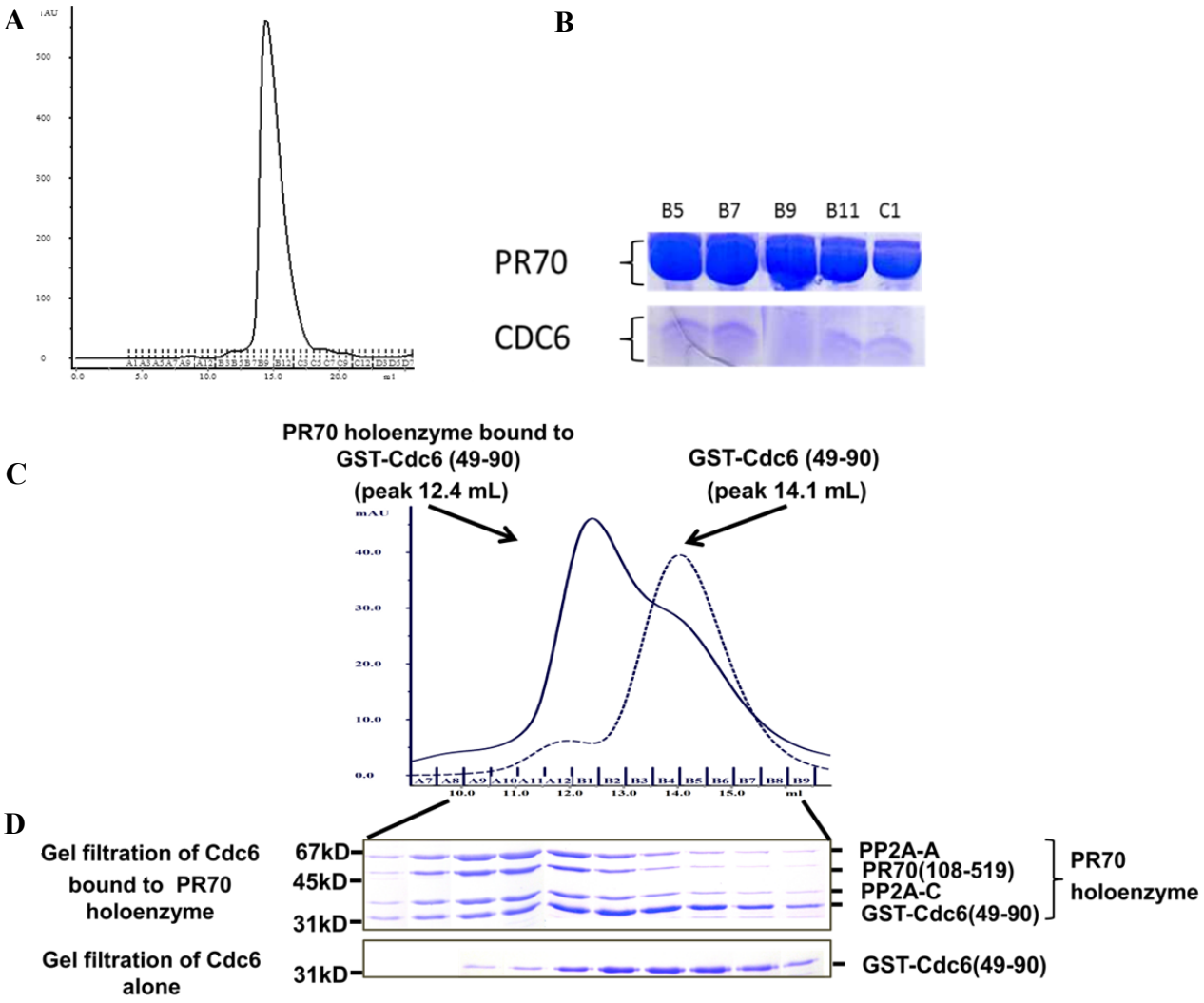


Figure 2.2. Cdc6 co-migrates with PR70 on size exclusion chromatography. **A.** PR70 108-519 was mixed with Cdc6 70-90 in a 1:1 molar ratio and submitted to size exclusion chromatography. The resulting chromatogram indicates the position of PR70 and bound Cdc6 70-90. **B**. SDS-PAGE shows the Cdc6 peptide eluted in the same fractions as PR70. No free Cdc6 was detected in later fractions as would be expected based on its size. **C**. GST-Cdc6 49-90 was submitted to size exclusion chromatography alone or mixed with PR70 holoenzyme. The peak for GST-Cdc6 with PR70 holoenzyme is shifted forward 1.7 mL more than compared to the position of Cdc6 position alone. **D**. SDS-PAGE fractions of peaks. Cdc6 travels with PR70 holoenzyme. Assays in Panel C and D were performed in collaboration with Dr. Feng Guo.

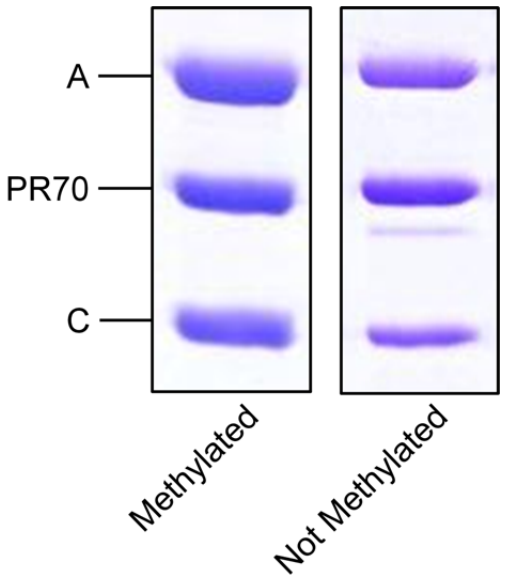


Figure 2.3. PR70 can form holoenzymes *in vitro* with both methylated C subunit and unmethylated C subunit. PR70 holoenzyme was assembled as described in the methods. Unmethylated or methylated PP2Ac did not affect assembly and purification of the holoenzyme. SDS-PAGE gels of final assembled and purified holoenzyme are shown.

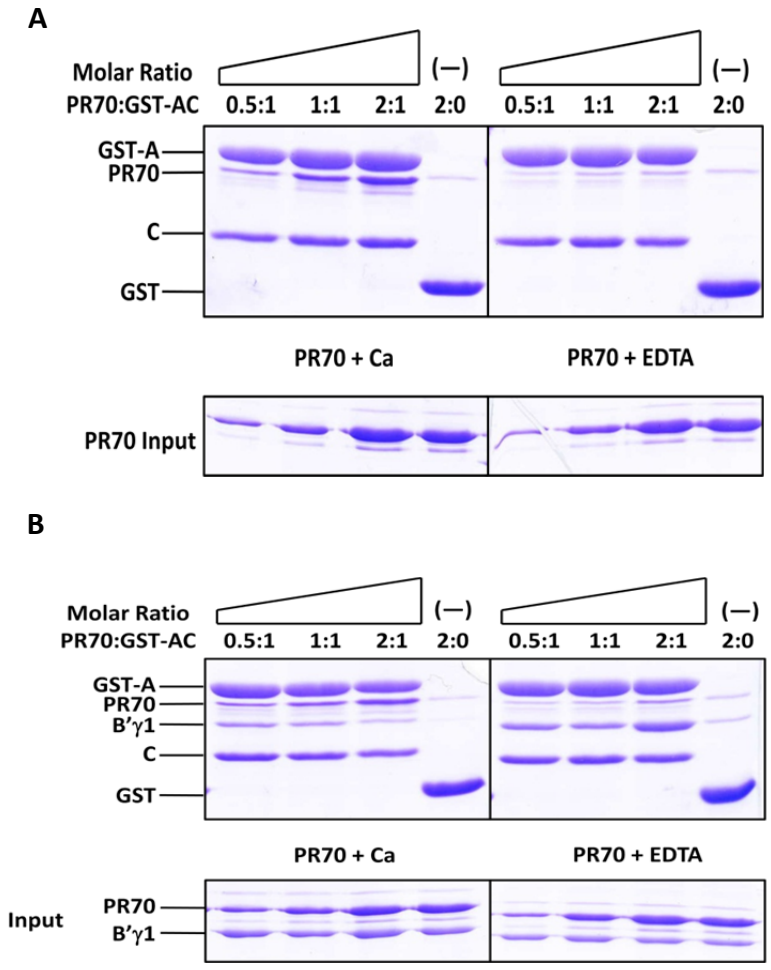


Figure 2.4. PR70 requires Ca^{2+} to bind to PP2A-AC. **A**. GST-AC was used to pull-down PR70 (108-575) with 1mM Ca^{2+} or PR70 treated with EDTA to remove Ca^{2+}. PR70 with Ca^{2+} binds stoichiometrically to AC, but PR70 does not bind AC at all when Ca^{2+} is chelated by EDTA. **B**. The same experiment as panel A was performed with the addition of an equivalent molar ratio of B'γ1. PR70 competes with B'γ1 to bind AC but cannot compete without Ca^{2+}. B'γ1 binding to AC is not affected by loss of Ca^{2+}.

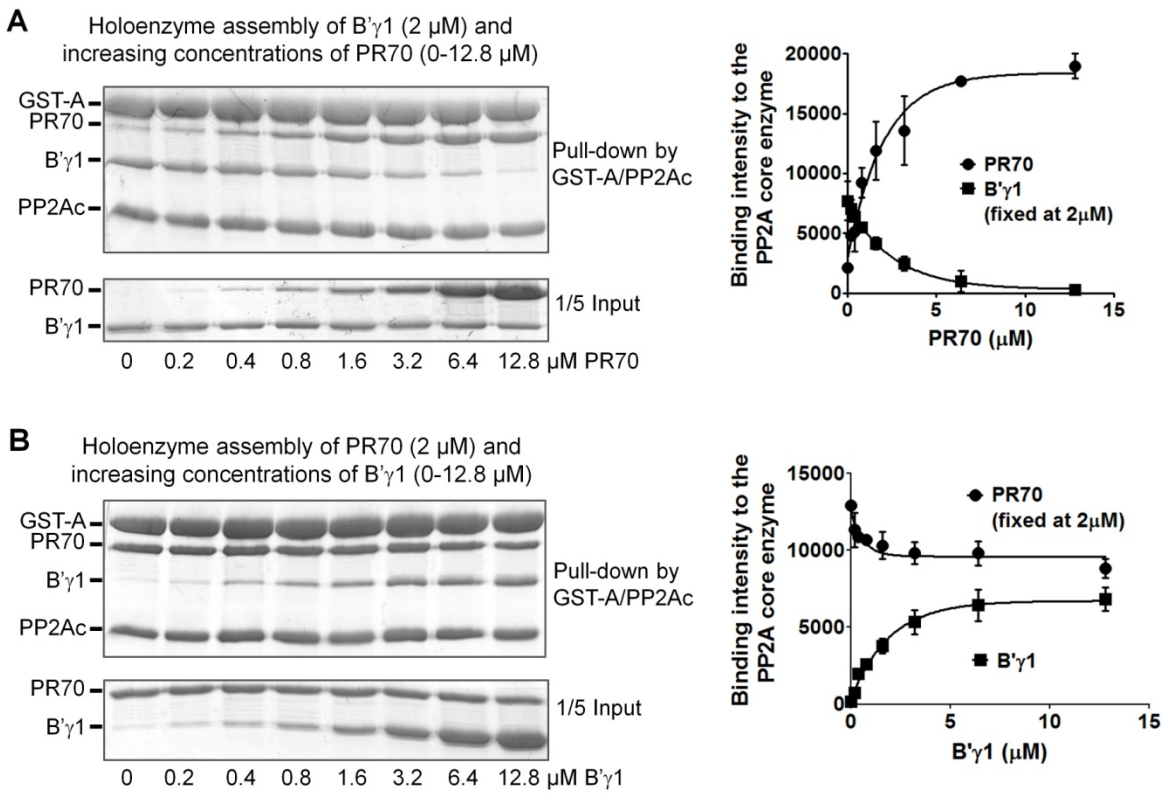


Figure 2.5. PR70 can out-compete B'γ1 for holoenzyme assembly. **A**. GST-AC was used to pull down a mixture of B'γ1 at fixed concentration (2 μM) and various concentrations of PR70 indicated below the gel. Increasing concentrations of PR70 can quickly out-compete fixed B'γ1 for AC binding. **B**. The experiment in A was performed with a fixed concentration of PR70 (2 μM) and various concentrations of B'γ1 indicated below the gel. Increasing B'γ1 cannot out-compete PR70 for holoenzyme binding. Three replicates of the assays in A and B were quantified with ImageJ and graphed with Prism software with the standard errors of the mean indicated. Detailed experimental conditions can be found in the methods section.

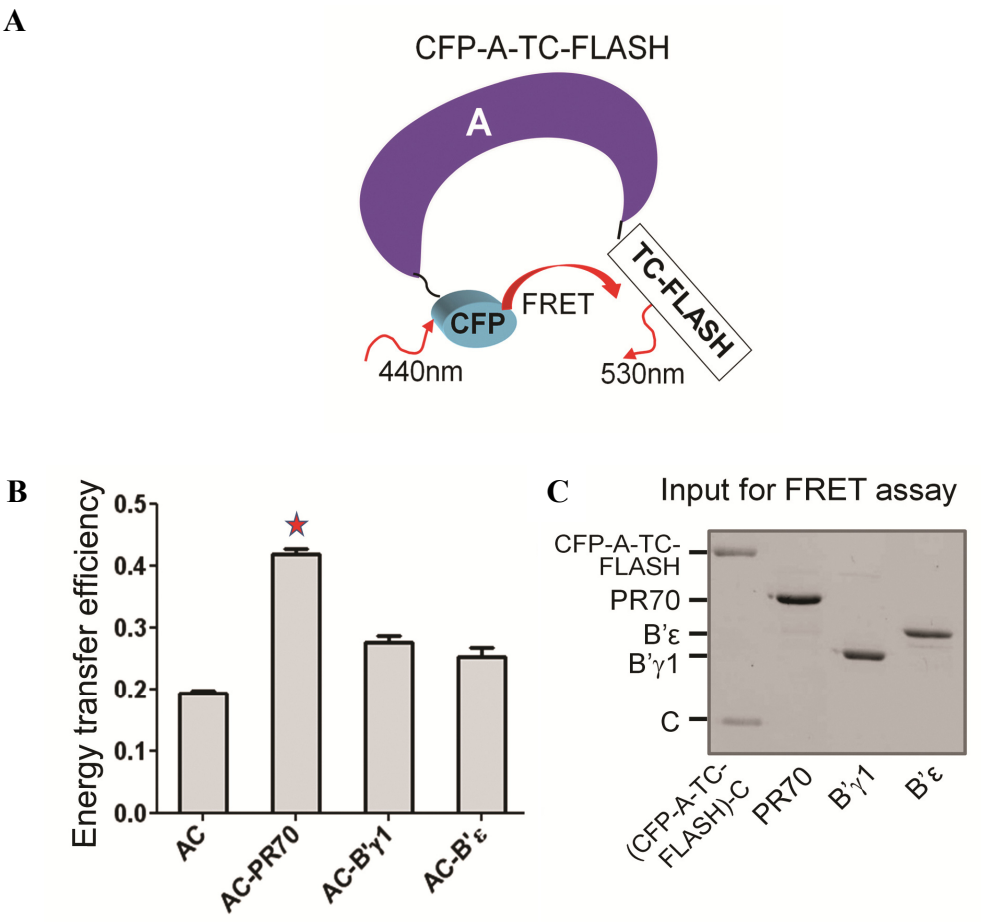
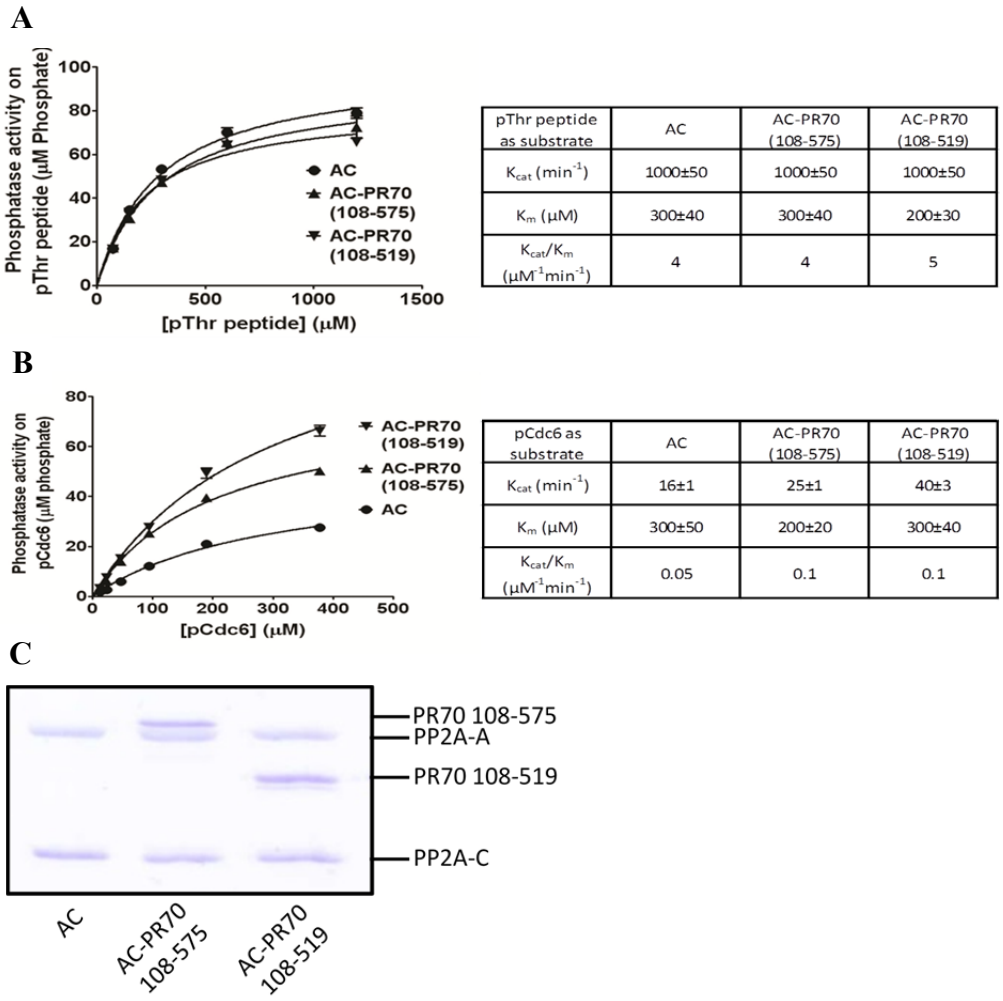


Figure 2.6. The PR70 holoenzyme forms the most compact holoenzyme structure known to date as measured by scaffold subunit flexibility. **A**. Diagram of FRET assay. The scaffold (A) subunit is highly flexible and can accommodate diverse B subunits. This flexibility can be measured by placing fluorophores on the N and C-terminus of the A subunit. CFP and TC-FlAsH were used as donor and acceptor respectively. **B**. (CFP-A-TC)C was mixed with 5 fold excess B subunits to ensure saturation, and FRET efficiency was measured and calculated as described in the methods. The assay indicated a marked increase in efficiency, as measured by donor loss, for the PR70 holoenzyme relative to B' holoenzymes or AC, indicating a more closed A conformation. **C**. Input for the FRET assay. Protein was normalized using the Bradford assay, then visualized on SDS-PAGE. Experiments were done in triplicate.



pThr peptide as substrate	AC	AC-PR70 (108-575)	AC-PR70 (108-519)
K_{cat} (min^{-1})	1000±50	1000±50	1000±50
K_m (μM)	300±40	300±40	200±30
K_{cat}/K_m ($\mu M^{-1}min^{-1}$)	4	4	5

pCdc6 as substrate	AC	AC-PR70 (108-575)	AC-PR70 (108-519)
K_{cat} (min^{-1})	16±1	25±1	40±3
K_m (μM)	300±50	200±20	300±40
K_{cat}/K_m ($\mu M^{-1}min^{-1}$)	0.05	0.1	0.1

Figure 2.7. PR70 enhances PP2A substrate specificity. **A**. The phosphatase activity of AC and PR70 holoenzyme on a small universal substrate was measured and steady-state kinetics were calculated as described in the methods. AC and PR70 holoenzyme have similar kinetics toward the universal pT peptide substrate. **B**. The assay described in A was performed with a peptide of pCdc6 49-90 (details in the methods section). PR70 enhances the dephosphorylation of pCdc6 relative to AC alone. Holoenzymes assembled with two different PR70 truncations have similar steady-state kinetics. **C**. The proteins for the assays in **A** and **B** were normalized by the Bradford assay then visualized on SDS-PAGE. All experiments were done in triplicate and mean values with error are summarized in the tables to the right. The phosphatase assay were performed in collaboration with Dr. Feng Guo.

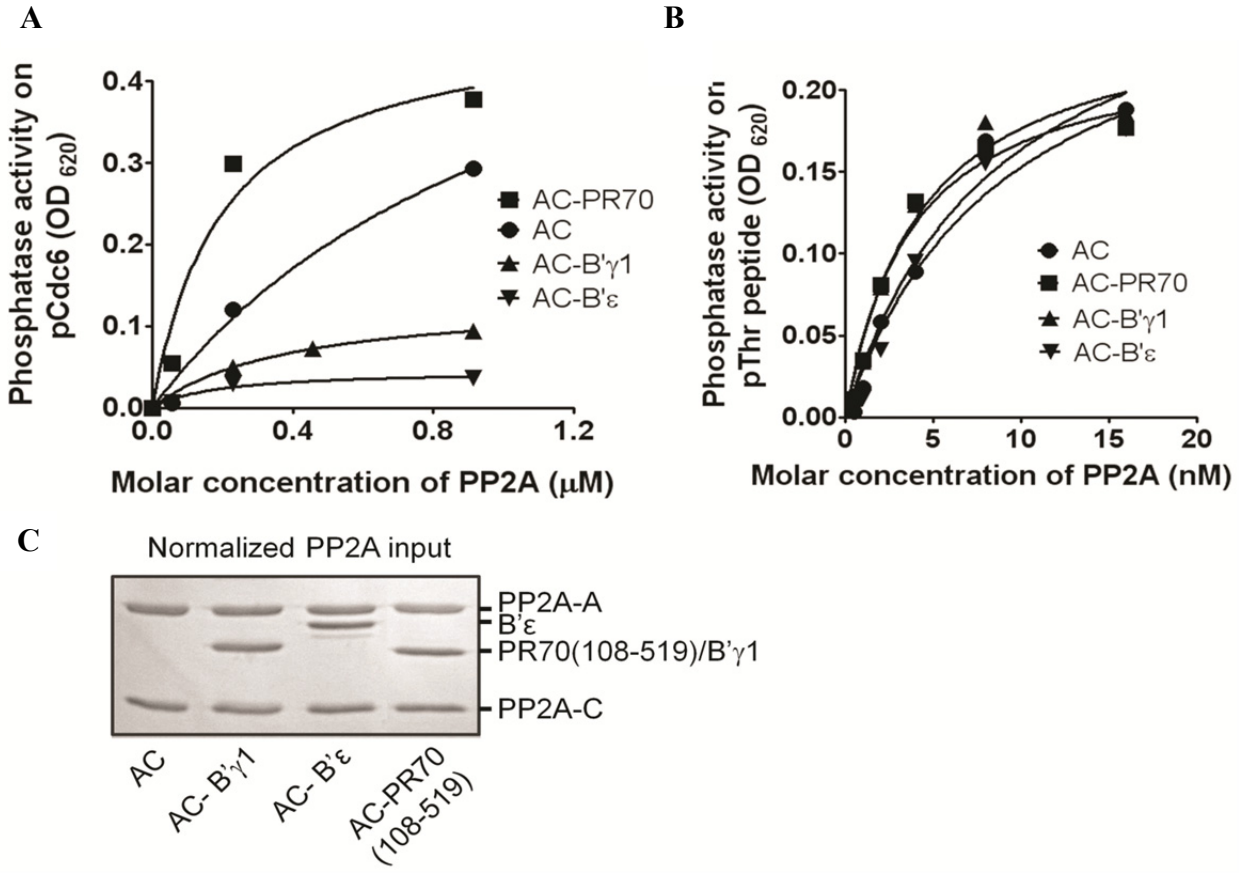


Figure 2.8. PR70 enhances Cdc6 dephosphorylation and B' family holoenzymes cannot dephosphorylate Cdc6. **A**. The phosphatase activity of AC and holoenzymes containing PR70 or B' subunits on pCdc6 49-90 was measured at various concentrations as described in the methods. PR70 can enhance phosphatase activity on pCdc6 relative to AC alone, whereas the B'γ1 and B'ε holoenzymes cannot dephosphorylate Cdc6 even at μM concentrations. **B**. A similar phosphatase assay against a universal pT substrate indicates the same holoenzymes do not show a difference in the dephosphorylation of this universal peptide. **C**. Input for assays in **A** and **B**. Protein was normalized using the Bradford assay, then visualized by SDS-PAGE. Experiments were done in triplicate. The phosphatase assay was performed in collaboration with Dr. Feng Guo.

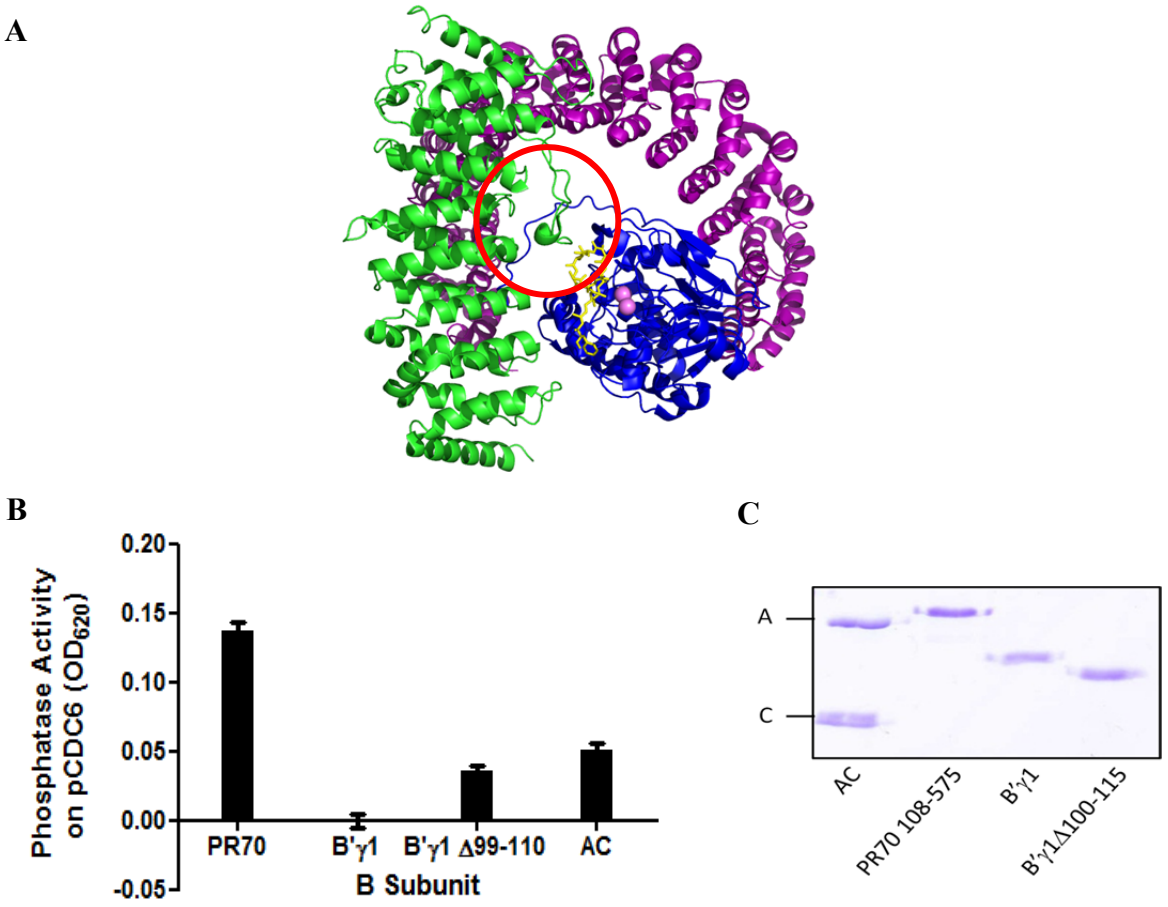


Figure 2.9. A loop near the active site in the B'γ1 holoenzyme prevents dephosphorylation of Cdc6 by the B'γ1 holoenzyme. **A**. The structure of the B'γ1 holoenzyme (2NPP) reveals a loop (99-110, circled in red) near the C subunit active site. A subunit is shown in purple, B subunit is shown in green, and C subunit is shown in blue. Active site metals are indicated in violet, and MCLR is shown in yellow sticks. **B**. A B'γ1 subunit with residues 99-110 removed was used in a phosphatase assay as described in the methods. Removal of this loop allowed the B'γ1 holoenzyme to non-specifically dephosphorylate pCdc6. The restoration of activity is comparable to the non-specific activity of AC alone. Phosphatase activity is indicated relative to basal activity of the B'γ1 holoenzyme. **C**. Input for phosphatase assay. Protein was normalized using the Bradford assay, then visualized by SDS-PAGE. Experiments were done in triplicate.

References

1 Hunter T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 1995; **80**:225-236.

2 Wurzenberger C, Gerlich DW. Phosphatases: providing safe passage through mitotic exit. *Nat Rev Mol Cell Biol* 2011; **12**:469-482.

3 Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2001; **353**:417-439.

4 Virshup DM. Protein phosphatase 2A: a panoply of enzymes. *Curr Opin Cell Biol* 2000; **12**:180-185.

5 Ahn JH, Sung JY, McAvoy T *et al.* The B"/PR72 subunit mediates Ca2+-dependent dephosphorylation of DARPP-32 by protein phosphatase 2A. *Proc Natl Acad Sci U S A* 2007; **104**:9876-9881.

6 Creyghton MP, Roel G, Eichhorn PJ, Vredeveld LC, Destree O, Bernards R. PR130 is a modulator of the Wnt-signaling cascade that counters repression of the antagonist Naked cuticle. *Proc Natl Acad Sci U S A* 2006; **103**:5397-5402.

7 Creyghton MP, Roel G, Eichhorn PJ *et al.* PR72, a novel regulator of Wnt signaling required for Naked cuticle function. *Genes Dev* 2005; **19**:376-386.

8 Hall DD, Feekes JA, Arachchige Don AS *et al.* Binding of protein phosphatase 2A to the L-type calcium channel Cav1.2 next to Ser1928, its main PKA site, is critical for Ser1928 dephosphorylation. *Biochemistry* 2006; **45**:3448-3459.

9 Davare MA, Horne MC, Hell JW. Protein phosphatase 2A is associated with class C L-type calcium channels (Cav1.2) and antagonizes channel phosphorylation by cAMP-dependent protein kinase. *J Biol Chem* 2000; **275**:39710-39717.

10 Sablina AA, Hector M, Colpaert N, Hahn WC. Identification of PP2A complexes and pathways involved in cell transformation. *Cancer Res* 2010; **70**:10474-10484.

11 Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochimica et biophysica acta* 2009; **1795**:1-15.

12 Magenta A, Fasanaro P, Romani S, Di Stefano V, Capogrossi MC, Martelli F. Protein phosphatase 2A subunit PR70 interacts with pRb and mediates its dephosphorylation. *Mol Cell Biol* 2008; **28**:873-882.

13 Yan Z, Fedorov SA, Mumby MC, Williams RS. PR48, a novel regulatory subunit of protein phosphatase 2A, interacts with Cdc6 and modulates DNA replication in human cells. *Mol Cell Biol* 2000; **20**:1021-1029.

14 Davis AJ, Yan Z, Martinez B, Mumby MC. Protein phosphatase 2A is targeted to cell division control protein 6 by a calcium-binding regulatory subunit. *J Biol Chem* 2008; **283**:16104-16114.

15 Bell SP, Dutta A. DNA replication in eukaryotic cells. *Annu Rev Biochem* 2002; **71**:333-374.

16 Duncker BP, Chesnokov IN, McConkey BJ. The origin recognition complex protein family. *Genome Biol* 2009; **10**:214.

17 Harper JW, Burton JL, Solomon MJ. The anaphase-promoting complex: it's not just for mitosis any more. *Genes Dev* 2002; **16**:2179-2206.

18 Mailand N, Diffley JF. CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. *Cell* 2005; **122**:915-926.

19 Xing Y, Xu Y, Chen Y *et al.* Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell* 2006; **127**:341-353.

20 Pevarello P, Brasca MG, Amici R *et al.* 3-Aminopyrazole inhibitors of CDK2/cyclin A as antitumor agents. 1. Lead finding. *J Med Chem* 2004; **47**:3367-3380.

21 Chao Y, Xing Y, Chen Y *et al.* Structure and mechanism of the phosphotyrosyl phosphatase activator. *Mol Cell* 2006; **23**:535-546.

22 Davis AJ, Yan Z, Martinez B, Mumby MC. Protein phosphatase 2A is targeted to cell division control protein 6 by a calcium-binding regulatory subunit. *The Journal of biological chemistry* 2008; **283**:16104-16114.

23 Mailand N, Diffley JF. CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. *Cell* 2005; **122**:915-926.

24 Yu XX, Du X, Moreno CS *et al.* Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen. *Mol Biol Cell* 2001; **12**:185-199.

25 Longin S, Zwaenepoel K, Louis JV, Dilworth S, Goris J, Janssens V. Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic Subunit. *J Biol Chem* 2007; **282**:26971-26980.

26 Xu Y, Xing Y, Chen Y *et al.* Structure of the protein phosphatase 2A holoenzyme. *Cell* 2006; **127**:1239-1251.

27 Xu Y, Chen Y, Zhang P, Jeffrey PD, Shi Y. Structure of a protein phosphatase 2A holoenzyme: insights into B55-mediated Tau dephosphorylation. *Molecular cell* 2008; **31**:873-885.

28 Cheng KY, Noble ME, Skamnaki V *et al.* The role of the phospho-CDK2/cyclin A recruitment site in substrate recognition. *The Journal of biological chemistry* 2006; **281**:23167-23179.

29 Kahl CR, Means AR. Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. *Endocr Rev* 2003; **24**:719-736.

30 Virshup DM, Shenolikar S. From promiscuity to precision: protein phosphatases get a makeover. *Molecular cell* 2009; **33**:537-545.

31 Stanevich V, Jiang L, Satyshur KA *et al.* The structural basis for tight control of PP2A methylation and function by LCMT-1. *Molecular cell* 2011; **41**:331-342.

Chapter III

The Structure of the PP2A-PR70 Holoenzyme

The data discussed here is published in Cell Research (Wlodarchak et. al. 2013)

The data in figure 3.10 A-C was obtained and analyzed in collaboration with Dr. Feng Guo. The structure of the PR72 regulatory subunit was determined by Dr. Yongna Xing in collaboration with Dr. Philip Jeffery and Dr. Yigong Shi.

Abstract

The B"/PR72 family of protein phosphatase 2A (PP2A) is an important PP2A family involved in diverse cellular processes and uniquely regulated by calcium binding to the regulatory subunit. The PR70 subunit in this family interacts with cell division control 6 (Cdc6), a cell cycle regulator important for control of DNA replication. Here I report the crystal structure of the trimeric PR70 holoenzyme at a resolution of 2.4 Å, and *in vitro* characterization of the PP2A-PR70 holoenzyme. The holoenzyme structure reveals that one of the PR70 calcium-binding motifs directly contacts the scaffold subunit which, together with two other contacts at separate locations, leads to the most compact scaffold subunit conformation among all PP2A holoenzymes. PR70 also binds distinctively to the catalytic subunit near the active site, which is required for PR70 to enhance phosphatase activity toward Cdc6. These studies provide a structural basis for unique regulation of B"/PR72 holoenzymes by calcium ions, and suggest mechanisms for precise control of substrate specificity among PP2A holoenzymes.

Introduction

As discussed in Chapter II, control of reversible protein phosphorylation is critical for regulating cellular function, and PP2A is one of the phosphatases involved in many of these processes[1, 2, 3, 4]. The three-dimensional structures of two PP2A holoenzymes have been determined[5-7], but no structure has been reported for B"'/striatin family of PP2A holoenzymes. The structures of the B"/PR72 family of regulatory subunits and holoenzymes were only recently reported[8], in part derived from the work described here. Furthermore, regulation of the dephosphorylation of cellular proteins remains poorly understood, as it is unclear how PP2A regulatory subunits restrict the access to the PP2A active site, allowing only selected substrates to be dephosphorylated. Several lines of evidence suggest that the PR70 regulatory subunit binds to Cdc6 and plays a role in controlling its cellular level[9]. How the phosphatase activity toward Cdc6 is regulated, however, remains largely unclear.

The A-subunit of PP2A contains 15 HEAT (huntingtin-elongation- A-subunit -TOR) repeats[10, 11]. The catalytic subunit specifically recognizes HEAT repeats 11–15[12] and regulatory subunits from the B/PR55 and B'/PR61 families interact with the N-terminal HEAT repeats[6, 7, 13]. The B"/PR72 family has no sequence similarity to B/PR55 or B'/PR61 regulatory subunits. All members of the B"/PR72 family have two conserved EF hand calcium-binding motifs. Calcium binding was shown to enhance interaction of these subunits with the PP2A core enzyme and affect the phosphatase activity[14-16]. However, the lack of structural information for this family of regulatory subunits and holoenzymes has prevented mechanistic understanding of PP2A regulation by B"/PR72 regulatory subunits.

Here I report the crystal structure of the trimeric PP2A holoenzyme containing PR70 in comparison with that of the isolated PR72. There is a distinct positioning of two PR70 EF-hand

calcium-binding motifs in the holoenzyme. The first EF-hand (EF1) is located on the top surface facing the phosphatase active site, and the second EF-hand (EF2) directly contacts the scaffold subunit. This architecture provides a possible explanation for the role of calcium-binding in holoenzyme assembly and substrate recognition. PR70 contacts the catalytic subunit near the phosphatase active site distinctly different from other families of regulatory subunits. These studies fill an important gap in knowledge on the structural basis of the B"/PR70 holoenzymes and adds structural insight into PP2A-PR70 substrate recognition.

Materials and Methods

Protein Preparation. All constructs and point mutations were generated using a standard PCR-based cloning strategy. Expression and purification of PP2A Aα (9-589), Cα (1-309), and PR72 subunits, and assembly of the PP2A core enzyme (Aα-Cα heterodimer) followed procedures described previously[12]. Expression and purification of CFP-Aα (9-589)-TC fusion protein and the core enzyme containing this protein were similar to Aα (9-589). Cdc6 and PR70 with different boundaries were cloned in pQlink vector (Addgene) harboring a GST-tag and a His_8-tag, respectively, and a TEV cleavage site between the affinity tag and the protein. The proteins were overexpressed at 23°C in *E. coli* strain DH5α. The soluble fraction of the *E. coli* cell lysate was purified over GS4B resin (GE Healthcare) or Ni-NTA resin (Qiagen), and further fractionated by anion exchange chromatography (Source 15Q, GE Healthcare) and gel filtration chromatography (Superdex 200, GE Healthcare). PR70, PR72 and their holoenzymes were purified in the presence of 1-2mM $CaCl_2$.

For crystallization of the PR70 holoenzyme, the PP2A core enzyme was fully methylated by PP2A specific methyltransferase (LCMT-1) in the presence of PTPA (PP2A phosphatase activator) at 1:1.5:0.2 molar ratio[17], followed by association with PR70 (122-490) fused to the Cdc6 peptide (70-90) via a flexible linker (STGNASDSSSDSSSSEGDGTV). The sample was fractionated by anion exchange and gel filtration chromatography to remove LCMT-1, PTPA, and the excess amount of PR70. The purified PR70 holoenzyme was mixed with MCLR in a 1:1.2 molar ratio prior to crystallization.

Crystallization and Data Collection. Crystals of the holoenzyme containing the PR70-Cdc6 fusion protein bound to MCLR were grown at 18°C by the sitting-drop vapor-diffusion method by mixing ~6 mg/ml of the protein complex with two thirds volume of a reservoir

solution containing 7% PEG3350 (v/v), 0.03 M succinic acid at pH4.75. The crystals appear in three days and grew to full within two weeks. Crystals of the holoenzyme with the seleno-methionine-substituted scaffold and PR70 subunits were grown similarly except that the reservoir solution contains 12% PEG3350 (v/v), 0.1 M sodium malonate pH5.0. Crystals were equilibrated in a cryoprotectant buffer and flash frozen as described above. Native and anomalous datasets for the PR70 holoenzyme were collected at APS LS-CAT sector 21 using beamline F and D, respectively, and processed using the software HKL2000[18].

Structure Determination. The structure of the holoenzyme containing PR70 were determined by MAD and SAD phasing, using the program CRANK[19] in the CCP4 package[20]. Selenium atoms were located by program AFRO/CRUNCH2[21] and refined using BP3[22]. Following phase improvement and density modification, a large fraction of the scaffold and PR70 subunits were automatically built using Buccaneer[23]. Model errors were corrected manually based on the electron density map followed by manual building of the rest of the model. The structures were built using Coot[24] and refined using REFMAC restraints with TLS[25]. The structures of the seleno-methionine-labeled and native PR70 holoenzymes were refined to 2.8 Å and 2.4 Å, respectively (Table 3.1).

GST-Mediated Pull-Down Assay. Approximately 10 μg of GST-AC (core enzyme) or GST-Cdc6 was bound to 10 μl of glutathione resin via GST tag. The resin was washed with 200 μl assay buffer three times to remove the excess unbound protein. Then, 10 μg or the indicated amount of PR70 constructs, wild type or mutant PR70 (108-575) was added to the resin in a 200 μl volume suspended in the assay buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, and 3 mM DTT. The mixture was washed three times with the assay buffer. The proteins remained bound to resin were examined by SDS-PAGE, and visualized by Coomassie blue

staining. All experiments were repeated three times. For determination of the binding affinity, titration of PR70 (108-575) wild type and mutants (1/32-4 μM) were added to the immobilized GST-AC for pull-down. The immobilized GST was used as control. The level of binding was quantified using Image J, and results from three separate experiments were fitted in GraphPad Prism (GraphPad Software, Inc.) after background subtraction to estimate Kd.

FRET Assay. The FRET assay was performed as described in Chapter II. For this assay, wild type and mutant PR70 subunits were used in place of B subunits from different families. The effect of regulatory subunits on the rate of energy transfer was determined similarly in the presence of five time molar concentration of wild type or mutant PR70 (108-575).

Phosphatase Assay. Phosphorylated Cdc6 was prepared as described in Chapter II. The phosphatase activity of PP2A core enzyme or holoenzyme containing wild type or mutant PR70 (108-575) was measured using 60 μM pCdc6 peptide in a buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, 3mM DTT, 50 μM $MnCl_2$ and 1 mM $CaCl_2$. The reaction was performed as described in Chapter II. The phosphatase activity of PP2A samples (0.4-20 nM) toward a universal phosphopeptide substrate (K-R-pT-I-R-R) was measured similarly as previous described[26]. All experiments were performed in triplicate and repeated three times. Mean ± SEM were calculated.

Results

Crystallization of the trimeric PR70 holoenzyme. The recombinant full-length PR72 and PR70 subunits eluded crystallization after extensive effort. An elastase-resistant core of the PR72 subunit (residues 165-443) was defined, and this protein was readily crystallized by previous efforts in our laboratory.

PP2A holoenzymes assembled with various PR72/PR70 fragments failed to produce diffracting crystals. PR70 was previously shown to interact with the N-terminal fragment of Cdc6[9, 27]; therefore, the PR70 holoenzyme was assembled using PR70 fused to Cdc6 fragments, which facilitated crystallization of this holoenzyme (Figure 3.2). Diffracting crystals were obtained for the PR70 holoenzyme bound to microcystin-LR (MCLR) containing PR70 (122-490) fused to Cdc6 (70-90) via a flexible linker (STGNASDSSSDSSSSEGDGTV).

Structural determination by molecular replacement using the structure of PR72 and the structural models of the PP2A core enzyme from other holoenzymes (PDB codes: 2NPP (B'γ1 holoenzyme), 3DW8 (B55α holoenzyme)) was not successful, indicating there are significant structural differences between the PR70 holoenzyme and holoenzymes containing other regulatory subunits. The structure was eventually determined by Selenium SAD (single-wavelength anomalous dispersion) phasing, and refined to 2.4 Å with the native dataset (Table 3.1). The electron density map identified a majority of the residues in all three PP2A subunits (residues 9-589 of the A-subunit, residues 2-296 of PP2Ac, and residues 122-478 of the PR70 subunit).

Overall structure of PR70 holoenzyme. The structure of the PR70 holoenzyme measures 110 Å in width, 100 Å in height, and 60 Å in thickness (Figure 3.2). The interhelical loops of the A-subunit serve as the docking sites for the regulatory and catalytic subunits, as seen

in holoenzymes containing the Bα and B'γ1 families of PP2A regulatory subunits[6, 7, 13]. In the holoenzyme containing PR70, the catalytic subunit associates with the C-terminal five HEAT repeats, and PR70 binds to HEAT repeats 1-7 at the N-terminus of the A-subunit (Figure 3.2). The PR70 binding region overlaps with the binding sites of the Bα and B'γ1 regulatory subunits, indicating that binding is mutually exclusive.

The PR70 subunit is an elongated, mostly α-helical protein harboring an N-terminal hydrophobic motif and two EF hand calcium-binding motifs (Figure 3.2). The N-terminal hydrophobic motif (125-129) and a broad surface in the protein core encompassing EF2 directly contacts the A-subunit (Figure 3.2 & 3.5). In addition to an A-B interface, a helix (439-446) of PR70 interacts directly with the catalytic subunit near the active site (Figure 3.2 & 3.5).

Comparison between PR70 and PR72. Sequence alignment of various B'' family subunits from different species indicated that the N and C-terminal sequences are quite different, but the core structure is conserved across the family and species. In particular, the hydrophobic motif, EF hands, and contact residues in the C-subunit binding helix are highly conserved (Figure 3.3A). An overlay of the structures of free PR72 and PR70 from the holoenzyme revealed that the core structure is very similar and only small conformational changes are seen in the core upon holoenzyme assembly (Figure 3.3B). The EF hands are also positioned similarly in both structures, suggesting they are important in holoenzyme assembly.

Comparison between holoenzymes from three different families. The PP2A-PR70 holoenzyme was the first to be crystallized in its family. The B'γ1 and Bα holoenzymes have been crystallized previously, and reveal distinct differences between families of regulatory subunits. In all of the holoenzymes, the C-subunit interacts with the C-terminal HEAT repeats of the A-subunit and induces a conformational change bringing it in closer proximity to the B

subunits (Figure 3.4). The B subunits all interact with the N-terminal HEAT repeats of the A-subunit. The Bα subunit consists of a 7 bladed β-propeller with a hairpin that extends down across the face of the N-terminal HEAT repeats of the scaffold subunit. The B'γ1 subunit is an elongated HEAT repeat protein that interacts with the top ridge of the N-terminal HEAT repeats of the A-subunit. PR70 consists of a linear arrangement of domains running diagonally across the top of the N-terminal HEAT repeats of the A-subunit, with a hydrophobic domain running along the face of the N-terminal HEAT repeats (Figure 3.4A). PR70, Bα, and B'γ1 interact with the C-subunit at different extents (Figure 3.4A & B), likely in part contributing to the differences in the conformation of the A-subunit in these holoenzymes (Figure 3.4C). Alignment of the scaffold subunits derived from the holoenzyme structures revealed the PR70 holoenzyme A-subunit is considerably more compact than that in the Bα and B'γ1 holoenzymes or AC alone (Figure 3.4C). This structural observation corroborated the biochemical evidence from the FRET assay (Figure 2.6). The holoenzymes also have distinct charge differences in their putative substrate binding regions. The Bα holoenzyme has a long acidic groove facing the active site, as does the B'γ1 holoenzyme (Figure 3.4B). The PR70 holoenzyme has alternating patches of positively and negatively charged residues near the active site, which matched the pattern seen in the binding region of its substrate, Cdc6 (KALPL**S**PRKRLGDDNLCNTPHLPPC**S**PPKQGKKENGPPHSHT).

Interfaces of PR70 with the scaffold and catalytic C-subunits. The PR70 subunit interacts with the A-subunit at two distinct interfaces (Figure 3.5A). At the first interface (AB1) the N-terminal hydrophobic (FYF) motif interacts along the side of HEAT repeat 1 of the A-subunit, with F128 binding in a hydrophobic pocket in the A-subunit (Figure 3.5B). The second interface (AB2) harbors extensive contacts between the PR70 protein core and the top ridge of

HEAT repeats 2-7 that are enriched in hydrogen bond, salt bridge, and stacking interactions (Figure 3.5A). Two acidic residues in HEAT repeats 2 and 3, D61 and E100, make hydrogen bond and salt bridge contacts to R288 and R398 of PR70 (Figure 3.5D). Two basic residues in repeats 5 and 7, R183 and R258, make hydrogen bond and salt bridge interactions to D403 in PR70 (Figure 3.5E). π-π stacking interactions were found in the middle of the interface, formed between W140 in HEAT repeat 4 and PR70 residues, W277, R362, and R398 (Figure 3.5E). D403 also participates in chelation of Ca^{2+} ions by EF2 of PR70. This architecture places the EF2 calcium-binding motif in direct contact with the A-subunit of PP2A, and supports the observation that PR70 will not bind to AC in the absence of Ca^{2+} (Figure 2.4). The interfaces between the PR70 subunit and the A-subunit bury a surface area of 3,200 $Å^2$.

PR70 interacts with the catalytic C-subunit near the phosphatase active site with a direct contact with the β12-β13 loop that hangs over the active site (Figure 3.5C). A PR70 helix (residues 439-446) is nestled into a shallow groove of the C-subunit, formed by Y91, Y267 (on the β12-β13 loop), R294, and R295. These residues make extensive hydrogen bond and salt bridge interactions with PR70 residues C439, Q440, D443, and K446 (Figure 3.5C). The interface between the PR70 subunit and the C-subunit buries a surface area of 1,300 $Å^2$.

Validation of residues required for PR70 holoenzyme assembly. In order to validate the structural observations and determine which interactions are biologically relevant, point mutations were made that would potentially disrupt protein binding. GST-AC pull-down of PR70 mutants revealed that mutations which disrupted hydrophobic interactions in the AB1 interface (F128A) and mutations that disrupt salt bridge interactions in the AB2 interface (R288D & R398D) all reduced PR70-AC binding (Figure 3.6). Mutations in the BC interface (D443K) appeared to have a minimal effect on binding, where double mutants including two

interfaces (F128A & R288D, F128A & D443K, and R288D & D443K) completely prevented binding. All of the mutants tested showed no difference in Cdc6 binding (Figure 3.7).

To determine whether the mutations at the inter-subunit interfaces have any effect on holoenzyme conformation, the FRET assay established in Chapter II was used to probe the differences in the conformation of the A-subunit in holoenzymes containing WT and mutant PR70 subunits. It was expected that the mutants with reduced binding affinity would give a reduced FRET efficiency reflecting a mixture of free AC (wider conformation) and bound PR70 holoenzyme (tighter conformation) (Figure 3.8). The mutants which have significantly reduced binding (F128A and R398D) were found to have correspondingly wider average conformations. D443K had somewhat reduced binding, but more drastic reduction in FRET efficiency, indicating that the weakened contacts with the C-subunit have an effect on the holoenzyme conformation (Figure 3.8).

The PR70 can enhance dephosphorylation of pCdc6 (Figure 2.7 & 2.8), therefore, mutant PR70 subunits were assayed on their ability to retain their phosphatase activity enhancement toward pCdc6. The double mutants (F128A & R288D, F128A & D443K, and R288D & D443K) and R398D did not enhance pCdc6 dephosphorylation at all relative to AC alone (Figure 3.9). F128A, despite having a reduction in binding comparable to R398D, did not impact the phosphatase activity as severely as did R398D or the double mutants. In contrast to mutations on the AB interfaces, D443K had minimal impacts on holoenzyme assembly but completely abolished enhanced PR70 holoenzyme dephosphorylation toward pCdc6 (Figure 3.9).

To further probe the differences in binding and activity between WT, F128A, and D443K, an *in vitro* pull-down assay was used employing various concentrations of PR70 mutant or WT, and the K_d was calculated by quantifying the bands on SDS-PAGE with ImageJ and

using nonlinear regression in GraphPad Prism to calculate the K_d (Figure 3.10C). The assay revealed that F128A reduced PR70-AC binding by 75% but only had a phosphatase activity reduction of 40%, whereas D443K reduced PR70-AC binding by only 50% and completely negated any enhanced phosphatase activity (Figure 3.10A & B). The WT and D443K PR70 subunits had comparable activity towards a synthetic pT peptide, indicating that only specific enhanced phosphatase activity was lost (Figure 3.10D). This result suggests that the BC interface is not strictly required for holoenzyme assembly, but perturbation of this interface greatly reduced the efficiency of PP2A-PR70 holoenzyme dephosphorylation of pCdc6.

Discussion

Despite recent advances in PP2A structural biology, many questions remain about the mechanisms underlying PP2A holoenzyme assembly, substrate specificity, and regulation. Since the diversity of PP2A holoenzyme functions are dictated by its distinctly different regulatory subunits, a complete understanding of this crucial regulatory enzyme requires structural information about each holoenzyme family. In this chapter, I described the crystallization and structure determination of the PR70-PP2A holoenzyme and discussed the differences between known holoenzyme structures and how B'' family holoenzymes are bound together.

The core of the bound PR70 and free PR72 subunits are highly similar, and most of the required residues for holoenzyme assembly are found in this region. This finding suggests that the binding of the B'' family of holoenzymes likely occurs by similar mechanisms and that the PR70 holoenzyme structure is representative of the B'' family. The structure revealed a highly compact scaffold subunit conformation, which corroborates the FRET data presented in chapter II. The structure also exhibited a large amount of buried surface interfaces with numerous residues participating in salt bridge interactions, hydrogen bonding, and stacking interactions. This tight multiple interface interaction provides evidence for the compaction of the A-subunit as well as the high binding affinity of PR70 over B'γ1 (Figure 2.5). The structure also showed the two EF hands suggested by the primary sequence. EF1 is located on the top of the PR70 subunit, whereas EF2 is located deep within the AB2 binding interface. This result supports the known importance of calcium for activity toward pCdc6[14] and PP2A-PR70 holoenzyme assembly (Figure 2.4).

The structure also revealed the importance of the interaction between a helix (439-446) on PR70 and the C-subunit in correctly positioning pCdc6 for dephosphorylation. A mutation in

this helix that disrupted interaction at this interface (D443K) completely abolished the enhanced phosphatase activity on pCdc6 despite having modest effect on holoenzyme assembly. This helix is at the beginning of the known PR70-Cdc6 binding region (440-575)[13,14], and data presented here narrowed that down to 446-519, as the helix is buried and the 519 truncation has similar binding and activity as FL (Figures 2.1 & 2.7). It should be noted, however, that the N-terminal portion of the B'' subunits, and particularly EF1, may still have some role in substrate dephosphorylation, as EF1 of PR72 has been implicated in enhancing the dephosphorylation of DARPP32[6].

Despite being necessary for crystallization, the fused Cdc6 fragment (70-90) was not visible in the structure. Since the interaction between PR70 and Cdc6 is weak, it may have been required to initiate crystallization but was displaced by crystal packing. Similarly, crystal formation may have cleaved the Cdc6 chain from PR70. The pH of the crystallization buffer was significantly below physiological pH which also may have contributed to dissociation or destruction of the Cdc6 peptide. Some surface side chains sustained light radiation damage; however, this fragment may have been present in the crystal initially but was more severely damaged by radiation leading to a loss in electron density for the chain. The holoenzyme does not crystallize at room temperature without the addition of MCLR, and this active site inhibitor may be responsible for displacing the peptide. Crystals do form at 4^{o}C without MCLR; however, they do not diffract X-rays, indicating that there is some instability around the active site even with the added peptide. Further experiments will be needed to test whether a co-crystal structure of Cdc6 and PP2A-PR70 can be obtained.

The differences in the PP2A holoenzyme structures provide insight as to the regulation of substrate dephosphorylation. Steric hindrance by different B subunits, such as B'γ1, may be

responsible for the observed lack of phosphatase activity toward pCdc6 (Figures 2.8, 2.9, & 3.4). In addition to steric hindrance, previous studies have suggested that PP2A B' holoenzymes regulate substrate specificity by charge-charge interactions[28]. Observations of the electrostatics near the putative substrate binding sites on the three families of holoenzymes show the Bα and B'γ1 holoenzymes have a fairly acidic groove and would favor more positively charged substrates, whereas the PR70 holoenzyme has alternating patches of positively and negatively charged regions. Although the electron density was not present for the fused 70-90 fragment of Cdc6, the residues in the Cdc6-binding region have alternating charges which would match the pattern of charges on the putative PR70 substrate-binding region (Figure 3.4). In addition, the closely related phosphatase, PP1, has been shown to regulate substrate binding and specificity by electrostatic interactions[29, 30]. Collectively, these observations suggest a mechanism for the regulation of substrate specificity by both electrostatic interactions and steric hindrance.

Observations of the structure of the PP2A-PR70 holoenzyme gave important insights as to the regulation of holoenzyme assembly and the regulation of substrate specificity. The enhanced binding of PR70 to PP2A-AC, due to extensive buried interface area, likely explains the increased competition between B' subunits, and the position of the EF2 hand in the AB interface provides a mechanism by which Ca^{2+} can regulate B'' holoenzyme assembly. Analysis of the steric interferences between different holoenzymes as well as their different electrostatic potentials suggests mechanisms by which substrate specificity is regulated.

The concept of substrate restriction by regulatory subunits is an emerging concept in the control of cellular signalling by PP2A[25,31]. Many PP2A holoenzymes have been implicated in the regulation of the cell cycle and, taken together, the observations in this study support the idea

that PP2A activity is highly regulated throughout the cell cycle by diverse mechanisms involving competition, steric hindrance, and specific electrostatic interactions.

Figures and Tables

Table 3.1. Crystallographic data collection, phasing, and refinement for PP2A holoenzyme containing PR70.

Data Collection and Phasing		
Crystal	PR70 Holoenzyme	PR70 Holoenzyme
Data set	SeMet SAD	Native
Space group	$P2_12_12_1$	$P2_12_12_1$
Wavelength (Å)	0.9792	0.9792
Resolution (Å)	49-2.8	50-2.4
Unique observations	119018	123017
Redundancy	7.1 (7.4)	7.0 (6.7)
R-symm[1]	0.157 (0.94)	0.068 (.592)
Completeness (%)	99.8 (100)	98.4 (89.7)
Phasing		
Figure of merit	0.68	
Refinement		
Resolution (Å)	49-2.8	50-2.4
No. reflections (free)	79333 (4113)	116785 (6193)
Completeness (%)	99.9	98.6
R-factor (%)	18.3	17.9
R-free (%)	24.9	22.8
Number of atoms (total)	19933	20419
Protein	19662	19886
Water	174	533
RMSD bond lengths (Å)	0.009	0.008
RMSD bond angles (^{o})	1.4	1.3
Average B-factors (Å^2)		
With TLS contribution	54.5	27.6 (42.1)
Ramachandran plot:		
Preferred regions (%)	94.8	97.0
Allowed regions (%)	4.4	2.5
Outliers (%)	0.9	0.6

X-ray diffraction data were collected on one crystal. Values in parentheses are for highest-resolution shell.

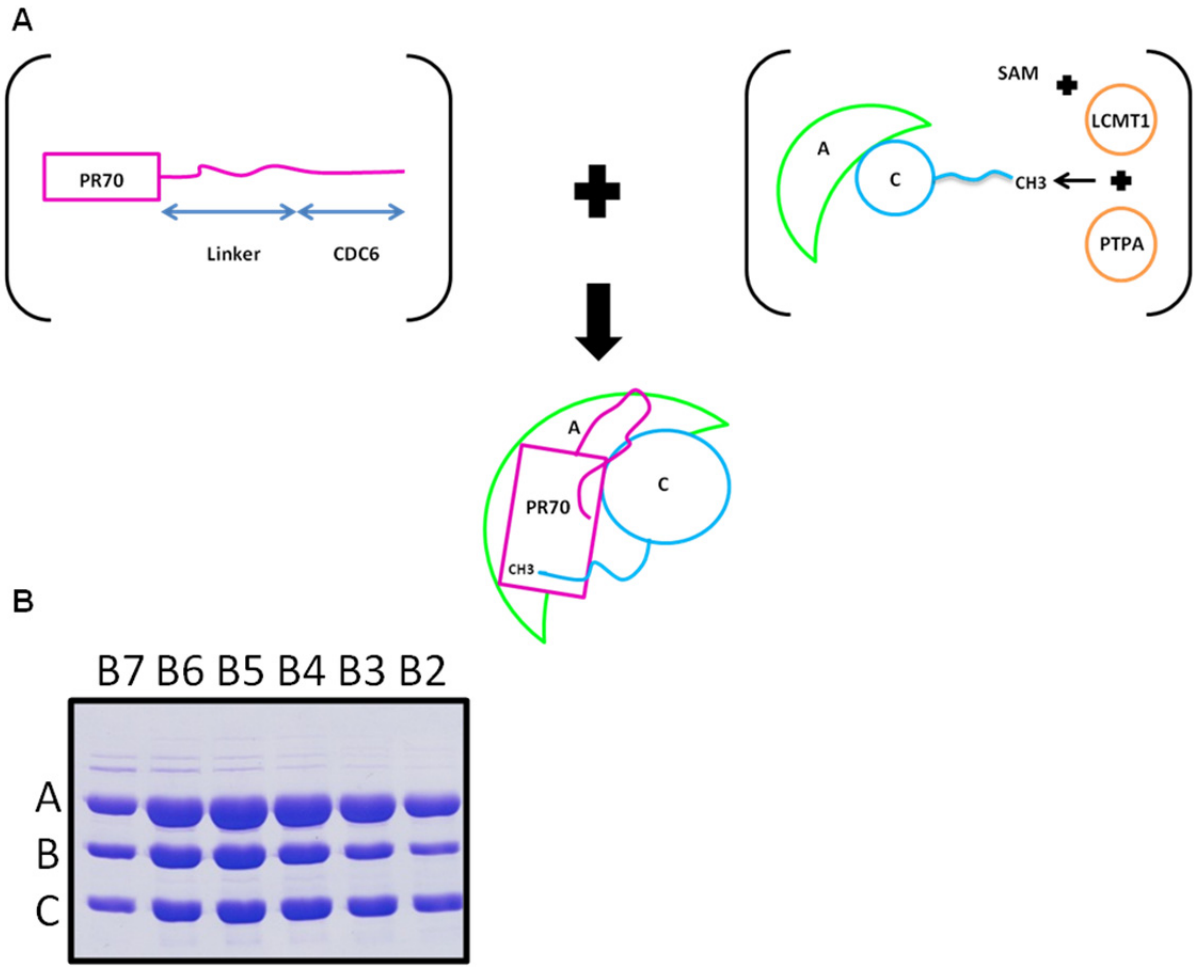


Figure 3.1. Preparation of PP2A-PR70 holoenzyme for crystallization. **A**. A PR70 holoenzyme prepared for crystallization as described in the methods. Briefly, a fusion protein was constructed consisting of the PR70 core (122-490) fused to Cdc6 70-90 with a flexible linker derived from JMJD6 (22 AAs). AC dimer was methylated with LCMT-1 and PTPA and mixed with the PR70 fusion protein. The resulting holoenzyme was purified and used for crystallization. **B**. An SDS-PAGE gel of the final holoenzyme after purification by size exclusion chromatography. Fraction numbers are given at the top of the gel and PP2A subunits are indicated on the left.

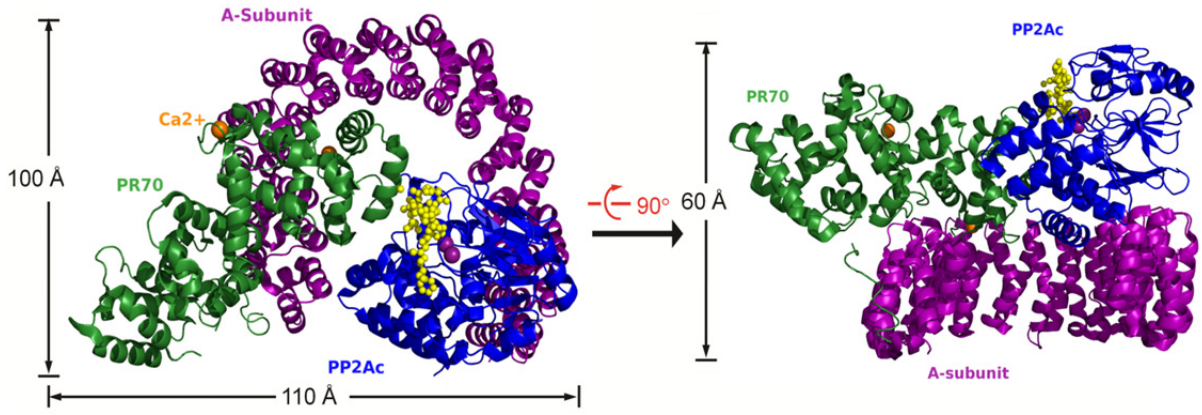


Figure 3.2. Overview of PP2A-PR70 holoenzyme structure. Two views are shown in cartoon ribbon and dimensions indicated in angstroms. The A-subunit, PR70, and C-subunit are shown in purple, forest, and blue, respectively. The inhibitor, MCLR, is shown in yellow sphere. Catalytic metal ions are shown in violet, and calcium ions are shown in orange. The holoenzyme measures 110Å wide, 60Å tall, and 100Å deep. The A and C subunits have a structure similar to other holoenzyme structures (2NPP and 3DW8). PR70 is an elongated multi-domain protein which binds to the N-terminus of the A subunit and interacts with the C subunit near the active site.

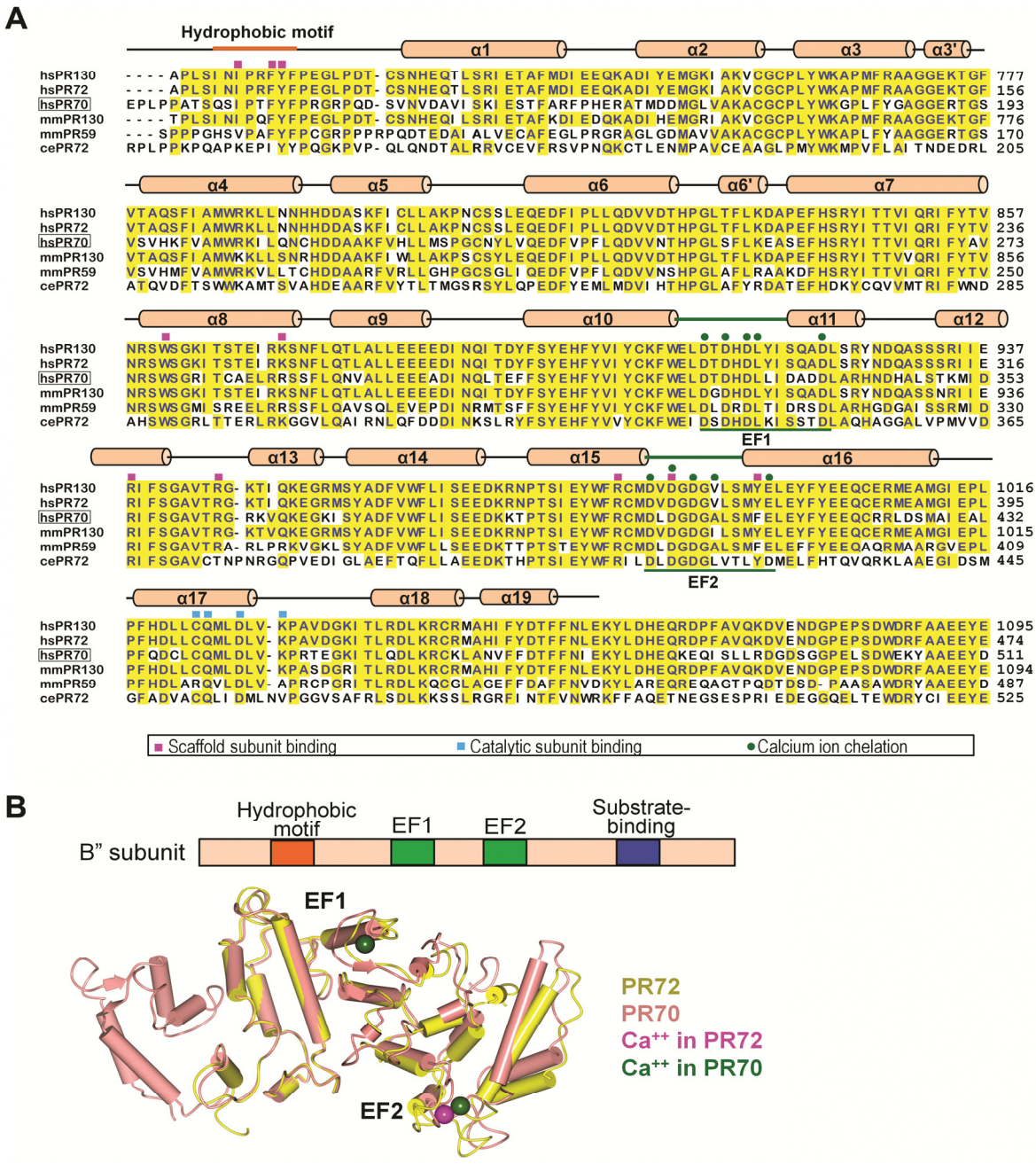


Figure 3.3. Alignment of PR70 and PR72. **A**. Primary sequence alignment of PR70 and PR72 from human (hs), mouse (mm), and C. elegans (ce). Secondary elements are indicated above the sequence. Conserved residues are highlighted in yellow and PR70 holoenzyme contacts are indicated by squares. Calcium binding residues are indicated by circles. The alignment shows the core region is highly conserved within the B'' family. The hydrophobic domain, EF hands,

and other residues involved in holoenzyme assembly are also highly conserved. **B**. Structural alignment of PR70 (orange) and PR72 (yellow) subunits. The conformation of the PR70 subunit as assembled in the holoenzyme shows only minor differences with the core structure of free PR72 (4I5J) and similar positioning of the EF hands. Taken together, this data indicate that the B'' family of holoenzymes likely bind to AC via similar mechanisms of interaction.

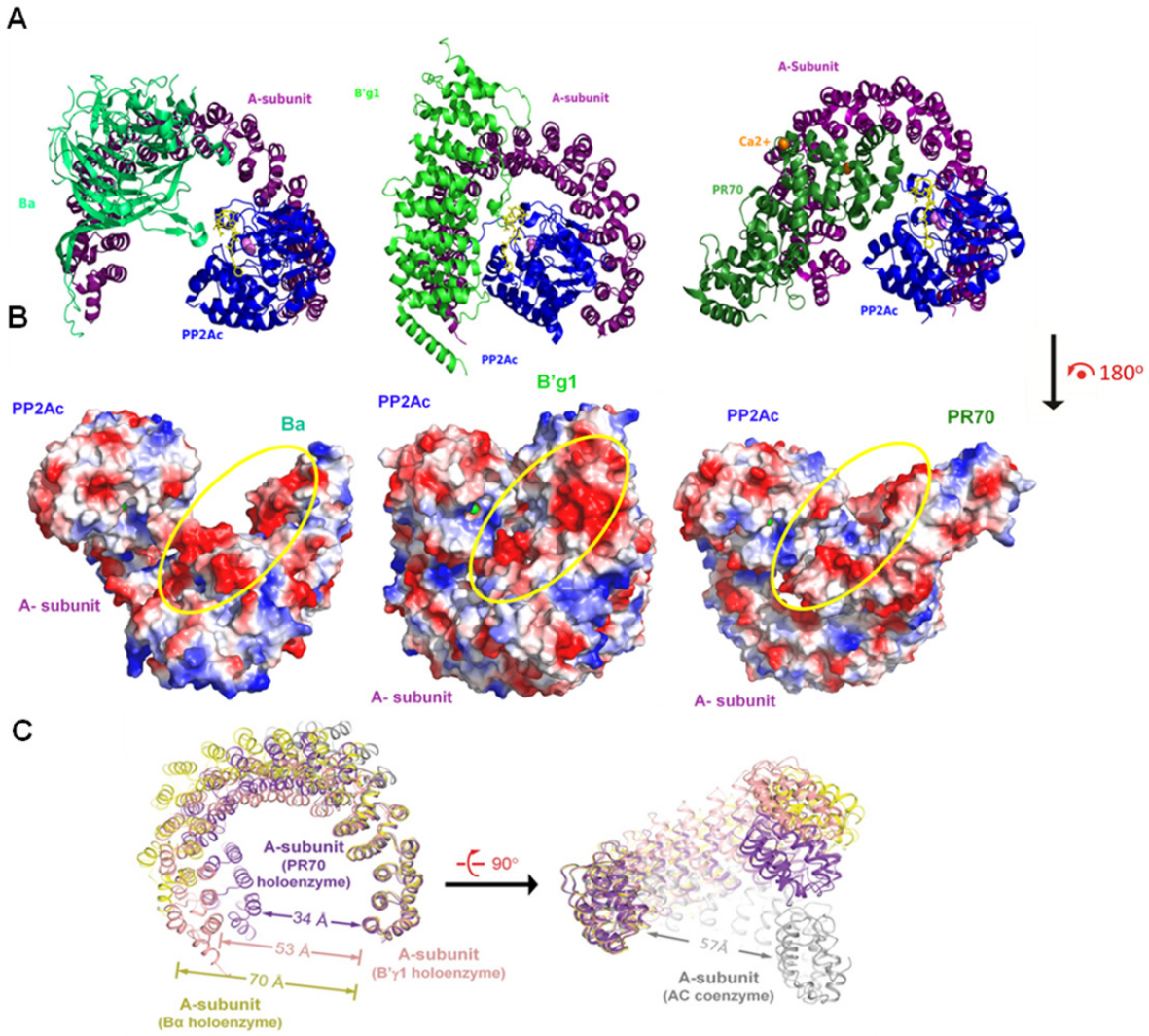


Figure 3.4. Differences in holoenzyme structures from three families. **A**. Overall view of holoenzymes of PP2A with Bα (3DW8), B'γ1 (2NPP), and PR70 (4I5L) shown in cartoon ribbon. The A-subunit is shown in purple, the C-subunit is shown in blue, and Bα, B'γ1, and PR70 are shown in lime, green, and forest, respectively. The active site metals are shown in violet, and calcium ions are shown in orange. The Bα subunit is a β-propeller protein, the B'γ1 subunit is an extended HEAT repeat protein, and the PR70 subunit consists of a linear arrangement of various domains (hydrophobic, helical, EF hands). **B**. Qualitative surface charge representations of the same holoenzymes. Blue is positively charged, red is negatively charged, and white is neutral. Active site metals are shown in green for contrast. The B and B' subunits

have a large negatively charged channel in the putative substrate binding region. The PR70-binding region consists of more or less even patches of alternating positive and negative charge. **C**. An alignment of the A-subunit s as observed in the Bα (yellow), B'γ1(pink), PR70 (purple) holoenzyme, and AC (grey) structures. The conformation of the A-subunit in the PR70 holoenzyme is considerably more compact than all other structures known. This data also corroborated the FRET data in figure 2.6.

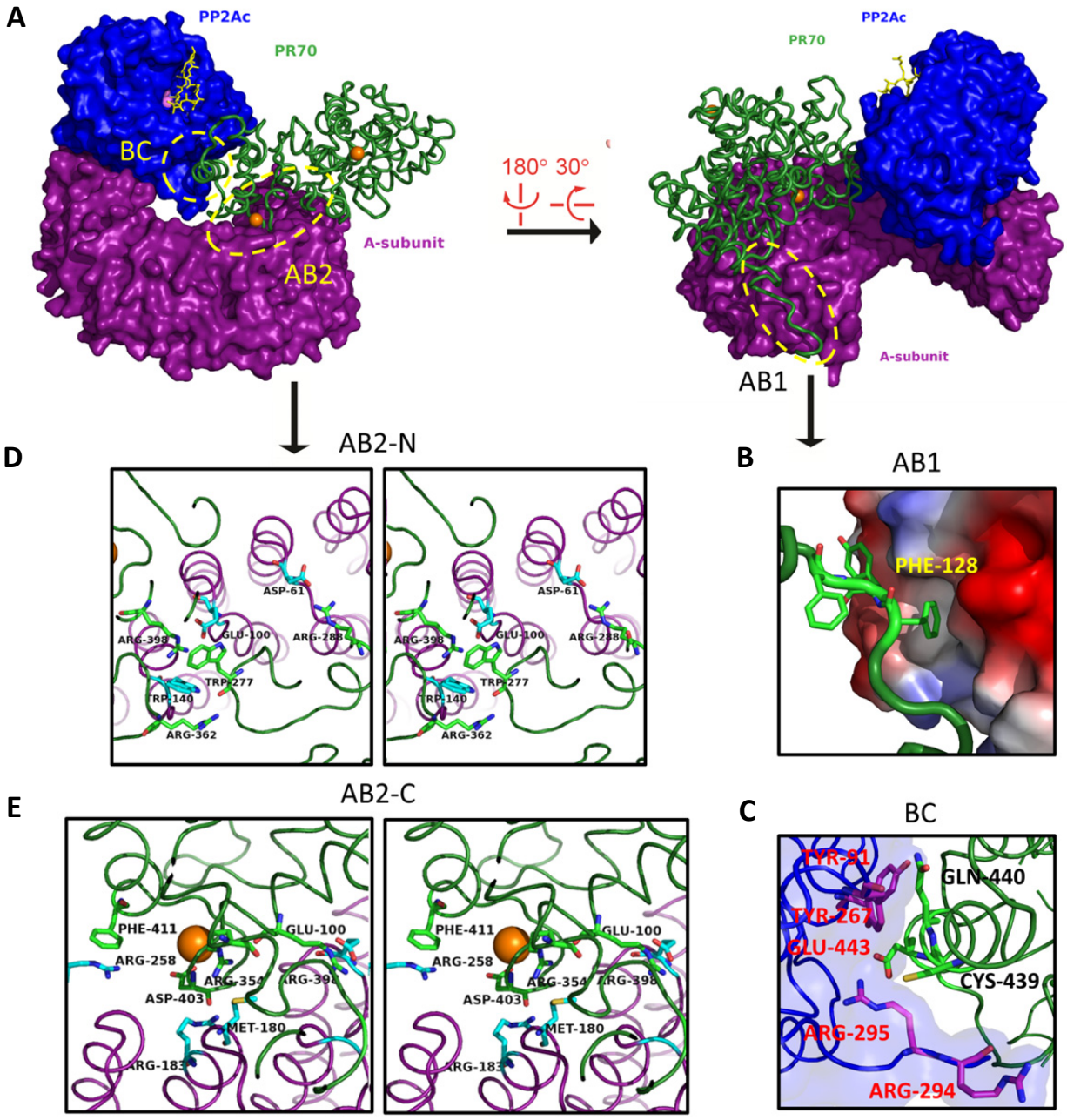


Figure 3.5. Inter-subunit interfaces of the PP2A-PR70 holoenzyme. **A**. Overview of the PP2A-PR70 holoenzyme with three PR70-AC interface regions indicated (AB1, AB2, BC). Two rotated views are shown. Color scheme is the same as in Figure 3.2. **B**. Close-up view of the AB1 interface containing the FYF motif. PR70 is shown in sticks and tube, and A is represented as charge density. F128 (center) sits in a hydrophobic pocket on the A-subunit. **C**. Close-up of the BC interface. PR70 is shown in tube with green sticks, C is shown in blue tube with

transparent surface and magenta sticks. PR70 participates in stacking interactions with C and salt bridge interactions, most notably PR70-D443K to C-R294. **D & E**. Close-up stereo views of the AB2 interface. Due to length of the interface, the view was broken into two overlapping parts. The A-subunit is in purple with cyan sticks, and the PR70 subunit is in forest with green sticks. Extensive salt bridges, hydrogen bonds, and stacking interactions are evident in this interaction. **D**. The N-terminal region of the interface starting with PR70-R288 and ending with PR70-R398. **E**. The C-terminal region of the interface starting with PR70-R398 and ending with PR70-F411.

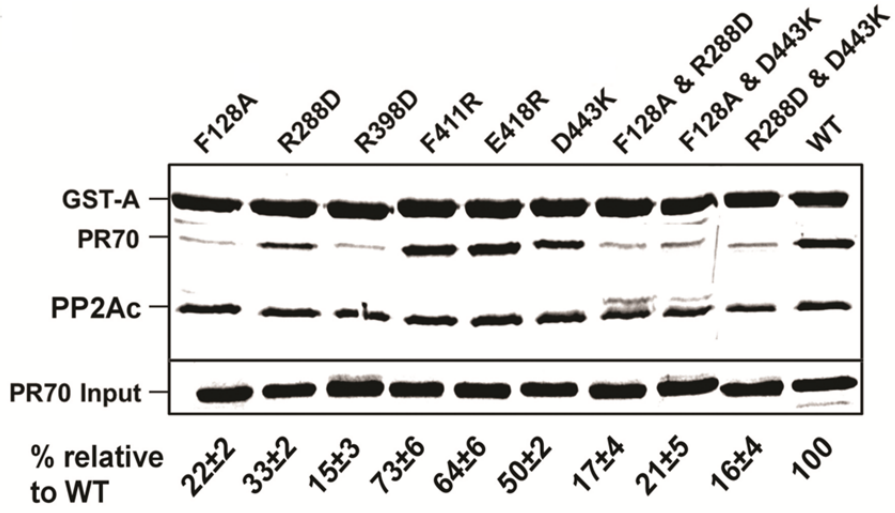


Figure 3.6. *In vitro* pull-down of wild type and mutant PR70 by GST-AC. PR70 mutants were generated to disrupt PR70 holoenzyme binding (except E418R was used as a control). GST-AC was used to pull-down PR70 WT and mutants as described in the methods. F128A, R398D, and double mutants (F128A & R288D, F128A & D443K, and R288D & D443K) largely reduced binding. F411R and D443K, unexpectedly, did not greatly reduce binding. Results from three experiments were quantified using ImageJ and analyzed using GraphPad Prism, and relative binding is quantified below the gel.

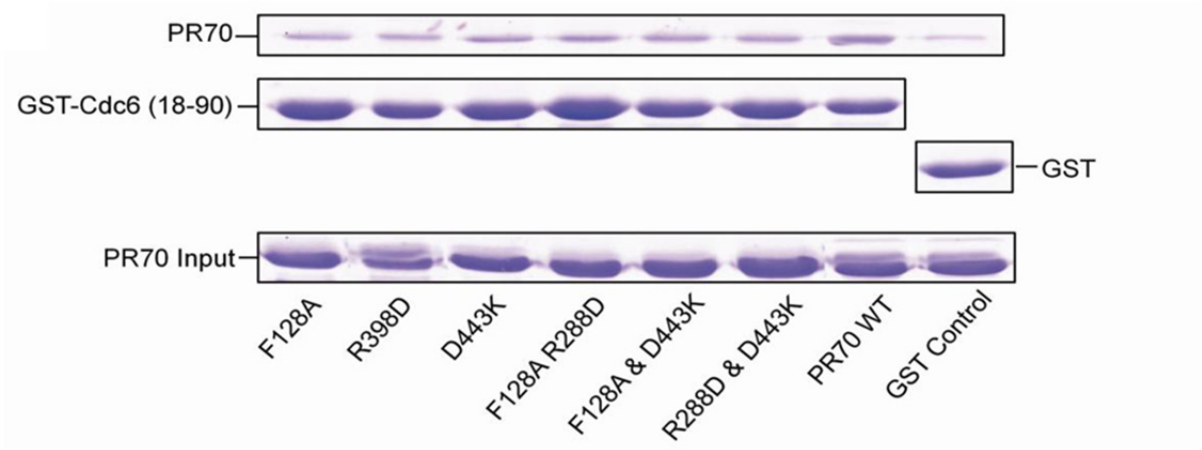


Figure 3.7. PR70 mutant protein binds Cdc6 similar to WT. GST-Cdc6 18-90 was used to pull-down PR70 mutants as described in the methods. All mutants bound Cdc6 comprably. GST-49-90 does not bind PR70 when the C-terminal residues are present (519-575). GST-18-90 was chosen to avoid steric hindrance due to the GST tag.

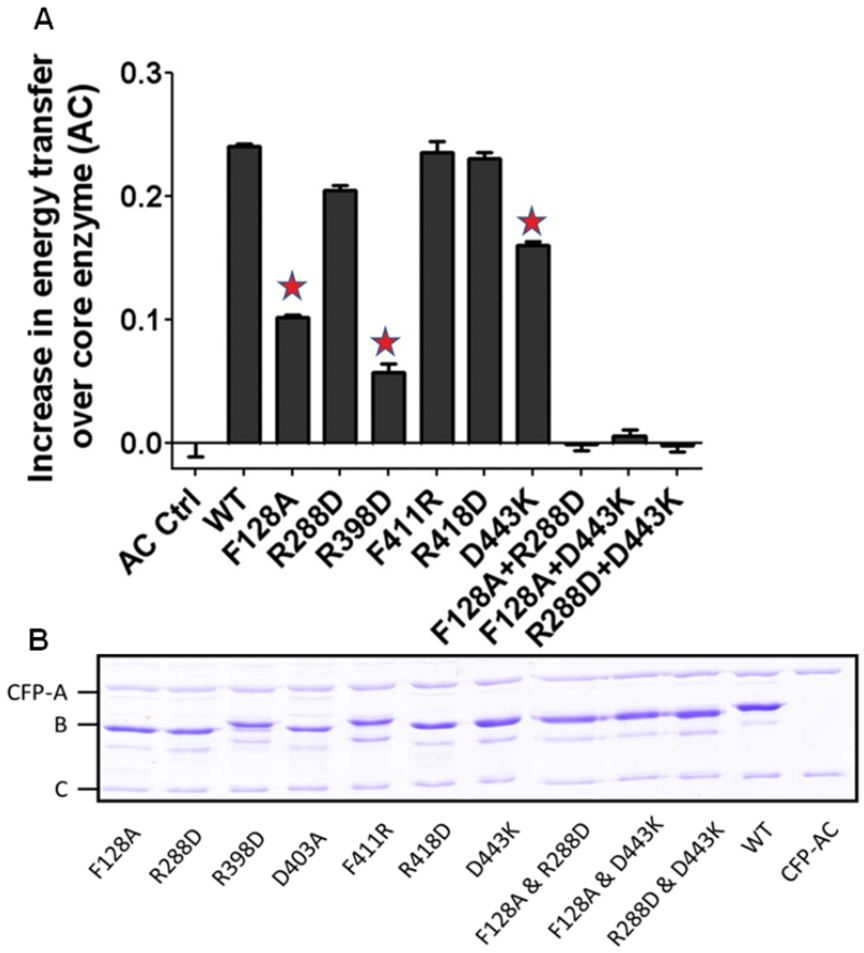


Figure 3.8. Changes in A-subunit conformation with mutant PR70 proteins. **A**. The FRET assay described in chapter II was used to quantify the conformation of the A-subunit by energy transfer efficiency. All measurements are shown relative to AC alone. Mutants with lower binding efficiency show an expanded conformation of the A-subunit (increased free AC in solution relative to holoenzyme) based on transfer efficiency. **B**. Input protein was normalized by the Bradford assay then visualized by SDS-PAGE.

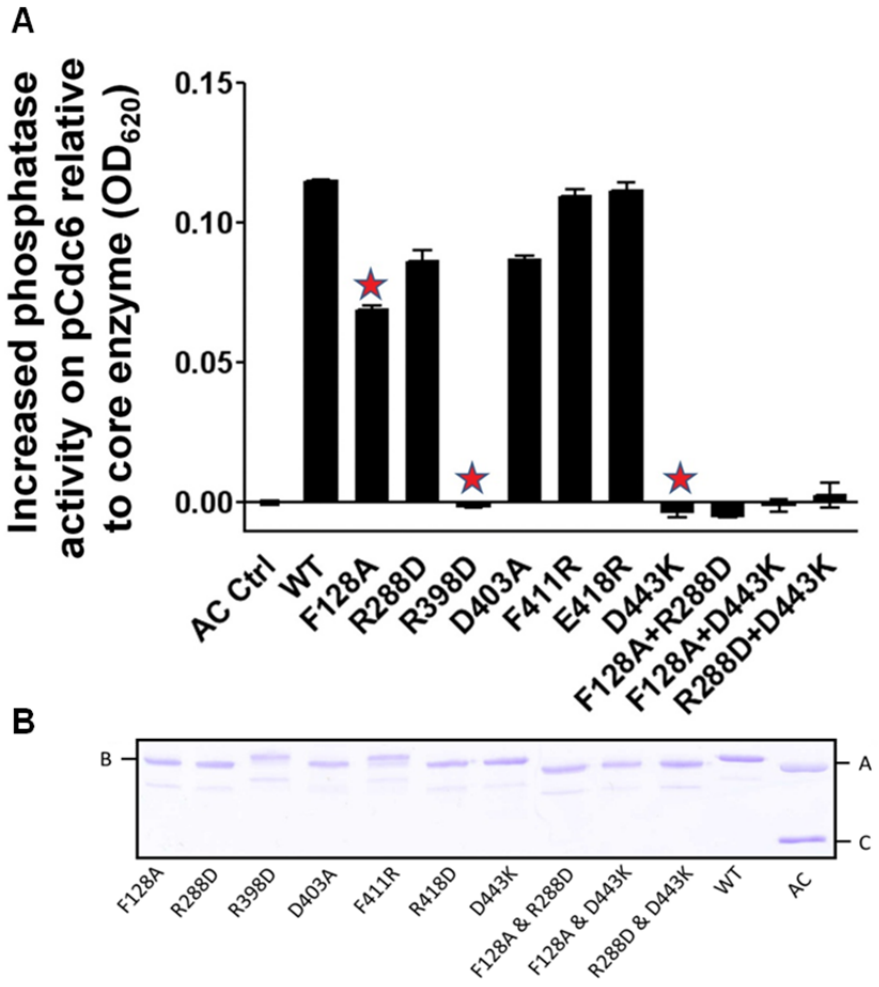
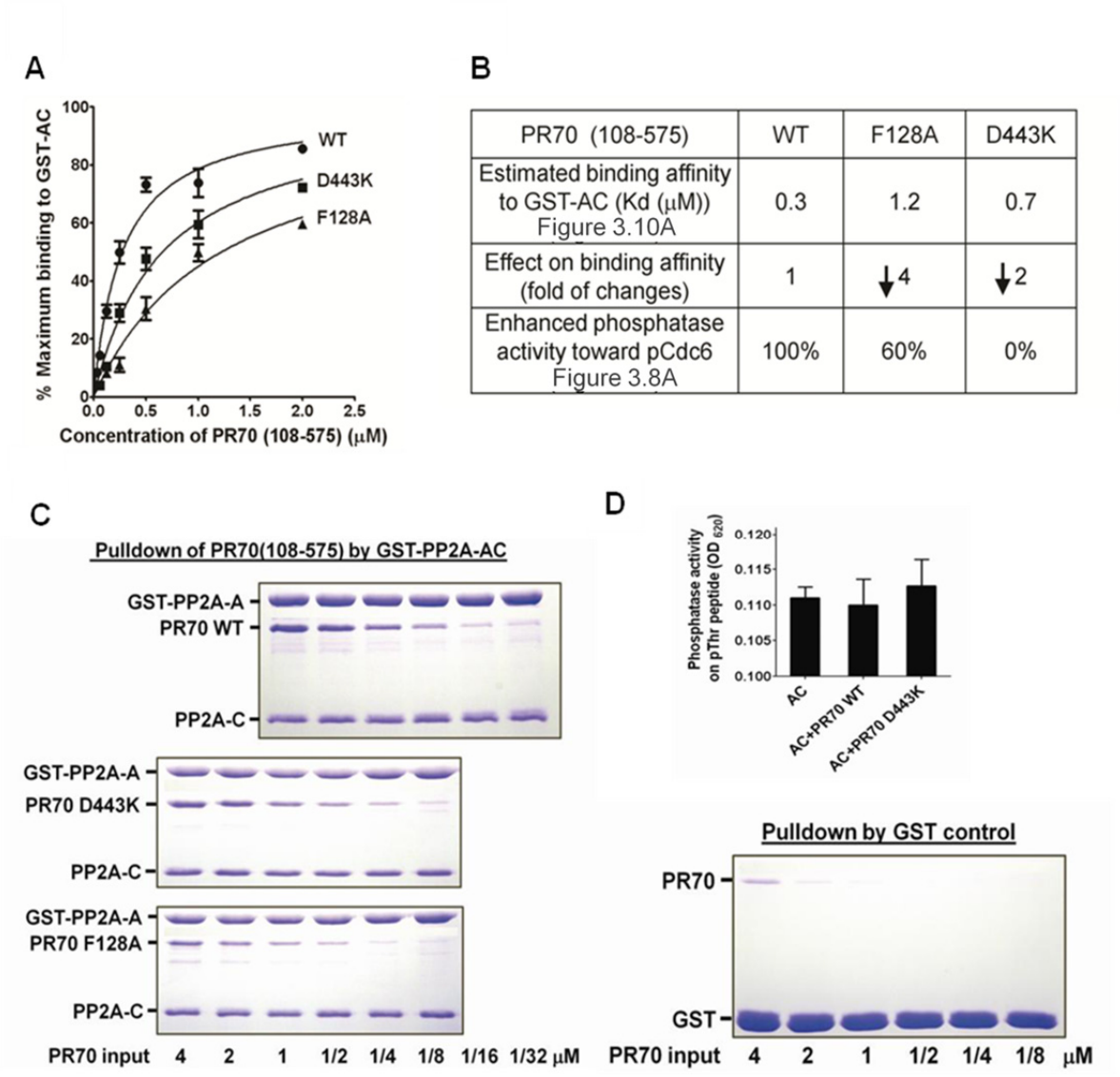


Figure 3.9. Phosphatase activity of PR70 mutants and wild type on pCdc6 49-90. **A**. Phosphatase activity was measured using the assay described in chapter II. Activity is indicated relative to AC activity. Mutants that still bind to AC have higher activity, and those that did not bind have similar activity to AC alone. The exception is D443K, in the B-C interface. This mutant bound well (Figure 3.6, 3.7) but does not have enhanced activity on pCdc6. **B**. Input protein was normalized by the Bradford assay then visualized on SDS-PAGE.



PR70 (108-575)	WT	F128A	D443K
Estimated binding affinity to GST-AC (Kd (μM)) Figure 3.10A	0.3	1.2	0.7
Effect on binding affinity (fold of changes)	1	↓4	↓2
Enhanced phosphatase activity toward pCdc6 Figure 3.8A	100%	60%	0%

Figure 3.10. Quantification of PR70 mutant binding to GST-AC. **A**. GST-AC was used to pull down various concentrations of PR70 WT, F128A, and D443K as described in the methods. A graphical representation of PR70 WT and mutant binding indicates that D443K has somewhat reduced binding and F128A has more reduced binding. **B**. The data in A and Figure 3.8A and 3.9A are summarized with regard to changes in binding affinity and phosphatase activity. The data indicate that although F128A has reduced binding far greater than D443K, D443 is absolutely essential for enhanced phosphatase activity. **C.** Representative SDS-PAGE gels of the data represented in **A** and **B**. The bands were quantified with ImageJ, and three sets were

analyzed in GraphPad Prism to create the figure in **A**. **D**. The phosphatase activity of PP2A WT and D443K holoenzyme on the artificial pT peptide was measured as described in the methods. The holoenzymes show equal activity toward the universal substrate. Assays in panel A-C were performed in collaboration with Dr. Feng Guo.

References

1 Hunter T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 1995; **80**:225-236.

2 Wurzenberger C, Gerlich DW. Phosphatases: providing safe passage through mitotic exit. *Nat Rev Mol Cell Biol* 2011; **12**:469-482.

3 Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2001; **353**:417-439.

4 Virshup DM. Protein phosphatase 2A: a panoply of enzymes. *Curr Opin Cell Biol* 2000; **12**:180-185.

5 Shi Y. Serine/threonine phosphatases: mechanism through structure. *Cell* 2009; **139**:468-484.

6 Xu Y, Xing Y, Chen Y *et al.* Structure of the protein phosphatase 2A holoenzyme. *Cell* 2006; **127**:1239-1251.

7 Xu Y, Chen Y, Zhang P, Jeffrey PD, Shi Y. Structure of a protein phosphatase 2A holoenzyme: insights into B55-mediated Tau dephosphorylation. *Mol cell* 2008; **31**:873-885.

8 Wlodarchak N, Guo F, Satyshur KA *et al.* Structure of the Ca2+-dependent PP2A heterotrimer and insights into Cdc6 dephosphorylation. *Cell Res* 2013; **23**:931-946.

9 Davis AJ, Yan Z, Martinez B, Mumby MC. Protein phosphatase 2A is targeted to cell division control protein 6 by a calcium-binding regulatory subunit. *J Biol Chem* 2008; **283**:16104-16114.

10 Ruediger R, Hentz M, Fait J, Mumby M, Walter G. Molecular model of the A subunit of protein phosphatase 2A: interaction with other subunits and tumor antigens. *J Virol* 1994; **68**:123-129.

11 Ruediger R, Roeckel D, Fait J, Bergqvist A, Magnusson G, Walter G. Identification of binding sites on the regulatory A subunit of protein phosphatase 2A for the catalytic C subunit and for tumor antigens of simian virus 40 and polyomavirus. *Mol Cell Biol* 1992; **12**:4872-4882.

12 Xing Y, Xu Y, Chen Y *et al.* Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell* 2006; **127**:341-353.

13 Xu Z, Cetin B, Anger M *et al.* Structure and function of the PP2A-shugoshin interaction. *Mol Cell* 2009; **35**:426-441.

14 Ahn JH, Sung JY, McAvoy T *et al.* The B"/PR72 subunit mediates Ca2+-dependent dephosphorylation of DARPP-32 by protein phosphatase 2A. *Proc Natl Acad Sci U S A* 2007; **104**:9876-9881.

15 Davis AJ, Yan Z, Martinez B, Mumby MC. Protein phosphatase 2A is targeted to cell division control protein 6 by a calcium-binding regulatory subunit. *J Biol Chem* 2008; **283**:16104-16114.

16 Magenta A, Fasanaro P, Romani S, Di Stefano V, Capogrossi MC, Martelli F. Protein phosphatase 2A subunit PR70 interacts with pRb and mediates its dephosphorylation. *Mol Cell Biol* 2008; **28**:873-882.

17 Stanevich V, Jiang L, Satyshur KA *et al.* The Structural Basis for Tight Control of PP2A Methylation and Function by LCMT-1. *Mol Cell* 2011; **41**:331-342.

18 Otwinowski Z, Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997; **276**:307-326.

19 Ness SR, de Graaff RA, Abrahams JP, Pannu NS. CRANK: new methods for automated macromolecular crystal structure solution. *Structure* 2004; **12**:1753-1761.

20 Winn MD, Ballard CC, Cowtan KD *et al.* Overview of the CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* 2011; **67**:235-242.

21 de Graaff RA, Hilge M, van der Plas JL, Abrahams JP. Matrix methods for solving protein substructures of chlorine and sulfur from anomalous data. *Acta Crystallogr D Biol Crystallogr* 2001; **57**:1857-1862.

22 Pannu NS, Read RJ. The application of multivariate statistical techniques improves single-wavelength anomalous diffraction phasing. *Acta Crystallogr D Biol Crystallogr* 2004; **60**:22-27.

23 Cowtan K. The Buccaneer software for automated model building. 1. Tracing protein chains. *Acta Crystallogr D Biol Crystallogr* 2006; **62**:1002-1011.

24 Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 2004; **60**:2126-2132.

25 Winn MD, Murshudov GN, Papiz MZ. Macromolecular TLS refinement in REFMAC at moderate resolutions. *Methods Enzymol* 2003; **374**:300-321.

26 Chao Y, Xing Y, Chen Y *et al.* Structure and mechanism of the phosphotyrosyl phosphatase activator. *Mol Cell* 2006; **23**:535-546.

27 Yan Z, Fedorov SA, Mumby MC, Williams RS. PR48, a Novel Regulatory Subunit of Protein Phosphatase 2A, Interacts with Cdc6 and Modulates DNA Replication in Human Cells. *Mol cell biol* 2000; **20**:1021 <last_page> 1029.

28 Saraf A, Oberg EA, Strack S. Molecular determinants for PP2A substrate specificity: charged residues mediate dephosphorylation of tyrosine hydroxylase by the PP2A/B' regulatory subunit. *Biochemistry* 2010; **49**:986-995.

29 Terrak M, Kerff F, Langsetmo K, Tao T, Dominguez R. Structural basis of protein phosphatase 1 regulation. *Nature* 2004; **429**:780-784.

30 O'Connell N, Nichols SR, Heroes E *et al.* The molecular basis for substrate specificity of the nuclear NIPP1:PP1 holoenzyme. *Structure* 2012; **20**:1746-1756.

31 Virshup DM, Shenolikar S. From promiscuity to precision: protein phosphatases get a makeover. *Mol cell* 2009; **33**:537-545.

Chapter IV

A High-throughput Peptide Chip Assay to Characterize PP2A-Substrate Interactions

The proteomic assay was performed by Dr. Yongna Xing in collaboration with Dr. Heng Zhu at Johns Hopkins

A review article is in preparation which will include table 4.1.

Unpublished data from the proteomic chip assay is withheld pending publication.

Abstract

Protein Phosphatase 2A has been highly implicated in many processes involved in neurological disease, heart disease, and cancer. PP2A is an assembly of diverse heterotrimeric holoenzymes. Regulatory subunits confer substrate specificity to the various holoenzymes, and they bind substrates via various structural interactions, nullifying the possibility of consensus sequences for substrate recognition. As such, identification and characterization of genuine PP2A substrates has occurred via traditional low-throughput methods such as yeast-two-hybrid assays and co-immunoprecipitation assays. Given the vast amount of processes and pathways in which PP2A is involved, novel high-throughput methods are needed to efficiently identify and characterize PP2A substrates *in vitro*. Built on a preliminary proteomic assay performed in our laboratory, which was able to identify 24 previously unknown PP2A substrates, I participated in the design of a peptide assay for the characterization of PP2A-substrate interactions. The peptide assay promises to be useful in expeditious characterization of PP2A-interacting proteins. These assays have considerable promise in providing large amounts of high-throughput data which will be useful in characterizing the mechanisms by which PP2A in involved in various critical pathways and provide potential targets for therapeutic treatment.

Introduction

Protein phosphatase 2A is one of the major S/T phosphatases in the cell. PP2A has been implicated in numerous human diseases such as cancer[1, 2], heart disease[3], and neuronal diseases[4]. While PP2A is believed to have an anti-proliferative, pro-apoptotic effect and is found suppressed in many cancers, quite a few PP2A holoenzymes were indicated to be important for progression through mitosis. The role of phosphorylation in heart disease is also regulated by complex phosphatase regulation, with some proteins contributing to heart failure when hypophosphorylated and some proteins contributing when hyperphosphorylated[5]. As such, PP2A has been known or suggested to act on some of these substrates. The impact of PP2A in neuronal disease has best been studied by its ability to dephosphorylate Tau, a key protein that when hyperphosphorylated can lead to neurofibrillary tangles and may be a contributing factor in Alzheimer's disease[4, 6].

PP2A is known or suspected to interact with nearly 300 substrates and likely many more (Table 4.1). PP2A can act on such a large array of substrates because of the diverse holoenzymes it can form. PP2A is comprised of a catalytic "C" subunit, a scaffold "A" subunit, and various regulatory "B" subunits[7]. The B subunits contribute the most to variation on holoenzyme formation and are divided into four families: the B (PR55), B' (PR56), B'' (PR72) and B''' (Striatin)[8]. Currently, the identified regulatory subunits are encoded for by 15 genes which can be alternatively spliced to yield 26 different B subunits[2]. These subunits share sequence homology within each family, but have little to no sequence homology between the families. As such, there is no specific "recognition motif" that PP2A substrates contain, and the recognition is likely conferred by structural elements inherent in each subunit, necessitating the need for structural information on the PP2A holoenzymes.

Since there are no consensus sequences for PP2A substrates, proteome-wide data mining is not able to easily identify PP2A substrates. In the past, the identification of PP2A substrates has mostly occurred via low-throughput, time-consuming processes such as yeast two-hybrid, co-immunoprecipitation, and mass spectrometric analyses. Proteomic chip microarrays offer newer, more powerful approaches for substrate identification.

Although many PP2A substrates have been identified, few have been completely characterized (Table 4.1). Most experiments have identified PP2A substrates based on interaction with the common C subunit; however, the B subunits direct substrate specificity, and this critical information is missing in many studies. In addition to a lack of information on which B subunits are responsible for activity, the PP2A-interacting regions of most of these substrates remain to be mapped. The process for mapping these interacting boundaries is traditionally done by expressing truncations of the substrates and using an *in vitro* pull-down assay to determine binding. This is a time-consuming and costly one-by-one process. Cross linking and identification by MS is another attractive method, but still requires expression of individual proteins and examining each interacting pair.

Developing methodologies to simultaneously and rapidly identify substrates and characterize the PP2A-substrate binding regions and the requisite B subunits would be of great benefit to the PP2A field. Modern high-throughput approaches are attractive options to simultaneously identify substrates and their interacting regulatory B subunits and to characterize their binding regions. In this chapter, I described the information based on data acquired from a proteomic assay that was used to identify several PP2A substrates and their participating B subunits. In addition to substrate identification, I will discuss the large-scale data mining of PP2A substrates and bioinformatic characterization of these substrates with the intention of

assembling a peptide array chip which will be used to determine the PP2A-binding regions of known substrates, as well as the required B subunits. These high-throughput assays have the potential to greatly reduce time and increase the throughput of PP2A substrate identification and characterization. Extensive knowledge of PP2A substrates will be helpful in identification of the mechanisms by which these interactions are involved in disease and possible development of targeted therapies to intervene in these processes.

Materials and Methods

Proteomic Chip Assay. A proteomic chip assay was performed by Dr. Yongna Xing in collaboration with Dr. Heng Zhu at John's Hopkins. Full experimental details are withheld here pending publication. In brief, a chip containing 17,000 human proteins synthesized in yeast and printed in duplicate was phosphorylated by a mixture of over 400 kinases was used with ATP to phosphorylate the proteins on the array. The kinase mixture was removed and the chip was washed three times with assay buffer. Holoenzymes containing B'γ1 or B'α were applied to the chip to selectively dephosphorylate the substrates present on the chip. This mixture was removed and the chip washed three times. Radiolabeled [γ-^{33}P] ATP was then used to re-phosphorylate the substrates that had been dephosphorylated. The mixture was removed and the array washed three times, then the array was imaged using a microarray scanner. A negative control chip was obtained by using the kinase cocktail and ATP alone then adding kinases with [γ-^{33}P] ATP. A positive control chip was obtained by using the kinase cocktail and [γ-^{33}P] ATP. In order to score potential substrates, the signal of the negative control, n, was subtracted from the positive control, p, and the experimental assay, a, and the ratio of the assay to the positive control was calculated [(a-n)/(p-n)]. Candidates with the highest scores indicated increased likelihood that they were PP2A substrates.

Design of a peptide array for identification of PP2A substrates. For a diagrammatic representation of this procedure, see figure 4.1. In order to create a comprehensive list of candidate substrates for inclusion on the peptide array, several sources of information were aggregated. Known substrates were collected from lists included in reviews and the primary sources vetted. Additional known substrates were mined from the BioGrid database (http://thebiogrid.org/), and PubMed was extensively queried for additional new substrates. In

addition to substrates identified in the literature, known CDK1 substrates, phospho-proteins known to be modified in heart disease, and proteins identified in the lab by the proteomic chip assay were also included.

Most phosphorylation sites occur in the unstructured regions of proteins that are likely on the surface and involved in PP2A interaction. Therefore, inclusion of only those areas would greatly reduce the amount of peptide necessary for investigating PP2A interaction. Secondary structure analysis of the candidate substrates was performed using the XtalPred server (http://ffas.burnham.org/XtalPred-cgi/xtal.pl) which aggregates several different prediction algorithms. The outputs were collected, and known phosphorylation sites retrieved from the PhosphoSite database (http://www.phosphosite.org/) were annotated on the sequences. Sequences were individually reviewed, and unstructured regions, areas enriched in phosphorylation sites, and/or areas known to interact with PP2A were selected for the peptide chip (Figure 4.2). These regions were divided into 20-amino acid long peptides with a step of 5 amino acids to generate the list of peptides that cover the entire unstructured region using Excel (Microsoft).

Peptide chip assay (proposed). The peptide chip will be printed using PEPperPRINT technology (B-Bridge International) at a rate of 4290 substrate peptides in duplicate per chip. In order to test binding of PP2A to peptide substrate fragments, a cy3 labeled scaffold subunit will be used (Figure 4.3). A will be labeled using a Cy3 mono-S ester labeling kit (GE Life Sciences) and assembled with C as described above. B subunits from three different families will then be assembled with the labeled AC similar to as described above. The labeled protein complexes will be applied to the chip and incubated for 5-30 min at RT. The protein solution will be removed and the chip washed 3x with assay buffer. The chip will be read by a GC3000 7G

microarray scanner (Affymetrix Inc.) and the data analyzed using the PEPperMAP software. Experiments will be conducted and analyzed similar to that described above for the proteomic chip.

Results

Identification of substrates by proteomic chip. The proteomic chip assay successfully identified 24 novel candidate substrates (data not shown). The 24 identified substrates were dephosphorylated by holoenzymes containing B'α, B'γ1, or both. These candidate substrates were included with the known and predicted PP2A substrates for the proteomic chip.

Assembly of the peptide chip. In order to assemble the peptide chip, all groups of substrates and potential substrates were thoroughly vetted. Substrates identified in the proteomic assay in our laboratory and potential substrates from lists of known CDK substrates or other phosphoproteins were queried in Pubmed or BioGrid to search for known interactions. All literature identifying proteins known to interact with PP2A was analyzed and summarized (Table 4.1). Phosphoproteins of special interest in heart disease, neuronal disease, or the cell cycle (but not known to interact with PP2A) were also analyzed and summarized (Table 4.2 & 4.3). All of the above-mentioned proteins were subjected to secondary analysis by XtalPred, and the results were annotated with known phosphosites or interacting boundaries. The aggregate information was used to manually select and narrow down boundaries of unstructured or highly modified regions, as well as known PP2A-interacting boundaries. Over 250 proteins were analyzed and divided into over 800 selected boundaries. Peptides of 20-mer length were generated with 5-mer overlaps. A set of peptides with phospho-mimicing S/T→E mutations were also generated to account for any interactions that require secondary modifications. In total, over 40,000 peptides were generated for the array.

Discussion

Our proteomic array-based phosphatase assay revealed several unknown PP2A substrates, with 24 selected for further analysis (data not shown). The assay revealed some substrates are specific to different holoenzymes, whereas others can be dephosphorylated by redundant holoenzymes from the same family. Interestingly, PP2A was found to dephosphorylate three different B subunits included on the chip: Bα, B'β, and B'δ (data not shown). The B' subunits can be phosphorylated by various kinases such as ERK[9], and other PP2A holoenzymes may be able to dephosphorylate different B subunits and modulate holoenzyme assembly and activity. The assay produced some substrates with scores well above 1. This likely indicates that some of these substrates were phosphorylated when they were produced in yeast; however, the large difference only strengthens the case that these candidates are PP2A substrates. Although all of the substrates have yet to be confirmed, it is highly likely that most, if not all, will be due to the specificity of the assay. In addition to traditional verification, the peptide chip assay may be a viable option for high throughput verification, further enhancing the efficiency of substrate discovery and characterization.

The assembly of the peptide chip required extensive effort. If algorithms were written to select boundaries to test based on secondary analysis and known information, the manual analysis time would be considerably reduced. This idea would also need to be tested, but based on subjective observations of known binding sites and phosphosites in relation to unstructured regions, this sort of algorithm would be practical. The costs of the chips are fairly reasonable (~$1,400/~4,000 peptides), and new technology will deliver more peptides at decreased cost in the future. One major concern for this peptide assay is the transient nature of PP2A binding to some of its substrates. Interactions with low binding affinity may be washed off easily or drift

off in the short interval between final wash and measurement. The engineers at PEPperPRINT do have concern that binding affinities weaker than 10 μM may be very difficult to observe. Some preliminary data on PP2A-Cdc6 binding affinity revealed a binding affinity in this range. Other substrates may bind tighter, but this assay has the potential to miss proteins with weaker, transient interaction. In the future, some method involving crosslinking or changes in washing procedures may be able to overcome this hurdle. This assay may also miss interactions which involve large structured surface areas. The method of selecting peptides in unstructured regions would preclude detection of this mode of interaction. It may be possible that PP2A binding requires phosphorylation on a residue separate from the one to be dephosphorylated. To account for this possibility, phospho-mimic peptides (where S/T are mutated to E) were also included. These peptides may also be useful for increasing binding affinity by binding to the active site in the case of peptides containing target residues.

In order to test proof-of-concept for this assay, a test array of 100 peptides will be synthesized. It will include some peptides of known binding regions as positive controls and some experimental peptides. A labeled AC dimer will serve to give amplified binding signals, possibly assisting in cases of low binding affinity. The assay will be conducted as described in the methods and Figure 4.3. If the assay is successful, it will be scaled up to include all of the peptides derived from the proteins in the tables below. The assay has a potential to yield a large amount of information and follow-up verification on individual substrates will be a time consuming process. In addition, since legitimate substrates may have widely varying binding affinities and this may affect the fluorescent binding signal, a simple signal cutoff may miss important substrates. One proposed way to score substrates is to split them into two groups—a high affinity group and a low affinity group and select those with the highest signal in each

group. The groups can be defined by identifying signals of positive controls consisting of representative high and low affinity substrate peptides and then choosing experimental substrates based on comparable signals. In all cases, it should be noted that the method of scoring substrates to choose for further analysis will be highly dependent on the success of the assay.

A quick glance at the known information relating to PP2A interacting proteins (Table 4.1) reveals the large gap of missing information regarding the nature of these associations. Traditional methods are costly in time and money and generally put out low volumes of information. High-throughput style assays, such as those proposed and tested here, have the potential to radically accumulate this vital information. Given the massive importance of PP2A in regulating many critical cellular processes, this information may provide a valuable resource for discovering predictive markers or developing drugs that modify these interactions to prevent or treat disease.

Figures and Tables

Table 4.1

Interacting Protein	B subunit	PhosphoSite	Interaction Boundary	Reference
AC8	-	-	-	[10]
ADRENRGIC FACTOR	-	-	-	[11]
AKT	B**α**	T308	-	[12]
APC	B', B'''	bCaternin, -	302-625, 188-774	[13, 14]
ApI 1 &2/μ	Bα	T156	-	[15]
APP	B'γ, B'ε	-	-	[16]
AR	-	-	-	[17]
ARL2	Bα	-	-	[18]
ATM	-	S1981	2427-2841	[19]
ATR	-	-	-	[20]
ATXN7L2	PR72	-	-	[21]
Aurora A	-	S51	46-56	[22]
AXIN1	B'α	-	595-726	[23, 24]
BAF-Lern4	Bα	-	59-938	[25]
β-Arrestin II	-	-	-	[26]
Bax	-	S184	-	[27]
Baz	B'	S1085	-	[28]
β-Caternin	B', Bα	S33,S37,T41,S45,S552,S675	APC, Mult.	[29, 30]
BCL2	B'γ	T69, S70, S87	-	[31, 32]
Bestorphin	-	-	-	[33]
BLNK	Bγ	-	-	[34]
BRCA1	B'γ	-	-	[35]
BUBR1	B'	-	630-720	[36]
CACNA1S	Bα	-	-	[37]
Calpain	-	-	-	[38]
caM kinase IV	-	-	-	[39]
CamkII	-	T253	-	[40]
Carpoxypeptidase D	-	-	-	[41]
CAS	-	-	-	[42]
Caspase-3	-	-	-	[43]
Cav1.2	Bα,Bβ,PR70	S1928	1927-2029	[38]
CBX1	-	-	-	[44]
CCT2	Bγ	-	-	[45]
CDC25	B'	T138	-	[46, 47, 48, 49]
Cdc6	PR70	S54/S74*	49-90*	[50, 51]
CDK16	Bα	-	-	[52]
Cdk4	-	-	-	[53]

Cdk9	-	-	-	[54]
CFTR	-	-	-	[55]
CG-NAP	PR130	-	-	[56]
Chk1	-	S317 & S345	-	[57]
CIP2A	-	-	-	[58]
c-MET	-	S985	-	[59]
CmyC	B'α	S62	40-179	[60]
Cofilin	-	-	-	[61]
Connexin-43	-	-	-	[62, 63]
CSNK1E	-	-	-	[64]
CTLA4	B'α	-	151-159	[65]
CUL3	B'β	-	315-374	[66, 67]
CXCR2	-	-	-	[68]
CyclinG1	B'α	-	-	[69, 70]
CyclinG2	B'β, B'γ	-	-	[71]
DARPP32	PR72	T75	-	[72]
Dlg4	B'δ	-	-	[73]
DNA polymerase A	-	-	-	[74]
E4orf3	-	-	-	[75]
E-CADHERIN	-	-	-	[76]
EGFR	PR130, B'ε	-	-	[77, 78]
EIF1AK2	B'α	-	-	[79]
EIF4EBP1	Bα	-	-	[80]
ELAVL1	B'ε	-	-	[81]
Emi2	-	-	319-375	[82]
Era	-	S18	-	[83]
Erf1	-	-	-	[84]
ERK1/2	B'β, B'γ	T202/T185	IEK-1	[9]
FAM107A	Bα	-	-	[85]
FMRP	-	-	-	[86]
Glutamate receptor	-	-	-	[87]
Gp130	-	S782	-	[88]
GRB2	Bα	-	-	[89]
Greatwall	B "α"	T194	-	[90]
GRK5	Bα	-	-	[91]
GSK3β	B'δ	S9	-	[92]
Gα12	-	-	-	[93]
H2AX	-	-	-	[94]
HAND-1	B'δ	-	150-216	[95]
HAND-2	B'δ	-	-	[95]
HCP-6	B	-	-	[96]

HDAC4	Bα	S298	1-289	[97]
HDAC5	Bα	-	-	[98, 99]
HDM2	CyG	S166	400-489	[70]
HOX11	-	-	-	[100]
HRX	-	-	-	[101]
HSF2	-	-	-	[102]
IEX-1	-	-	-	[9]
IKKB	-	S77 &S181	121-179	[103, 104]
IKKG	-	-	-	[105]
IQGAP1	-	-	-	[106]
IRAK1	-	-	-	[107]
JAK2	-	-	-	[108]
JNK	-	-	-	[109]
KCNQ2	B’γ,	-	E12-14	[110]
Keratin 8	-	-	-	[111]
Keratin18	-	-	-	[111]
KSR1	Bα	S392	249-320	[112]
LATS2	Bα	-	-	[35]
LNX1	-	-	-	[113]
Mdm2	B’α/CyG	T216	100-280, 400-489	[70]
MEK3	α4	T193	-	[114]
MEKK3	Bα, Bδ	S526	-	[115]
Mid-1	-	-	-	[116]
MKK4	-	-	-	[117]
MLH1	Bβ&Bδ	-	-	[118]
Mst3	B’’’	T178, T182	-	[119]
MTOR	Bα	-	-	[120]
Naked	PR72	-	-	[121, 122]
NDRG1	Bα	-	-	[123]
NEK1	B’α	S109	1-267	[124]
Nemo	-	S68	-	[125]
NHE3	B’δ	-	651-839	[126]
NKCC1	-	-	-	[127]
NM23H2	-	-	-	[128]
NMDA receptor	-	-	-	[129]
NOD2	B’ε	-	-	[130]
NorepinephrineXporter	-	-	-	[131]
NOTCH1	Bα	-	-	[132]
NR3A	-	-	-	[133]
NRF1	B’γ	-	-	[134]
Occludin	-	-	-	[135]

OSBP	-	-	-	[136]
P107	PR59	Mult.	-	[137]
p35	B'δ	-	-	[138]
P38	-	-	-	[43]
P53	-,B'γ1/3	S37, T55	-	[139, 140]
P70 S6 Kinase	-	-	-	[141]
PACS1	-	S278	-	[142]
PAK1	-	-	-	[141]
PAK3	-	-	-	[141]
Paxillin	B'γ1/2	-	-	[143]
PDE4D3/mAKAP	B'δ	-	2083-2319	[144]
Period	-	-	-	[145]
PIM1	B'β	-	70-139	[146]
Pin1	B'β	-	-	[147, 148]
PKA	-	-	-	[149]
PKR	B'α	B'α	B'α	[79]
PPFIA1	B'δ	-	-	[150]
PTPN14	-	-	-	[151]
Rack1	-	-	138-317	[152]
Raf1	Bα/δ	S259	-	[153]
RalA	Aβ	S183, S184	-	[53]
Rb	PR70	T826	792-928*	[154, 155]
Rec8	B'-sgo	Mult.	-	[156]
RelA	A	S536	1-155, 354-551	[104, 108, 157]
REPOman	B'α	S893	586-595	[158]
Rev	Bα& Bδ	-	-	[159]
RHEB	-	-	-	[160]
Rho-B	-	-	-	[160]
R-RAS	-	-	-	[160]
RRN3	Bα	S44	-	[161]
RSA1&2	-	-	-	[162]
Runx2	-	-	-	[163]
Securin	-	-	-	[164]
Separase	B'α-ε	-	1419-1474	[165]
Seratonin- R	-	-	-	[166]
SET	-	-	-	[167]
SG2NA	-	-	-	[168]
SHC	-	Y317	-	[169]
Sk1	B'α	S225	451-470	[170]
Smad3	-	-	1-232	[171]
SMAD9	B'ε	-	-	[172]

SMURF1	Bδ	-	-	[173]
SOX2	B'γ	-	-	[174]
Sp1	-	T739	-	[175]
Sprouty	A	S112, S115	50-60	[176]
SRC	Bγ	S12		[177]
STAT5	-	-	-	[178]
STE20	-	-	-	[127]
SUMO1	-	-	-	[179]
TAU	Bα	Mult.	197-259, 265-328	[5]
TAX	-	-	-	[105]
TBC1D3	B'γ	S6K	-	[180]
TCEAL1	Bα& Bδ	-	-	[181]
TGFBR1	Bα	-	-	[182]
TH	B'β	S19,S31, S40	R37 & R38	[183]
TIP	-	-	-	[184]
TOM22	Bβ	-	-	[185]
TOP1	Bβ	-	-	[186]
TRAF2	B'γ	T117	272-501	[104]
TSC2	-	-	-	[160]
TTP	-	-	-	[187]
UBD	-	-	-	[188]
UBR5	Bγ	-	-	[189]
UPF1	-	-	-	[190]
Vimentin	Bα	-	-	[191]
Vpu	B'ε	-	-	[192]
ZRANB1	B'''	-	-	[193]

Table 4.1. PP2A-interacting proteins identified by literature search. Reviews, Pubmed, and Biogrid results represented. The first row is the abbreviated interacting protein, the second is B subunit if known, the third is dephosphorylation site if known, the fourth is interacting boundary if known, and the last row indicates the reference. * Indicates experimental data from this thesis.

Table 4.2

Substrate	Reference
ALB	[5]
Branched chain ketoacid dehydr. E1α	[5]
CapZ Interacting Protein	[5]
Cysteine-rich secretory Protien 2	[5]
Eef2	[5]
Enolase3	[5]
Heatschock B1	[5]
Mapkepk2	[5]
Mib-CK	[5]
Myosin reg. light chain 2	[5]
PEA-15	[5]
Prkar1a	[5]
Pyruvate dehydr. E1α form subunit 1	[5]
Tropmyosin alpha-4 chain	[5]
Troponin 1	[5]
Tubulin alpha	[5]
Tubulin alpha 1A	[5]
Δ(3,5)-Δ(2,4)-dienoyl-CoA isomerase	[5]

Table 4.2. Predicted PP2A substrates implicated in heart disease. Substrates were included in the chip. The first row indicates the substrate, and the second row indicates the reference.

Table 4.3

Substrate	Reference
Actopaxin	[194]
Amphiphysin	[195]
BAD	[196]
BARD1	[197]
Beta-tubulin	[198]
B-myb	[199]
BRCA2	[200]
Calesmon	[201]
Cdc20	[202]
CDK7	[203]
CK2	[204]
Dab2	[205]
DNA polymerase Lambda	[206]
Drc1	[207]
Dynein light intermediate chain	[208]
Dystophin	[209]
ECT2	[210]
Elongation factor1	[211]
FANCG	[212]
FOXM1c	[213]
FOXO1	[214]
GM130(Golgi Protein)	[215]
GRASP-65	[216]
HCN1	[217]
hHR6A	[218]
HIRA	[219]
Histone H1	[220]
HMG-1	[221]
HPV E1 protein	[222]
hRad9	[223]
HuCDC7	[224]
Kar9	[225]
KID	[226]
KIF11	[227]
LaminA	[228]
LaminB	[228]
MAP4	[229]
MARCKS	[230]

MCM2	231
MdmX	232
MEF	233
Mps1 p	234
MyoD	235
NDEL	236
Net1	237
NF-Y	238
Nir2	239
NPAT	240
NUCKS	241
P21- activated Kinase	242
P27kip	243
Protein Phosphatse 1 Inhibitor (I-2)	244
Rab4	245
RCC1	246
RI aplha subunit pf PKA	247
Ribonucleotide Reductase R2	248
Ski oncoprotein	249
Stathmin	250
Stem-Loop binding protein	251
Survivin	252
Swi5	236
TOPK	253
Upstream binding factor	254
WARTS tumor suppressor	255
Spc42	234
SRs2 DNA Helicase	256
Wee1	257

Table 4.3. Known CDK1 substrates that are potential, but not confirmed, PP2A substrates. Substrates were included in the peptide chip. Row one lists the substrate, row two indicates the reference.

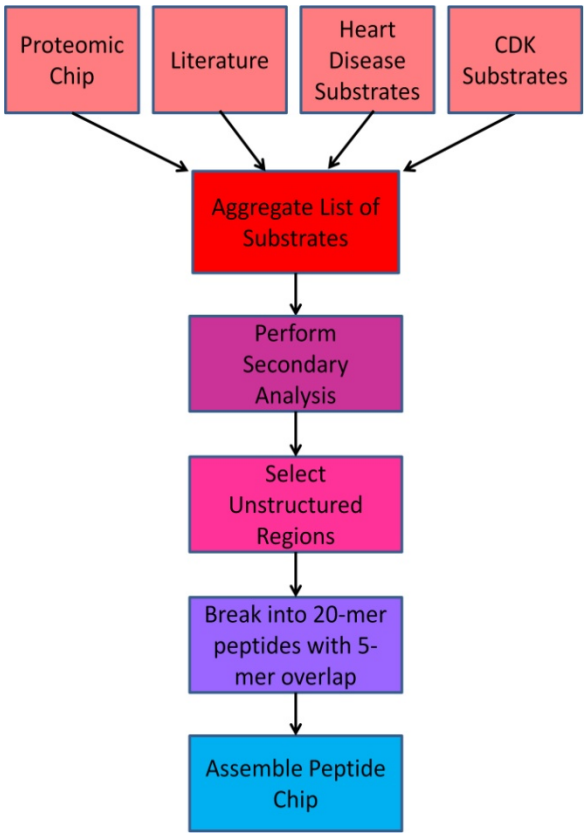


Figure 4.1. Flow chart depicting the selection of peptides for the assay. Candidate substrates were selected from known substrates in the literature, potential substrates from literature (CDK substrates and phospho-proteins implicated in heart disease), and the proteomic assay. A secondary structural analysis was then performed on the candidate substrates using the XtalPred server. Unstructured regions were manually selected and these areas were broken into 20mer peptides with 5mer overlaps by Microsoft Excel. The candidate peptides were submitted for printing on a chip in duplicate by a commercial company (B-bridge International).

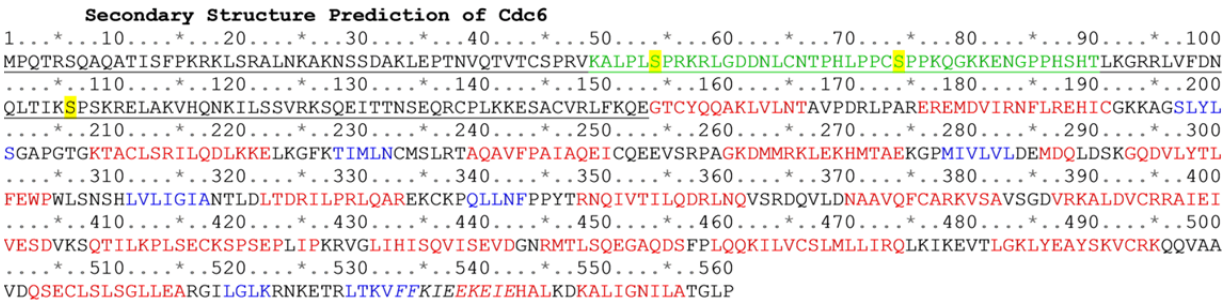


Figure 4.2. An example of secondary structure prediction used to select candidate regions for the peptide chip. Secondary structure was predicted by the XtalPred server. α-helices are indicated in red, and β-strands are indicated in blue. The underlined region was chosen for the peptide chip. The Cdc6-PR70-binding region that was determined experimentally is indicated in green. Known phosphorylation sites are highlighted in yellow. The experimental data for Cdc6 binding along with the secondary analysis corroborates the hypothesis that PP2A binding occurs in largely unstructured and modified regions.

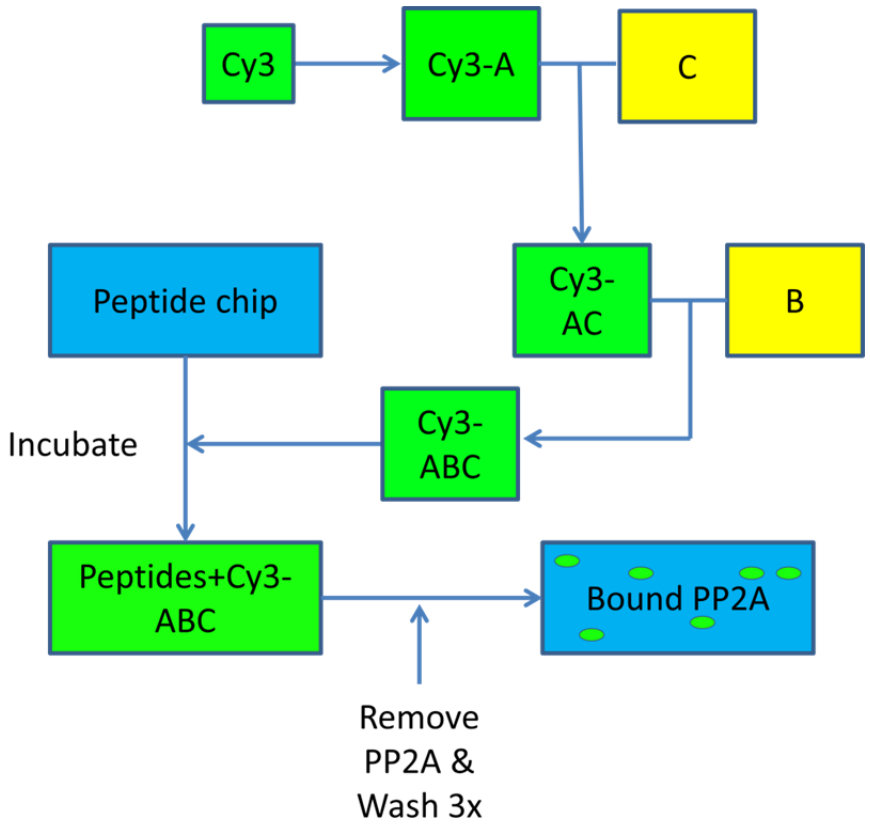


Figure 4.3. Diagrammatic representation of the proposed peptide chip assay. Candidates for the peptide chip were chosen as described in the methods. The chip will be synthesized using the PEPperPRINT technology (B-Bridge International). The A-subunit will be esterified with Cy3 using a commercially available kit then assembled with C as described in chapter II. The labeled AC will be assembled and purified with various B subunits from multiple families similar to that as described in chapter II and III. The complex will be overlaid onto the peptide chip, incubated, and removed. The chip will be washed 3x to remove excess enzyme, and the fluorescent signal on the chip will be visualized by a microarray scanner to detect bound PP2A. The resulting fluorescent image will be analyzed by the PEPperMAP software and compared to the map of peptides on the chip. Signals will be scored based on intensity, and overlapping peptides with high fluorescent intensity will be used to determine the PP2A binding motif of each substrate.

References

1 Sablina AA, Hector M, Colpaert N, Hahn WC. Identification of PP2A complexes and pathways involved in cell transformation. *Cancer Res* 2010; **70**:10474-10484.

2 Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochim et biophys acta* 2009; **1795**:1-15.

3 Heijman J, Dewenter M, El-Armouche A, Dobrev D. Function and regulation of serine/threonine phosphatases in the healthy and diseased heart. *J Mol Cell Cardiol* 2013; **64**:90-98.

4 Martin L, Latypova X, Wilson CM, Magnaudeix A, Perrin ML, Terro F. Tau protein phosphatases in Alzheimer's disease: the leading role of PP2A. *Ageing Res Rev* 2013; **12**:39-49.

5 Kotlo K, Johnson KR, Grillon JM, Geenen DL, deTombe P, Danziger RS. Phosphoprotein abundance changes in hypertensive cardiac remodeling. *J Proteomics* 2012; **77**:1-13.

6 Xu Y, Chen Y, Zhang P, Jeffrey PD, Shi Y. Structure of a protein phosphatase 2A holoenzyme: insights into B55-mediated Tau dephosphorylation. *Mol Cell* 2008; **31**:873-885.

7 Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2001; **353**:417-439.

8 Virshup DM. Protein phosphatase 2A: a panoply of enzymes. *Curr Opin Cell Biol* 2000; **12**:180-185.

9 Letourneux C, Rocher G, Porteu F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *Embo J* 2006; **25**:727-738.

10 Crossthwaite AJ, Ciruela A, Rayner TF, Cooper DM. A direct interaction between the N terminus of adenylyl cyclase AC8 and the catalytic subunit of protein phosphatase 2A. *Mol Pharmacol* 2006; **69**:608-617.

11 Krueger KM, Daaka Y, Pitcher JA, Lefkowitz RJ. The role of sequestration in G protein-coupled receptor resensitization. Regulation of beta2-adrenergic receptor dephosphorylation by vesicular acidification. *J Biol Chem* 1997; **272**:5-8.

12 Kuo YC, Huang KY, Yang CH, Yang YS, Lee WY, Chiang CW. Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. *J Biol Chem* 2008; **283**:1882-1892.

13 Galea MA, Eleftheriou A, Henderson BR. ARM domain-dependent nuclear import of adenomatous polyposis coli protein is stimulated by the B56 alpha subunit of protein phosphatase 2A. *J Biol Chem* 2001; **276**:45833-45839.

14 Breitman M, Zilberberg A, Caspi M, Rosin-Arbesfeld R. The armadillo repeat domain of the APC tumor suppressor protein interacts with Striatin family members. *Biochim Biophys Acta* 2008; **1783**:1792-1802.

15 Ricotta D, Hansen J, Preiss C, Teichert D, Honing S. Characterization of a protein phosphatase 2A holoenzyme that dephosphorylates the clathrin adaptors AP-1 and AP-2. *J Biol Chem* 2008; **283**:5510-5517.

16 Olah J, Vincze O, Virok D *et al.* Interactions of pathological hallmark proteins: tubulin polymerization promoting protein/p25, beta-amyloid, and alpha-synuclein. *J Biol Chem* 2011; **286**:34088-34100.

17 Yang CS, Xin HW, Kelley JB, Spencer A, Brautigan DL, Paschal BM. Ligand binding to the androgen receptor induces conformational changes that regulate phosphatase interactions. *Mol Cell Biol* 2007; **27**:3390-3404.

18 Shern JF, Sharer JD, Pallas DC *et al.* Cytosolic Arl2 is complexed with cofactor D and protein phosphatase 2A. *J Biol Chem* 2003; **278**:40829-40836.

19 Goodarzi AA, Jonnalagadda JC, Douglas P *et al.* Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. *Embo j* 2004; **23**:4451-4461.

20 Kim ST, Lim DS, Canman CE, Kastan MB. Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem* 1999; **274**:37538-37543.

21 Lim J, Hao T, Shaw C *et al.* A protein-protein interaction network for human inherited ataxias and disorders of Purkinje cell degeneration. *Cell* 2006; **125**:801-814.

22 Horn V, Thelu J, Garcia A, Albiges-Rizo C, Block MR, Viallet J. Functional interaction of Aurora-A and PP2A during mitosis. *Mol Biol Cell* 2007; **18**:1233-1241.

23 Arnold HK, Zhang X, Daniel CJ *et al.* The Axin1 scaffold protein promotes formation of a degradation complex for c-Myc. *Embo j* 2009; **28**:500-512.

24 Yamamoto H, Hinoi T, Michiue T *et al.* Inhibition of the Wnt signaling pathway by the PR61 subunit of protein phosphatase 2A. *J Biol Chem* 2001; **276**:26875-26882.

25 Asencio C, Davidson IF, Santarella-Mellwig R *et al.* Coordination of kinase and phosphatase activities by Lem4 enables nuclear envelope reassembly during mitosis. *Cell* 2012; **150**:122-135.

26 Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* 2005; **122**:261-273.

27 Xin M, Deng X. Protein phosphatase 2A enhances the proapoptotic function of Bax through dephosphorylation. *J Biol Chem* 2006; **281**:18859-18867.

28 Krahn MP, Egger-Adam D, Wodarz A. PP2A antagonizes phosphorylation of Bazooka by PAR-1 to control apical-basal polarity in dividing embryonic neuroblasts. *Dev Cell* 2009; **16**:901-908.

29 Seeling JM, Miller JR, Gil R, Moon RT, White R, Virshup DM. Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. *Science* 1999; **283**:2089-2091.

30 Zhang W, Yang J, Liu Y *et al.* PR55 alpha, a regulatory subunit of PP2A, specifically regulates PP2A-mediated beta-catenin dephosphorylation. *J Biol Chem* 2009; **284**:22649-22656.

31 Lin SS, Bassik MC, Suh H *et al.* PP2A regulates BCL-2 phosphorylation and proteasome-mediated degradation at the endoplasmic reticulum. *J Biol Chem* 2006; **281**:23003-23012.

32 Ruvolo VR, Kurinna SM, Karanjeet KB *et al.* PKR regulates B56(alpha)-mediated BCL2 phosphatase activity in acute lymphoblastic leukemia-derived REH cells. *J Biol Chem* 2008; **283**:35474-35485.

33 Marmorstein LY, McLaughlin PJ, Stanton JB, Yan L, Crabb JW, Marmorstein AD. Bestrophin interacts physically and functionally with protein phosphatase 2A. *J Biol Chem* 2002; **277**:30591-30597.

34 Oellerich T, Bremes V, Neumann K *et al.* The B-cell antigen receptor signals through a preformed transducer module of SLP65 and CIN85. *Embo j* 2011; **30**:3620-3634.

35 Woods NT, Mesquita RD, Sweet M *et al.* Charting the landscape of tandem BRCT domain-mediated protein interactions. *Sci Signal* 2012; **5**:rs6.

36 Xu P, Raetz EA, Kitagawa M, Virshup DM, Lee SH. BUBR1 recruits PP2A via the B56 family of targeting subunits to promote chromosome congression. *Biol Open* 2013; **2**:479-486.

37 Kristensen AR, Gsponer J, Foster LJ. A high-throughput approach for measuring temporal changes in the interactome. *Nat Methods* 2012; **9**:907-909.

38 Hall DD, Feekes JA, Arachchige Don AS *et al.* Binding of protein phosphatase 2A to the L-type calcium channel Cav1.2 next to Ser1928, its main PKA site, is critical for Ser1928 dephosphorylation. *Biochemistry* 2006; **45**:3448-3459.

39 Westphal RS, Anderson KA, Means AR, Wadzinski BE. A signaling complex of Ca2+-calmodulin-dependent protein kinase IV and protein phosphatase 2A. *Science* 1998; **280**:1258-1261.

40 Hoffmann C, Gaietta G, Bunemann M *et al.* A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat Methods* 2005; **2**:171-176.

41 Varlamov O, Kalinina E, Che FY, Fricker LD. Protein phosphatase 2A binds to the cytoplasmic tail of carboxypeptidase D and regulates post-trans-Golgi network trafficking. *J Cell Sci* 2001; **114**:311-322.

42 Yokoyama N, Miller WT. Protein phosphatase 2A interacts with the Src kinase substrate p130(CAS). *Oncogene* 2001; **20**:6057-6065.

43 Alvarado-Kristensson M, Andersson T. Protein phosphatase 2A regulates apoptosis in neutrophils by dephosphorylating both p38 MAPK and its substrate caspase 3. *J Biol Chem* 2005; **280**:6238-6244.

44 Nozawa RS, Nagao K, Masuda HT *et al.* Human POGZ modulates dissociation of HP1alpha from mitotic chromosome arms through Aurora B activation. *Nat Cell Biol* 2010; **12**:719-727.

45 Glatter T, Wepf A, Aebersold R, Gstaiger M. An integrated workflow for charting the human interaction proteome: insights into the PP2A system. *Mol Syst Biol* 2009; **5**:237.

46 Lammer C, Wagerer S, Saffrich R, Mertens D, Ansorge W, Hoffmann I. The cdc25B phosphatase is essential for the G2/M phase transition in human cells. *J Cell Sci* 1998; **111 (Pt 16)**:2445-2453.

47 Margolis SS, Walsh S, Weiser DC, Yoshida M, Shenolikar S, Kornbluth S. PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation. *EMBO J* 2003; **22**:5734-5745.

48 Margolis SS, Perry JA, Weitzel DH *et al.* A role for PP1 in the Cdc2/Cyclin B-mediated positive feedback activation of Cdc25. *Mol Biol Cell* 2006; **17**:1779-1789.

49 Margolis SS, Perry JA, Forester CM *et al.* Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell* 2006; **127**:759-773.

50 Petersen BO, Lukas J, Sorensen CS, Bartek J, Helin K. Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *The EMBO journal* 1999; **18**:396-410.

51 Davis AJ, Yan Z, Martinez B, Mumby MC. Protein phosphatase 2A is targeted to cell division control protein 6 by a calcium-binding regulatory subunit. *J Biol Chem* 2008; **283**:16104-16114.

52 Varjosalo M, Keskitalo S, Van Drogen A *et al.* The protein interaction landscape of the human CMGC kinase group. *Cell Rep* 2013; **3**:1306-1320.

53 Sablina AA, Chen W, Arroyo JD *et al.* The tumor suppressor PP2A Abeta regulates the RalA GTPase. *Cell* 2007; **129**:969-982.

54 Ammosova T, Washington K, Debebe Z, Brady J, Nekhai S. Dephosphorylation of CDK9 by protein phosphatase 2A and protein phosphatase-1 in Tat-activated HIV-1 transcription. *Retrovirology* 2005; **2**:47.

55 Vastiau A, Cao L, Jaspers M *et al.* Interaction of the protein phosphatase 2A with the regulatory domain of the cystic fibrosis transmembrane conductance regulator channel. *FEBS Lett* 2005; **579**:3392-3396.

56 Takahashi M, Shibata H, Shimakawa M, Miyamoto M, Mukai H, Ono Y. Characterization of a novel giant scaffolding protein, CG-NAP, that anchors multiple signaling enzymes to centrosome and the golgi apparatus. *J Biol Chem* 1999; **274**:17267-17274.

57 Leung-Pineda V, Ryan CE, Piwnica-Worms H. Phosphorylation of Chk1 by ATR is antagonized by a Chk1-regulated protein phosphatase 2A circuit. *Mol Cell Biol* 2006; **26**:7529-7538.

58 Junttila MR, Puustinen P, Niemela M *et al.* CIP2A inhibits PP2A in human malignancies. *Cell* 2007; **130**:51-62.

59 Hashigasako A, Machide M, Nakamura T, Matsumoto K. Bi-directional regulation of Ser-985 phosphorylation of c-met via protein kinase C and protein phosphatase 2A involves c-Met activation and cellular responsiveness to hepatocyte growth factor. *J Biol Chem* 2004; **279**:26445-26452.

60 Arnold HK, Sears RC. Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. *Mol Cell Biol* 2006; **26**:2832-2844.

61 Samstag Y, Nebl G. Interaction of cofilin with the serine phosphatases PP1 and PP2A in normal and neoplastic human T lymphocytes. *Adv Enzyme Regul* 2003; **43**:197-211.

62 Kanemitsu MY, Jiang W, Eckhart W. Cdc2-mediated phosphorylation of the gap junction protein, connexin43, during mitosis. *Cell Growth Differ* 1998; **9**:13-21.

63 Meilleur MA, Akpovi CD, Pelletier RM, Vitale ML. Tumor necrosis factor-alpha-induced anterior pituitary folliculostellate TtT/GF cell uncoupling is mediated by connexin 43 dephosphorylation. *Endocrinology* 2007; **148**:5913-5924.

64 Varjosalo M, Sacco R, Stukalov A *et al.* Interlaboratory reproducibility of large-scale human protein-complex analysis by standardized AP-MS. *Nat Methods* 2013; **10**:307-314.

65 Baroja ML, Vijayakrishnan L, Bettelli E *et al.* Inhibition of CTLA-4 function by the regulatory subunit of serine/threonine phosphatase 2A. *J Immunol* 2002; **168**:5070-5078.

66 Bennett EJ, Rush J, Gygi SP, Harper JW. Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. *Cell* 2010; **143**:951-965.

67 Oberg EA, Nifoussi SK, Gingras AC, Strack S. Selective proteasomal degradation of the B'beta subunit of protein phosphatase 2A by the E3 ubiquitin ligase adaptor Kelch-like 15. *J Biol Chem* 2012; **287**:43378-43389.

68 Fan GH, Yang W, Sai J, Richmond A. Phosphorylation-independent association of CXCR2 with the protein phosphatase 2A core enzyme. *J Biol Chem* 2001; **276**:16960-16968.

69 Okamoto K, Kamibayashi C, Serrano M, Prives C, Mumby MC, Beach D. p53-dependent association between cyclin G and the B' subunit of protein phosphatase 2A. *Mol Cell Biol* 1996; **16**:6593-6602.

70 Okamoto K, Li H, Jensen MR *et al.* Cyclin G recruits PP2A to dephosphorylate Mdm2. *Mol Cell* 2002; **9**:761-771.

71 Bennin DA, Don AS, Brake T *et al.* Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B' subunits in active complexes and induces nuclear aberrations and a G1/S phase cell cycle arrest. *J Biol Chem* 2002; **277**:27449-27467.

72 Ahn JH, Sung JY, McAvoy T *et al.* The B"/PR72 subunit mediates Ca2+-dependent dephosphorylation of DARPP-32 by protein phosphatase 2A. *Proc Natl Acad Sci U S A* 2007; **104**:9876-9881.

73 Arbuckle MI, Komiyama NH, Delaney A *et al.* The SH3 domain of postsynaptic density 95 mediates inflammatory pain through phosphatidylinositol-3-kinase recruitment. *EMBO Rep* 2010; **11**:473-478.

74 Dehde S, Rohaly G, Schub O *et al.* Two immunologically distinct human DNA polymerase alpha-primase subpopulations are involved in cellular DNA replication. *Mol Cell Biol* 2001; **21**:2581-2593.

75 Shtrichman R, Sharf R, Barr H, Dobner T, Kleinberger T. Induction of apoptosis by adenovirus E4orf4 protein is specific to transformed cells and requires an interaction with protein phosphatase 2A. *Proc Natl Acad Sci U S A* 1999; **96**:10080-10085.

76 Gotz J, Probst A, Mistl C, Nitsch RM, Ehler E. Distinct role of protein phosphatase 2A subunit Calpha in the regulation of E-cadherin and beta-catenin during development. *Mech Dev* 2000; **93**:83-93.

77 Zwaenepoel K, Goris J, Erneux C, Parker PJ, Janssens V. Protein phosphatase 2A PR130/B"alpha1 subunit binds to the SH2 domain-containing inositol polyphosphate 5-phosphatase 2 and prevents epidermal growth factor (EGF)-induced EGF receptor degradation sustaining EGF-mediated signaling. *FAESB J* 2010; **24**:538-547.

78 Foerster S, Kacprowski T, Dhople VM *et al.* Characterization of the EGFR interactome reveals associated protein complex networks and intracellular receptor dynamics. *Proteomics* 2013; **13**:3131-3144.

79 Xu Z, Williams BR. The B56alpha regulatory subunit of protein phosphatase 2A is a target for regulation by double-stranded RNA-dependent protein kinase PKR. *Mol Cell Biol* 2000; **20**:5285-5299.

80 Bishop JD, Nien WL, Dauphinee SM, Too CK. Prolactin activates mammalian target-of-rapamycin through phosphatidylinositol 3-kinase and stimulates phosphorylation of p70S6K and 4E-binding protein-1 in lymphoma cells. *J Endocrinol* 2006; **190**:307-312.

81 Abdelmohsen K, Srikantan S, Yang X *et al.* Ubiquitin-mediated proteolysis of HuR by heat shock. *EMBO J* 2009; **28**:1271-1282.

82 Wu JQ, Hansen DV, Guo Y *et al.* Control of Emi2 activity and stability through Mos-mediated recruitment of PP2A. *Proc Natl Acad Sci U S A* 2007; **104**:16564-16569.

83 Keen JC, Zhou Q, Park BH *et al.* Protein phosphatase 2A regulates estrogen receptor alpha (ER) expression through modulation of ER mRNA stability. *J Biol Chem* 2005; **280**:29519-29524.

84 Lechward K, Zolnierowicz S, Hemmings BA. Eukaryotic translation termination factor 1 associates with protein phosphatase 2A and targets it to ribosomes. *Biochemistry (Mosc)* 1999; **64**:1373-1381.

85 Ewing RM, Chu P, Elisma F *et al.* Large-scale mapping of human protein-protein interactions by mass spectrometry. *Mol Syst Biol* 2007; **3**:89.

86 Narayanan U, Nalavadi V, Nakamoto M *et al.* FMRP phosphorylation reveals an immediate-early signaling pathway triggered by group I mGluR and mediated by PP2A. *J Neurosci* 2007; **27**:14349-14357.

87 Mao L, Yang L, Arora A *et al.* Role of protein phosphatase 2A in mGluR5-regulated MEK/ERK phosphorylation in neurons. *J Biol Chem* 2005; **280**:12602-12610.

88 Mitsuhashi S, Shima H, Tanuma N *et al.* Protein phosphatase type 2A, PP2A, is involved in degradation of gp130. *Mol Cell Biochem* 2005; **269**:183-187.

89 Bisson N, James DA, Ivosev G *et al.* Selected reaction monitoring mass spectrometry reveals the dynamics of signaling through the GRB2 adaptor. *Nat Biotechnol* 2011; **29**:653-658.

90 Hegarat N, Vesely C, Vinod PK *et al.* PP2A/B55 and Fcp1 Regulate Greatwall and Ensa Dephosphorylation during Mitotic Exit. *PLoS Genet* 2014; **10**:e1004004.

91 Wu Z, Chen Y, Yang T, Gao Q, Yuan M, Ma L. Targeted ubiquitination and degradation of G-protein-coupled receptor kinase 5 by the DDB1-CUL4 ubiquitin ligase complex. *PLoS One* 2012; **7**:e43997.

92 Liu L, Eisenman RN. Regulation of c-Myc Protein Abundance by a Protein Phosphatase 2A-Glycogen Synthase Kinase 3beta-Negative Feedback Pathway. *Genes Cancer* 2012; **3**:23-36.

93 Zhu D, Kosik KS, Meigs TE, Yanamadala V, Denker BM. Galpha12 directly interacts with PP2A: evidence FOR Galpha12-stimulated PP2A phosphatase activity and dephosphorylation of microtubule-associated protein, tau. *J Biol Chem* 2004; **279**:54983-54986.

94 Chowdhury D, Keogh MC, Ishii H, Peterson CL, Buratowski S, Lieberman J. gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Mol Cell* 2005; **20**:801-809.

95 Firulli BA, Howard MJ, McDaid JR *et al.* PKA, PKC, and the protein phosphatase 2A influence HAND factor function: a mechanism for tissue-specific transcriptional regulation. *Mol Cell* 2003; **12**:1225-1237.

96 Yeong FM, Hombauer H, Wendt KS *et al.* Identification of a subunit of a novel Kleisin-beta/SMC complex as a potential substrate of protein phosphatase 2A. *Curr Biol* 2003; **13**:2058-2064.

97 Paroni G, Cernotta N, Dello Russo C *et al.* PP2A regulates HDAC4 nuclear import. *Mol Biol Cell* 2008; **19**:655-667.

98 Joshi P, Greco TM, Guise AJ *et al.* The functional interactome landscape of the human histone deacetylase family. *Mol Syst Biol* 2013; **9**:672.

99 Greco TM, Yu F, Guise AJ, Cristea IM. Nuclear import of histone deacetylase 5 by requisite nuclear localization signal phosphorylation. *Mol Cell Proteomics* 2011; **10**:M110.004317.

100 Kawabe T, Muslin AJ, Korsmeyer SJ. HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. *Nature* 1997; **385**:454-458.

101 Adler HT, Nallaseth FS, Walter G, Tkachuk DC. HRX leukemic fusion proteins form a heterocomplex with the leukemia-associated protein SET and protein phosphatase 2A. *J Biol Chem* 1997; **272**:28407-28414.

102 Xing H, Hong Y, Sarge KD. Identification of the PP2A-interacting region of heat shock transcription factor 2. *Cell Stress Chaperones* 2007; **12**:192-197.

103 Kray AE, Carter RS, Pennington KN *et al.* Positive regulation of IkappaB kinase signaling by protein serine/threonine phosphatase 2A. *J Biol Chem* 2005; **280**:35974-35982.

104 Li S, Wang L, Berman MA, Zhang Y, Dorf ME. RNAi screen in mouse astrocytes identifies phosphatases that regulate NF-kappaB signaling. *Mol Cell* 2006; **24**:497-509.

105 Fu DX, Kuo YL, Liu BY, Jeang KT, Giam CZ. Human T-lymphotropic virus type I tax activates I-kappa B kinase by inhibiting I-kappa B kinase-associated serine/threonine protein phosphatase 2A. *J Biol Chem* 2003; **278**:1487-1493.

106 Takahashi K, Suzuki K. Regulation of protein phosphatase 2A-mediated recruitment of IQGAP1 to beta1 integrin by EGF through activation of Ca2+/calmodulin-dependent protein kinase II. *J Cell Physiol* 2006; **208**:213-219.

107 Dobierzewska A, Giltiay NV, Sabapathi S, Karakashian AA, Nikolova-Karakashian MN. Protein phosphatase 2A and neutral sphingomyelinase 2 regulate IRAK-1 protein ubiquitination and degradation in response to interleukin-1beta. *J Biol Chem* 2011; **286**:32064-32073.

108 Fuhrer DK, Yang YC. Complex formation of JAK2 with PP2A, P13K, and Yes in response to the hematopoietic cytokine interleukin-11. *Biochem Biophys Res Commun* 1996; **224**:289-296.

109 Shanley TP, Vasi N, Denenberg A, Wong HR. The serine/threonine phosphatase, PP2A: endogenous regulator of inflammatory cell signaling. *J Immunol* 2001; **166**:966-972.

110 Borsotto M, Cavarec L, Bouillot M *et al.* PP2A-Bgamma subunit and KCNQ2 K+ channels in bipolar disorder. *Pharmacogenomics J* 2007; **7**:123-132.

111 Tao GZ, Toivola DM, Zhou Q *et al.* Protein phosphatase-2A associates with and dephosphorylates keratin 8 after hyposmotic stress in a site- and cell-specific manner. *J Cell Sci* 2006; **119**:1425-1432.

112 Ory S, Zhou M, Conrads TP, Veenstra TD, Morrison DK. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr Biol* 2003; **13**:1356-1364.

113 Guo Z, Song E, Ma S *et al.* Proteomics strategy to identify substrates of LNX, a PDZ domain-containing E3 ubiquitin ligase. *J Proteome Res* 2012; **11**:4847-4862.

114 Prickett TD, Brautigan DL. Cytokine activation of p38 mitogen-activated protein kinase and apoptosis is opposed by alpha-4 targeting of protein phosphatase 2A for site-specific dephosphorylation of MEK3. *Mol Cell Biol* 2007; **27**:4217-4227.

115 Fritz A, Brayer KJ, McCormick N, Adams DG, Wadzinski BE, Vaillancourt RR. Phosphorylation of serine 526 is required for MEKK3 activity, and association with 14-3-3 blocks dephosphorylation. *J Biol Chem* 2006; **281**:6236-6245.

116 Short KM, Hopwood B, Yi Z, Cox TC. MID1 and MID2 homo- and heterodimerise to tether the rapamycin-sensitive PP2A regulatory subunit, alpha 4, to microtubules: implications for the clinical variability of X-linked Opitz GBBB syndrome and other developmental disorders. *BMC Cell Biol* 2002; **3**:1.

117 Avdi NJ, Malcolm KC, Nick JA, Worthen GS. A role for protein phosphatase-2A in p38 mitogen-activated protein kinase-mediated regulation of the c-Jun NH(2)-terminal kinase pathway in human neutrophils. *J Biol Chem* 2002; **277**:40687-40696.

118 Cannavo E, Gerrits B, Marra G, Schlapbach R, Jiricny J. Characterization of the interactome of the human MutL homologues MLH1, PMS1, and PMS2. *J Biol Chem* 2007; **282**:2976-2986.

119 Gordon J, Hwang J, Carrier KJ *et al.* Protein phosphatase 2a (PP2A) binds within the oligomerization domain of striatin and regulates the phosphorylation and activation of the mammalian Ste20-Like kinase Mst3. *BMC Biochem* 2011; **12**:54.

120 Peterson RT, Desai BN, Hardwick JS, Schreiber SL. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. *Proc Natl Acad Sci U S A* 1999; **96**:4438-4442.

121 Creyghton MP, Roel G, Eichhorn PJ *et al.* PR72, a novel regulator of Wnt signaling required for Naked cuticle function. *Genes Dev* 2005; **19**:376-386.

122 Creyghton MP, Roel G, Eichhorn PJ, Vredeveld LC, Destree O, Bernards R. PR130 is a modulator of the Wnt-signaling cascade that counters repression of the antagonist Naked cuticle. *Proc Natl Acad Sci U S A* 2006; **103**:5397-5402.

123 Tu LC, Yan X, Hood L, Lin B. Proteomics analysis of the interactome of N-myc downstream regulated gene 1 and its interactions with the androgen response program in prostate cancer cells. *Mol Cell Proteomics* 2007; **6**:575-588.

124 Surpili MJ, Delben TM, Kobarg J. Identification of proteins that interact with the central coiled-coil region of the human protein kinase NEK1. *Biochemistry* 2003; **42**:15369-15376.

125 Palkowitsch L, Leidner J, Ghosh S, Marienfeld RB. Phosphorylation of serine 68 in the IkappaB kinase (IKK)-binding domain of NEMO interferes with the structure of the IKK

complex and tumor necrosis factor-alpha-induced NF-kappaB activity. *J Biol Chem* 2008; **283**:76-86.

126 Bobulescu IA, Quinones H, Gisler SM *et al.* Acute regulation of renal Na+/H+ exchanger NHE3 by dopamine: role of protein phosphatase 2A. *Am J Physiol Renal Physiol* 2010; **298**:F1205-1213.

127 Liedtke CM, Wang X, Smallwood ND. Role for protein phosphatase 2A in the regulation of Calu-3 epithelial Na+-K+-2Cl-, type 1 co-transport function. *J Biol Chem* 2005; **280**:25491-25498.

128 Chen GI, Tisayakorn S, Jorgensen C, D'Ambrosio LM, Goudreault M, Gingras AC. PP4R4/KIAA1622 forms a novel stable cytosolic complex with phosphoprotein phosphatase 4. *J Biol Chem* 2008; **283**:29273-29284.

129 Chan SF, Sucher NJ. An NMDA receptor signaling complex with protein phosphatase 2A. *J Neurosci* 2001; **21**:7985-7992.

130 Nimmo ER, Stevens C, Phillips AM *et al.* TLE1 modifies the effects of NOD2 in the pathogenesis of Crohn's disease. *Gastroenterology* 2011; **141**:972-981.e971-972.

131 Sung U, Jennings JL, Link AJ, Blakely RD. Proteomic analysis of human norepinephrine transporter complexes reveals associations with protein phosphatase 2A anchoring subunit and 14-3-3 proteins. *Biochem Biophys Res Commun* 2005; **333**:671-678.

132 Yatim A, Benne C, Sobhian B *et al.* NOTCH1 nuclear interactome reveals key regulators of its transcriptional activity and oncogenic function. *Mol Cell* 2012; **48**:445-458.

133 Ma OK, Sucher NJ. Molecular interaction of NMDA receptor subunit NR3A with protein phosphatase 2A. *Neuroreport* 2004; **15**:1447-1450.

134 Satoh J, Kawana N, Yamamoto Y. Pathway Analysis of ChIP-Seq-Based NRF1 Target Genes Suggests a Logical Hypothesis of their Involvement in the Pathogenesis of Neurodegenerative Diseases. *Gene Regul Syst Bio* 2013; **7**:139-152.

135 Seth A, Sheth P, Elias BC, Rao R. Protein phosphatases 2A and 1 interact with occludin and negatively regulate the assembly of tight junctions in the CACO-2 cell monolayer. *J Biol Chem* 2007; **282**:11487-11498.

136 Wang PY, Weng J, Anderson RG. OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. *Science* 2005; **307**:1472-1476.

137 Voorhoeve PM, Hijmans EM, Bernards R. Functional interaction between a novel protein phosphatase 2A regulatory subunit, PR59, and the retinoblastoma-related p107 protein. *Oncogene* 1999; **18**:515-524.

138 Louis JV, Martens E, Borghgraef P *et al.* Mice lacking phosphatase PP2A subunit PR61/B'delta (Ppp2r5d) develop spatially restricted tauopathy by deregulation of CDK5 and GSK3beta. *Proc Natl Acad Sci U S A* 2011; **108**:6957-6962.

139 Dohoney KM, Guillerm C, Whiteford C *et al.* Phosphorylation of p53 at serine 37 is important for transcriptional activity and regulation in response to DNA damage. *Oncogene* 2004; **23**:49-57.

140 Li HH, Cai X, Shouse GP, Piluso LG, Liu X. A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *Embo j* 2007; **26**:402-411.

141 Westphal RS, Coffee RL, Jr., Marotta A, Pelech SL, Wadzinski BE. Identification of kinase-phosphatase signaling modules composed of p70 S6 kinase-protein phosphatase 2A (PP2A) and p21-activated kinase-PP2A. *J Biol Chem* 1999; **274**:687-692.

142 Scott GK, Gu F, Crump CM *et al.* The phosphorylation state of an autoregulatory domain controls PACS-1-directed protein traffic. *Embo j* 2003; **22**:6234-6244.

143 Ito A, Kataoka TR, Watanabe M *et al.* A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation. *Embo j* 2000; **19**:562-571.

144 Dodge-Kafka KL, Bauman A, Mayer N *et al.* cAMP-stimulated protein phosphatase 2A activity associated with muscle A kinase-anchoring protein (mAKAP) signaling complexes inhibits the phosphorylation and activity of the cAMP-specific phosphodiesterase PDE4D3. *J Biol Chem* 2010; **285**:11078-11086.

145 Sathyanarayanan S, Zheng X, Xiao R, Sehgal A. Posttranslational regulation of Drosophila PERIOD protein by protein phosphatase 2A. *Cell* 2004; **116**:603-615.

146 Ma J, Arnold HK, Lilly MB, Sears RC, Kraft AS. Negative regulation of Pim-1 protein kinase levels by the B56beta subunit of PP2A. *Oncogene* 2007; **26**:5145-5153.

147 Michniewicz M, Zago MK, Abas L *et al.* Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. *Cell* 2007; **130**:1044-1056.

148 Huang HK, Forsburg SL, John UP, O'Connell MJ, Hunter T. Isolation and characterization of the Pin1/Ess1p homologue in Schizosaccharomyces pombe. *J Cell Sci* 2001; **114**:3779-3788.

149 Ahn JH, McAvoy T, Rakhilin SV, Nishi A, Greengard P, Nairn AC. Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56delta subunit. *Proc Natl Acad Sci U S A* 2007; **104**:2979-2984.

150 Rual JF, Venkatesan K, Hao T *et al.* Towards a proteome-scale map of the human protein-protein interaction network. *Nature* 2005; **437**:1173-1178.

151 Wang W, Huang J, Wang X *et al.* PTPN14 is required for the density-dependent control of YAP1. *Genes Dev* 2012; **26**:1959-1971.

152 Kiely PA, O'Gorman D, Luong K, Ron D, O'Connor R. Insulin-like growth factor I controls a mutually exclusive association of RACK1 with protein phosphatase 2A and beta1 integrin to promote cell migration. *Mol Cell Biol* 2006; **26**:4041-4051.

153 Adams DG, Coffee RL, Jr., Zhang H, Pelech S, Strack S, Wadzinski BE. Positive regulation of Raf1-MEK1/2-ERK1/2 signaling by protein serine/threonine phosphatase 2A holoenzymes. *J Biol Chem* 2005; **280**:42644-42654.

154 Lees JA, Buchkovich KJ, Marshak DR, Anderson CW, Harlow E. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. *EMBO J* 1991; **10**:4279-4290.

155 Magenta A, Fasanaro P, Romani S, Di Stefano V, Capogrossi MC, Martelli F. Protein phosphatase 2A subunit PR70 interacts with pRb and mediates its dephosphorylation. *Mol Cell Biol* 2008; **28**:873-882.

156 Ishiguro T, Tanaka K, Sakuno T, Watanabe Y. Shugoshin-PP2A counteracts casein-kinase-1-dependent cleavage of Rec8 by separase. *Nat Cell Biol* 2010; **12**:500-506.

157 Yang J, Fan GH, Wadzinski BE, Sakurai H, Richmond A. Protein phosphatase 2A interacts with and directly dephosphorylates RelA. *J Biol Chem* 2001; **276**:47828-47833.

158 Qian J, Beullens M, Lesage B, Bollen M. Aurora B defines its own chromosomal targeting by opposing the recruitment of the phosphatase scaffold Repo-Man. *Curr Biol* 2013; **23**:1136-1143.

159 Naji S, Ambrus G, Cimermancic P *et al.* Host cell interactome of HIV-1 Rev includes RNA helicases involved in multiple facets of virus production. *Mol Cell Proteomics* 2012; **11**:M111.015313.

160 Lee WJ, Kim DU, Lee MY, Choi KY. Identification of proteins interacting with the catalytic subunit of PP2A by proteomics. *Proteomics* 2007; **7**:206-214.

161 Mayer C, Zhao J, Yuan X, Grummt I. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev* 2004; **18**:423-434.

162 Schlaitz AL, Srayko M, Dammermann A *et al.* The C. elegans RSA complex localizes protein phosphatase 2A to centrosomes and regulates mitotic spindle assembly. *Cell* 2007; **128**:115-127.

163 Rajgopal A, Young DW, Mujeeb KA *et al.* Mitotic control of RUNX2 phosphorylation by both CDK1/cyclin B kinase and PP1/PP2A phosphatase in osteoblastic cells. *J Cell Biochem* 2007; **100**:1509-1517.

164 Gil-Bernabe AM, Romero F, Limon-Mortes MC, Tortolero M. Protein phosphatase 2A stabilizes human securin, whose phosphorylated forms are degraded via the SCF ubiquitin ligase. *Mol Cell Biol* 2006; **26**:4017-4027.

165 Holland AJ, Bottger F, Stemmann O, Taylor SS. Protein phosphatase 2A and separase form a complex regulated by separase autocleavage. *J Biol Chem* 2007; **282**:24623-24632.

166 Bauman AL, Apparsundaram S, Ramamoorthy S, Wadzinski BE, Vaughan RA, Blakely RD. Cocaine and antidepressant-sensitive biogenic amine transporters exist in regulated complexes with protein phosphatase 2A. *J Neurosci* 2000; **20**:7571-7578.

167 Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem* 1996; **271**:11059-11062.

168 Moreno CS, Park S, Nelson K *et al.* WD40 Repeat Proteins Striatin and S/G2 Nuclear Autoantigen Are Members of a Novel Family of Calmodulin-binding Proteins That Associate with Protein Phosphatase 2A. *J Biol Chem* 2000; **275**:5257-5263.

169 Ugi S, Imamura T, Ricketts W, Olefsky JM. Protein phosphatase 2A forms a molecular complex with Shc and regulates Shc tyrosine phosphorylation and downstream mitogenic signaling. *Mol Cell Biol* 2002; **22**:2375-2387.

170 Pitman MR, Barr RK, Gliddon BL, Magarey AM, Moretti PA, Pitson SM. A critical role for the protein phosphatase 2A B'alpha regulatory subunit in dephosphorylation of sphingosine kinase 1. *Int J Biochem Cell Biol* 2011; **43**:342-347.

171 Heikkinen PT, Nummela M, Leivonen SK *et al.* Hypoxia-activated Smad3-specific dephosphorylation by PP2A. *J Biol Chem* 2010; **285**:3740-3749.

172 Colland F, Jacq X, Trouplin V *et al.* Functional proteomics mapping of a human signaling pathway. *Genome Res* 2004; **14**:1324-1332.

173 Xie Y, Avello M, Schirle M *et al.* Deubiquitinase FAM/USP9X interacts with the E3 ubiquitin ligase SMURF1 protein and protects it from ligase activity-dependent self-degradation. *J Biol Chem* 2013; **288**:2976-2985.

174 Cox JL, Wilder PJ, Gilmore JM, Wuebben EL, Washburn MP, Rizzino A. The SOX2-interactome in brain cancer cells identifies the requirement of MSI2 and USP9X for the growth of brain tumor cells. *PLoS One* 2013; **8**:e62857.

175 Chuang JY, Wang SA, Yang WB *et al.* Sp1 phosphorylation by cyclin-dependent kinase 1/cyclin B1 represses its DNA-binding activity during mitosis in cancer cells. *Oncogene* 2012; **31**:4946-4959.

176 Lao DH, Yusoff P, Chandramouli S *et al.* Direct binding of PP2A to Sprouty2 and phosphorylation changes are a prerequisite for ERK inhibition downstream of fibroblast growth factor receptor stimulation. *J Biol Chem* 2007; **282**:9117-9126.

177 Eichhorn PJ, Creyghton MP, Wilhelmsen K, van Dam H, Bernards R. A RNA interference screen identifies the protein phosphatase 2A subunit PR55gamma as a stress-sensitive inhibitor of c-SRC. *PLoS Genet* 2007; **3**:e218.

178 Yokoyama N, Reich NC, Miller WT. Involvement of protein phosphatase 2A in the interleukin-3-stimulated Jak2-Stat5 signaling pathway. *J Interferon Cytokine Res* 2001; **21**:369-378.

179 Grant MM. Identification of SUMOylated proteins in neuroblastoma cells after treatment with hydrogen peroxide or ascorbate. *BMB Rep* 2010; **43**:720-725.

180 Wainszelbaum MJ, Liu J, Kong C *et al.* TBC1D3, a hominoid-specific gene, delays IRS-1 degradation and promotes insulin signaling by modulating p70 S6 kinase activity. *PLoS One* 2012; **7**:e31225.

181 Sowa ME, Bennett EJ, Gygi SP, Harper JW. Defining the human deubiquitinating enzyme interaction landscape. *Cell* 2009; **138**:389-403.

182 Griswold-Prenner I, Kamibayashi C, Maruoka EM, Mumby MC, Derynck R. Physical and functional interactions between type I transforming growth factor beta receptors and Balpha, a WD-40 repeat subunit of phosphatase 2A. *Mol Cell Biol* 1998; **18**:6595-6604.

183 Saraf A, Oberg EA, Strack S. Molecular determinants for PP2A substrate specificity: charged residues mediate dephosphorylation of tyrosine hydroxylase by the PP2A/B' regulatory subunit. *Biochemistry* 2010; **49**:986-995.

184 McConnell JL, Gomez RJ, McCorvey LR, Law BK, Wadzinski BE. Identification of a PP2A-interacting protein that functions as a negative regulator of phosphatase activity in the ATM/ATR signaling pathway. *Oncogene* 2007; **26**:6021-6030.

185 Dagda RK, Barwacz CA, Cribbs JT, Strack S. Unfolding-resistant translocase targeting: a novel mechanism for outer mitochondrial membrane localization exemplified by the Bbeta2 regulatory subunit of protein phosphatase 2A. *J Biol Chem* 2005; **280**:27375-27382.

186 Trzcinska AM, Girstun A, Piekielko A, Kowalska-Loth B, Staron K. Potential protein partners for the N-terminal domain of human topoisomerase I revealed by phage display. *Mol Biol Rep* 2002; **29**:347-352.

187 Sun L, Stoecklin G, Van Way S *et al.* Tristetraprolin (TTP)-14-3-3 complex formation protects TTP from dephosphorylation by protein phosphatase 2a and stabilizes tumor necrosis factor-alpha mRNA. *J Biol Chem* 2007; **282**:3766-3777.

188 Aichem A, Kalveram B, Spinnenhirn V *et al.* The proteomic analysis of endogenous FAT10 substrates identifies p62/SQSTM1 as a substrate of FAT10ylation. *J Cell Sci* 2012; **125**:4576-4585.

189 Chen HW, Yang CC, Hsieh CL, Liu H, Lee SC, Tan BC. A functional genomic approach reveals the transcriptional role of EDD in the expression and function of angiogenesis regulator ACVRL1. *Biochim Biophys Acta* 2013; **1829**:1309-1319.

190 Ohnishi T, Yamashita A, Kashima I *et al.* Phosphorylation of hUPF1 induces formation of mRNA surveillance complexes containing hSMG-5 and hSMG-7. *Mol Cell* 2003; **12**:1187-1200.

191 Turowski P, Myles T, Hemmings BA, Fernandez A, Lamb NJ. Vimentin dephosphorylation by protein phosphatase 2A is modulated by the targeting subunit B55. *Mol Biol Cell* 1999; **10**:1997-2015.

192 Jager S, Cimermancic P, Gulbahce N *et al.* Global landscape of HIV-human protein complexes. *Nature* 2012; **481**:365-370.

193 Tran H, Bustos D, Yeh R *et al.* HectD1 E3 ligase modifies adenomatous polyposis coli (APC) with polyubiquitin to promote the APC-axin interaction. *J Biol Chem* 2013; **288**:3753-3767.

194 Curtis M, Nikolopoulos SN, Turner CE. Actopaxin is phosphorylated during mitosis and is a substrate for cyclin B1/cdc2 kinase. *Biochem J* 2002; **363**:233-242.

195 Floyd SR, Porro EB, Slepnev VI, Ochoa GC, Tsai LH, De Camilli P. Amphiphysin 1 binds the cyclin-dependent kinase (cdk) 5 regulatory subunit p35 and is phosphorylated by cdk5 and cdc2. *J Biol Chem* 2001; **276**:8104-8110.

196 Konishi Y, Lehtinen M, Donovan N, Bonni A. Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. *Mol Cell* 2002; **9**:1005-1016.

197 Hayami R, Sato K, Wu W *et al.* Down-regulation of BRCA1-BARD1 ubiquitin ligase by CDK2. *Cancer Res* 2005; **65**:6-10.

198 Fourest-Lieuvin A, Peris L, Gache V *et al.* Microtubule regulation in mitosis: tubulin phosphorylation by the cyclin-dependent kinase Cdk1. *Mol Biol Cell* 2006; **17**:1041-1050.

199 Saville MK, Watson RJ. The cell-cycle regulated transcription factor B-Myb is phosphorylated by cyclin A/Cdk2 at sites that enhance its transactivation properties. *Oncogene* 1998; **17**:2679-2689.

200 Esashi F, Christ N, Gannon J *et al.* CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. *Nature* 2005; **434**:598-604.

201 Mak AS, Carpenter M, Smillie LB, Wang JH. Phosphorylation of caldesmon by p34cdc2 kinase. Identification of phosphorylation sites. *J Biol Chem* 1991; **266**:19971-19975.

202 Yudkovsky Y, Shteinberg M, Listovsky T, Brandeis M, Hershko A. Phosphorylation of Cdc20/fizzy negatively regulates the mammalian cyclosome/APC in the mitotic checkpoint. *Biochem Biophys Res Commun* 2000; **271**:299-304.

203 Garrett S, Barton WA, Knights R, Jin P, Morgan DO, Fisher RP. Reciprocal activation by cyclin-dependent kinases 2 and 7 is directed by substrate specificity determinants outside the T loop. *Mol Cell Biol* 2001; **21**:88-99.

204 Bosc DG, Slominski E, Sichler C, Litchfield DW. Phosphorylation of casein kinase II by p34cdc2. Identification of phosphorylation sites using phosphorylation site mutants in vitro. *J Biol Chem* 1995; **270**:25872-25878.

205 He J, Xu J, Xu XX, Hall RA. Cell cycle-dependent phosphorylation of Disabled-2 by cdc2. *Oncogene* 2003; **22**:4524-4530.

206 Frouin I, Toueille M, Ferrari E, Shevelev I, Hubscher U. Phosphorylation of human DNA polymerase lambda by the cyclin-dependent kinase Cdk2/cyclin A complex is modulated by its association with proliferating cell nuclear antigen. *Nucleic Acids Res* 2005; **33**:5354-5361.

207 Noguchi E, Shanahan P, Noguchi C, Russell P. CDK phosphorylation of Drc1 regulates DNA replication in fission yeast. *Curr Biol* 2002; **12**:599-605.

208 Addinall SG, Mayr PS, Doyle S, Sheehan JK, Woodman PG, Allan VJ. Phosphorylation by cdc2-CyclinB1 kinase releases cytoplasmic dynein from membranes. *J Biol Chem* 2001; **276**:15939-15944.

209 Milner RE, Busaan JL, Holmes CF, Wang JH, Michalak M. Phosphorylation of dystrophin. The carboxyl-terminal region of dystrophin is a substrate for in vitro phosphorylation by p34cdc2 protein kinase. *J Biol Chem* 1993; **268**:21901-21905.

210 Hara T, Abe M, Inoue H *et al.* Cytokinesis regulator ECT2 changes its conformation through phosphorylation at Thr-341 in G2/M phase. *Oncogene* 2006; **25**:566-578.

211 Belle R, Cormier P, Poulhe R, Morales J, Huchon D, Mulner-Lorillon O. Protein phosphorylation during meiotic maturation of Xenopus oocytes: cdc2 protein kinase targets. *Int J Dev Biol* 1990; **34**:111-115.

212 Mi J, Qiao F, Wilson JB *et al.* FANCG is phosphorylated at serines 383 and 387 during mitosis. *Mol Cell Biol* 2004; **24**:8576-8585.

213 Luscher-Firzlaff JM, Lilischkis R, Luscher B. Regulation of the transcription factor FOXM1c by Cyclin E/CDK2. *FEBS Lett* 2006; **580**:1716-1722.

214 Huang H, Regan KM, Lou Z, Chen J, Tindall DJ. CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. *Science* 2006; **314**:294-297.

215 Lowe M, Rabouille C, Nakamura N *et al.* Cdc2 kinase directly phosphorylates the cis-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. *Cell* 1998; **94**:783-793.

216 Lin CY, Madsen ML, Yarm FR, Jang YJ, Liu X, Erikson RL. Peripheral Golgi protein GRASP65 is a target of mitotic polo-like kinase (Plk) and Cdc2. *Proc Natl Acad Sci U S A* 2000; **97**:12589-12594.

217 Yoon HJ, Feoktistova A, Chen JS, Jennings JL, Link AJ, Gould KL. Role of Hcn1 and its phosphorylation in fission yeast anaphase-promoting complex/cyclosome function. *J Biol Chem* 2006; **281**:32284-32293.

218 Sarcevic B, Mawson A, Baker RT, Sutherland RL. Regulation of the ubiquitin-conjugating enzyme hHR6A by CDK-mediated phosphorylation. *EMBO J* 2002; **21**:2009-2018.

219 Hall C, Nelson DM, Ye X *et al.* HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. *Mol Cell Biol* 2001; **21**:1854-1865.

220 Meijer L, Arion D, Golsteyn R *et al.* Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. *EMBO J* 1989; **8**:2275-2282.

221 Nissen MS, Langan TA, Reeves R. Phosphorylation by cdc2 kinase modulates DNA binding activity of high mobility group I nonhistone chromatin protein. *J Biol Chem* 1991; **266**:19945-19952.

222 Deng W, Lin BY, Jin G *et al.* Cyclin/CDK regulates the nucleocytoplasmic localization of the human papillomavirus E1 DNA helicase. *J Virol* 2004; **78**:13954-13965.

223 St Onge RP, Besley BD, Pelley JL, Davey S. A role for the phosphorylation of hRad9 in checkpoint signaling. *J Biol Chem* 2003; **278**:26620-26628.

224 Masai H, Matsui E, You Z, Ishimi Y, Tamai K, Arai K. Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 bY Cdks. *J Biol Chem* 2000; **275**:29042-29052.

225 Liakopoulos D, Kusch J, Grava S, Vogel J, Barral Y. Asymmetric loading of Kar9 onto spindle poles and microtubules ensures proper spindle alignment. *Cell* 2003; **112**:561-574.

226 Ohsugi M, Tokai-Nishizumi N, Shiroguchi K, Toyoshima YY, Inoue J, Yamamoto T. Cdc2-mediated phosphorylation of Kid controls its distribution to spindle and chromosomes. *EMBO J* 2003; **22**:2091-2103.

227 Blangy A, Lane HA, d'Herin P, Harper M, Kress M, Nigg EA. Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. *Cell* 1995; **83**:1159-1169.

228 Peter M, Nakagawa J, Doree M, Labbe JC, Nigg EA. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 1990; **61**:591-602.

229 Aizawa H, Kamijo M, Ohba Y *et al.* Microtubule destabilization by cdc2/H1 histone kinase: phosphorylation of a "pro-rich region" in the microtubule-binding domain of MAP-4. *Biochem Biophys Res Commun* 1991; **179**:1620-1626.

230 Manenti S, Yamauchi E, Sorokine O *et al.* Phosphorylation of the myristoylated protein kinase C substrate MARCKS by the cyclin E-cyclin-dependent kinase 2 complex in vitro. *Biochem J* 1999; **340 (Pt 3)**:775-782.

231 Montagnoli A, Valsasina B, Brotherton D *et al.* Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. *J Biol Chem* 2006; **281**:10281-10290.

232 Elias B, Laine A, Ronai Z. Phosphorylation of MdmX by CDK2/Cdc2(p34) is required for nuclear export of Mdm2. *Oncogene* 2005; **24**:2574-2579.

233 Miyazaki Y, Boccuni P, Mao S *et al.* Cyclin A-dependent phosphorylation of the ETS-related protein, MEF, restricts its activity to the G1 phase of the cell cycle. *J Biol Chem* 2001; **276**:40528-40536.

234 Jaspersen SL, Huneycutt BJ, Giddings TH, Jr., Resing KA, Ahn NG, Winey M. Cdc28/Cdk1 regulates spindle pole body duplication through phosphorylation of Spc42 and Mps1. *Dev Cell* 2004; **7**:263-274.

235 Kitzmann M, Vandromme M, Schaeffer V *et al.* cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. *Mol Cell Biol* 1999; **19**:3167-3176.

236 Mori D, Yano Y, Toyo-oka K *et al.* NDEL1 phosphorylation by Aurora-A kinase is essential for centrosomal maturation, separation, and TACC3 recruitment. *Mol Cell Biol* 2007; **27**:352-367.

237 Queralt E, Lehane C, Novak B, Uhlmann F. Downregulation of PP2A(Cdc55) phosphatase by separase initiates mitotic exit in budding yeast. *Cell* 2006; **125**:719-732.

238 Chae HD, Yun J, Bang YJ, Shin DY. Cdk2-dependent phosphorylation of the NF-Y transcription factor is essential for the expression of the cell cycle-regulatory genes and cell cycle G1/S and G2/M transitions. *Oncogene* 2004; **23**:4084-4088.

239 Litvak V, Argov R, Dahan N *et al.* Mitotic phosphorylation of the peripheral Golgi protein Nir2 by Cdk1 provides a docking mechanism for Plk1 and affects cytokinesis completion. *Mol Cell* 2004; **14**:319-330.

240 Ma T, Van Tine BA, Wei Y *et al.* Cell cycle-regulated phosphorylation of p220(NPAT) by cyclin E/Cdk2 in Cajal bodies promotes histone gene transcription. *Genes Dev* 2000; **14**:2298-2313.

241 Ostvold AC, Norum JH, Mathiesen S, Wanvik B, Sefland I, Grundt K. Molecular cloning of a mammalian nuclear phosphoprotein NUCKS, which serves as a substrate for Cdk1 in vivo. *Eur J Biochem* 2001; **268**:2430-2440.

242 Thiel DA, Reeder MK, Pfaff A, Coleman TR, Sells MA, Chernoff J. Cell cycle-regulated phosphorylation of p21-activated kinase 1. *Curr Biol* 2002; **12**:1227-1232.

243 Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev* 1997; **11**:1464-1478.

244 Li M, Stefansson B, Wang W, Schaefer EM, Brautigan DL. Phosphorylation of the Pro-X-Thr-Pro site in phosphatase inhibitor-2 by cyclin-dependent protein kinase during M-phase of the cell cycle. *Cell Signal* 2006; **18**:1318-1326.

245 van der Sluijs P, Hull M, Huber LA, Male P, Goud B, Mellman I. Reversible phosphorylation--dephosphorylation determines the localization of rab4 during the cell cycle. *EMBO J* 1992; **11**:4379-4389.

246 Hutchins JR, Moore WJ, Hood FE *et al.* Phosphorylation regulates the dynamic interaction of RCC1 with chromosomes during mitosis. *Curr Biol* 2004; **14**:1099-1104.

247 Gupte RS, Traganos F, Darzynkiewicz Z, Lee MY. Phosphorylation of RIalpha by cyclin-dependent kinase CDK 2/cyclin E modulates the dissociation of the RIalpha-RFC40 complex. *Cell Cycle* 2006; **5**:653-660.

248 Chan AK, Persad S, Litchfield DW, Wright JA. Ribonucleotide reductase R2 protein is phosphorylated at serine-20 by P34cdc2 kinase. *Biochim Biophys Acta* 1999; **1448**:363-371.

249 Marcelain K, Hayman MJ. The Ski oncoprotein is upregulated and localized at the centrosomes and mitotic spindle during mitosis. *Oncogene* 2005; **24**:4321-4329.

250 Beretta L, Dobransky T, Sobel A. Multiple phosphorylation of stathmin. Identification of four sites phosphorylated in intact cells and in vitro by cyclic AMP-dependent protein kinase and p34cdc2. *J Biol Chem* 1993; **268**:20076-20084.

251 Koseoglu MM, Graves LM, Marzluff WF. Phosphorylation of threonine 61 by cyclin a/Cdk1 triggers degradation of stem-loop binding protein at the end of S phase. *Mol Cell Biol* 2008; **28**:4469-4479.

252 O'Connor DS, Grossman D, Plescia J *et al.* Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc Natl Acad Sci U S A* 2000; **97**:13103-13107.

253 Fujibuchi T, Abe Y, Takeuchi T *et al.* Expression and phosphorylation of TOPK during spermatogenesis. *Dev Growth Differ* 2005; **47**:637-644.

254 Voit R, Hoffmann M, Grummt I. Phosphorylation by G1-specific cdk-cyclin complexes activates the nucleolar transcription factor UBF. *EMBO J* 1999; **18**:1891-1899.

255 Morisaki T, Hirota T, Iida S *et al.* WARTS tumor suppressor is phosphorylated by Cdc2/cyclin B at spindle poles during mitosis. *FEBS Lett* 2002; **529**:319-324.

256 Chiolo I, Carotenuto W, Maffioletti G, Petrini JH, Foiani M, Liberi G. Srs2 and Sgs1 DNA helicases associate with Mre11 in different subcomplexes following checkpoint activation and CDK1-mediated Srs2 phosphorylation. *Mol Cell Biol* 2005; **25**:5738-5751.

257 Watanabe N, Arai H, Iwasaki J *et al.* Cyclin-dependent kinase (CDK) phosphorylation destabilizes somatic Wee1 via multiple pathways. *Proc Natl Acad Sci U S A* 2005; **102**:11663-11668.

Chapter V

Future Directions, Discussion, and Overall Conclusions

Future Directions

Many open questions remain in the PP2A field. The experiments discussed in this thesis have shed a considerable amount of light on some of these questions and simultaneously brought up new ones. Three gaps are discussed here regarding the mechanism of holoenzyme in specific substrate recognition. Accordingly, three experiments are proposed to address the gaps in understanding how the regulatory subunits enhance phosphatase activity and control substrate specificity.

Structural basis of substrate recognition by PP2A holoenzymes. The structural basis for enhancement of PP2A holoenzyme phosphatase activity toward specific substrates remains to be elucidated. In the manuscript published from chapters II and III, we proposed that substrates interacted with PP2A based on patterns of charge recognition[1]. There is some evidence for this mechanism[2], and it has been suggested for other substrates as well[3], but more experimental evidence is needed to support this notion. Obtaining a co-crystal structure of a substrate bound to the regulatory subunit, and preferably with the C-subunit in the holoenzyme form, would be invaluable in ascertaining the mechanism of substrate binding. No holoenzyme structure has been solved to date without an inhibitor[3-5], making this a challenging prospect. The difficulty in finding suitable substrates for obtaining a co-crystal structure is hampered, in part, by a lack of biochemically characterized substrates. The assay described in chapter IV is one such method of characterizing these substrates in a high-throughput manner and may be useful for discovering ideal candidates for crystallization.

The design described in chapter III did not deliver a co-crystal structure as anticipated. The construct does produce crystals at 4^{o}C with no added inhibitor; however, these are quite fragile and do not diffract x-rays. With the success of obtaining the holoenzyme structure (even

if the peptide was not visible) and the success of obtaining crystals without an inhibitor, the method, with some modification, still holds promises to obtain the structure. I would propose the production and treatment of various constructs to be used for holoenzyme assembly (Figure 5.1). The current PR70 structure ends shortly after the interacting helix between it and the C-subunit. To provide more surface for substrate interaction and possibly increased binding affinity, the PR70 core to be used (122-519) will be slightly larger than that used for the current structure (122-490). Fused to this will be a fragment of Cdc6 from 49-90 which encompasses the PP2A binding motif as well as both phospho-sites. As it is unknown which phospho-site is dephosphorylated by PP2A and whether the other one is required to be dephosphorylated or phosphorylated prior to binding, non-phosphorylatable mutants as well as phospho-mimicing mutants will be used along with the wild-type. These constructs may also be phosphorylated with ATP-γS in order to see the substrate locked into the active site. The PR70 fusion proteins will be assembled with AC as described in chapter III except without the addition of MCLR. Constructs will be initially screened at 18^{o}C and possibly at 4^{o}C if needed. Any diffracting crystals will be processed as before and the structure determined by molecular replacement.

If a structure is obtained, it will likely produce much information as to the nature of PP2A-substrate binding. Based on the data obtained previously[1], I would predict that Cdc6 will make salt bridge and hydrogen bond contacts with various residues on the surface of PR70 facing the active site and a few residues might bind to the C subunit to help position a phospho-serine into the active site. Studies along this line can be applied to other regulatory subunit/holoenzyme-substrate interactions such that insights into general principles can be gained for guiding the understanding of PP2A function in cell signaling.

Biochemical Understanding of Substrate Restriction – Charge Interactions and Steric Hindrance. In addition to the enhancement of phosphatase activity, the mechanisms by which the regulatory subunits control substrate specificity need to be further investigated. The data shown here indicate that in the case of Cdc6, steric hindrance by the B' family of holoenzymes prevents dephosphorylation. It is unknown if a similar result would be obtained using the B or B''' families of holoenzymes. Furthermore, other substrates for other families of PP2A holoenzymes need to be tested to determine if this effect is unique to Cdc6 or if it is a general mechanism.

If the previous experiment produces a structure, the testing of the charged interactions between Cdc6 and PR70 will proceed quite easily in a similar fashion to the mutagenic analysis described in chapter III. If that is not the case, there are a limited number of residues in the PR70 binding motif of Cdc6 that could be mutated to test their interactions (Figure 5.2). An analysis of the residues on PR70 nearest to the binding site will be used to find candidate residues responsible for Cdc6 interaction. These residues will be mutated and tested for binding with pull-down assays as above.

One important question regarding charge interaction and steric hindrance is whether it is a conserved mechanism within and between families. PP2A-PR70 can also dephosphorylate Rb[6], and the same battery of assays described in chapters II and III would be useful in assessing if this is a conserved function with PR70. One difficulty in assessing Rb is the fact that other holoenzymes can dephosphorylate certain Rb residues as well[7]. Residues known to be dephosphorylated by other B subunits would need to be mutated to phospho-mimics (D) to account for non-specific dephosphorylation by B' subunits when comparing their activity to that of PR70. In addition to testing other PR70 substrates, holoenzymes from the B and B' family

will be tested against substrates from different families. For example, substrates such as tau and tyrosine hydroxylase would be tested with the PR70 holoenzyme and others to determine if they can also dephosphorylate these substrates.

I would predict that specific charge patterning for substrate binding is a conserved mechanism for enhancing substrate dephosphorylation, and that steric hindrance is a conserved mechanism by which the regulatory subunits restrict substrate specificity. I would predict that the patterns are family dependent but within the family there are enough specific changes to accommodate a wide range of substrates. These results combined with the high-throughput characterization described in chapter IV may be able to elucidate some general patterns of PP2A-substrate interactions. This would be useful in predicting new substrates and small molecule binding sites that would be useful for therapeutic intervention.

Elucidating Mechanisms of PP2A Regulation *in vivo*. In addition to biochemical characterization, the *in vivo* mechanisms of PP2A regulation require further investigation. One of the difficulties with *in vivo* investigation is the fact that some B subunits form holoenzymes that dephosphorylate multiple substrates. For example, Rb and Cdc6 are both dephosphorylated by PP2A-PR70[6, 8]. If PR70 is knocked down, it may be difficult to ascertain if the resulting observations are due to loss of dephosphorylation toward Rb or toward Cdc6. To further complicate matters, many PP2A substrates are involved in the same pathways; for example, Cdc6 is transcribed by E2F responsive genes therefore an effect on Rb activity may also affect Cdc6[9]. In order to design better *in vivo* experiments, more biochemical information is needed on specific PP2A-substrate interactions.

Many PP2A-substrate interactions have been discovered *in vivo*, but few have explored the *in vivo* mechanisms by which PP2A regulates each substrate. Furthermore, despite an

expanding body of *in vitro* biochemical characterization, few of these ideas have been tested in cells. Part of that is due to the difficulty of knocking-down or over-expressing PP2A subunits in cells. One alternative approach would be to use the information obtained by either the co-crystal study or the biochemical characterization experiments. I propose to use this information to create mutated B constructs which are defective in binding to very specific substrates. These mutant B subunits will be expressed in human cells in culture and the WT PR70 silenced using siRNA. The effects on substrate stability, changes in gene transcription or other known markers of substrate integrity, cell cycle stability, and transforming potential will be measured. I would predict that disruption of specific substrate dephosphorylation would cause subtle but significant effects. Global disruption of a protein affects multiple pathways and it can be hard to ascertain which pathway disruption is causing a specific phenotype; however, very specific disruption can identify which pathway led to the observed phenotype with more certainty. For example, if all Rb dephosphorylation is inhibited, it would be expected that many E2F responsive genes are transcribed and the cells will quickly move into S phase. If only PP2A-PR70 dephosphorylation is inhibited, the phenotype will likely be less dramatic, perhaps only being affected by minor changes in the timing of cell division, or a dramatically altered phenotype will only present itself under specific conditions such as oxidative stress. In the case of Cdc6, a specific disruption would determine at what stage of the cell cycle Cdc6 is dephosphorylated and destroyed without the disruption of Rb which might result in off-target effects. Although it is difficult to predict exact outcomes of specific changes in the cell cycle, experiments such as this offer very accurate ways of predicting which pathways are involved when a particular substrate is not dephosphorylated. It may be possible that redundant mechanisms exist in the cell, and the use of controls for multiple holoenzymes will be crucial in this regard.

General Conclusions

PP2A is a complex and diverse group of phosphatases. It has been globally implicated as a tumor suppressor and interacts with a large variety of substrates[10]. Since PP2A is deregulated in many cancers and some neural disease, the importance of studying it is of paramount significance. There are over 250 known PP2A substrates and more to be discovered (Chapter IV). The genes for PP2A regulatory subunits transcribe 26 different products and can maximally form 100 different holoenzymes. With a small group of phosphatases interacting with a large amount of substrates, some holoenzymes must dephosphorylate multiple substrates. As there is no known PP2A-binding consensus sequences, information regarding substrate binding must be obtained by looking at the holoenzyme structure. Only three holoenzyme structures have been discovered so far[1, 3, 4], and none of them with a substrate bound to the active site. In addition, the full characterization of PP2A substrates has proceeded at a slow pace and there are many gaps in knowledge (Table 4.1). Kinases have been more thoroughly studied than phosphatases, especially in the context of treatments for cancer. Although kinase inhibitors have shown some clinical promise, many are not the "silver bullet" many thought they would be. Part of this is the underlying complexity of the cell cycle and its redundant mechanisms, and part of this is failing to fully understand the complex regulation of phosphorylation in the cell. It is not possible to study the cell cycle without acknowledging the critical role that PP2A plays in its regulation. This role will likely become even more apparent as new studies implicate PP2A in an increasing amount of regulatory networks. For a long time, PP2A was thought of as a simple housekeeping enzyme, but this was only due to our inability to understand the complexities of its interactions and regulation[11]. We now have the tools to study this increasingly important enzyme in detail and use that information to design better treatments for human diseases.

Figures

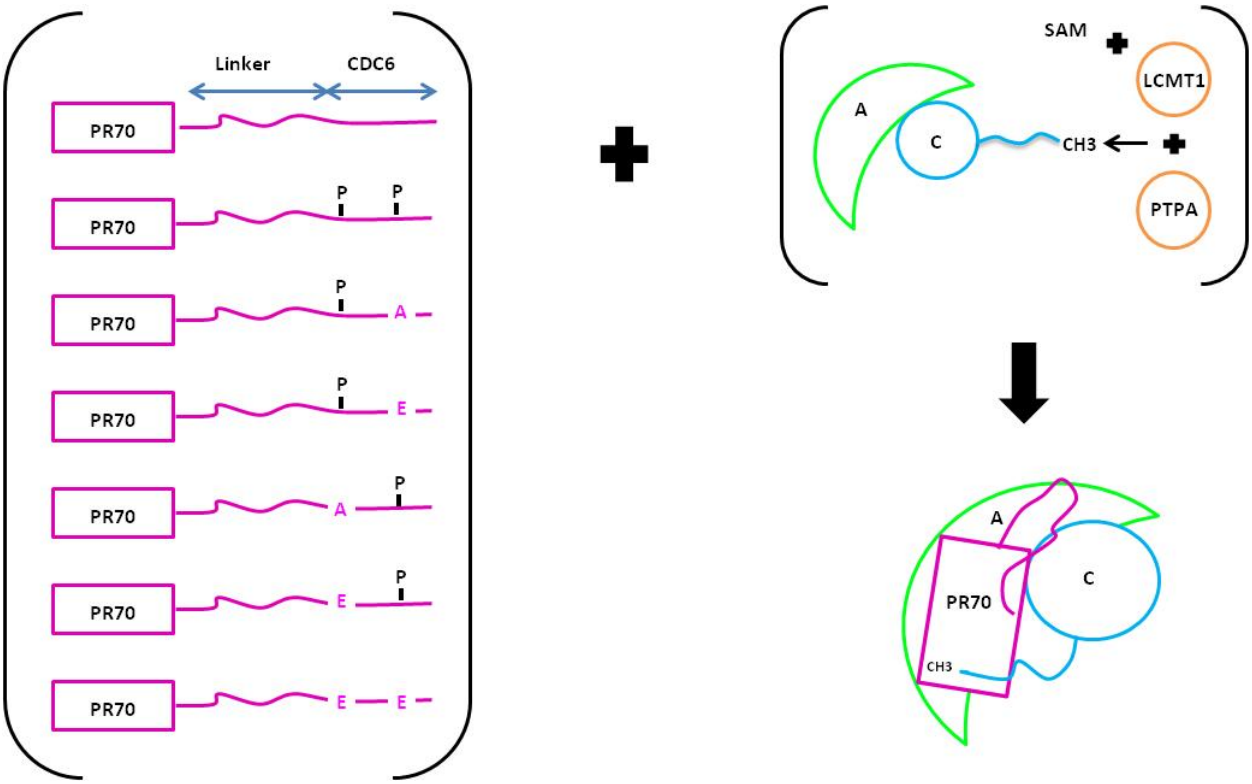


Figure 5.1. Proposed design of holoenzyme constructs for crystallization. PR70 holoenzyme will be prepared similarly to that as described in figure 3.1 with the following modifications. Instead of using PR70 122-490 and Cdc6 70-90, PR70 122-519 and Cdc6 49-90 will be used in the fusion constructs. The following modifications of the Cdc6 sequence will be used: WT, phosphorylated WT, phosphorylated S74A, phosphorylated S74E, phosphorylated S54A, phosphorylated S54E, S54E & S74E. All phosphorylation will be performed with ATPγS. These variations will hopefully account for potential differences in binding relative to phosphorylation status and will hopefully produce a structure with a non-hydrolyzable moiety in the active site.

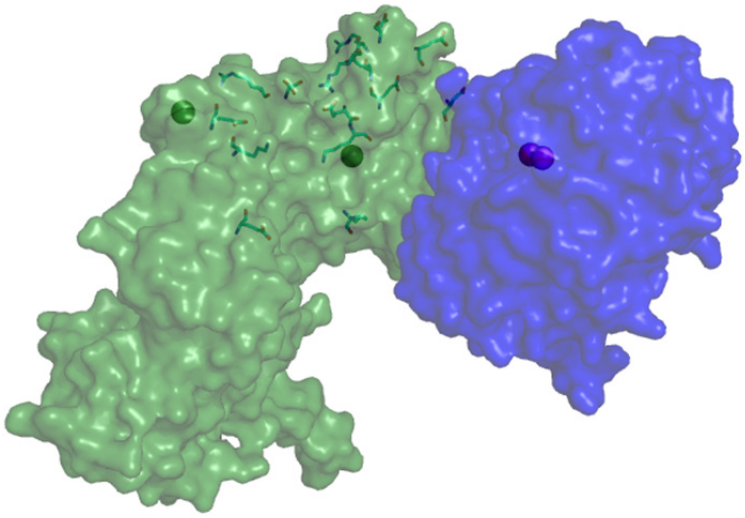


Figure 5.2. Potential residues involved in Cdc6 binding. The PR70 and C-subunits of the PR70 Holoenzyme (4I5L) are shown in transparent surface with selected charged residues shown as sticks. B is shown in forest and C is shown in blue. Calcium atoms and active site metals are shown in sphere. Analysis of the putative substrate binding surface reveals 15 charged residues: 9 acidic (D & E) and 6 basic (K & R). These residues would be the first candidates for mutagenesis to determine critical enzyme-substrate interactions.

References

1 Wlodarchak N, Guo F, Satyshur KA *et al.* Structure of the Ca2+-dependent PP2A heterotrimer and insights into Cdc6 dephosphorylation. *Cell Res* 2013; **23**:931-946.

2 Saraf A, Oberg EA, Strack S. Molecular determinants for PP2A substrate specificity: charged residues mediate dephosphorylation of tyrosine hydroxylase by the PP2A/B' regulatory subunit. *Biochemistry* 2010; **49**:986-995.

3 Xu Y, Chen Y, Zhang P, Jeffrey PD, Shi Y. Structure of a protein phosphatase 2A holoenzyme: insights into B55-mediated Tau dephosphorylation. *Mol Cell* 2008; **31**:873-885.

4 Xu Y, Xing Y, Chen Y *et al.* Structure of the protein phosphatase 2A holoenzyme. *Cell* 2006; **127**:1239-1251.

5 Xu Z, Cetin B, Anger M *et al.* Structure and function of the PP2A-shugoshin interaction. *Mol Cell* 2009; **35**:426-441.

6 Magenta A, Fasanaro P, Romani S, Di Stefano V, Capogrossi MC, Martelli F. Protein phosphatase 2A subunit PR70 interacts with pRb and mediates its dephosphorylation. *Mol Cell Biol* 2008; **28**:873-882.

7 Kolupaeva V, Janssens V. PP1 and PP2A phosphatases--cooperating partners in modulating retinoblastoma protein activation. *FEBS J* 2013; **280**:627-643.

8 Davis AJ, Yan Z, Martinez B, Mumby MC. Protein phosphatase 2A is targeted to cell division control protein 6 by a calcium-binding regulatory subunit. *J Biol Chem* 2008; **283**:16104-16114.

9 Borlado LR, Mendez J. CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. *Carcinogenesis* 2008; **29**:237-243.

10 Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochi Biophys Acta* 2009; **1795**:1-15.

11 Virshup DM, Shenolikar S. From promiscuity to precision: protein phosphatases get a makeover. *Mol Cell* 2009; **33**:537-545.