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## Investigating the interplay between protein kinases and caspases

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Graduate Program in Biochemistry  
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of  
Philosophy  
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**INVESTIGATING THE INTERPLAY BETWEEN PROTEIN  
KINASES AND CASPASES**

(Thesis Format: Integrated Article)

by

Jacob P. Turowec

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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## Abstract

The balance between cell survival and death is a crucial process in human development and tissue homeostasis, but is also misregulated in disease. In large part, apoptosis is controlled by caspases, a hierarchical series of cysteine aspartic acid proteases that demolish the cell by cleaving key structural and enzymatic proteins, but emerging paradigms have highlighted the ability of kinases to regulate caspase activity. One way in which kinases can control the progression of apoptosis is through phosphorylation of caspase substrates, which acts to prevent caspase cleavage of that target.

In this thesis, we develop new strategies to study this regulatory mechanism, and focus particularly on protein kinase CK2; a kinase with enigmatic regulation, an obvious, but undefined role in anti-apoptotic signaling, and demonstrated value as a candidate for targeted therapy of cancer. First, we used predictive strategies for identifying CK2 substrates that, when phosphorylated, regulate proteolysis by caspases. We predicted and validated that phosphorylation of caspase-3 itself by CK2 prevents its cleavage and activation, representing a putative, novel mechanism by which CK2 might control apoptosis in disease. We then utilized cell models that over-expressed different forms of CK2, and found that caspase-3 phosphorylation was specific only for the CK2 $\alpha'$  subunit in the absence of CK2 $\beta$  - a level of isozyme specificity not before seen for CK2. Interestingly, documented misregulation of this isoform in disease suggests that the phenotypes generated by varied CK2 levels in cancer may well depend on the form of CK2 present. We also establish the utility of proteomic applications for identifying novel caspase substrates whose cleavage is regulated by phosphorylation. We noted that phosphorylation can also promote caspase substrate cleavage, likely through a mechanism in which phosphorylation alters scissile bond accessibility.

Collectively, our work provides insight into the regulation of protein kinase CK2 and demonstrates that the specialized functions of different forms may define cellular phenotypes in disease. Furthermore, we identified a number of caspase substrates whose

cleavage is regulated by phosphorylation, thereby validating our novel approaches and delineating putative apoptotic control points.

**Keywords:** caspase regulation, kinase, CK2, free-catalytic CK2, apoptosis

## Co-authorship Statement

The chapters of this thesis were written by Jacob Turowec and edited by David Litchfield.

The data presented in chapter 2 contains figures from the published manuscripts: A peptide-based target screen implicates the protein kinase CK2 in the global regulation of caspase signalling. Duncan JS\*, Turowec JP\*, Duncan KE, Vilk G, Wu C, Luscher B, Li SS, Gloor GB, Litchfield DW. *Science Signaling* (2011) 4(172):ra30. \*equal contribution

Regulation of caspase pathways by protein kinase CK2: identification of proteins with overlapping CK2 and caspase consensus motifs. Turowec JP, Duncan JS, Gloor GB, Litchfield DW. *Mol Cell Biochem* (2011) 356(1-2): 159-67.

Versatile strategy for biochemical, electrochemical and immunoarray detection of protein phosphorylations. Martic S, Gabriel M, Turowec JP, Litchfield DW, Kraatz HB. *J Am Chem Soc.* (2012) 134(41): 17036-45.

All experiments were performed by JPT except the following: Gregory Gloor wrote and excuted the pearl script for the peptide-match program. James Duncan performed figure 2.3B and 2.6C. Greg Vilk performed experiments for 2.5B and C.

In chapter 3, JPT performed all experiments except the following: Greg Vilk performed the experiments for figure 3.4B. Michelle Gabriel cloned the CK2 $\alpha/\alpha'$  chimera constructs. Between initial and final thesis submission, a modified version of chapter 3 was accepted to *Oncotarget*: Characterizing the convergence of protein kinase CK2 and caspase-3 reveals isoform-specific phosphorylation of caspase-3 by CK2 $\alpha'$ : implications for pathological roles of CK2 in promoting cancer cell survival. Turowec JP\*, Vilk G\*, Gabriel M and Litchfield DW. *Oncotarget.* (2013) *in press* \*equal contribution

In chapter 4, JPT performed all experimentation except the following: David Smalley ran samples on the LTQ-Orbitrap. James Knight performed database searches of the raw mass spectrometer data, and Stephanie Zukowski synthesized peptide substrates.

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## List of Abbreviations

AML	acute myeloid leukemia
AMPPNP	Adenylyl-imidodiphosphate
ANOVA	analysis of variance
ARC	apoptosis repressor with caspase recruitment domain
ATP	adenosine triphosphate
BSA	bovine serum albumin
CARD	caspase activation and recruitment domains
CASBAH	caspase substrate database homepage
CDK1	cyclin dependent kinase 1
CK2	protein kinase CK2 (formely casein kinase 2)
CK2 holoenzyme	heterotetramer consisting of a CK2 $\beta$ dimer and two catalytic subunits
CKIP-1	CK2 interacting protein-1
CML	chronic myelogenous leukemia
CP $\alpha$	actin capping protein subunit $\alpha$
CX-4945	5-(3-chlorophenylamino) benzo[c][2,6]naphthyridine-8-carboxylic acid
DED domain	death effector domain
DISC	death inducing signalling complex
DMEM	Dulbecco's modified eagle medium
DNP	2,4-dinitrophenol
DRB	5,6-dichloro-1- $\beta$ -d- ribofuranosylbenzimidazole
DTT	dithiothreitol
DYRK1A	dual specificity tyrosine-phosphorylation-regulated kinase 1A
EGF	epidermal growth factor
EBV	Epstein-Barr virus
EMT	epithelial-mesenchymal transition

ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FHA	forkhead-associated domain
FLAG	DYKDDDDK epitope
FLIP	FLICE-inhibitory protein
Free-catalytic CK2	catalytic CK2 unbound to CK2 $\beta$
GAM	goat anti-mouse
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAR	goat anti-rabbit
GO	gene ontology
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	hemagglutinin epitope YPYDVPDYA
IAP	inhibitor of apoptosis protein
I $\kappa$ B	inhibitor of $\kappa$ B
HeLa	human cervical cancer cell line
IR	ionizing radiation
$\lambda$ phosphatase	recombinant, phage protein phosphatase
LC	liquid chromatography
LNCaP	human prostate cancer cell line
MAPK	mitogen-activated protein kinase
MDC1	mediator of DNA damage checkpoint protein 1
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS1	analysis performed by the first analyzer in tandem mass spectrometry
MST	mammalian STE20-like protein kinase
N-terminome	the complement of N-termini within a proteome
Neo N-terminome	new N-termini that result from protease treatment



NF- $\kappa$ B	nuclear factor $\kappa$ -light-chain-enhancer of activated B cells
n+1	one residue C-terminal to a phospho-acceptor
NABH <sub>3</sub> CN	sodium cyanoborohydride
NLS	nuclear localization sequence
ODC1	ornithine decarboxylase 1
P1	the amino acid that on the substrate that is covalently bound to a protease during the transition state
P1'	C-terminal to P1
P2	N-terminal to P1
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Phosida	phosphorylation site database
PI3K	phosphatidylinositide 3-kinase
PKA	protein kinase A
PLL	poly-L-lysine
PML	promyelocytic leukemia protein
PMSF	phenylmethanesulfonylfluoride
PP2A	protein phosphatase 2
PP5	protien phosphatase 5
PVDF	polyvinylidene fluoride
RIPK1	receptor-interacting serine/threonine-protein kinase 1
S1	pocket on proteases that bind the P1 residue on the substrate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRM	selected reaction monitoring
TAILS	terminal amino isotopic labeling of substrates

TBB	4,5,6,7-tetrabromo-1H-benzotriazole
TBBz	4, 5, 6, 7-tetrabromobenzotriazole
TBST	Tris-buffered saline and tween 20
TNF $\alpha$	tumour necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
Tris	tris(hydroxymethyl)aminomethane
U2-OS	human osteosarcoma cell line
UV	ultraviolet radiation
XRCC1	X-ray repair cross-complementing protein 1
YFP	yellow fluorescent protein

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## **CHAPTER 1 - INTRODUCTION**

### ***1.1 GENERAL INTRODUCTION***

Phosphorylation by protein kinases is an imperative process that orchestrates a number of cellular pathways (1). Via the transfer of phosphate from ATP to protein substrates, kinases mediate changes in transcription, protein structure and function and protein-protein interactions in response to diverse stimuli. Not surprisingly, the misregulation of kinases by overexpression, constitutive activation, or deactivation, can distort normal signaling events and lead to a diseased state. In light of these observations, protein kinases have emerged as promising candidates for targeted therapeutics to treat a wide variety of diseases, including cancer (2). The recent success of Imatinib – a specific inhibitor of the bcr-abl kinase found in chronic myelogenous leukemia – represents the most prominent example of a kinase inhibitor impacting disease management in the clinic (3). While Imatinib represents a ‘silver bullet’ for one specific form of cancer, these diseases remain remarkably heterogeneous – a fact highlighted by the multitude of kinases known to play a role in a number of other malignancies (2,4).

As well as contributing to diverse aspects of signaling that ultimately promote cellular fitness, a specific, direct function for protein phosphorylation in regulating apoptotic machinery is emerging (5,6)). Apoptosis, or programmed cell death, is a vital process that contributes to tissue homeostasis and development, but is often blunted in cancer (7). By avoiding apoptosis, malignant cells can persist inappropriately, thereby contributing to tumour progression (8). Major effectors of the apoptotic program are a family of proteases known as caspases. Once activated, they function by cleaving

structural, enzymatic and transcription factor proteins that collectively participate in the co-ordinated demolition of the cell (9). Interestingly, cross-talk between protein kinases and caspases has become apparent, with a growing number of examples highlighting how kinases alter caspase activity and vice versa (reviewed in (5,6). It is anticipated that understanding the convergence of these enzymes, especially in the context of the disease state, will benefit how the apoptotic pathway can be selectively activated in cancer cells.

Perhaps the most pertinent kinase implicated in the regulation of caspase pathways is protein kinase CK2. CK2 phosphorylates a number of caspases, caspase substrates, and caspase regulators (reviewed in (6,10) and 1.5 and 1.6 of this thesis). It is also overexpressed in a number of human cancers, and after pharmacologically inhibiting CK2 in disease models of CK2 overexpression, these same cells undergo programmed cell death (reviewed in (10,11) and 1.4.2 of this thesis). As such, CK2 has emerged as a candidate for molecular targeted therapy. Indeed, the number of patents for CK2 inhibitors has dramatically increased over recent years, and one, CX-4945, has even entered human clinical trials (12,13). Functionally, CK2 is constitutively active, and so over-expression in disease is believed to promote survival by hyper-phosphorylating its substrates; alternatively, high levels of CK2 may also lead to inappropriate phosphorylation of disease-specific substrates (11). However, gaps in our knowledge remain as to which substrates promote the anti-apoptotic phenotype emanating from CK2, and how this constitutively active kinase is regulated in cells.

## ***1.2 ENZYMATIC AND STRUCTURAL FEATURES OF CK2***

### **1.2.1 General**

Protein kinase CK2 was isolated almost 60 years ago on the basis of its ability to phosphorylate the milk protein casein (14). As a result, it was originally designated 'Casein kinase II', though this misnomer is less frequently used because of the lack of physiological significance for the phosphorylation of casein by CK2 (15). CK2 is ubiquitously expressed in eukaryotes, and in humans, is composed of a regulatory CK2 $\beta$  subunit and two catalytic subunits (CK2 $\alpha$  and CK2 $\alpha'$ ) (16). Catalytically, CK2 catalyzes the phosphorylation of Ser/Thr and even Tyr, albeit at much lower efficiency (17-19), when surrounded by acidic amino acids. Acidic residues, typically Glu or Asp at n+3 and, to a lesser extent, n+1 are the strongest determinants, though catalysis is generally improved upon addition of more acidic residues (20-23). CK2 $\alpha$  and  $\alpha'$  are roughly 85% identical within their catalytic domains but are encoded by distinct gene products (16,24-26) (Figure 1.1). The only considerable divergence in sequence between the two catalytic subunits is located outside the catalytic domain on the C-terminus; presumably, this results in the inability to distinguish CK2 $\alpha$  and  $\alpha'$  on the basis of their catalytic properties (16,27). Along these lines, there is no known substrate that displays preference for one catalytic subunit over the other, at least *in vitro*, as the residues dictating primary amino acid specificity are conserved (28).

The regulatory CK2 $\beta$  subunit is a 25 kDa protein in humans that has little sequence homology with any other protein, but is extremely conserved between species (29,30). Within the CK2 tetramer, or holoenzyme, CK2 $\beta$  forms a dimer core that is flanked by the two catalytic subunits (31-33). While the protein interaction interface between CK2 $\beta$  and the catalytic subunits are considered small at 830 Å<sup>2</sup> (33,34), the dissociation constants are quite strong (35); evidence for this is provided by the fact that

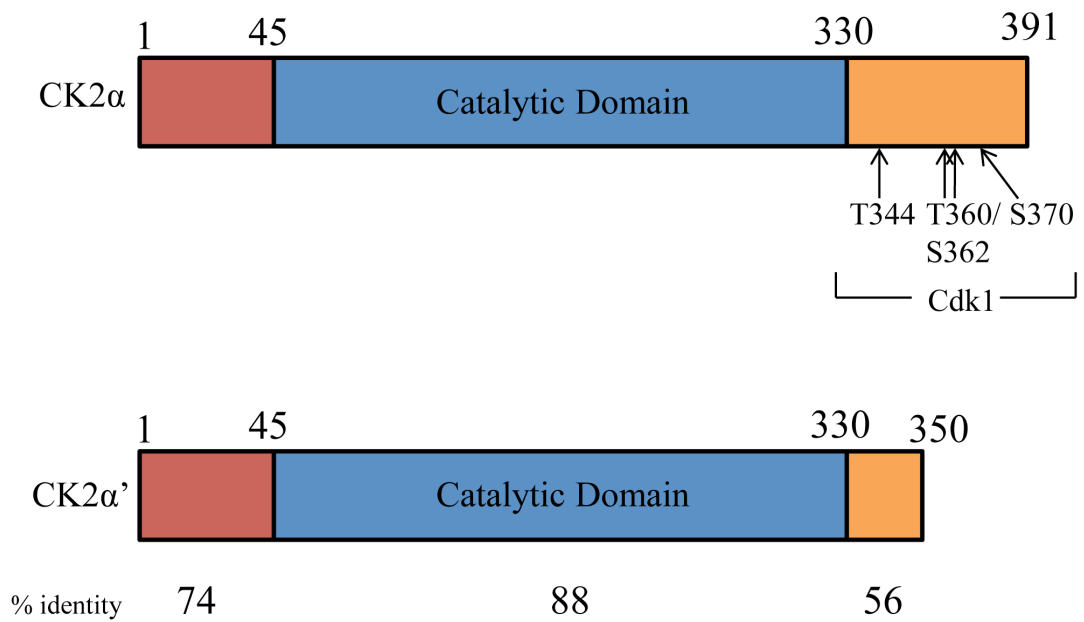


Figure 1.1 – Schematic representation of CK2α and α'. The N-terminal, catalytic and C-terminal sections of the catalytic isoforms and their percent identity are shown. This figure is a modified version from (233).

breaking the interaction *in vitro* is only possible under denaturing conditions (36). Although some of the enzymatic characteristics of tetrameric CK2 can be distinguished from that of the free catalytic subunits (discussed below), a striking feature of CK2 is that both of these forms are catalytically competent (27,37). In this respect, CK2 is distinct from cyclin-dependent kinases where catalytic activity is dependent on the presence of an activating cyclin and from second messenger-dependent kinases, such as PKA, where catalytic activity is suppressed by an autoinhibitory subunit (38).

### **1.2.2 Structural Features**

Structural studies have yielded many insights into the unique features of protein kinase CK2 (reviewed in (39)), the most important of which is the apparent active conformation in over 40 crystal structures to date. This is in contrast to MAPKs and tyrosine kinases, for example, where activation loop phosphorylation is required to achieve the open conformation and catalytic activity (40). Structures of CK2, however, show the activation loop stabilized in the open conformation in the absence of both regulatory phosphorylations and subunits (Figure 1.2). In large part, this is achieved by stabilizing forces exerted by hydrophobic interactions between the N-terminus and the activation loop (36). Furthermore, CK2 contains a ‘DWG’ motif just upstream of the activation loop that provides an extra hydrogen bond compared to most other kinases’ ‘DFG’ motif (41).

Structures of CK2 in complex with purine analogues have also revealed unique features of its ATP binding domain in relation to the ATP binding domain of other protein kinases. In CK2, the ATP binding domain is collapsed and relatively thin in one

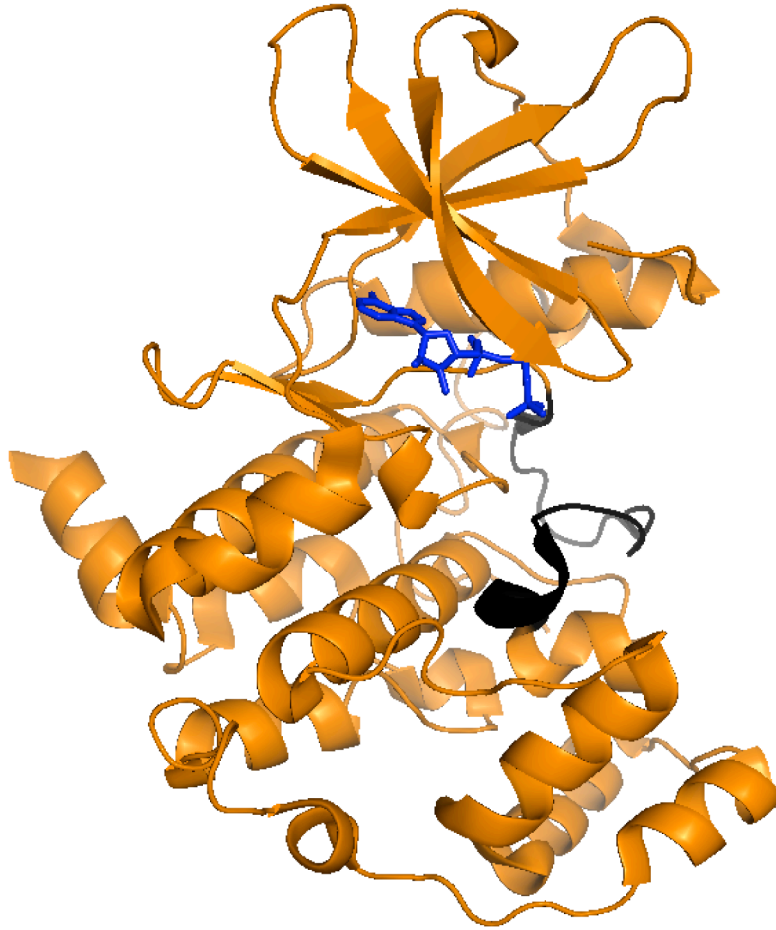


Figure 1.2 – Crystal Structure of CK2 $\alpha$ . Recombinant maize CK2 $\alpha$  is shown in complex with Mg<sup>2+</sup> and the ATP analogue AMPPNP (Adenylyl-imidodiphosphate) (PDB: 1DAW). The activation loop is shown in black and AMPPNP in blue.



plane, while being wider in the second plane (42). The widening allows for the utilization of GTP as a phosphate donor, a relatively unique characteristic amongst protein kinases, while the thinning in the other plane provides the opportunity for the rational design of specific, small molecule ATP competitive inhibitors that exploit Van der Waals interactions in CK2 (43,44). In large part, the ‘thinness’ is mediated by V66 and I174 in CK2 $\alpha$  – residues typically occupied by smaller amino acids in other protein kinases (45). Mutation of V66 and I174 to alanine results in at least a 10-fold decrease in sensitivity to competitive inhibitors, offering a strategy for rescue experiments in cell culture systems (45,46).

Another unique feature of CK2 revealed by crystallography was that the tetrameric configuration of the holoenzyme consists of a CK2 $\beta$  dimer core that is flanked and bound by two individual catalytic subunits (33). More recently, Lolli *et al.* (2012) demonstrate that the CK2 $\alpha$ 2/CK2 $\beta$ 2 holoenzyme is capable of forming inactive, supramolecular structures composed of 3 holoenzymes (47). In this instance, the N-terminus from one CK2 $\beta$  subunit and the C-terminus from another preclude the active site from substrate and nucleotide binding, while the other three catalytic subunits are sterically hindered. The observation that CK2 tetramers can form filamentous structures under crystallization conditions may clarify the finding that in cells, CK2 $\alpha$  and  $\beta$  belong to both fast and slow moving subpopulations (48).

CK2 $\alpha'$  structures of the catalytic subunit have begun to highlight isozymic differences between the two catalytic subunits. Bischoff *et al.* (2011) noted that the interdomain hinge region of CK2 $\alpha'$  aligns seamlessly with the tetrameric form of CK2 $\alpha$ , whereas this region of CK2 $\alpha$  in the absence of  $\beta$  is slightly collapsed and may interfere

with productive nucleotide binding (49). The CK2 $\beta$  interface of CK2 $\alpha$  is also quite malleable and changes upon addition of the regulatory subunit. Curiously, the  $\beta$  interface on CK2 $\alpha'$  resembles the holoenzyme form of CK2 $\alpha$ , offering an attractive explanation for the observed 12-fold lower affinity of CK2 $\alpha'$  for  $\beta$  compared to  $\alpha$  and  $\beta$  (49). Along these lines, gel filtration of CK2 $\alpha'$  holoenzyme suggests that CK2 $\alpha'$  predominately forms trimers consisting of one catalytic subunit and a dimer of CK2 $\beta$ , in contrast to the tetramers of CK2 $\alpha/\beta$  (50).

### **1.2.3 CK2 $\beta$ regulatory subunit: a modulator of CK2 function**

While CK2 $\beta$  is not strictly required to turn on or off the catalytic activity of CK2, the regulatory subunit appears to increase activity toward most substrates by enhancing thermal stability (typically 3-5 fold) (27,37). Along these lines, the majority of CK2 substrates can be effectively phosphorylated by either tetrameric CK2 or free catalytic CK2 subunits, but there are isolated examples of targets, such as eIF2 $\beta$ , where CK2 $\beta$  is required to enable efficient phosphorylation, or calmodulin, where the presence of CK2 $\beta$  leads to complete inhibition of phosphorylation (51-53). In instances where CK2 $\beta$  stimulates phosphorylation, there is evidence to suggest that it serves as a substrate docking protein (34); topoisomerase II and p53 both bind CK2 $\beta$  at its N-terminus independent of the catalytic subunit (54,55). Interestingly, in cases where CK2 $\beta$  blocks substrate phosphorylation, this can be rescued by treating the holoenzyme with polyamines (56). Polyamines act to bind CK2 $\beta$  at amino acids 55-64 – a particularly

acidic stretch (DLEPDEELED) – and, it is hypothesized, compete  $\beta$  away from a basic region of CK2 $\alpha$  effectively widening the substrate-binding region of CK2 $\alpha$  (57,58).

The role of the regulatory CK2 $\beta$  subunit in controlling substrate specificity in cells remains poorly understood, mostly because of the lack of a *bona fide* model system that exhibits clear pools of catalytic subunits of CK2 not bound to CK2 $\beta$ . The demonstration that calmodulin is phosphorylated at its CK2 sites in rat liver was amongst the first evidence provided to suggest that in cells, either functional free catalytic subunits exist, cellular holoenzyme is activated by polyamines and/or some other mechanism contributes to modulating CK2 substrate specificity (59). Since this time, a number of other anecdotal examples have emerged. For instance, CK2 $\alpha'$  can be separated from CK2 $\beta$  during ion-exchange of LNCaP cell lysates, suggesting the existence of catalytic CK2 devoid of the regulatory subunit. Further evidence for this form of CK2 was obtained when Vilks *et al.* (2001) observed an increase in CK2 activity upon ectopic expression of CK2 $\beta$  in U2-OS cells (60). The authors utilized a substrate peptide with a moderate preference for the holoenzyme form of CK2, and so they conclude that the over-expressed CK2 $\beta$  complexed catalytic CK2 previously not apart of the holoenzyme, thereby raising the measured activity. Similarly, Miyata and Nishida (2008) show that cdc37 phosphorylation is increased at a specific CK2 phosphorylation site upon ectopic expression of CK2 $\beta$  (61), and so is open to the same interpretation made by Vilks and colleagues. On the flip side, the Pinna group observed that in CHO cell lysates, ectopic expression of CK2 $\beta$  did not alter CK2 activity against a peptide substrate specific for the holoenzyme (62). The authors' interpretation was that had free-catalytic CK2 $\alpha$  or  $\alpha'$  existed, ectopic  $\beta$  would have quenched this population and been detected in lysate

kinase assays with the holoenzyme specific peptide. In this respect, Luscher and Litchfield noted that CK2 $\beta$  is synthesized in excess of the catalytic subunits in lymphoma cells and is quantitatively incorporated into the holoenzyme (63). Presumably, given the extremely tight interaction between the regulatory and catalytic subunits ( $K_D$  values of  $45.5 \times 10^{-9}$  M for CK2 $\alpha'$  and  $3.7 \times 10^{-9}$  M for CK2 $\alpha$  (35)), these latter observations suggest that unless as of yet unknown cellular mechanisms act to segregate  $\beta$  from  $\alpha$  and  $\alpha'$ , the holoenzyme will form. Interestingly, the Filhol group has observed fluctuating ratios of catalytic to regulatory CK2 subunits across a panel of breast cancer tissues and a correlation between low CK2 $\beta$  levels and epithelial-to-mesenchymal transition (64). The authors hypothesize that a dip in holoenzyme activity reduces phosphorylation of the holoenzyme-specific substrate SNAIL, which then drives the EMT (epithelial-mesenchymal transition) phenotype. This last example is the first hint that variations in the free-catalytic to holoenzyme ratio can control a cellular phenotype.

To further study CK2 $\beta$ -independent functions of the catalytic subunits, new tools are emerging. Raaf and colleagues have utilized the crystal structure of the CK2 holoenzyme to rationally design mutants of CK2 $\alpha$  that are deficient in binding to CK2 $\beta$ , though experiments exploring cellular phenotypes and substrates have not yet been performed (65). Furthermore, the Cochet group has designed short peptides and low-molecular weight chemicals that interfere with the holoenzyme formation of recombinant CK2 subunits (66,67). As well, French *et al.* (2007) have designed a CK2 $\beta$  mutant resistant to proteasomal degradation (68). Coupled with the fact that there is no known cellular mechanism for dis-assembly of the CK2 holoenzyme, extending the CK2 $\beta$  half-life may increase the proportion of catalytic CK2 bound to  $\beta$ .

Holoenzyme formation also appears to alter CK2 localization. Expression of GFP-CK2 $\alpha$  is almost completely nuclear, but co-expression of GFP-CK2 $\alpha$  and YFP-CK2 $\beta$  resulted in an even distribution of CK2 throughout the cell (69). Similar experiments have yet to be performed with CK2 $\alpha'$ , but both isoforms have an identical nuclear localization signal, suggesting similar behavior might be expected(15). While CK2 $\alpha'$  has not been studied with fluorescent fusions, defective spermatogenesis in CK2 $\alpha'$ -null mice has prompted localization studies of endogenous CK2 $\alpha'$  in testes (70). CK2 $\alpha'$  localization measured in a number of testes cell types across the rat life cycle revealed that localization depends on age and cell type (71). CK2 $\alpha'$  and  $\beta$  are nuclear in the Sertoli cells of 5-day old rats, but cytoplasmic in the same cells of older rats. Also, CK2 $\alpha'$  occupies a distinct cellular location from CK2 $\beta$  in epididymal spermatocytes;  $\alpha'$  is found predominately in the acrosome and  $\beta$  in the flagellum.

Collectively, these findings suggest that CK2 $\beta$  controls the substrate specificity of CK2, but also raise the specter that individual CK2 subunits exist outside the holoenzyme and are governed by unique modes of regulation. At this point however, the precise mechanisms that drive tetramer formation and dissociation in cells remain unclear. Furthermore, some of the evidence is acquired through low-resolution techniques, such as those performing kinases assays with cell lysates and CK2-form specific substrate peptides. Here, the field would be better served with phospho-specific antibodies targeted against CK2-form specific substrates that aim to measure cellular CK2, though no such materials exist.

### ***1.3 PHYSIOLOGICAL REGULATION OF CK2***

### 1.3.1 Overview of CK2 regulation

CK2 is accredited with the phosphorylation of a large number of proteins; estimates comparing a CK2 motif, or ‘weblogo’, and the known human phosphoproteome suggest approximately 20% of known phosphorylation sites conform to the CK2 consensus motif (72). Fittingly, CK2 has been implicated in a diverse set of functions, including development, cell cycle progression, circadian rhythms, viral infection, DNA damage response, autophagy and apoptosis. Given the diversity of biological processes CK2 is associated with, it is not unexpected that the regulation of CK2 is complex and incompletely defined. The prevalence of CK2 substrates in various cellular compartments, including the nucleus, cytoplasm, mitochondria and plasma membrane, has led to the prevailing notion that discrete populations of CK2 are ultimately controlled by the regulated accessibility to the substrates (73). Furthermore, emerging evidence is beginning to highlight divergent roles for the catalytic isozymes; a function usually, but not exclusively, attributed to their divergent C-terminus.

### 1.3.2 Catalytic isozyme specific functions

Despite extensive sequence similarity and catalytically identical characteristics *in vitro*, CK2 $\alpha$  and  $\alpha'$  appear to have unique physiological functions. Isozyme specific functions have been observed in lower eukaryotes, such as *Saccharomyces cerevisiae*. Temperature sensitive alleles of CKA1 and CKA2 – the two catalytic isoforms of yeast CK2 – grown at the non-permissive temperature exhibit defects in cell polarity and cell cycle arrest, respectively (74,75). Unique phenotypes are also observed in knockout-mice. CK2 $\alpha'$  knockouts exhibit defective spermatogenesis due to increased apoptosis,

suggesting that CK2 $\alpha$  is unable to compensate for this function (70). Likewise, CK2 $\alpha$  knockout mice are embryonic lethal and abort at day E11.5 (76). As well, forced expression of kinase-inactive CK2 $\alpha'$ , but not inactive CK2 $\alpha$ , contributes to a proliferative defect in U2-OS cells. Collectively, these data reinforce the notion that the catalytic isoforms of CK2 occupy distinct nodes in signaling.

The most obvious explanation for functional divergence of the two catalytic subunits is the completely unrelated C-terminus of CK2 $\alpha$  (amino acids 330-391) (Figure 1.1). Interestingly, within this tail region are four proline-directed phospho-acceptors whose phosphorylation during prophase and metaphase is required for cell survival through mitosis (77). These phosphorylation sites generate a binding module for Pin1, which targets CK2 $\alpha$  to the mitotic spindle (78,79). Furthermore, Pin1 binding to CK2 $\alpha$  can alter substrate specificity (80), most notably towards topoisomerase II (78). Similarly, a recent role for the ERK-mediated phosphorylation of two of these sites, T360 and S362, has been proposed to function in promoting  $\alpha$ -catenin phosphorylation by CK2 in an EGF-dependent manner (81). CK2 $\alpha'$  also has a unique substrate in the nuclear transcription factor NKX3.1 (82). Using in-gel kinase assays, the Bieberich group demonstrated that only CK2 $\alpha'$  fractions devoid of CK2 $\beta$  phosphorylate NKX3.1 and that phosphorylation may increase the half-life of this tumour suppressor in cell line models of prostate cancer.

The catalytic domain of CK2 also controls specific functions of the different isozymes. Under the presumption that specific interacting proteins control unique roles for  $\alpha$  and  $\alpha'$ , Bosc *et al.* (2000) performed yeast two-hybrid screens to identify interactors of CK2 (83). CKIP-1 (CK2 interacting protein-1) is a PH-domain containing protein that

localizes CK2 $\alpha$ , but not CK2 $\alpha'$ , to the plasma membrane (83,84). Changes in the expression of CKIP-1 induce alterations in cell morphology and the actin cytoskeleton (85). Since CKIP-1 interacts with CP $\alpha$  (actin capping protein subunit  $\alpha$ ), a subunit of the heterodimeric actin capping protein that can be phosphorylated by CK2, these observations are consistent with a working model suggesting that CKIP-1 could participate in the regulation of the actin cytoskeleton by modulating the CK2 catalyzed phosphorylation of CP $\alpha$ . Interestingly, C-terminal  $\alpha$  truncations (ie CK2 $\alpha^{1-330}$ ) are just as able to interact with CKIP-1 as wild-type  $\alpha$ , suggesting that specific interactions are guided by residues within the highly conserved region of  $\alpha$  and  $\alpha'$ , though the structural or sequence determinants that dictate specific binding remain elusive (83,86). PP2A is another cytoplasmic substrate that, like CKIP-1, interacts with CK2 $\alpha$  at regions located within the catalytic domain (87). Here, interactions are governed by  $_{176}\text{HEHRKL}_{181}$ , which is a region slightly divergent in CK2 $\alpha'$  (HQQKKL) and, therefore may dictate isoform specific interactions, though this has not been rigorously tested. Furthermore, because CK2 $\alpha$ /PP2A interactions only occur in the absence of CK2 $\beta$ , the observation that endogenous CK2 $\alpha$  and PP2A interact supports the hypothesis that CK2 has distinct, non-holoenzymatic functions within the cell.

CK2 $\alpha$  and  $\alpha'$  also differ in their subcellular localization. Despite identical nuclear localization signals (15), Brown *et al.* (2010) observed an high CK2 $\alpha$ :CK2 $\alpha'$  ratio in the nucleus and low ratio in the cytoplasm of head and neck squamous cell carcinoma cell lines(88). Further supporting differential mechanisms that control localization is the finding that CK2 $\alpha'$ , but not  $\alpha$ , shuttles to the nucleus in response to ionizing radiation in HeLa cells (89). The molecular mechanisms controlling re-



localization in this case remain enigmatic. What is interesting, though, is that CK2 $\beta$  appears to control nuclear localization of CK2 $\alpha$ ; expression of GFP-CK2 $\alpha$  (green fluorescent protein-CK2 fusion) is almost completely nuclear in HeLa cells, but co-expression of GFP-CK2 $\alpha$  and YFP-CK2 $\beta$  (yellow fluorescent protein-CK2 fusion) resulted in an even distribution of CK2 throughout the cell (69). Furthermore, a small population of yeast CK2A2 dissociate from CK2 $\beta$  after UV stress, perhaps supporting a mechanism by which CK2 $\beta$  is a controller of nuclear CK2 $\alpha$ ' in response to DNA damage (90).

### **1.3.3 CK2 in cell stress**

Functionally, the phosphorylation of CK2 substrates has implications in controlling diverse cellular events. For instance, CK2 is an emerging, major player in the DNA damage response. CK2 constitutively phosphorylates XRCC1 (X-ray repair cross-complementing protein 1) (91,92) and MDC1 (93), which generates binding sites for FHA (fork-head associated) domains, and increases cell survivability after DNA damage (94). Evidence for CK2 stimulation after DNA damage has also been observed. Phospho-proteomics experiments investigating kinase activity after double-stranded DNA breaks noted an increase in the number of phospho-peptides containing sequences that conform to the CK2 consensus motif (95). One specific substrate that is dynamically controlled after DNA damage is HP $\beta$ -1 as phosphorylation occurs within seconds of double stranded breaks (96). After phosphorylation, HP $\beta$ -1 is mobilized and the recruitment of other proteins, such as  $\gamma$ -H2AX, to sites of DNA damage is promoted. In

both the phospho-proteomic and HP $\beta$ -1 study, how CK2 activity is stimulated remains elusive.

Mechanistic explanations for the control of CK2 activity against other substrates are beginning to emerge. After exposing HeLa cells to UV radiation, NF- $\kappa$ B induction is blocked by either CK2 $\beta$  knockdown or alanine mutations of six CK2 phospho-acceptors within the C-terminal domain of I $\kappa$ B – a negative regulator of NF- $\kappa$ B (97). This signal appears to emanate from p38-induced activation of CK2, as I $\kappa$ B phosphorylation is lost in when p38 $\alpha$ <sup>-/-</sup> MEFs are stimulated with UV (97). A role for p38 in stimulating CK2 activity has also been observed in response to other cell stressors such as anisomycin, TNF $\alpha$  (tumour necrosis factor-alpha) and arsenite, and appears to occur via direct binding of active p38 to CK2 (98). CK2 also participates in hierarchical phosphorylation which represents an emerging paradigm that can partly explain how a generally constitutively active kinase is controlled (99). Given the tendency of CK2 to phosphorylate residues downstream of acidic amino acids, it is perhaps not surprising that phospho-residues also promote phosphorylation by CK2. For example, following double-stranded DNA breaks, S14 phosphorylation of Rad51 by Plk1 generates a phospho-acceptor for CK2 at T13 (100). Similarly, Src phosphorylation of C-Raf at Y340/Y341 promotes CK2 catalyzed phosphorylation at S338 (101). In this way, hierarchical phosphorylation has the interesting characteristic of subjecting CK2 to the same regulatory control as the priming kinase (99).

#### ***1.4 EVIDENCE FOR PATHOLOGICAL FUNCTIONS OF CK2***

CK2 has garnered interest as a candidate for targeted cancer therapeutics, especially of late, with the number of patents for CK2 inhibitors being filed dramatically increasing over the past 5 years (reviewed in (12)). Largely, interest in CK2 as an inhibitor target is rooted in a body of literature that explores, via genetic and pharmacological means, the impact of manipulating CK2 activity on cell survival and proliferation (reviewed in (102)). Below, we will highlight work that supports the establishment of CK2 as a candidate for targeted cancer therapy.

#### **1.4.1 CK2 over-expression in disease and model systems**

Evidence acquired as early as the 1980s indicated upwards of 3 to 8-fold greater CK2 activity in human breast and colorectal carcinoma samples compared to healthy tissue (103,104). Since then, increased CK2 levels and/or activity have been observed in large variety of human cancers, including: breast (64,105), prostate (106), lung (107), head and neck (108), colorectal (109), gastric (110), leukemia (111), and kidney (112). Upon the development of transgenic mouse models that overexpress CK2 $\alpha$ , a causative relationship between increased CK2 and the development of cancer was born. Transgenic CK2 $\alpha$  overexpressed in T-cells or the breast showed an increase in lymphoma and mammary tumorigenesis, respectively (105,113). Furthermore, oncogenic co-operativity was observed when mice with T-cell specific overexpression of CK2 $\alpha$  were cross-bred with mice over-expressing the tal-1 or myc oncogene or those that were p53<sup>-/-</sup> (113-115).

In cell culture, ectopic expression of CK2 appears to have only slight effects on proliferation. Over-expression of CK2 $\alpha$  or  $\alpha'$  does not appear to overtly alter growth rate

in U2-OS (116), Chinese Hamster Ovary or 3T3 L1 cells (117), though cooperation between catalytic CK2 and Ras was observed when Balb/c 3T3 co-transfections exhibited significantly quicker growth compared to transfection of single genes (118). Similarly, over-expression of CK2 $\alpha$  in U937 leukemic cells had a slight, albeit significant reduction of cells in G0/G1, which reinforces the pro-survival role of CK2 (119).

#### **1.4.2 Neutralizing CK2 activity in disease models**

The functionality of high CK2 activity in cancer is most apparent when this activity is neutralized. Indeed, a positive correlation between CK2 levels and cellular sensitivity to various small-molecule CK2 inhibitors has been established. Cell viability of T cell acute lymphoblastic leukemia was significantly decreased by TBB and DRB – two chemically unrelated CK2 inhibitors – whereas healthy thymus cells were not affected by these drugs (120) (Figure 1.3). Similarly, Kim and colleagues (2007) found that primary samples of AML (acute myeloid leukemia) characterized by high CK2 $\alpha$  had a significantly greater apoptotic response to apigenin, a moderately selective CK2 inhibitor (45), compared to CK2-low AML and bone marrow mononuclear cells (119). Also, Donor patients of the CK2-high AML had significantly lower survival and disease-free survival. Furthermore, and perhaps not surprisingly, inhibition of CK2 signaling cooperates with a plethora of other apoptotic or chemotherapeutic agents to reduce cell viability, including nocodazole (121), gemcitabine (94), cisplatin (94), erlotinib (122), neocarzinostatin (123), thioguanine (124), IR (125), and TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) (126,127). CK2 inhibition is also effective against cell lines that have a selected resistance against chemotherapeutic agents such as

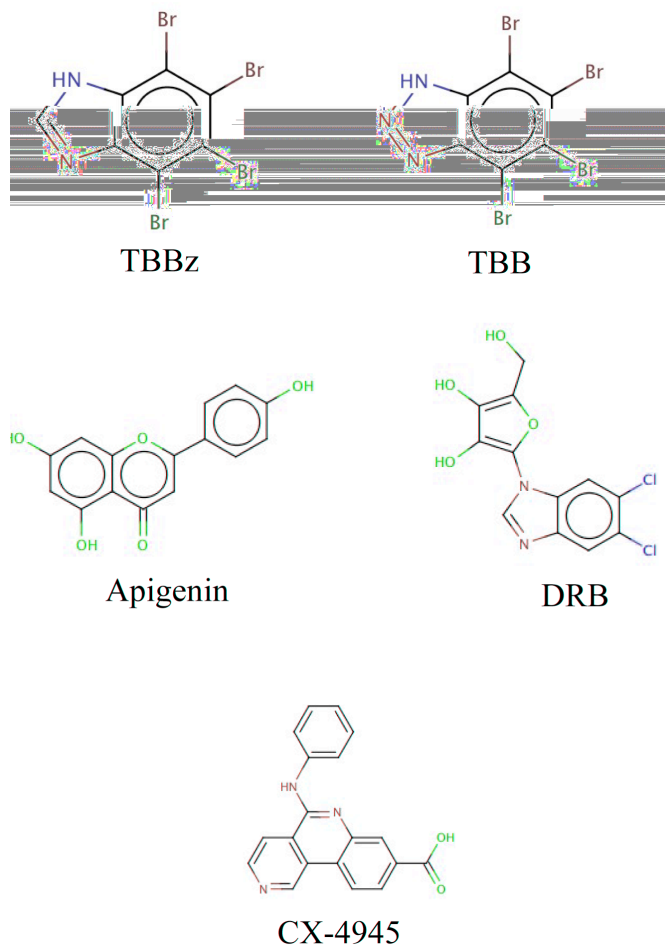


Figure 1.3 Selected examples of CK2 inhibitors. Five commonly used CK2 inhibitors are depicted, including the current ‘gold standard’, CX-4945. A complete review of CK2 inhibitors is beyond the scope of this thesis and can be found elsewhere (234).

vinblastine or imatinib (128,129). In these instances, resistant cell lines are actually more sensitive to CK2 inhibitors than cells sensitive to vinblastine or imatinib, consistent with the notion that CK2 activity plays a key role in supporting cell viability under stress.

### **1.4.3 Perspectives: CK2 as a therapeutic target**

The protective role of CK2 in cancer cell survival suggests that these cells can become ‘addicted’ to CK2 activity (130). Furthermore, CK2 appears to fit the classification of a non-oncogene (131). Conceptually, non-oncogenes encode a class of proteins that are not mutated in cancer, nor sufficient to transform cells in isolation, but nevertheless appear to be high-jacked and perform a specific function that for a given tumour type increases fitness – a concept termed ‘non-oncogene addiction’ (132). In this regard, CK2 inhibition has the peculiar characteristic of generating different phenotypes depending on the genetic background. Transient knockdown of CK2 causes apoptosis in MiaPaCa2 cells (133), numerous prostate cancer cell lines in culture and in mouse xenografts (134,135), and PANC-1 cells (136), but autophagy in glioblastoma cells (137). Similarly, treatment of BT-474 cell with CX-4945 (Figure 1.3), the most specific CK2 inhibitor generated to date, causes a G2 arrest, but a G1 arrest in BxPC-3 cells (138). Nevertheless, targeting non-oncogenes using small-molecules has the added benefit that resistance mutations, either at the amino acid level or gross over-expression by gene duplication, are much less likely to occur over the course of treatment. Indeed, chronic imatinib treatment for CML (chronic myelogenous leukemia) has led to resistance mutations in the oncogenic driver kinase Bcr-Abl (139,140) and similarly, B-raf inhibitors can lead to copy number gains in B-raf of some patients (141).

Taken together, it is clear that CK2 levels are increased in cancer and that targeting CK2 activity quells cancer cell survival and represents a powerful cancer targeting strategy. It follows that CX-4945 has recently entered human clinical trials for the treatment of ovarian cancer and non-small cell lung carcinoma and is the first CK2 inhibitor to do so. Somewhat less satisfying are the unknown regulatory mechanisms controlling increased CK2 levels in cancer. As mentioned earlier, there are no known mutations of CK2, either activating or inhibitory, that occur in disease suggesting that the high activity of CK2 in cancer results from altered levels of CK2 itself. Transcriptionally, all three CK2 genes have Ets response elements located upstream of the coding region (142,143). However, that CK2 mRNA expression is ubiquitous throughout mouse tissue (144), while protein expression is not supports the existence of control mechanisms affecting translation and/or protein stability (34). With respect to the latter, CK2 has a long half-life (24 h) (145) and the CK2 $\beta$  subunit is turned-over by ubiquitination (68), but how these processes are altered in cancer remains unknown.

### ***1.5 CK2 SUBSTRATES THAT MEDIATE THE PATHOLOGICAL PHENOTYPE***

The correlation between CK2 activity and cancer cell survivability indicates that the selective advantage mediated by CK2 is driven by the phosphorylation of CK2 substrates – presumably the hyper-phosphorylation of physiological targets and possibly phosphorylation of pathological substrates. As mentioned earlier, CK2 has a large repertoire of documented substrates implicated in cancer signaling pathways such as PI3K/Akt, NF- $\kappa$ B, Wnt, Hsp90, caspase cascades and DNA damage. Below we will

highlight a handful of *bona fide* substrates whose hyper-phosphorylation has a documented role in contributing to cell survival.

The negative correlation between CK2 activity and PML (promyelocytic leukemia protein) levels represents one of the most clearly defined links in CK2 and disease. PML is a scaffolding protein that coordinates nuclear bodies responsible for transmitting pro-apoptotic signals (146). Importantly, PML protein levels are low in a number of cancer lines as a result of ubiquitin-mediated degradation that is signaled only after phosphorylation at S517 by CK2 (147). Furthermore, Scaglioni and colleagues not only correlate PML levels to CK2 inhibition using small molecule inhibitors, but also show that S517A mutants resist degradation and are tumour suppressive in mouse models of lung cancer. Interestingly, PML phosphorylation by CK2 is also mediated by EBV infection (148). Another CK2 substrate believed to be functionally relevant in cancer is cdc37. As a kinase-specific chaperone, cdc37 acts in conjunction with Hsp90 to stabilize client kinases (149), most notably Raf-1 (150), but only when phosphorylated at S13 by CK2 as S13A mutants fail to bind kinase clients (151). Kinase stabilization is particularly relevant to cancer treatment, as a number of oncogenic kinases require the Hsp90/cdc37 chaperone system for stabilization and, therefore, activity (149,152). pS13 is negatively regulated by protein phosphatase 5 (PP5) and so may be susceptible to hyper-phosphorylation by CK2 in cancer cells with high CK2 activity, though this has only been tested in models utilizing ectopic CK2 (153). Here, expression of CK2 $\alpha$ , CK2 $\beta$  or both increased pS13 in COS7 cells, providing evidence that high CK2 activity can disrupt the kinase/phosphatase balance of pCDC37 and may be relevant in cancer



(61). Along these lines, CK2 inhibition appears to cause decreased levels of a number of CDC37 client kinases in multiple myeloma cells (154).

A direct role for CK2 in regulating the apoptotic machinery has also been described and is an emerging theme in the pathological functionality of CK2 in cancer. CK2 phosphorylates ARC (apoptosis repressor with caspase recruitment domain) at T149 which leads to ARC binding to and inactivation of caspase-8 (155). Furthermore, ARC over-expression, but not the T149A mutant, completely blocks apoptosis caused by caspase-8 over-expression in HEK293 cells. While ARC expression is constrained to the heart and skeletal muscle in human physiology (156), ARC is also prevalent in numerous human cancer cell lines (157), is a prognostic indicator of poor outcome for AML patients (158), and promotes chemoresistance in mouse models of breast cancer (159). Whether ARC function in cancer is dependent on pT149 has not been tested, but the inactivity of T149A against caspase-8 suggests CK2 is required for the disease-driving functions of ARC. CK2 also phosphorylates Bid (160) – a pro-apoptotic protein that participates in mitochondrial permeabilization only after proteolysis by caspase-2 or -8 (161). Subsequent release of cytochrome c and smac/Diablo results in caspase-9 activation and a blockade of the inhibitory effect of IAPs on caspase activity, respectively (162). Interestingly, CK2 phosphorylates Bid proximally to the scissile bond cleaved by caspases and functions by blocking the proteolytic generation of tBid and, therefore, mitochondrial permeabilization (160). Indeed, non-phosphorylatable mutants of Bid are more quickly cleaved in response to caspase-8 activation. Moreover, that the temporal separation between caspase-8 activation and tBid generation can be destroyed by CK2

inhibitors suggests that Bid phosphorylation may act as a check to incoming apoptotic stimulus (163).

## **1.6 CK2 AND CASPASES**

The observation of high CK2 activity in cancer cells, and the identification of CK2 substrates that are intimately involved in regulating apoptosis are consistent with notion that CK2 functions as a blockade to apoptotic signaling in cancer, thereby increasing cellular fitness for those cancer cells. Substrates such as Bid and ARC reinforce this link, but evidence for a direct regulation of caspase activity by CK2 is also emerging and will be highlighted below.

### ***1.6.1 Caspases: General***

Caspases are a family of 14 proteases best known for their role in inflammation and apoptosis. Their name is derived from a mnemonic of cysteine-dependent aspartate-specific protease and they function by catalyzing the proteolytic cleavage of scissile bonds C-terminal to an aspartic acid through use of a conserved, catalytic cysteine residue (164). The apoptotic caspases are further classified into two sub-groups based on both sequence conservation and hierarchy within the caspase cascade (165) (Figure 1.4). Initiator caspases differ from executioners because of a larger pro-domain on the N-terminus that mediate protein-protein interactions required for extrinsic and intrinsic apoptotic initiation (Figure 1.4). The extrinsic apoptotic program relies on ligand engagement to receptors that co-ordinate the formation of the DISC (death inducing signaling complex). Here, the N-terminal DED (death effector domain) of caspase-8 and

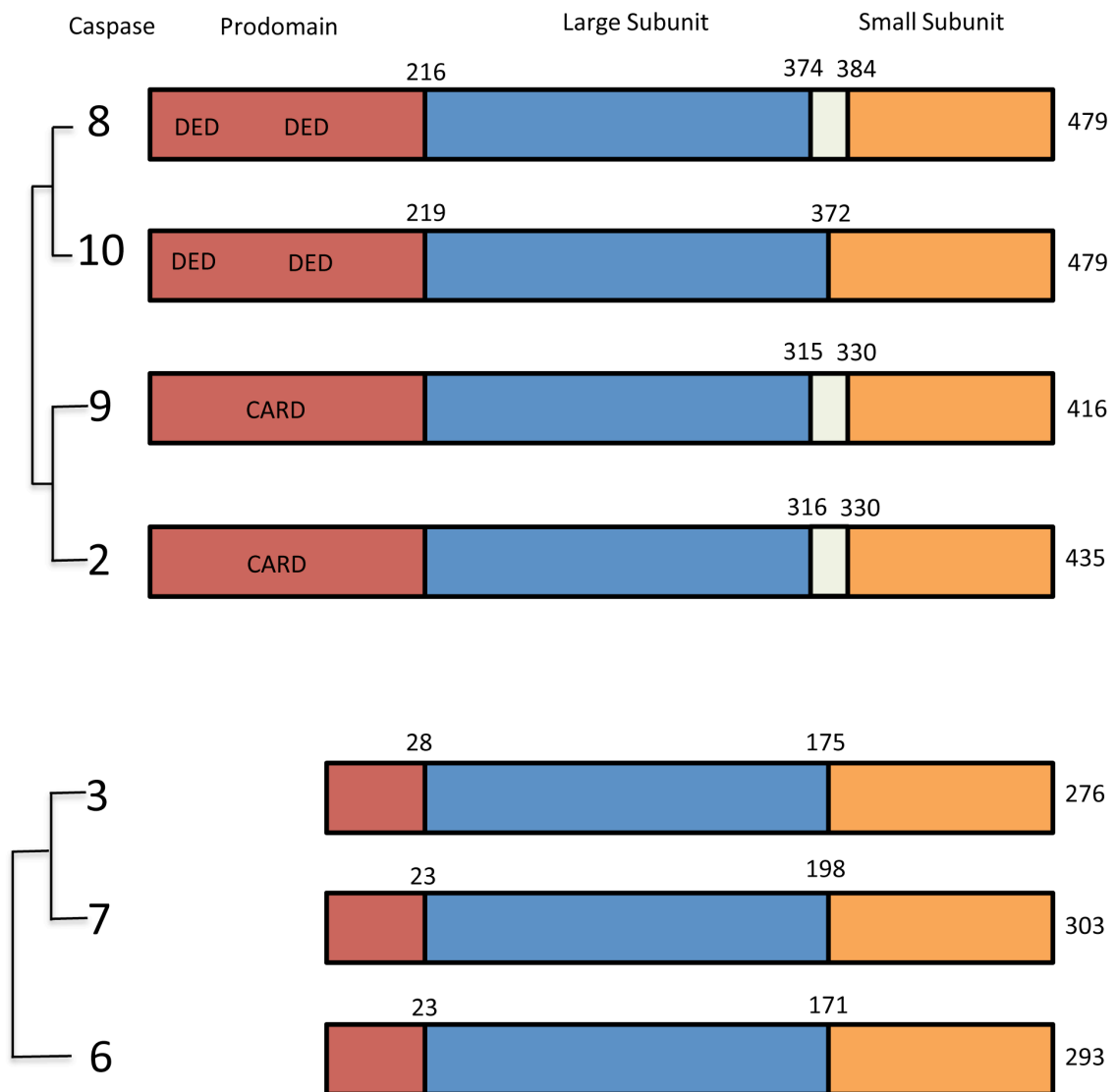


Figure 1.4 – Schematic representation of the apoptotic caspases. Effector caspases, with the larger prodomain containing protein binding domains, are shown on top, and the smaller effector caspases shown below. Processing sites are indicated at domain junctions, relatedness indicated on the left, and length in amino acids on the right. This figure is an adaptation from (235) and (236).

-10 are recruited to the DISC and achieve catalytic competency through proximity-induced dimerization (166-168). The intrinsic pathway is induced by ER stress or DNA damage (169). Ultimately, pro and anti-apoptotic Bcl2 protein family members (which themselves are heavily regulated, but beyond the scope of this thesis) act as sentinels for apoptotic stimuli and transmit their signal by modulating mitochondrial membrane integrity (reviewed in (170)). Breach of the mitochondrial membrane releases cytochrome c which causes oligomerization of Apaf-1 and formation apoptosome (171), a so-called soluble receptor capable of binding the CARD (caspase activation and recruitment domain) containing pro-domain of caspase-9. Like caspase-8, recruitment to the signaling complex mediates proximity-induced dimerization and activation of caspase-9 (172). In contrast, the executioner caspases become activated only after cleavage by initiator caspases at conserved aspartic acid residues between the large and small subunit (Figure 1.4). Cleavage does not alter greatly the overall structure of dimerized caspase-7, for instance, but permits maturation and exposure of the active site (173,174). Once active, the executioners deliver the apoptotic message through proteolysis of key structural and enzymatic proteins (9). Importantly, caspases generate a limited digestome; caspase substrates appear to have co-evolved and are not driven solely by the presence of an exposed aspartic acid and consensus motif determinants, but are affected by structural elements of the substrate as well (175).

Given the dire cellular consequences of caspase activation, it is not surprising that caspases are exquisitely regulated by a number of mechanisms. As mentioned above, the intrinsic pathway controls caspase activation indirectly through the integrity of the mitochondrial membrane. Mitochondrial permeabilization results in the release of the

aforementioned cytochrome c, but also Smac and Omi, which function by binding and inactivating inhibitors of apoptosis proteins (IAPs) (176,177). IAPs are a family of proteins characterized by containing at least one baculoviral repeat domain which bind to and inactivate mature caspases -3, -7 or -9 (reviewed in (178)). IAPs themselves are often over-expressed in cancer (179,180), and therefore Smac mimetics that bind to and inhibit IAPs are currently being pursued as a therapy for inducing apoptosis of various cancers (reviewed in (181)). Another endogenous inhibitor of caspase activity is FLIP (FLICE-inhibitory protein) (182). In this case, inhibition is mediated by blocking the maturation of caspase-8, as FLIP functions as a decoy molecule that prevents the formation caspase-8 dimers, and therefore the proximity induced dimerization and apoptotic activity of caspase-8 (183). Interestingly, CK2 inhibition has been implicated in reducing FLIP expression, and sensitizing endometrial cancer cells to extrinsic apoptotic inducers (184). Finally, phosphorylation of caspases by a variety of kinases is also implicated in both positively and negatively regulating caspase activity (reviewed in (5)). Perhaps the most well studied example is the inhibitory phosphorylation of caspase-9 at Thr125 by ERK (185), CDK1 (cyclin dependent kinase 1) (186), DYRK1A (dual specificity tyrosine-phosphorylation-regulated kinase 1A) and p38 $\alpha$  (187). Phosphorylation of caspase-9 occurs under resting conditions (187), but also in response to activated MAPK signaling (185), mitosis (186) and osmotic stress (187) and reduces caspase-9 activation and apoptosis. pT125 may also have clinical relevance in cancers such as gastric carcinoma, where tissue microarray staining suggested that 50% of samples tested stained positively for this post-translational modification (188). Other examples include caspase-8 phosphorylation by Src family tyrosine kinases at Y380

which inhibits activation in response to Fas ligand (189), and caspase-2 phosphorylation by CDK1 at S340 which safeguards against caspase-2 activation during mitosis (190). Taken together, apoptotic progression is regulated at the level of both initiator and executioner caspases by a number of diverse mechanisms.

It should be noted that caspase activation and the execution of apoptosis are not mutually exclusive. In one instance, caspase inhibition of apoptotic cells only forces mature B lymphocytes to undergo necrosis (191), a form of cell death characterized by organelle and cell swelling that leads to plasma membrane rupture (192). As mentioned above, the activation of the intrinsic apoptotic pathway is controlled by mitochondrial integrity. That caspase blockage is downstream of mitochondrial permeabilization led to the conclusion that caspase-independent cell death occurs largely through toxicity accompanied by disrupting mitochondrial function (193,194). However, and pertinent to the survivability of cancer cells, caspase-independent cell death induced by mitochondrial disruption can be overcome by GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (195). Here, GAPDH serves a protective function by increasing ATP production and perhaps promoting the autophagic clearance of damaged mitochondria.

On the flip side of cell death in the absence of caspase activity, limited caspase activation has a role in functions beyond apoptosis, such as differentiation and cell survival. In the case of cell survival, caspase-8 activity is controlled by heterodimerization with the decoy molecule FLIP and found to alter the recognition motif when compared with homodimers (183). These heterodimers result in cleavage of CYLD (196) and protection of cell death induced by RIPK1 (197). Limited caspase-3 activity also directs differentiation of hematopoietic (198), embryonic (199), neural (200),

skeletal (201), bone marrow stromal (202). Most pertinent for our discussion was the observation that caspase-cleaved connexin 45.6 only occurs in differentiated chick lens cells, but previous phosphorylation by CK2 at S363 is sufficient to block proteolysis of connexin 45.6 (203). Taken together, caspase activity performs diverse functions and is exquisitely regulated by a number of mechanisms.

### ***1.6.2 CK2 regulates caspase activity***

The examples of ARC, Bid, and even PML, phosphorylation represents clear transmittance of an anti-apoptotic signal that emanate from CK2. Via the phosphorylation of caspases themselves, CK2 is also able to control apoptosis by regulating caspase activity (Figure 1.5). In one example, CK2 phosphorylates caspase-2 in the pro-domain at S157 (204). Phospho-caspase-2 is unable to dimerize and achieve catalytic competency, whereas S157A mutants auto-activate. Furthermore, CK2 $\alpha$  knockdown is sufficient to drive caspase-2 auto-activation, as is the CK2 inhibitor DRB. CK2 also phosphorylates murine caspase-9 proximally to the processing site between the large and small subunit (205). Much like Bid, phosphorylation of caspase-9 is sufficient to block proteolysis catalyzed by caspase-8 and delay apoptosis cued by the DISC. The phospho-acceptor responsible for cleavage blockade is not conserved in human caspase-9, but nonetheless represents another intriguing example by which phosphorylation prevents caspase-mediated degradation. Indeed, a number of other CK2 substrates are also controlled in this way (6) – a regulatory process we have named ‘phospho-blocking’ – and represents the over-arching theme of this thesis (Figure 1.5).

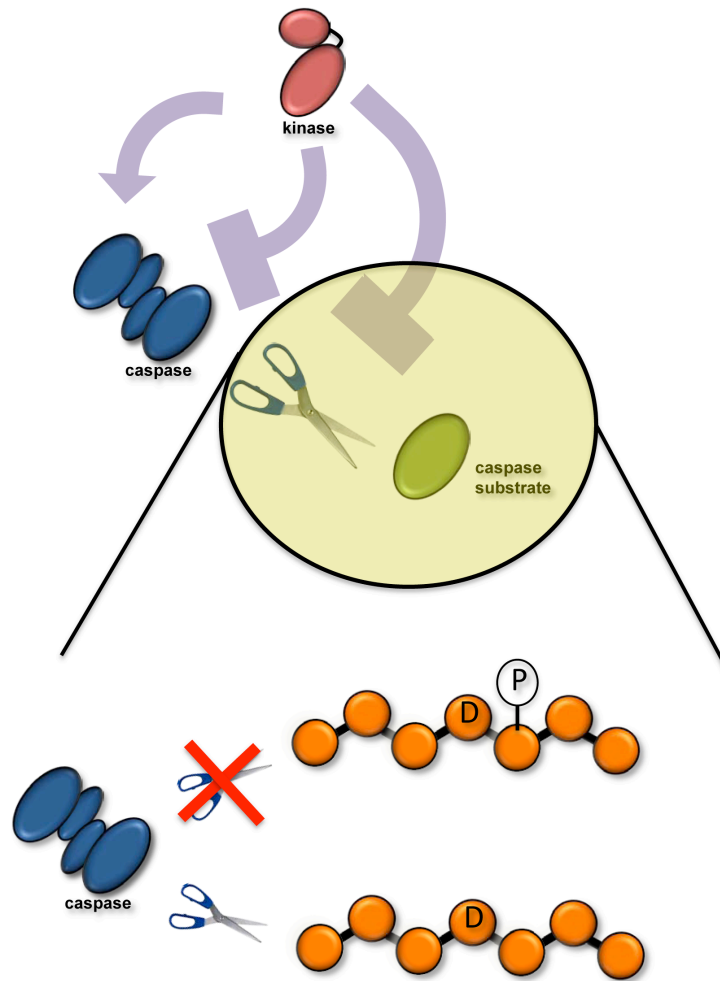


Figure 1.5 – Overview of kinase governance of caspase activity. Caspase activity is controlled in a number of ways by kinases. Most obviously, kinase phosphorylation of caspases can have a direct effect on the catalytic activity of caspases (reviewed in (5)). As well, indirect modulation of caspase activity can occur through phosphorylation of caspase substrates. Here, phosphorylation near the scissile bond blocks proteolytic processing. In this example, phosphorylation at P1' is depicted, but inhibitory phosphorylations at P2 and P4 have also been observed (215).



### ***1.6.3 Regulation of caspases by phospho-blocking***

By definition, caspases hydrolyze the peptide bond on the C-terminal side of the aspartic acid, or P1, residue. This P1 residue is recognized by the caspase S1 binding pocket – an oxyanion hole that hydrogen bonds with remarkable specificity to the carboxyl moiety of aspartic acid (206) (4 orders of magnitude more specific for Asp than Glu (207)). Similarly, substrate residues that reside roughly four amino acids upstream of P1, termed P2-P4, also make significant contacts with binding sites S2-S4 on the active caspase. Structural alignments of caspase-1, -3, -7 and -8 reveal that substrate recognition is guided by the size and chemical nature of the binding pockets (208). The P4-P1 determinants have also been rigorously characterized through positional scanning peptide libraries (207,209), and motif analysis of caspase substrate cleavage sites either curated from the literature (210) or generated from proteomic datasets (211-214). Amongst the trends emerging from a superficial analysis of caspase consensus motifs is the presence of acidic determinants for at least two of the four positions within the recognition sequence (207,209). In spite of this, but consistent with the observation that phosphorylation of Bid and caspase-9 blocks proteolysis by upstream caspases, phospho-residues located at P4, P2 and P1' reduces proteolysis of peptide substrates catalyzed by caspase-3 and -7 and phospho-P2 impairs cleavage by caspase-8 (215). Furthermore, and as alluded to earlier, phospho-blocking also regulates a number of protein substrates (Table 1.1), and in some instances, alters apoptotic progression.

Prominent examples of phospho-blocking affecting apoptotic progression include the aforementioned Bid. The location of Bid within the apoptotic cascade makes its cleavage and activation a powerful control point; only after processing by caspase-8 does

Protein	Site	Kinase	Block	Promote	Alter Apoptosis	Reference
Bid	T58, S61	CK2, CK1	X		X	(160)
Caspase-9 (mouse)	S348	CK2	X		X	(205)
PTEN	S370, S385	CK2	X			(218)
Max	S11	CK2	X			(219)
Connexin 45.6	S363	CK2	X			(203)
HS1		CK2	X			(220)
YY1	S118	CK2	X			(221)
Presenilin-2	S327, S330	CK2, CK1	X		X	(216,222)
Presenilin-1	S346	PKC			X	(223)
Acinus*	S422, S573	Akt	X		X	(224)
Caspase-2	S340	Cdk1	X <sup>†</sup>		X	(225)
Caspase-8	S387	Cdk1	X <sup>†</sup>		X	(226)
Caspase-8	S380	Src	X <sup>†</sup>		X	(189)
MST1*	S387	Akt	X		X	(227)
MST1	S327	MST1	X			(228)
PLC- $\gamma$ 1	Y771	EGFR	X			(229)
I $\kappa$ B- $\alpha$ (chicken)	S35	I $\kappa$ B- $\alpha$ kinase	X			(230)
Nogo-B	S16	Cdk1	X			(231)
PKC $\delta$	Y332	Src		X	X	(232)

Table 1.1 – Previously demonstrated examples of phosphorylation-dependent regulation of caspase-mediated cleavage

\*phosphorylation distant (>10 amino acids) from scissile bond

<sup>†</sup>Direct evidence does not exist for blockage, but phosphorylation sites are located in the interdomain linker region, and apoptosis is reduced.

it promote mitochondrial permeabilization and effector caspase activation. Interestingly, targets downstream of executioner caspases can also impair apoptotic progression. Phospho-mimetic mutants of Presenilin-2 block its own cleavage by caspase-3, but also the cleavage of PARP, another executioner caspase substrate (216). DNA fragmentation, another metric for apoptotic progression is also delayed in this instance. Collectively, these examples, and others from Table 1.1, highlight phospho-blocking as an important regulator of apoptotic progression.

## **1.7 RATIONALE FOR STUDY**

Much like caspases, the CK2 consensus motif consists predominately of acidic residues, especially at the n+1 and +3 positions, and more generally at positions anywhere from -2 to +7 (20-23). Given the shared features of the caspase and CK2 recognition motifs, and the constitutive activity of CK2, it may come as little surprise that a number of caspase substrates are phosphorylated by CK2 near the scissile bond and alters caspase cleavage (Table 1.1). Furthermore, the hyperactivity of CK2 in cancer and the general anti-apoptotic role of this activity implies that phospho-blocking of caspase substrates by CK2 may globally regulate the caspase degradome on a larger scale than previously appreciated (6,217). This presumption is also supported by the observation that caspase inhibition during cell stress can rescue cell death, and that phospho-blocking of other caspase substrates reduces apoptotic progression.

Another important consideration when studying CK2 relates to its enigmatic regulation. It is constitutively active, yet is controlled in cells through subtle mechanisms (73). Perhaps the most perplexing regulatory feature governing CK2 activity is the

regulatory subunit itself – CK2 $\beta$ . CK2 $\beta$  controls substrate specificity of a few substrates, but is thought to not affect phosphorylation of the majority of targets (52). As well, the holoenzyme forms readily *in vitro* and will dissociate only under denaturing conditions (36). However, there is indirect evidence for free-catalytic specific functionality in cells, yet these studies are often anecdotal and provide indirect evidence. Therefore, biomarkers that can unambiguously probe for the presence of different forms of CK2 in cells will bolster the understanding of CK2 regulation and how it contributes to disease processes such as apoptosis.

More generally, the emergence of an integrated network of phosphorylation and caspase-mediated proteolysis suggests apoptotic regulation extends beyond just CK2 (5,6). Indeed, a number of phospho-blocked substrates are phosphorylated by other kinases also implicated in oncogenesis (Table 1.1). However, a remaining challenge in studying this signaling mechanism is its complexity; the dynamic nature of phosphorylation networks in cells make identifying hierarchically regulated cleavage events difficult when studying entire proteomes. Indeed, the only phospho-blocked caspase substrates identified to date have been found serendipitously. Moving forward, unbiased strategies that aim to identify novel phospho-blocking events will be crucial in defining this important signaling mechanism.

## **1.8 SCOPE OF THESIS**

Given the documented integration of CK2 within the apoptotic and caspase signaling networks, we first set out to identify CK2 phosphorylation events that function to block caspase-mediated degradation. In chapter 2, we utilized peptide-match programs

that aligned putative CK2 phospho-acceptors directly proximal to predicted caspase scissile bonds. Interestingly, we uncovered and validated that CK2 phosphorylation of caspase-3 itself prevents its activating cleavage by initiator caspases -8 and -9 using recombinant proteins. Furthermore, we found that caspase-3 is phosphorylated exclusively by free-catalytic CK2, prompting development of phospho-specific antibodies to study caspase-3 phosphorylation in cells. These antibodies represent the first biomarker for a free-catalytic specific substrate of CK2.

In chapter 3, we utilized our phospho-specific caspase-3 antibodies to study phosphorylation of a free-catalytic specific substrate in cells. Interestingly, ectopic expression of only CK2 $\alpha'$ , but not CK2 $\alpha$ , resulted in phosphorylation of caspase-3, highlighting divergent functions for these extremely similar kinases. We also provide evidence that isozyme preference is not the result of differences in binding to endogenous, cellular CK2 $\beta$ , supporting a role for other regulatory factors in controlling substrate access. Given the unbalanced expression of CK2 subunits in disease tissues from cancer patients, our work provides evidence that the CK2 phospho-proteome may depend on the complement of CK2 subunits present. Furthermore, in cancer cell lines or tissues where CK2 $\alpha'$  is high and CK2 $\beta$  is low, phosphorylation of caspase-3 may represent a mechanism by which CK2 promotes anti-apoptotic signaling.

Finally, in chapter 4, we sought to develop unbiased methods to identify phosphorylation events that alter cleavage by caspases. To this end, we utilized a proteomic strategy that enriches proteomes for neo-N-termini generated by lysate protease treatment. In our case, HeLa lysates were treated with or without  $\lambda$  phosphatase, followed by caspase reactions. We validated three substrates as having altered cleavage

kinetics as a result of lysate dephosphorylation; Golgin-160 and Yap1 were phospho-blocked, whereas MST3 was actually cleaved less after dephosphorylation. We supplemented this work with a systematic investigation of the determinant properties of phosphorylated-Ser when positioned throughout the entirety of an extended caspase motif on model substrate peptides. Phosphorylation was generally inhibitory towards proteolysis, even at residues beyond the canonical caspase motif. Collectively, this work suggests that phosphorylation events that positively regulate cleavage do so by mechanisms controlling scissile bond accessibility. Furthermore, we demonstrate the utility of unbiased approaches to identify novel cleavage events regulated by phosphorylation.

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## **CHAPTER 2 – PREDICTION AND CHARACTERIZATION OF CASPASE-3 AS A SUBSTRATE OF PROTEIN KINASE CK2**

### ***2.1 INTRODUCTION***

Protein kinase CK2 is a highly conserved, constitutively active Ser/Thr kinase that is overexpressed in a number of human cancers (1,2). Illustrating its emergence as a cancer therapeutic target, inhibition of CK2 by genetic or pharmacological means is detrimental to cancer, but not healthy cell proliferation and survival (3-5). As a result of its constitutive activity and high abundance in cells, CK2 phosphorylates a large number of substrates – on the order of thousands, according to some estimates (6) – and, not surprisingly, participates in a number of cellular processes including anti-apoptotic signaling (2,7). Given the ability of cancer cells to evade apoptosis and perpetuate inappropriate cell growth, current dogma holds that the role of CK2 in cancer pathology is likely carried out by the inappropriate or hyperphosphorylation of apoptotic signaling proteins, which then act to sustain an anti-apoptotic stimulus. Identifying hyperphosphorylated CK2 substrates that perpetuate pro-survival signaling will be crucial in delineating how best to exploit CK2 inhibitors for targeted therapeutics.

Apoptotic stimuli ultimately impinge upon and activate caspases – a family of cysteine-dependent, aspartate-specific proteases that execute cell death by cleaving key structural and enzymatic proteins (8). Perhaps not surprisingly, caspases are regulated by kinases at multiple levels (9). Phosphorylation of proteins controlling apoptotic initiation, such as the Bcl2 family members Bid (10) and Bad (11), for example, and caspases themselves are well documented to alter caspase activity. Interestingly, caspase

activity is also controlled indirectly via the phosphorylation of their substrates, a number of which are targets of CK2. Here, phospho-residues in close proximity to the scissile bond, most notably at P2 or P1', hamper substrate cleavage (12). Furthermore, not only does phosphorylation of Bid (10) and Presinilin-2 (13) by CK2 reduce their susceptibility to caspase cleavage, but it also dulls the apoptotic response.

The identification of a number of CK2/caspase substrates has led us and others to hypothesize that CK2 may phosphorylate a number of other caspase substrates (1,14). In large part, this postulation is strengthened by the observed consensus motifs for CK2 and caspases. By definition, caspases cleave the scissile bond at a P1 Asp, but also prefer Asp or Glu at P3 and P4, for executioner caspases-3, and -7, and Glu at P3 for initiator caspase-8 and -9 (15). Similarly, CK2 phosphorylates substrates preferentially at Ser/Thr residues that are upstream of acidic amino acid determinants. Asp or Glu residues at n+3 and, to a slightly lesser extent, n+1 are the strongest determinants, but phosphorylation is generally increased by acidic amino acids from n-2 to +7 (16-19). Taken together, the constitutive activity of CK2 and the general similarity between the caspase and CK2 consensus motifs may explain the relatively large number of previously identified CK2/caspase substrates whose phosphorylation blocks caspase cleavage (12). Furthermore, the anti-apoptotic role of CK2 upon over-expression highlights the possibility that the convergence between CK2 and caspase signaling represents a global, previously underappreciated mechanism by which CK2 promotes cell survival.

In an effort to identify CK2 substrates that are phosphorylated proximally to a caspase cleavage site, we interrogated the proteome for sequences containing an overlapping CK2/caspase motif. To the best of our knowledge, this is the first predictive

analysis investigating the hierarchical regulation of kinases and caspases. Comparative analysis between our predictions and phospho-proteomic or caspase databases identified a number of candidates that contained one of the predicted post-translational modifications. Significantly, we found that caspase-3 itself contains a putative CK2 phosphorylation site directly adjacent to the scissile bond whose cleavage by upstream caspases is required for activation. We validated that recombinant caspase-3 is phosphorylated by CK2 at the predicted sites and that phosphorylation does indeed block proteolysis by caspase-8 and-9. In characterizing caspase-3 phosphorylation, we found that it is actually blocked by the regulatory CK2 $\beta$  subunit, a rare characteristic of CK2 substrates that may have implications for the physiological regulation of caspase-3 by CK2. Finally, we developed phospho-specific antibodies of caspase-3, which lay the groundwork for testing the pathological significance of caspase-3 phosphorylation in cells expressing high levels of CK2.

## ***2.2 MATERIALS AND METHODS***

### **2.2.1 Bioinformatics and database searching**

We designed a Perl based peptide match program to search the human proteome databases (Swiss-Prot and National Center for Biotechnology Information, 07/10/12) for overlapping CK2 and caspase consensus sequences. Overlapping motifs were designed such that the putative phospho-acceptor (S/T-X-X-D) is positioned at P2 or P1' of the predicted scissile bond for caspase-3/-7 (D-E-X-D) and caspase-8/-9 (I/V/L-E-X-D). After a proteome-wide search for matching peptides was performed, the peptide match software returned the GI number, Swiss-Prot primary accession number, and the Swiss-



Prot protein ID, as well as the sequence of the matching peptide ( $\pm 10$  amino acid residues on either side of the recognized peptide pattern). Subsequently, database mining was performed by manually searching a caspase substrate database (CASBAH) (20), and phosphorylation databases (Phosida and UniProt KB) (21) for the predicted CK2/caspase modifications.

### **2.2.2 Generation and purification of caspase-3 and related mutants**

Catalytically inactive His-tagged caspase-3 (ATCC) was engineered using a QuickChange II Site-Directed Mutagenesis Kit (Stratagene). Human caspase-3 pET23b was PCR amplified to make the (C163A) mutation to abolish catalytic activity thereby allowing for purification of full-length caspase-3 using the primer  $5'$ -TTCATTATTCAGGCCCGCCGTGGTACAGAA- $3'$ . In all cases, recombinant caspase-3 used in this study contains C163A mutation and, therefore, is the full-length, zymogen form. Phosphorylation mutants (T174A), (S176A) and (T174A S176A) of caspase-3 were generated using the following primers  $5'$ -GACTGTGGCATTGAGGCAGACAGTGGTGGTGAT- $3'$ ,  $5'$ -GGCATTGAGACAGACGCTGGTGGTGATGAC- $3'$ ,  $5'$ -GACTGTGGCATTGAGGCAGACGCTGGTGGTGATGATGAC- $3'$ , respectively. caspase-3 (C163A) and the phosphorylation mutants were purified with a HiTrap SP HP column (Amersham Biosciences) using the AKTA Purifier FPLC (Amersham Biosciences). Purified, recombinant caspase was then dialyzed into 50% glycerol, 50 mM Tris (tris(hydroxymethyl)aminomethane) (pH 7.5), and 150 mM NaCl and stored at  $-20^{\circ}\text{C}$ .

### **2.2.3 In vitro phosphorylation of caspase-3 by GST-CK2 $\alpha$**

In vitro kinase assays were performed using recombinant, 6×His-tagged, full-length, inactive (C163A) caspase-3. For phosphorylation, 0.5 – 2 µg of recombinant caspase was incubated with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.1 mM ATP, 0.2 µCi [ $\gamma$ -<sup>32</sup>P]-ATP and 35 ng of recombinant GST-CK2 $\alpha$  for 20 min at 30°C. Assays investigating stoichiometry of phosphorylation or being used for downstream 2D-peptide mapping were allowed to run for 2 h. Reactions were stopped by the addition of SDS sample buffer and proteins resolved by 10% SDS-PAGE gel electrophoresis. Gels were then dried and radiolabeled proteins visualized using a phosphorimager. The relative amount of <sup>32</sup>P incorporation was determined using ImageQuant TL software.

For kinetic analysis of caspase-3, calmodulin and  $\alpha$ -casein, 0.5 – 8 µM purified protein substrate was reacted with CK2 and terminated by adding 2× SDS sample buffer. Reactions were performed for 5 and 10 minutes to verify linearity. The holoenzyme was reconstituted by incubating equimolar amounts of His-CK2 $\beta$  and GST-CK2 $\alpha$  for 5 minutes prior to the assay. Samples were processed by SDS-PAGE, stained with coomassie blue and then dried. Phosphorylation was detected using a phosphorimager and absolute <sup>32</sup>P incorporation was determined using ImageQuant TL software. Kinetic constants were determined using GraphPad Prism software version 4.0.

#### **2.2.4 2D phospho-peptide mapping and phospho-amino acid analysis**

2D phospho-peptide mapping was performed as previously described (22). Briefly, reactions were resolved by SDS-PAGE, the gel dried and bands corresponding to caspase 3 were excised. Proteins were digested by trypsin overnight and then separated on thin-layer chromatography plates at 1000V (horizontal dimension) and by

chromatography in Scheidtmann buffer (vertical dimension). Plates were analyzed by a phosphorimager. Next, the indicated spots were scraped off the plate and processed for phospho-amino acid analysis. Briefly, recovered peptides were partially hydrolyzed in constant boiling HCl as previously described (23). Next, two-dimensional separation of partially hydrolyzed products was carried out as previously described (24). Briefly, phospho-amino acids were spotted on thin layer chromatography plates and separated in pH 1.9 buffer (2.5% formic acid/7.8% glacial acetic acid) in the first dimension (horizontal in figures) and pH 3.5 buffer (0.5% pyridine/5.0% glacial acetic acid) in the second. <sup>32</sup>P-amino acids were visualized using a phosphorimager and the presence or absence of phospho-amino acids was determined empirically.

### **2.2.5 Cleavage of caspase-3 by caspase-8 and -9**

To test the effect of caspase-3 phosphorylation on caspase-mediated cleavage, 2 µg of purified caspase-3 were incubated with GST-CK2α in a kinase assay for 2 h as described above. Following the kinase assay, 6 U/µl of caspase-8 (BIOMOL) or caspase-9 (BIOMOL) was added to the kinase reaction in caspase assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol and 10 mM DTT) for caspase-8 or (100 mM MES, pH 6.5, 10% PEG (Polyethylene Glycol, average MW 8000), 0.1% CHAPS, 10 mM DTT) for caspase-9 reactions. Cleavage reactions were incubated overnight at 30 °C with gentle agitation to obtain optimal cleavage and then stopped by addition of SDS sample buffer. Radiolabeled proteins were resolved on 12% SDS-PAGE electrophoresis, and gels were dried and visualized on a phosphorimager. Determination of the amount of <sup>32</sup>P incorporated was assessed using ImageQuant TL

software. Non-radiolabeled cleavage reactions were performed in parallel to verify caspase-8 and -9 activity. Cold kinase assays (non-radiolabelled ATP) were performed as above except were resolved on 12% SDS-PAGE, transferred to PVDF and probed with anti-caspase-3 antibodies.

### **2.2.6 Western blotting**

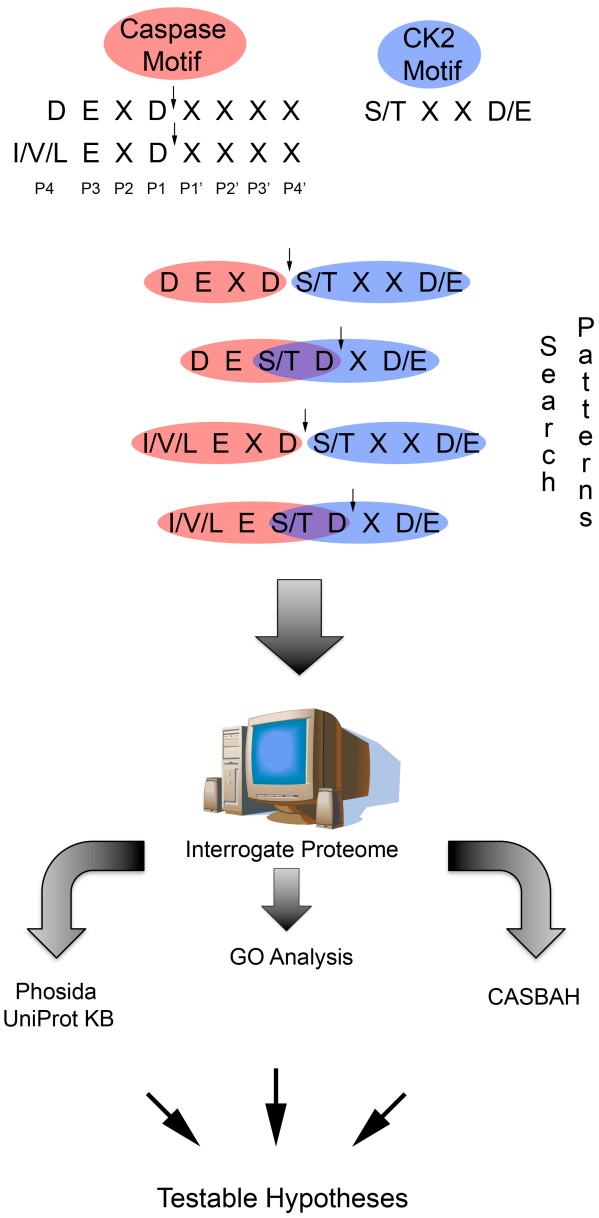
Samples were separated by SDS-PAGE and transferred to PVDF using standard procedures. Antibodies against caspase-3 (Cell Signaling Technology) and Phosphothreonine (Cell Signaling Technology) were used. Phospho-specific caspase-3 antibodies were generated against GIEpTDpSGVDDMAC by YenZym Antibodies, LLC, San Francisco, Ca using proprietary methods. Secondary antibodies used were GAR-680 (LI-COR Biosciences). Densitometric analysis was performed using Odyssey software (version 3.0) from LI-COR Biosciences.

## **2.3 RESULTS**

### **2.3.1 Identification of proteins with overlapping CK2 and caspase consensus motifs**

To test the hypothesis that CK2 phosphorylation sites frequently overlap caspase cleavage motifs, we undertook a bioinformatics approach that employed a novel peptide match strategy (Figure 2.1). Using perl script, we developed a program to interrogate the human proteome for proteins containing overlapping CK2 and caspase consensus motifs that positioned the CK2 phospho-acceptor directly proximal to the scissile bond at P2 and P1', sites previously shown to block cleavage of caspase substrate at both the protein and peptide level (12,25,26). To determine which of the putative CK2/caspase substrates had

Figure 2.1 – Workflow for the identification of caspase substrates whose cleavage is regulated by CK2 phosphorylation. Phospho-acceptors from a minimal CK2 consensus motif (blue) are positioned at P2 or P1' within a caspase consensus motif (red) and the proteome searched for these sequences using perl script. Hits were cross analyzed using Phosida and UniProt KB to determine if putative phospho-acceptors were previously identified as phosphorylated in cells, and CASBAH to assess if there was precedent for the predicted scissile bonds to actually be cleaved by caspases.



Pattern	Protein ID	Sequence	
DE.D[ST]..[DE]	MPP10	sdeditnvhdDELDSNKEddeiaeeae	
	CBPD	kksllshefqDETDTEEEtlyssk	
	UB2R1	eeeadscfgdDEDDSGTEe	
	NEK3	ldperlepglDEEDTDFEeednpdwvs	
	NFIA	sstkrksveDEMDSPGEepfytgqgrs	
	CV019	lfkppedsqdDESDDAEeeqtkrrrp	
	NOLC1	qpvedsedssDESDDSSSEekkpptkav	
	UT14C	pknypplseneDEGDSDGERkhqkllaai	
	NOL8	sgklfdssddDESDDSEDDsnrfkikpqf	
	ZC3H8	tatdsderidDEIDTEVEetqEEKikle	
	TANK	tsvtprglcrDEEDTSFEslskfnvkfp	
	CHD8	srsvppvkleDEDDSDSEldlslklspss	
	CF203	kstkkslqkvDEEDSDEEshhdemseqe	
	BAZ1B	rkkfpdrlaeDEGDSEPEavgqsrgrrrq	
	DE[ST]D.[DE]	DDEF2	yewrllhedlDESDDMdekllqpspn
		CBPD	kksllshefqDETDTEEetlyssk
		PSA3	kyakeslkeeDESDDN
MRE11		tknysevievDESDDVEedifpttstkt	
CV019		lfkppedsqdDESDDAEeeqtkrrr	
GSE1		gyyylddsyDESDEEevrahlrcva	
NIPBL		knntaaetedDESDGEDrgggtsgsl	
NOL8		sgklfdssddDESDDSEDDsnrfkikp	
MYPN		iefrlertpvDESDDDEiqhdeiptgk	
OSBL8		ksdkendqehDESDDNEvmgkseesdt	
UN93B		gyryleednsDESDDAEgehgdgae	
ZF106		kiefqvhaleDESDDGETsdtekhgk	
MST4		krwkaeghsdDESDDSEgdssestsre	
CPSF2		qskeadidssDESDDIEedidqpsahk	
MAN1		gskvllgfssDESDDVEasprdqaggg	

Table 2.1A. Amino acid sequences of putative CK2 and caspase-3/-7 substrates that have been previously identified to be phosphorylated in cells. The identified motifs are highlighted in capitals. Phosphorylation sites were identified by cross-referencing Phosida (21) and Expsy.

Pattern	Protein ID	Sequence	
[IVL]E.D[ST]..[DE]	VAMP4	gsvkserrnlLEDDSD <sup>E</sup> Eedfflrgpsg	
	NUCL	pkkmapppkeVEEDSEDE <sup>E</sup> emsedeeds	
	BMI1	qrdgltnageLES <sup>D</sup> SGSDkanspaggip	
	RBL2	pasttrrrl <sup>f</sup> VENDSPSDggtppgrmppq	
	NOL8	ddrfrmdsr <sup>f</sup> LET <sup>D</sup> SEEEqeevnekkta	
	MY18A	vtkyqkrknkLE <sup>G</sup> SDSDV <sup>D</sup> seledrvdgv	
	SCNM1	grgrwv <sup>k</sup> denVE <sup>F</sup> DSDEE <sup>e</sup> ppdlpl	
	EHMT1	asslhvngesLE <sup>M</sup> SDDED <sup>d</sup> seeleeddg	
	AVEN	ggwgagasapVE <sup>D</sup> SDA <sup>E</sup> tygeendeeg	
	SDA1	apgkcqkrkyIE <sup>I</sup> DSDEE <sup>p</sup> rgells <sup>l</sup> rd	
	SPAT7	ltdretsv <sup>n</sup> vIE <sup>G</sup> SD <sup>P</sup> E <sup>k</sup> veisngl <sup>c</sup> g	
	MBD2	sraadteem <sup>d</sup> IE <sup>M</sup> DSG <sup>D</sup> E	
	RRP15	kdhfys <sup>d</sup> ddaIE <sup>A</sup> DSE <sup>G</sup> Daepcd <sup>k</sup> enen	
	[IVL]E[ST]D.[DE]	MPP10	nlkyk <sup>d</sup> ffdpVE <sup>S</sup> DE <sup>D</sup> itnv <sup>h</sup> d <sup>e</sup> ld
		PHF2	fkdsdyv <sup>y</sup> psLE <sup>S</sup> DE <sup>D</sup> npif <sup>k</sup> sr <sup>s</sup> kk
ATRX		krkpsiv <sup>t</sup> kyVE <sup>S</sup> DDE <sup>k</sup> pl <sup>d</sup> det <sup>v</sup> ne	
HDAC4		qagvq <sup>v</sup> kqepIE <sup>S</sup> DEE <sup>e</sup> a <sup>e</sup> pp <sup>r</sup> ev <sup>e</sup> p	
WRN		ndnend <sup>t</sup> syvIE <sup>S</sup> DE <sup>D</sup> lem <sup>e</sup> ml <sup>k</sup> h <sup>l</sup> s	
CHD4		r <sup>s</sup> ssed <sup>d</sup> ldVE <sup>S</sup> DF <sup>D</sup> dasins <sup>y</sup> sv <sup>s</sup>	
SF3A1		f <sup>g</sup> esee <sup>v</sup> emeVE <sup>S</sup> DEE <sup>d</sup> kq <sup>e</sup> ka <sup>e</sup> ep	
TTBK2		p <sup>g</sup> ete <sup>e</sup> ksilLE <sup>S</sup> DNE <sup>d</sup> ekl <sup>r</sup> sg <sup>q</sup> hc	
NOL8		ddrfrmdsr <sup>f</sup> LET <sup>D</sup> SE <sup>e</sup> eqeev <sup>n</sup> ekk	
CEP68		tesrwk <sup>s</sup> eeeVE <sup>S</sup> DDE <sup>y</sup> lal <sup>p</sup> ar <sup>l</sup> t <sup>q</sup>	
CA052		ar <sup>l</sup> lpe <sup>g</sup> eetLE <sup>S</sup> DDE <sup>k</sup> deht <sup>s</sup> kk <sup>r</sup> k	
RSF1		g <sup>k</sup> psr <sup>k</sup> rl <sup>h</sup> rIET <sup>D</sup> EE <sup>s</sup> cd <sup>n</sup> ah <sup>g</sup> da	
SETD2		rg <sup>p</sup> lk <sup>r</sup> rr <sup>q</sup> eIE <sup>S</sup> DSE <sup>s</sup> d <sup>g</sup> el <sup>q</sup> dr <sup>k</sup> k	
WDR70		kt <sup>q</sup> p <sup>k</sup> tm <sup>f</sup> aqVE <sup>S</sup> DDE <sup>e</sup> ak <sup>n</sup> ep <sup>w</sup> kk	
PHF8		fk <sup>d</sup> aey <sup>i</sup> ypsLE <sup>S</sup> DD <sup>D</sup> d <sup>p</sup> alk <sup>s</sup> rp <sup>k</sup> k	
WNK2	gp <sup>q</sup> r <sup>f</sup> l <sup>r</sup> rs <sup>v</sup> VE <sup>S</sup> DQ <sup>E</sup> pp <sup>g</sup> lea <sup>a</sup> ea		
UBP15	kg <sup>a</sup> saat <sup>g</sup> ipLE <sup>S</sup> DE <sup>D</sup> Snd <sup>n</sup> d <sup>n</sup> d <sup>i</sup> en		

Table 2.1B. Amino acid sequences of putative CK2 and caspase-8/-9 substrates that have been previously identified to be phosphorylated in cells. The identified motifs are highlighted in capitals. Phosphorylation sites were identified by cross-referencing Phosida (21) and Expsy.



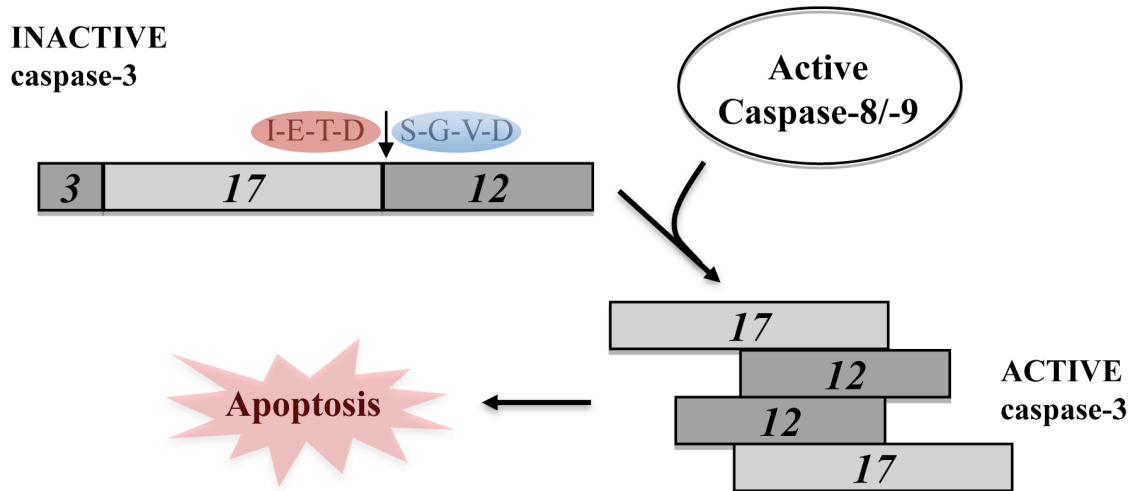
Protein ID	Sequence
CASP3	acrgtel <sup>I</sup> dcgIETDSGVD <sup>dd</sup> machkipv
CASP8	qgdnyqkgipVETDSE <sup>eq</sup> pyl <sup>em</sup> dl <sup>s</sup>
IF4H	kfk <sup>g</sup> fcyvefDEVDSLKE <sup>al</sup> tydgall <sup>g</sup>
MST4	krwkaeghsdDES <sup>DSE</sup> gsdsestsre
SATB1	khfkk <sup>t</sup> k <sup>d</sup> mmVEMDSLSE <sup>l</sup> sq <sup>g</sup> ganhvn

Table 2.2. Amino acid sequences of putative CK2 and caspase substrates that have been previously identified to be cleaved by caspases. The identified motifs are highlighted in capitals. Caspase cleavage sites were identified by cross-referencing CASBAH (20).

the predicted post-translational modification, we cross-referenced our bioinformatics data set against existing databases for phosphorylation (Phosida and UniProtKB) and caspase cleavage (CASBAH) (20,21). 59 of the 335 (17.6%) putative CK2/caspase substrates have previously been reported as phosphorylated in cells (Table 2.1A and 2.1B) illustrating the validity of the predictive approaches. Furthermore, our peptide match strategy also identified 5 previously known caspase substrates (Table 2.2). In establishing the highest priority candidates for testing if phosphorylation blocks cleavage, we gave precedence to proteins that: i) were previously identified as having at least one of the predicted post-translational modifications, and ii) has a pro-apoptotic function in cells upon caspase-catalyzed proteolysis. Application of both of these criteria identified caspase-3 and -8 as candidate caspase substrates regulated by CK2 phosphorylation.

Caspase-3 is an executioner caspase that is cleaved and activated by initiator caspases upon extrinsic or intrinsic apoptotic stimuli, and is responsible for the majority of caspase activity after engagement of the mitochondrial apoptotic pathway (27,28). Importantly, the phosphorylation site predicted by our screen reside at P1' within the cleavage site responsible for caspase-3 activation (29), and therefore, could act to block proteolysis by upstream caspases, and hinder apoptotic execution (Figure 2.2A). Interestingly, this CK2 consensus motif (S/T-X-X-D/E) is conserved from humans to frogs, but is not present in caspase-6 or -7 – the other executioner caspases – implying specialized and conserved regulation for caspase-3 (Figure 2.2B). The effect of caspase-8 phosphorylation on activation was previously studied, and so will not be further discussed here (G. Poggenpoel, personal communication).

A



B

CASP3(HUMAN)	IETDSGVD
CASP3(MOUSE)	IETDSGTD
CASP3(FROG)	IETDSCSE
CASP7(HUMAN)	IQADSGPI
CASP6(HUMAN)	DVVDNQTE

Figure 2.2 – Mechanism of caspase-3 activation. (A) The CK2 consensus site identified is located at the junction between the large and small subunit of caspase-3, where cleavage by caspase-8 or -9 is required to activate caspase-3. (B) An alignment of the scissile bond between the large and small subunit of human executioner caspases and frog and mouse caspase-3 is presented.

### 2.3.2 CK2 phosphorylates caspase-3 at T174 and S176 *in vitro*

As a prelude to assessing the regulatory effects of caspase-3 phosphorylation on its cleavage, we first tested the hypothesis that caspase-3 is a substrate for CK2. When reactions with GST-CK2 $\alpha$  and caspase-3 were performed for 2 h, stoichiometry of phosphorylation approached 1.5 pmol of phosphate per pmol of caspase-3, indicating the presence of multiple phospho-acceptors (Figure 2.3A). Next, we tested if CK2 phosphorylated caspase-3 at the predicted CK2 phospho-acceptor, S176. S176A caspase-3 mutants resulted in decreased, but not abolishment of, phospho-incorporation (Figure 2.3B). Interestingly, T174 also conforms to a minimal consensus motif – albeit not the motif used in our bioinformatics analysis – as  $n + 1$  acidic residues (16-19) are also positive determinants for CK2 phosphorylation. Indeed, T174A mutants exhibited reduced phosphorylation compared to wild-type, and S176A/T174A double mutants were not phosphorylated (Figure 2.3B). Phosphorylation of T174 was also tested using a generic P-Thr antibody. Antibody reactivity was lost upon mutation of T174, but not S176, to alanine (Figure 2.3C). Therefore, we successfully predicted S176 and serendipitously identified T174 as prominent CK2 phosphorylation sites on caspase-3. Like the Ser at P1', T174 at P2 is unique amongst the most closely related executioner caspases -6 and -7 and is conserved from *Homo sapiens* to *Xenopus laevis* (Figure 2.2B), suggesting the presence of specialized regulatory mechanisms.

In an effort to further evaluate the presence of pT174 and pS176, we performed 2-dimensional, thin layer chromatography on tryptic peptides of phosphorylated mutant and wild-type caspase-3. As seen in Figure 2.4A, mapping patterns of the single mutants are identical and do not yield new spots when samples are mixed and run together (data not

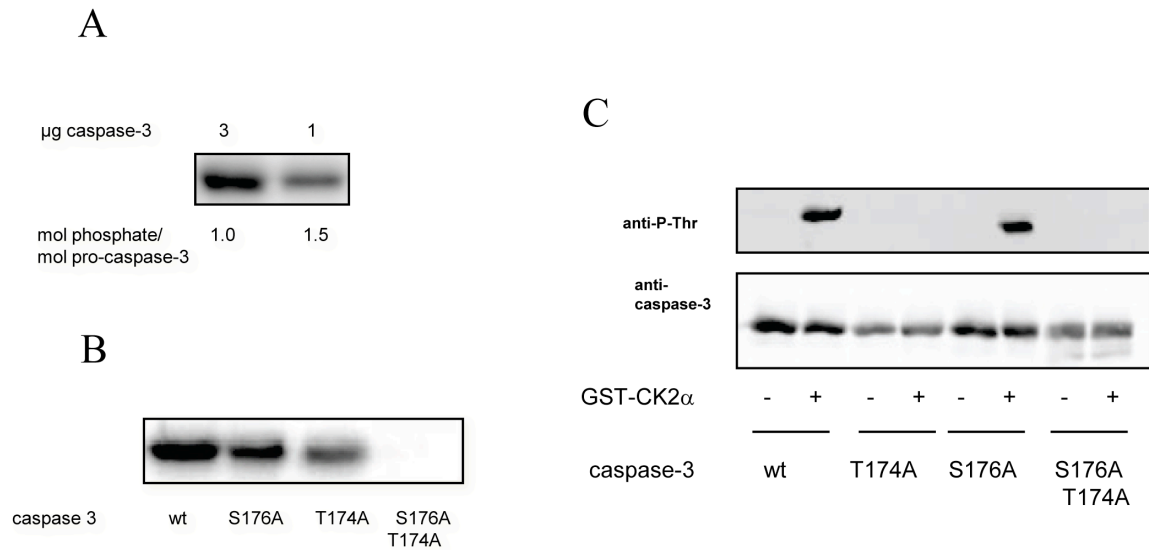


Figure 2.3 – Caspase-3 is phosphorylated by GST-CK2α at T174 and S176. (A) Different amounts of recombinant caspase-3 were phosphorylated by GST-CK2α for 2 hours and separated by SDS-PAGE. The gel was dried and stoichiometry of phosphorylation was calculated after exposure to a phosphorimager. (B) The indicated recombinant caspase-3 was phosphorylated by GST-CK2α and processed as above or (C) western blotted using anti-P-Thr or anti-caspase-3 antibodies as indicated.

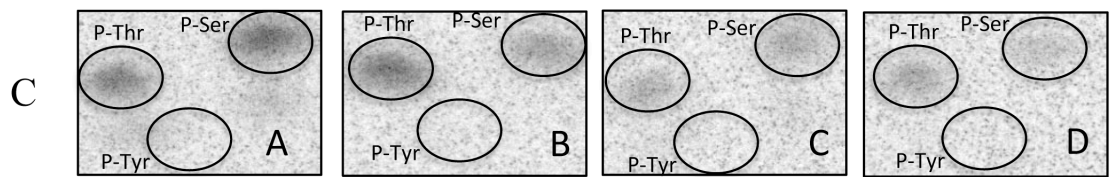
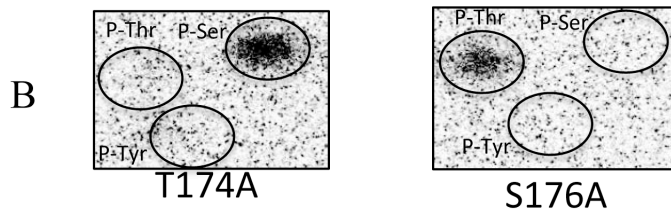
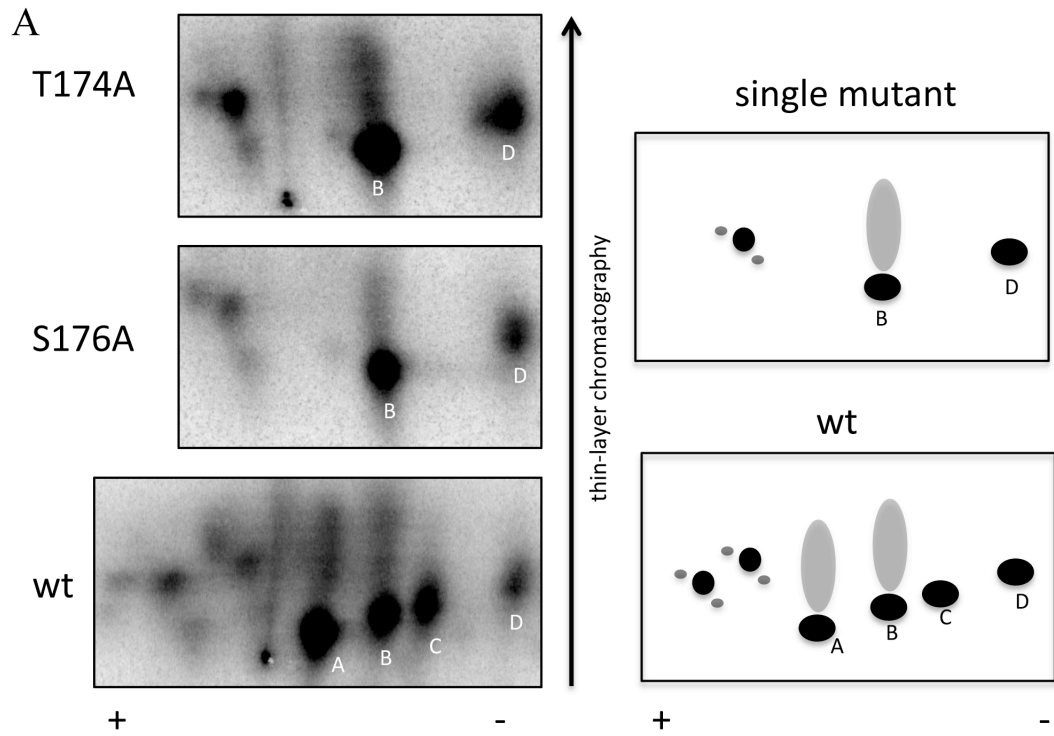
shown). In contrast, phospho-peptide maps of wild-type caspase-3 reveal two more prominent spots (labeled A and C in Figure 2.4A). Given the characteristic upward streak in the liquid chromatography phase of spot ‘A’ and ‘B’, and that ‘A’ and ‘C’ are equidistant from each other as are ‘B’ and ‘D’, we reasoned that ‘A’ is the same peptide as ‘B’ – only doubly, rather than singly, phosphorylated. Likewise, we hypothesized that ‘C’ is the doubly phosphorylated counterpart of ‘D’.

To test if spot ‘B’ contained singly phosphorylated peptides, we retrieved phospho-peptides from the mapping plates of mutant caspase-3 and performed phospho-amino acid analysis. Spot ‘B’ contained almost exclusively pSer and pThr for T174A and S176A mutants, respectively (Figure 2.4B), suggesting that this spot indeed contains singly phosphorylated peptide. Similar results were also observed for spot ‘D’ of both mutants (data not shown). Interestingly, phospho-amino acid analysis of singly phosphorylated spot ‘B’ from wild-type caspase-3 mapping plates revealed the presence of an approximately equal amount of pSer and pThr. The presence of both phospho-acceptors in a spot that co-migrates with peptides from single phosphorylation site mutants suggests that phosphorylation does not occur in a sequentially ordered fashion. Not surprisingly, spots ‘A’ and ‘C’, the predicted doubly phosphorylated peptides, also contained both pThr and pSer.

### **2.3.3 CK2 $\beta$ inhibits phosphorylation of caspase-3 by GST-CK2 $\alpha$**

In an effort to fully characterize the phosphorylation of caspase-3 by CK2 *in vitro*, we performed kinase assays in the presence or absence of the regulatory CK2 $\beta$  subunit. Generally, CK2 $\beta$  stimulates the catalytic subunits of CK2 by 3-5 fold on most substrates

Figure 2.4 – 2D-peptide mapping and phospho-amino acid analysis reveals non-ordered phosphorylation of T174 and S176. (A) Recombinant caspase-3 was phosphorylated by GST-CK2 $\alpha$  for 2 h, digested with trypsin and separated by electrophoresis (horizontal) and chromatography (vertical). Thin layer chromatography plates were imaged by autoradiography. On the right is a schematic representation of the spot patterns generated by the wild-type and single mutant phospho-caspase-3. (B) Spot ‘B’ from T174A and S176A plates were scraped off, partially hydrolyzed by boiling in acid and phospho-amino acids separated by 2D-electrophoresis as described in the materials and methods. Plates were imaged using a phosphorimager. (C) Phospho-amino acid analysis of spots ‘A’ – ‘D’ of wild-type phospho-caspase-3 was carried out as described in (B).





(30), but we found that CK2 $\beta$  almost completely abolished phosphorylation of caspase-3 (Figure 2.5A). Interestingly, when comparing GST-CK2 $\alpha$  to commercially available holoenzyme or GST-CK2 $\alpha$  supplemented with stoichiometric amounts of recombinant His-CK2 $\beta$ , we observed a decrease in the ratio of phospho- caspase-3 to phospho-casein. A detailed kinetic analysis using reconstituted CK2 holoenzyme confirmed these results and revealed that caspase-3 phosphorylation behaved similarly to calmodulin – a CK2 substrate that was previously shown to be specific only for the catalytic subunit (Figure 2.5B) (30). In both cases, addition of the  $\beta$  subunit reduced the  $k_{cat}/K_m$  ( $\text{sec}^{-1}\mu\text{M}^{-1}$ ) by a factor of 20, while casein expectedly showed more favourable phosphorylation, further validating holoenzyme reconstitution after  $\beta$  addition. Also like calmodulin, poly-L-lysine treatment of the holoenzyme rescues the inhibitory effect of CK2 $\beta$  on caspase-3 phosphorylation (Figure 2.5C) (30,31).

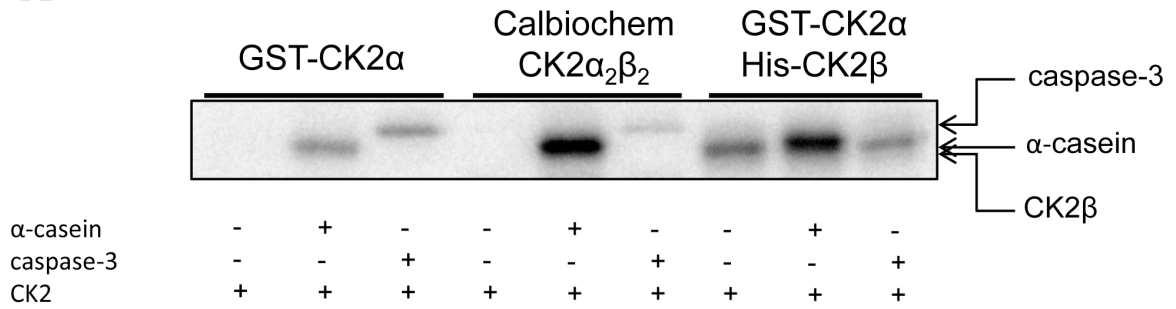
#### **2.3.4 Phosphorylation of caspase-3 by GST-CK2 inhibits cleavage by initiator caspases 8 and 9**

Following the identification of CK2 phosphorylation sites that flank the scissile bond on caspase-3, we next tested if phosphorylation blocks caspase-3 cleavage by upstream initiator caspases. As judged by figure 2.6A and B, pre-phosphorylation of caspase-3 by CK2 significantly reduces the appearance of cleaved caspase-3 (17 kDa fragment) after incubation with initiator caspases-8 and -9. Furthermore, we ran an experiment in parallel in which pre-phosphorylation of caspase-3 was performed with radiolabelled ATP- $\gamma$ -P<sup>32</sup> (Figure 2.6A). Here, the absence of a radiolabelled 17-kDa caspase-3 fragment after initiator caspase treatment further reinforces the notion that

Figure 2.5 – Phosphorylation of caspase-3 is inhibited by the regulatory CK2 $\beta$  subunit.

(A) Three different preparations of CK2 were used to phosphorylate recombinant caspase-3 for 20 minutes. In the case of the reconstituted holoenzyme, equimolar His-CK2 $\beta$  was added to GST-CK2 $\alpha$  and incubated for 5 minutes prior to starting the reaction to allow the holoenzyme to form. Reactions were separated by SDS-PAGE, the gels dried and visualized by a phosphorimager. (B) Kinetic analysis of different CK2 substrates was performed with GST-CK2 $\alpha$  or GST-CK2 $\alpha$ /His-CK2 $\beta$  (hCK2). Reconstitution of the holoenzyme and reaction visualization was performed as in (A) and the standard error from the mean calculated from three kinase reactions. CAS =  $\alpha$ -casein, C3 = caspase-3 and CaM = calmodulin. (C) Kinase assays were performed as in (A). Poly-L-Lysine (PLL) was added to the holoenzyme 5 minutes prior to starting the kinase reaction.

A



B

	C3 (CK2 $\alpha$ )	C3 (hCK2)	CaM (CK2 $\alpha$ )	CaM (hCK2)	CAS (CK2 $\alpha$ )	CAS (hCK2)
Vmax (nmol min <sup>-1</sup> mg <sup>-1</sup> )	52.26	7.859	30.99	5.711	39.8	52.65
Km ( $\mu$ M)	1.437	1.719	9.217	21.35	3.664	3.061
kcat (sec <sup>-1</sup> )	2.90	0.44	1.72	0.32	2.21	2.93
kcat/Km (sec <sup>-1</sup> $\mu$ M <sup>-1</sup> )	2.02	0.25	0.19	0.01	0.60	0.96
Vmax (SEM)	3.669	1.868	4.406	3.421	4.813	3.659
Km (SEM)	0.2988	1.138	2.091	16.51	0.9534	0.4865

C

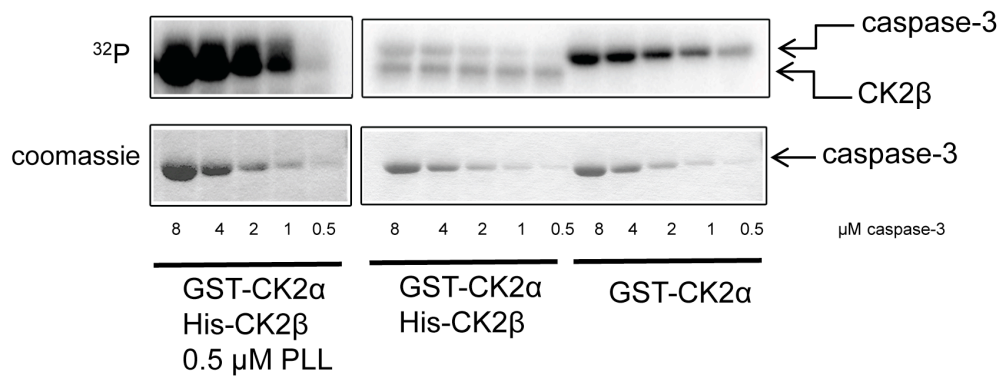
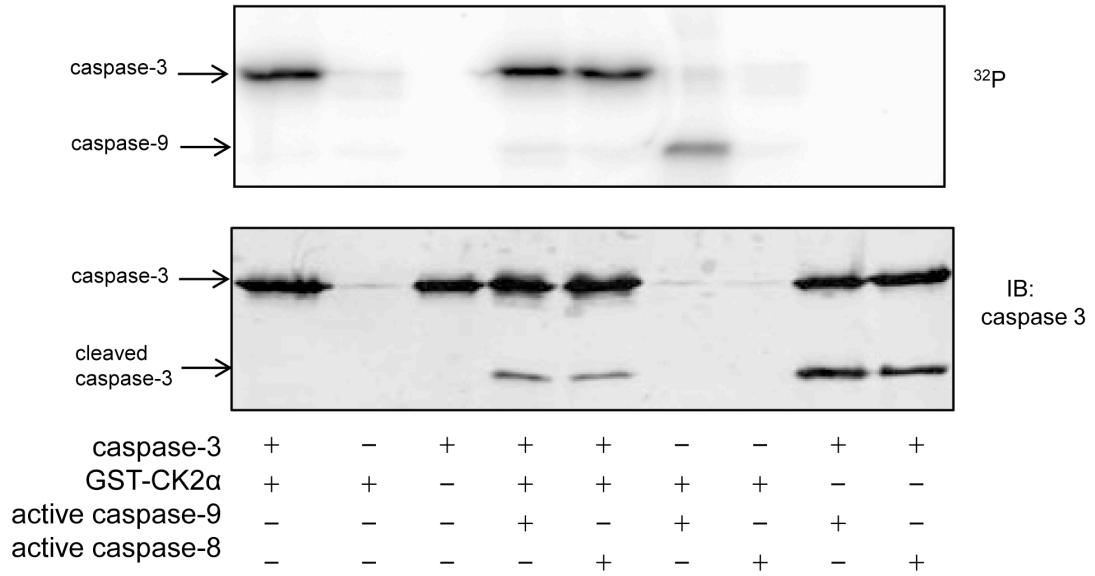
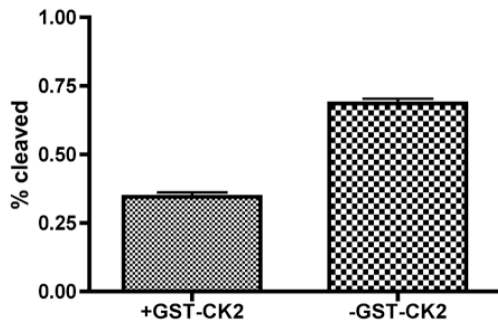


Figure 2.6 – Phosphorylation of caspase-3 by GST-CK2 $\alpha$  blocks proteolytic processing by caspase-8 and -9. (A) Kinase assays with GST-CK2 $\alpha$  and caspase-3 were performed for 2 h, followed by addition of active caspase-8 or -9 for an additional hour. Reactions were performed with both ( $\gamma$ - $^{32}$ P) ATP, to visualize phospho-caspase-3 (top panel), and ‘cold’ ATP to visualize total caspase-3 (bottom panel). (B) Cold kinase assays were performed as in (A) in triplicate and the percent cleavage, as determined by densitometric analysis of the cleaved caspase-3 fragment, plotted. Error bars represent the standard error from the mean. (C) Cold kinase assays were performed as in (A) using either wild-type caspase-3 or the double phospho-acceptor mutant and TBB as indicated.

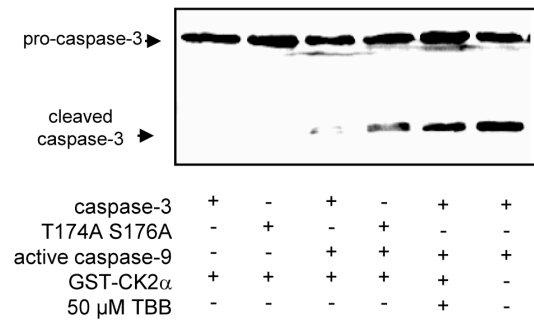
A



B



C



caspase-3 phosphorylated by GST-CK2 is resistant to proteolysis. To test if binding of GST-CK2 to caspase-3, as opposed to phosphorylation, is responsible for the proteolytic blockage, we compared the cleavage of caspase-3 to a double phospho-acceptor mutant after pre-incubation with GST-CK2 and ATP. That more cleavage appears with the T174A/S176A mutant is consistent with the hypothesis that phosphorylation, rather than binding by GST-CK2 $\alpha$ , is responsible for inhibiting proteolysis of caspase-3 (Figure 2.6C). Furthermore, protection from cleavage can be rescued by co-treating pre-phosphorylating assays with the CK2 ATP competitive inhibitor, TBB.

### **2.3.5 Characterization of phospho-specific antibodies targeted against pT174/pS176 of caspase-3**

As a prelude to studying caspase-3 phosphorylation in cells, we undertook the development of affinity purified pT174/pS176 caspase-3 antibodies through a contract supplier. To validate antibody specificity and sensitivity, we probed various forms of caspase-3, including wild-type, both single mutants and the double mutant after treatment with GST-CK2 $\alpha$  (Figure 2.7). Densitometric analysis revealed a time dependent increase in phospho-caspase-3 signal that mirrored that of  $^{32}\text{P}$  incorporation into caspase-3. At a 1:500 dilution, the anti-phospho-caspase-3 was able to detect approximately picomolar levels of phosphorylated caspase-3. Furthermore, these antibodies were capable of detecting both phosphorylated T174A and S176A, but not the non-phosphorylatable mutant, validating antibody specificity.

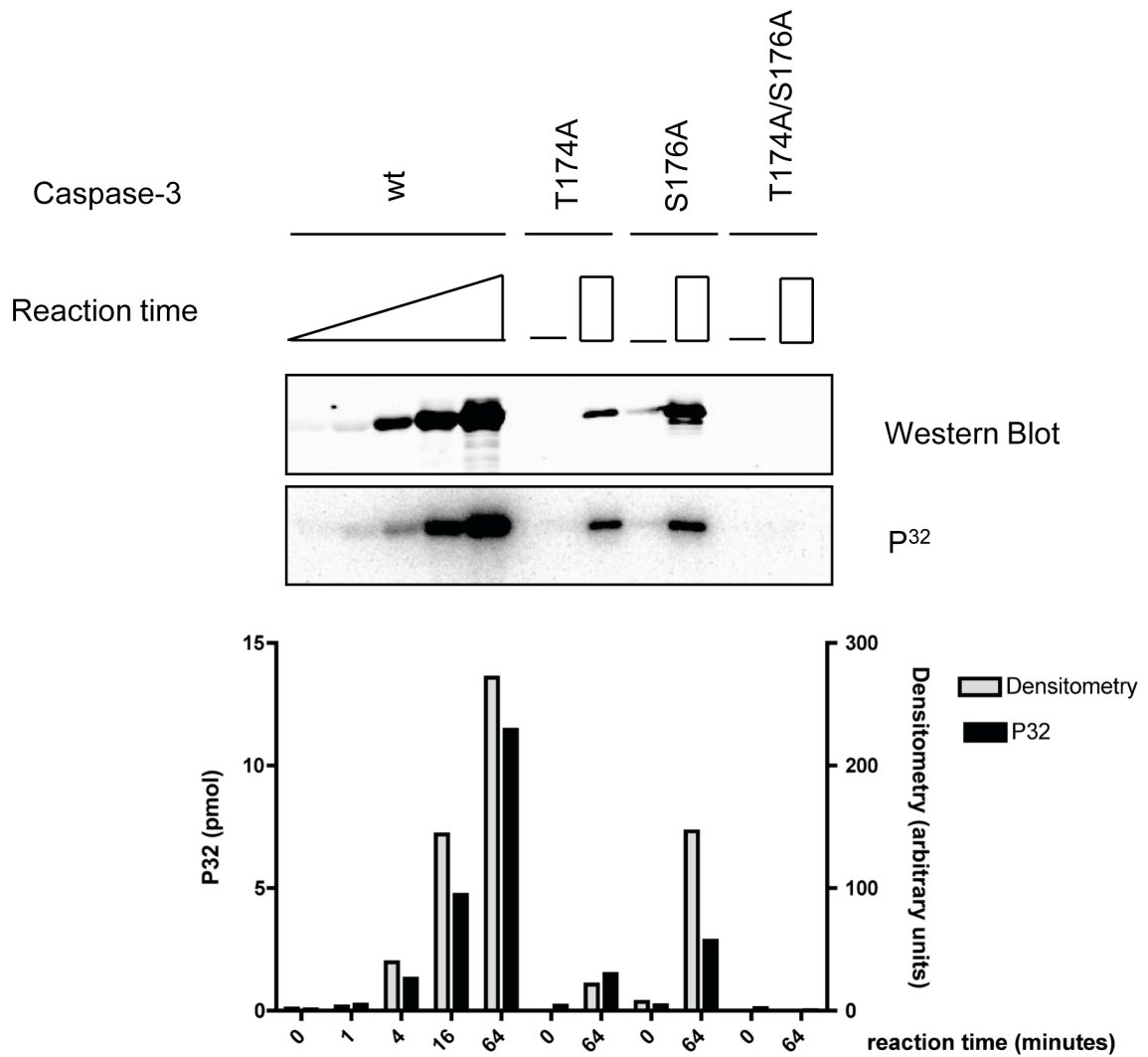


Figure 2.7 – Characterization of pT174/pS176 phospho-specific caspase-3 antibodies. Recombinant caspase-3 and the corresponding single and double phosphorylation site mutants were phosphorylated by recombinant GST-CK2 $\alpha$  with  $\gamma$ -<sup>32</sup>P-ATP. Samples were separated by gel electrophoresis, transferred to PVDF and visualized by phosphorimaging. Next, the same membrane was immunoblotted with pT174/pS176 phospho-caspase-3 antibodies.

## 2.4 DISCUSSION

Evidence of a structural mechanism for phosphorylation-dependent protection of caspase substrates, as well as the protection of a wide variety of caspase targets by phosphorylation suggests a role for phosphorylation in the negative regulation of apoptosis (9,25,32). Perhaps the most pertinent protein kinase that indirectly regulates the cleavage of caspase substrates by phosphorylation is CK2. CK2 is over-expressed in human cancers, participates in anti-apoptotic signaling, is constitutively active, and has a consensus motif that, like caspases, shows preference for acidic amino acids (33). Therefore, we were prompted to undertake the first bioinformatics analysis of clustered CK2 and caspase motifs and, in doing so, identified 64 proteins previously reported to have at least one of the predicted post-translational modifications. One protein, MST4, had reports of both modifications, but upon further literature review it was clear that its suitability as a caspase substrate was controversial, and so was omitted from being a high priority candidate (34,35). With no candidates known to be both cleaved by caspases and phosphorylated in cells, we opted to focus on putative substrates previously reported to have at least one of the predicted post-translational modifications and/or a *bona fide* role in apoptotic signaling upon caspase-mediated cleavage and identified caspase-3 itself.

The significance of identifying regulatory phosphorylation sites on caspase-3 is underscored by its critical role in the apoptotic cascade (Figure 2A). The extrinsic and intrinsic apoptotic pathways both converge on caspase-3, where considerable evidence suggests that caspase-3 is the primary executioner caspase responsible for carrying out the demolition phase of the late apoptotic response (36-38). Caspase-3 is subject to genetic alterations in cancer, including deletions, which abolish expression in MCF-7



breast cancer cells or point mutations in HL60/ADR cells that impair activation (39-41). In both instances, inhibition of caspase-3 activation diminishes the apoptotic response to chemotherapeutic agents, and sensitivity to cytotoxic drugs is rescued by expression of wild-type caspase-3, suggesting that post-translational modifications that act to block caspase-3 activity may also be detrimental to an apoptotic response. Along these lines, a number of other post-translational modifications have been identified as contributors to the regulation of caspase-3, including ubiquitination (42), nitrosylation (43), and glutathiolation (44). That CK2 blocks caspase-3 activation *in vitro* highlights a novel, albeit still theoretical, CK2 substrate that could perpetuate an anti-apoptotic signal.

Kinetic studies revealed that caspase-3 is preferentially phosphorylated in the absence of the regulatory CK2 $\beta$  subunit, and that CK2 displays better kinetic parameters against caspase-3 than  $\alpha$ -casein and calmodulin. Interestingly, CK2 has hundreds of known substrates, but only a small fraction – Calmodulin, CapZ, S100B and NKX3.1 – have shown reduced phosphorylation in the presence of CK2 $\beta$  (6,19,30,45,46). One postulation for the inhibitory effect of CK2 $\beta$  on a subset of substrates is that an acidic stretch on  $\beta$  (amino acids 55-64) binds the catalytic subunit and partially occludes substrate binding (47,48). Under this model, relaxation of restricted substrates can be achieved with polyamines that act by binding to and competing CK2 $\beta$ (55-64) off of the catalytic subunit, thereby rescuing substrate phosphorylation. That poly-L-lysine treatment of the holoenzyme permitted caspase-3 phosphorylation suggests that the structural/binding features governing phosphorylation of other substrates of CK2 inhibited by CK2 $\beta$ , such as calmodulin, may also dictate phospho-transfer onto caspase-

3. Along these lines, in both instances the presence of CK2 $\beta$  appears not to affect  $K_m$ , but instead catalytic efficiency.

The development of a phospho-caspase-3 antibody represents the first biomarker for a CK2 substrate phosphorylated preferentially by the free catalytic subunit. In this regard, the cellular function of catalytic CK2 devoid of CK2 $\beta$  remains relatively unexplored. One major component of this is the extremely tight interaction between the regulatory and catalytic subunits (49), and a critical role in cell viability for CK2 $\beta$  that makes knockdown or knockout studies impractical (50). The CK2 holoenzyme, made up of a CK2 $\beta$  dimer core bound to two catalytic subunits, only dissociates under denaturing conditions *in vitro* (51). It seems to follow that unless the regulatory and catalytic subunits are excluded from one another, are actively dissociated, or are disproportionally expressed, the holoenzyme will form in cells. Despite the tight association between CK2 $\alpha$  or  $\alpha'$  and  $\beta$ , evidence for free catalytic functionality in cells has been observed. The phosphorylation of calmodulin at the sites phosphorylated *in vitro* by CK2 were identified from rat liver (52). In a related vein, evidence for misbalanced expression between the catalytic and regulatory subunits has been observed in breast (53) and head and neck cancers (54). While the absolute levels of catalytic and regulatory CK2 were not measured in these samples, it is tempting to speculate that sub-stoichiometric amounts of CK2 $\beta$  preclude holoenzyme formation and drive free-catalytic specific functions. Indeed, in Chapter 3, we aim to expand on these studies by utilizing our antibodies to examine the presence of free-catalytic CK2 in HeLa cells, and test the hypothesis that caspase-3 phosphorylation blocks apoptotic progression.

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## **CHAPTER 3 – CHARACTERIZING THE CONVERGENCE OF PROTEIN KINASE CK2 AND CASPASE-3 REVEALS ISOFORM-SPECIFIC PHOSPHORYLATION OF CASPASE-3 BY CK2 $\alpha'$ : IMPLICATIONS FOR THE PATHOLOGICAL ROLES OF CK2**

### ***3.1 INTRODUCTION***

Protein kinase CK2 is a ubiquitously expressed, highly conserved, constitutively active Ser/Thr kinase implicated in a plethora of cellular functions, including cancer progression (1). High transcript expression (2,3), protein levels and kinase activity drive the pathological functions of CK2 in a number of human tumors and lymphomas (reviewed in (4)). Indeed, this causative role for CK2 in transformation was observed in mice where tissue-specific overexpression of CK2 $\alpha$  in breast and T-cells increased cancer burden (5,6). As such, interest in CK2 as a target for cancer therapeutics has swelled of late, with the number of patents filed for CK2 inhibitors greatly increasing in recent years (7) and one inhibitor, CX-4945, even reaching clinical trials (8). Implicit in understanding how CK2 inhibition can be most successfully translated to the clinic will be a careful dissection of CK2 function in disease. Interestingly, recent studies have observed mis-balanced expression of the CK2 subunits in breast (9) and head and neck cancer (10), though how the relative levels of different subunits can impact disease progression remains largely unexplored.

The two catalytic subunits of CK2 – CK2 $\alpha$  and  $\alpha'$  – are almost 88% identical within their catalytic domain, but exhibit divergent C-termini (11-14). Not surprisingly, CK2 $\alpha$  and  $\alpha'$  display a shared minimal consensus phosphorylation site of S/T-X-X-D/E,

suggesting that isozyme-specific, cellular functions/substrates are governed through subtle regulatory processes. The unique of CK2 $\alpha$  C-terminus has a number of mitotic phosphorylation sites and dictates specific interactions with Pin-1 and the mitotic spindle (15,16), but its catalytic domain also appears able to govern isozyme-specific interactions with CKIP-1 (17), PP2A (18), and a tighter interaction with the CK2 $\beta$  subunit compared to CK2 $\alpha'$  (19). Interestingly, while CK2 $\beta$  generally promotes phosphorylation of CK2 substrates by increasing thermal stability, a small subset of targets – most notably calmodulin – are phosphorylated only in the absence of CK2 $\beta$  (20,21). Despite a divergence in binding affinities between CK2 $\beta$  and CK2 $\alpha$  or  $\alpha'$ , the phosphorylation of substrates specific to the catalytic form of CK2 has not been systematically investigated in cells. Furthermore, the observation that CK2 $\beta$  modulates substrate specificity may be particularly important with respect to CK2-related disease pathology, as a high CK2 $\alpha$ :CK2 $\beta$  ratio drives epithelial-to-mesenchymal transition in human breast cancer (9) and transgenic mice that over-express only CK2 $\alpha$  in the mammary gland are more prone to adenocarcinomas than control litter mates (5).

The functional consequence of CK2 over-expression in human cancer remains enigmatic, likely due to the huge number (thousands) of substrates predicted to be phosphorylated by CK2 (22). With respect to the cancer promoting functions of CK2, an anti-apoptotic role has been observed in a number of different studies (reviewed in (4)). Interestingly, CK2 $\alpha'^{-/-}$  mice display a hyper-apoptotic phenotype in spermatocytes (23) and forced expression of kinase-inactive CK2 $\alpha'$  in U2-OS cells leads to defects in growth (24), but the cancer/CK2 literature remains largely biased to CK2 $\alpha$ . Mechanistically, a number of CK2 substrates, such as Bid (25) and YY1 (26), are also caspase substrates,

and are phosphorylated proximally to their scissile bond such that phosphorylation blocks both caspase cleavage, and the progression of apoptosis (reviewed in (27)). This phenomena appeared phenotypically relevant to CK2 over-expression and cancer progression, so we previously explored the ability of CK2 to phosphorylate caspase substrates *en masse* proximally to their scissile bond, and identified the caspase-3 zymogen as a lead candidate (28). Furthermore, we found that caspase-3 was phosphorylated by recombinant CK2 catalytic subunits at the residues directly flanking the scissile bond that directs cleavage and caspase-3 activation. Phosphorylation at these positions blocked caspase-3 cleavage by caspase-8 and -9 *in vitro*, validating our predictive strategy for identifying CK2 substrates that experience differential caspase-mediated degradation that depends on phosphorylation.

Given the importance of caspase-3 in the apoptotic response, we developed phospho-specific antibodies and employed systematic over-expression of the different forms of CK2. We found that caspase-3 phosphorylation was specific for CK2 $\alpha'$  in cells, but not *in vitro* kinase assays, and that the catalytic domain drove this preference. Furthermore, we show that CK2 $\beta$  negatively regulates caspase-3 phosphorylation, though the isozymic specificity was not a result of differential binding between  $\alpha$  and  $\alpha'$  to endogenous CK2 $\beta$ . Instead, our data support a model by which the free catalytic form of CK2 $\alpha$  and  $\alpha'$  are differentially regulated by a non-CK2 $\beta$  mechanism that differentially controls substrate access.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Cell culture and transfections**

HeLa (tet-Off) cells (Clontech) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) on 10-cm dishes. Cells were transfected using 40 µg of total plasmid DNA via the calcium phosphate method (29). For phosphatase inhibition studies, cells were treated with okadaic acid (Bioshop).

### 3.2.2 Plasmid Construction

CK2 $\alpha$ -HA, CK2 $\alpha'$ -HA, caspase-3-FLAG (C163A), caspase-3-his (C163A) and myc-CK2 $\alpha'$  were prepared as described elsewhere (28,30). CK2 $\alpha$ -HA (1-45 CK2 $\alpha'$ ) and CK2 $\alpha$ -HA (300-350 CK2 $\alpha'$ ) were constructed by first subcloning CK2 $\alpha$ -HA from pRC/CMV into pet28a using HindIII and ApaI. CK2 $\alpha'$ -HA was cut with BstBI / Bsu36I, to obtain the 1-45 amino acid fragment of CK2 $\alpha'$ , and ligated into the pet28a-CK2 $\alpha$ -HA. This subclone was then moved back to pRC/CMV using HindIII / XbaI. For 300-350 CK2 $\alpha'$ , CK2 $\alpha'$ -HA was ligated into pRC/CMV-CK2 $\alpha$ -HA after digestion with Bsu36I and ApaI. 45-300 CK2 $\alpha'$  was cloned using overlapping DNA sub-fragments generated by PCR reactions with T7 (F 5'-TAA TAC GAC TCA CTA TAG GG-3') and reverse 5'-CCC CGA CCA AGT TTT CGA ACC AGC TGG TAG TCA TC-3' (for 1-45 CK2 $\alpha$ ), forward 5'-GAT GAC TAC CAG CTG GTT CGA AAA CTT GGT CGG GG-3' and reverse 5'-GTC CAG GAA ATC CAA GGC CTC AGG GCT GAC AAG GTG-3' (for 45-300 CK2 $\alpha'$ ) and forward 5'-CAC CTT GTC AGC CCT GAG GCC TTG GAT TTC CTG GAC-3' and Sp6 (R 5'-CAG CGA GCT CTA GCA TTT AGG TGA CAC TAT AG-3') (for 300-391 CK2 $\alpha$ -HA). Sub-fragments were gel purified, mixed, PCR

amplified for 2 cycles, amplified for another 20 cycles with T7 and Sp6, ligated into pCR-BLUNT, and cloned back into pRC/CMV using HindIII and ApaI. Caspase-3-FLAG-NLS-(C163A) was generated by performing PCR reactions on caspase-3-FLAG-(C163A) with T7 (F) and a reverse primer that included an NLS (nuclear localization sequence) tag (PKKKRKV) and a XhoI restriction site downstream of the FLAG tag (5'-TTC TCG AGC TAC ACC TTA CGC TTT TTC TTT GGC TTG TCG TCG TCG TCC TT-3'). The PCR product was ligated into PCR-Blunt, digested out using BamHI and XhoI and ligated into pCDNA3.1(+). All constructs were sequence verified.

### **3.2.3 Cell lysis, immuno-precipitations and Western blotting**

Cells were lysed in NP-40 alternative lysis buffer [1% NP-40 alternative, 150 mM NaCl, 50 mM Tris (tris(hydroxymethyl)aminomethane) (pH 7.5), leupeptin (10 µg/mL), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin (10 µg/mL), aprotinin (5 µg/mL), 25 mM NaF, 1 µM okadaic acid and 1 mM sodium orthovanadate]. Lysates were sonicated 2 × 5 s, spun for 15 minutes at 4°C at 13 000 × g, and protein concentrations determined by bicinchoninic acid (BCA) assay (Thermo Scientific). For immuno-precipitations, 0.5 – 2 mg of lysate was tumbled 4 µg of anti-Flag M2 (Sigma) and 30 µL of protein A/G sepharose beads (GE Healthcare) for 1 H. For CK2β immuno-precipitations, anti-CK2β sera (31) was tumbled with 200 µg of lysate for 1 H, then 30 µL of protein A sepharose beads added and tumbled for an additional hour. Endogenous caspase-3 was immuno-precipitated from 10 mg of lysate with 5 µg of anti-caspase-3 (BD Biosciences clone 19) and 30 µL of protein A/G sepharose beads. Immune-complexes were washed 2 × 500 µL NP-40 alternative lysis buffer and proteins separated

by 12% SDS-PAGE, transferred to PVDF membranes and blotted using antibodies to: caspase-3 (BD biosciences clone 19), HA (3F10, Roche), PARP (Cell Signaling Technology), Vinculin (Sigma), phospho-caspase-3 (pT174/pS176) (32), CK2 $\alpha$  (33), CK2 $\alpha'$  (33), CK2 $\beta$  (33), SAV-680, GAM-680 and GAR-800 (LI-COR Biosciences). Quantitation of western blots was performed using Odyssey software (version 3.0) from LI-COR Biosciences.

### **3.2.4 Nuclear and Cytoplasmic fractionation of HeLa cells**

CK2-HA was assessed in different cellular departments using the NE-PER Nuclear-Cytoplasmic extraction kit (Thermo Scientific). Briefly, transfected HeLa cells were lysed and subsequently treated according to manufacturer's recommendations in order to obtain purified whole cell extracts of nuclear or cytoplasmic origin. Using a BCA protein assay kit (Thermo Scientific), 40 or 80  $\mu$ g of whole cell extract was loaded onto 10 or 15 % SDS-PAGE gels and the resolved proteins were transferred to PVDF membrane for subsequent Western Blotting using monoclonal and polyclonal antibodies targeted against caspase-3, phosphorylated caspase-3, CK2 $\alpha$ , CK2 $\alpha'$ , and CK2 $\beta$ . Vinculin and PARP antibodies were used to monitor for purity of each cellular compartment.

### **3.2.5 Kinase assays**

Kinase assays using recombinant proteins were performed largely as described in (28). Caspase-3-his-(C163A) and catalytic subunits of GST-tagged CK2 were purified from bacterial sources as previously noted in (28) and (34), respectively. Holoenzyme

preparations of CK2 were prepared by expressing catalytic and regulatory subunits of CK2 in separate preparations of *E. coli*, mixing the pellets during lysis, and isolating the holoenzyme by purification on a heparin column and then by size exclusion chromatography. The activity of purified CK2 was characterized (34) by performing kinase assays with the substrate peptide RRRDDDSDDD (EZBiolab) (100  $\mu$ M) so that equal units of enzyme could be added to kinase assays containing 1  $\mu$ g of recombinant caspase-3-his-(C163A). All assays were performed in 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 100  $\mu$ M ATP and 0.2  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP per 30  $\mu$ L of reaction.

When using cell extracts as the kinase source, lysates were prepared by scraping cells into ice-cold 50 mM  $\beta$ -glycerophosphate, 1.5 mM EGTA, leupeptin (10  $\mu$ g/mL), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin (10  $\mu$ g/mL), aprotinin (5  $\mu$ g/mL), 1  $\mu$ M okadaic acid and 1 mM sodium orthovanadate, followed by sonicating for 2  $\times$  5 s and removing debris by centrifuging for 15 minutes. After a Bradford assay, kinase assays were performed using 5  $\mu$ g of lysate, 100  $\mu$ M RRRDDDSDDD or 100  $\mu$ M of eIF2 $\beta$  peptide (MSGDEMIFDPTMSKKKKKKKKP) in 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 100  $\mu$ M ATP and 0.2  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P] ATP per 30  $\mu$ L of reaction. Assays were performed for 5 or 10 minutes, to verify linearity, and stopped by spotting on P81 paper. <sup>32</sup>P was detected using a STORM phosphorimager and quantitated with ImageQuant TL software.

### **3.2.6 Lysate dephosphorylation assay**

Phospho-caspase-3 was generated by phosphorylating caspase-3-his (C163A) with excess GST-CK2 $\alpha$  in 150 mM NaCl, 50 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, and 100

$\mu\text{M}$  ATP. Reactions were diluted 1/50 into 100  $\mu\text{g}$  of lysate such that 1  $\mu\text{g}$  of phospho-caspase-3 was in each reaction. Fifty  $\mu\text{M}$  of the CK2 inhibitor TBBz (calbiochem) was included to stop the kinase assay. 500, 50 or 5 nM of okadaic acid (Bioshop) or tautomycin (calbiochem) was also added to the lysate dephosphorylation assay. DMSO was used as a carrier control.

### **3.2.7 Microscopy**

Cells were grown on sterile glass cover slips, washed with PBS, fixed with 3.7% paraformaldehyde for 30 minutes, washed in 0.1 M glycine for 20 minutes at 37°C and permeabilized with 0.1% Triton X-100 for 5 minutes. All solutions were made in PBS and incubations were performed at room temperature unless otherwise stated. After permeabilization, cells were incubated for 45 minutes with a 1:50 dilution of anti-caspase-3 in 5% BSA/PBS, washed 3 $\times$  PBS and then incubated with GAM-AlexaFluor 488 (1:1000) (Invitrogen) for 45 minutes. Samples were stained with Hoechst 33258 before final PBS washes and mounting with ProLong Gold (Invitrogen). Slides were visualized using an Axiovert inverted fluorescence microscope.

## **3.3 RESULTS**

### **3.3.1 The catalytic domain of CK2 $\alpha'$ directs phosphorylation of caspase-3**

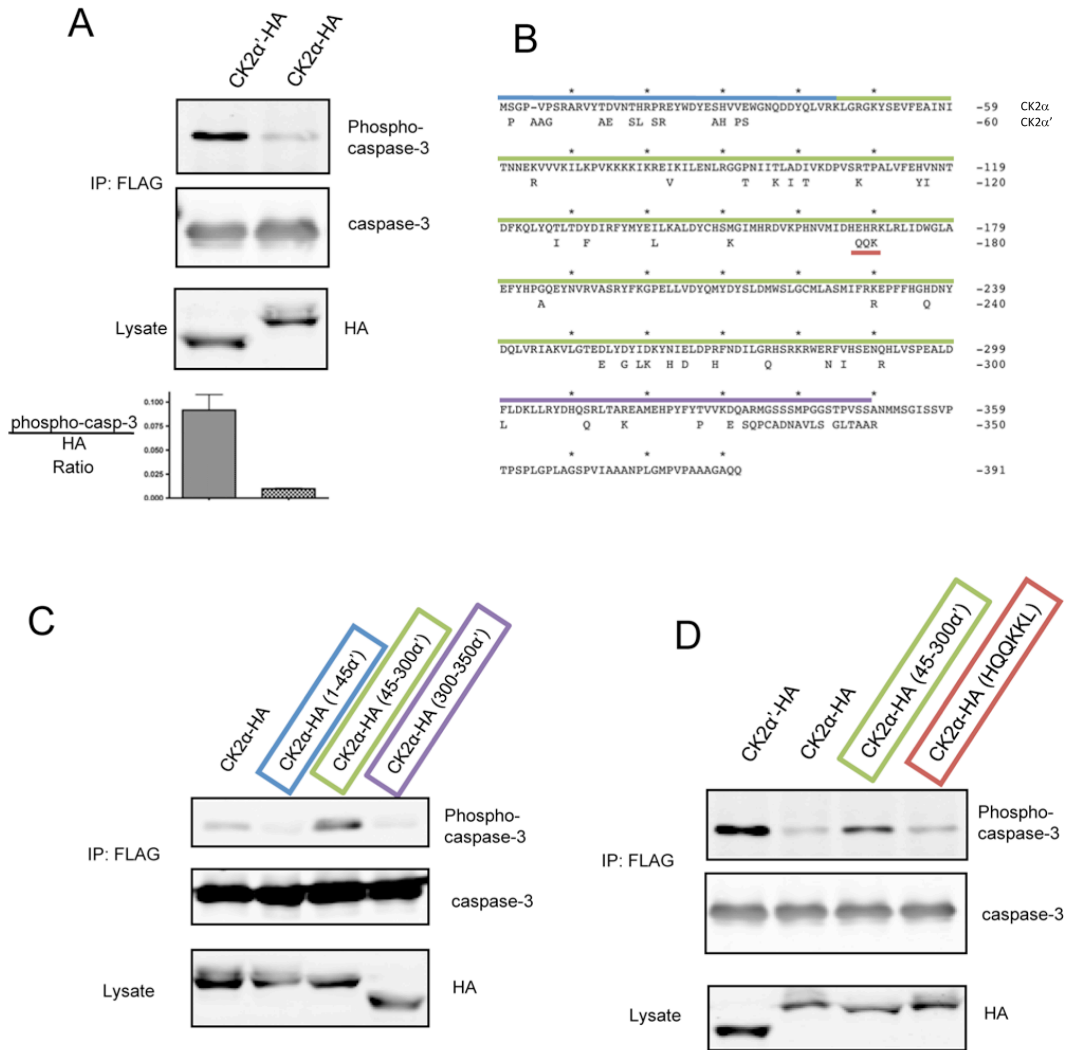
We previously reported that caspase-3 is phosphorylated by recombinant CK2 at T174 and S176 – a phosphorylation event that mitigates activation by upstream caspases (28). To further investigate caspase-3 phosphorylation, we raised antibodies against synthetic peptides of the doubly phosphorylated caspase-3 peptide (32) and systematically modulated expression of the CK2 catalytic isoforms to test



phosphorylation of caspase-3-FLAG-(C163A). Interestingly, despite equal expression and activity of CK2 $\alpha$ -HA and  $\alpha'$ -HA, caspase-3 phosphorylation was induced only in the presence of CK2 $\alpha'$ -HA (Figure 3.1A, Supplemental Figure 3.1) (11-14). To the best of our knowledge, this is the first demonstration that the cellular isozymes of CK2 can exhibit different activities against a substrate – an observation especially remarkable given the 86% identity within their catalytic domain (residues 45-312) (Figure 3.1B). In an effort to avoid unwanted caspase-3 activation, we utilized caspase-3-FLAG-(C163A) which replaces the catalytic cysteine with alanine. We found that caspase-3-FLAG was less efficiently phosphorylated than caspase-3-FLAG-(C163A) (hereafter referred to as C3-FLAG) and so emphasize the characterization of isozymic specificity in this study (Supplemental Figure 3.1C).

To identify the sequence determinants responsible for isozymic preference of caspase-3 phosphorylation, we constructed CK2 $\alpha$  /  $\alpha'$  chimeras whereby three different segments of CK2 $\alpha'$  were swapped into CK2 $\alpha$ -HA (Figure 3.1 C, upper panel). Segments consisted of the N-terminus (1-45), the catalytic domain that contains the most of the conserved subdomains for kinase family members (45-300), and the divergent C-terminus (300-350). Upon co-transfection of the different chimeras with C3-FLAG, we were surprised to learn that the catalytic domain of CK2 dictates specificity for  $\alpha'$ , as only CK2 $\alpha$ -HA constructs containing amino acids 45-300 of CK2 $\alpha'$  showed phosphorylation of caspase-3 greater than CK2 $\alpha$ -HA (Figure 3.1C). Furthermore, though considerable divergence of the C-terminus exists, it does not play a role in dictating substrate specificity in this instance. Interestingly, amongst the primary sequence differences between the catalytic domain of CK2 $\alpha$  and  $\alpha'$  is the conspicuous presence of

Figure 3.1 – C3-FLAG is phosphorylated by CK2 $\alpha'$ -HA, but not CK2 $\alpha$ -HA in cells. (A) HeLa-Tet-Off cells were transfected with C3-FLAG and CK2 $\alpha$ -HA or CK2 $\alpha'$ -HA, lysed, and C3-FLAG isolated by immunoprecipitation. Lysates and immunoprecipitations were blotted as indicated. Densitometric analysis represents the ratio of pC3:HA signal with error bars generated from the standard deviation of 3 independent experiments. (B) Sequence alignment of human CK2 $\alpha$  (above) and  $\alpha'$  (lower). Residues that are not identical in CK2 $\alpha'$  are indicated and the segments used for chimeras are highlighted: residues 1-45 in blue, 45-300 in green, and 300-350 in purple and the motif responsible for PP2A binding by CK2 $\alpha$  in red. (C and D) Cells were co-transfected with CK2 chimeras and C3-FLAG, lysed, and C3-FLAG enriched by immunoprecipitation. Lysates and immunoprecipitated samples were blotted as indicated.



a highly conserved HEHRKL motif in CK2 $\alpha$  that is absent in CK2 $\alpha'$  and previously reported to be required for binding PP2A (18) (Figure 3.1B). Multiple authors have hypothesized that this region could serve as a distinguishing feature for PP2A interaction, and isoform specific functions of catalytic CK2 (35,36). Therefore, we hypothesized that a PP2A/CK2 $\alpha$ -HA interaction could deter C3-FLAG phosphorylation, and so aimed to test this by swapping the CK2 $\alpha'$  HQQKKL sequence into CK2 $\alpha$ -HA and probing for C3-FLAG phosphorylation. However, CK2 $\alpha$ -HA(HQQKKL) was unable to rescue C3-FLAG phosphorylation, arguing against a PP2A-CK2 $\alpha$  complex that dictates isoform specific phosphorylation of caspase-3 (Figure 1D).

### 3.3.2 CK2 $\beta$ controls caspase-3 phosphorylation

The regulatory CK2 $\beta$  subunit is capable of controlling substrate specificity despite having no known catalytic activity (37). Most substrates are phosphorylated more readily by the CK2 holoenzyme *in vitro* – a characteristic likely driven by the improved stability of CK2 $\alpha$  or  $\alpha'$  in complex with CK2 $\beta$  – though a small subset of substrates, most notably calmodulin, are phosphorylated only in the absence of CK2 $\beta$  (20,21). Interestingly, CK2 $\alpha'$  binds CK2 $\beta$  10 $\times$  more weakly *in vitro* than does CK2 $\alpha$  as a result of altered folding of the  $\beta$ 4/ $\beta$ 5 loop – a structural feature within the catalytic domain that makes significant contacts with CK2 $\beta$  (19). Therefore, we were driven to test first the hypothesis that CK2 $\beta$  regulates phosphorylation of caspase-3, and second, that the differential affinity for CK2 $\beta$  and the two catalytic subunits might drive isozymic substrate preferences. Upon co-transfection of myc-CK2 $\beta$  with either catalytic isozyme

and C3-FLAG, we found that CK2 $\beta$  dramatically attenuated C3-FLAG phosphorylation by CK2 $\alpha'$ -HA and further reduced the low level of phosphorylation achieved by CK2 $\alpha$ -HA expression (Figure 3.2A). CK2 $\beta$  also blocked phosphorylation of C3-FLAG by myc-CK2 $\alpha'$  in U2-OS cells (data not shown). The ability of CK2 $\beta$  to block caspase-3 phosphorylation in cells prompted us to test all forms of CK2 for their ability to phosphorylate caspase-3 in kinase assays using recombinant proteins. Figure 3.2B shows that isozymic specificity was lost when *in vitro* kinase assays were performed, but the inhibitory effect of CK2 $\beta$  remained. Furthermore, like other CK2 substrates that are phosphorylated only in the absence of CK2 $\beta$ , treatment of the holoenzyme with polyamines resulted in hyperphosphorylation of caspase-3 in *in vitro* kinase assays (data not shown).

### **3.3.3 Endogenous CK2 $\beta$ binds CK2 $\alpha$ -HA and CK2 $\alpha'$ -HA equally and stoichiometrically in lysates**

In an effort to investigate if pools of CK2 $\alpha'$  devoid of CK2 $\beta$  were forming after over-expression, we utilized a CK2 substrate peptide that displays little preference for catalytic subunits or the holoenzyme (DSD in Figure 3.3A) and an eIF2 $\beta$  substrate peptide that is specific for the holoenzyme (38). An increase in the DSD:eIF2 $\beta$  ratio indeed suggested an increase in CK2 $\beta$ -free myc-CK2 $\alpha'$  (Figure 3.3A). Of particular interest was the observation that C3-FLAG phosphorylation actually preceded measurable differences in DSD:eIF2 $\beta$  phosphorylation, suggesting that our assay either lacks the required sensitivity to detect small changes in the ratio of free catalytic subunits and holoenzyme or that a cellular, CK2 $\beta$ -refractory population of CK2 $\alpha'$  becomes

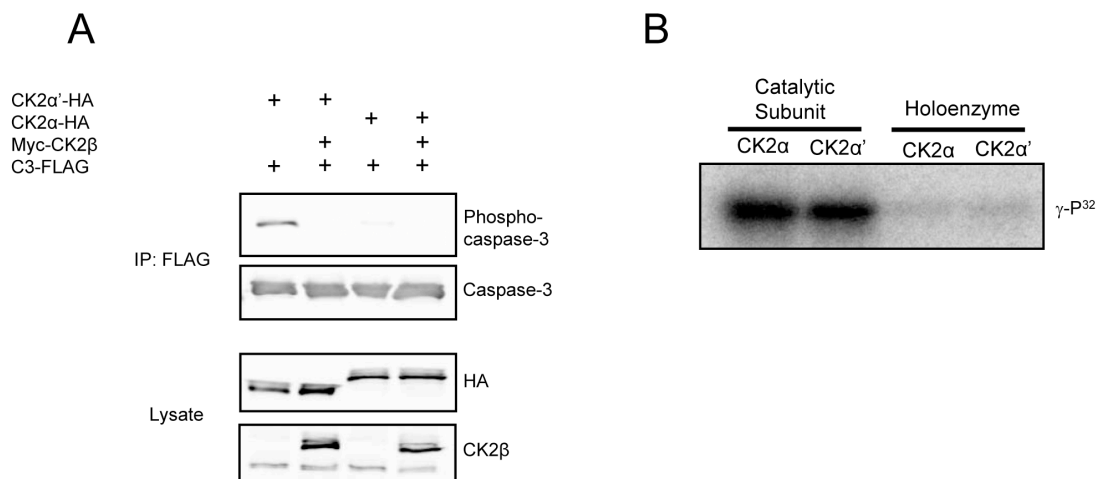


Figure 3.2 – CK2 $\beta$  inhibits caspase-3 phosphorylation. (A) Cells were co-transfected with the indicated CK2 constructs and C3-FLAG. Lysates were immunoprecipitated with anti-FLAG to isolate caspase-3, separated by SDS-PAGE and immunoblotted as indicated. (B) Equal units of the indicated forms of recombinant CK2 were used in kinase reactions with caspase-3-His (C163A) and ATP- $\gamma$ -P<sup>32</sup>. Reactions were separated by SDS-PAGE, the gels dried, and visualized using a phosphorimager.

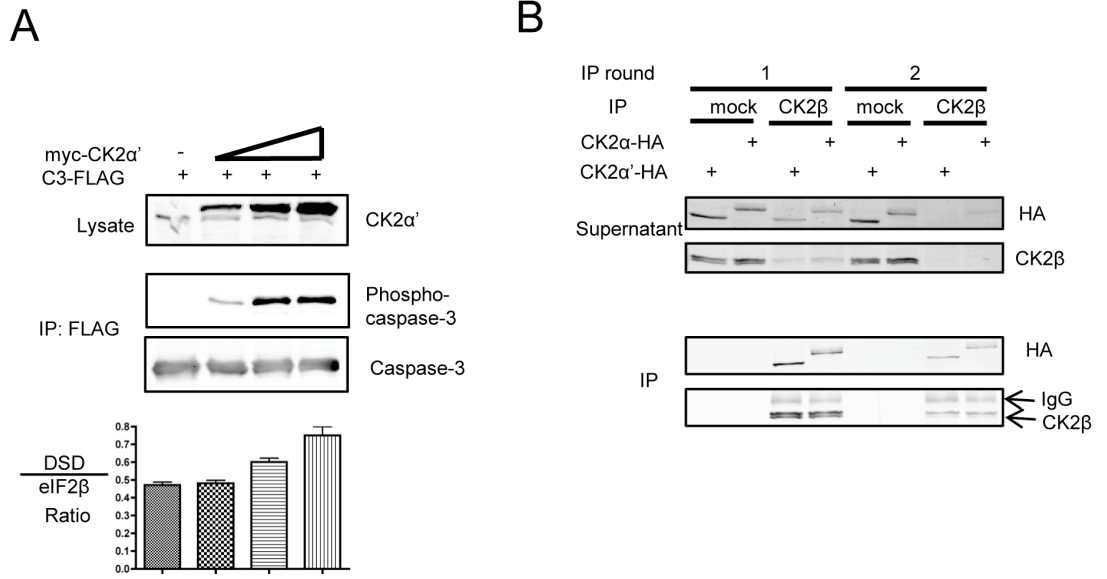


Figure 3.3 – Investigation of CK2 form in cell lysates reveals a predominately holoenzyme form. (A) Cells were transfected with C3-FLAG and varying amounts of myc-CK2α', and harvested after 40 h. Lysates and FLAG immunoprecipations were probed as indicated. Lysates were assessed in kinase assays along with two CK2 substrate peptides. The DSD peptide (RRRDDSDSD) is phosphorylated by both catalytic free CK2 and holoenzyme while the eIF2β peptide (MSGDEMIFDPTMSKKKKKKKKP) is phosphorylated exclusively by the CK2 holoenzyme. Graphs represent the ratio of DSD:eIF2β activity with error bars representing the standard deviation of triplicate kinase assays. (B) Cells transfected with CK2α-HA or CK2α'-HA were harvested and subjected to consecutive rounds of immunoprecipitation using a CK2β antibody or mock control. Both the supernatant from the immunoprecipitation and the immunoprecipitation itself were western blotted for HA and CK2β as indicated.

complexed with CK2 $\beta$  upon cell lysis. In support of the latter, we also observed complete complex formation between HA-tagged CK2 catalytic subunits with endogenous CK2 $\beta$  (Figure 3.3B). Here, lysates subjected to immunoprecipitation with CK2 $\beta$  antibodies show over 90% depletion of CK2 $\beta$ , CK2 $\alpha$ -HA and CK2 $\alpha'$ -HA after two rounds of immunoprecipitation, with no substantial difference between the catalytic subunits remaining in the supernatant. That HA-tagged CK2 $\alpha$  and  $\alpha'$  bound endogenous CK2 $\beta$  further reinforces the notion that both isozymes of ectopic CK2 are fully functional, and that the difference in caspase-3 phosphorylation may arise from differences in the cellular regulation of CK2 $\alpha$ -HA versus CK2 $\alpha'$ -HA that extend exclusively beyond regulation by CK2 $\beta$ .

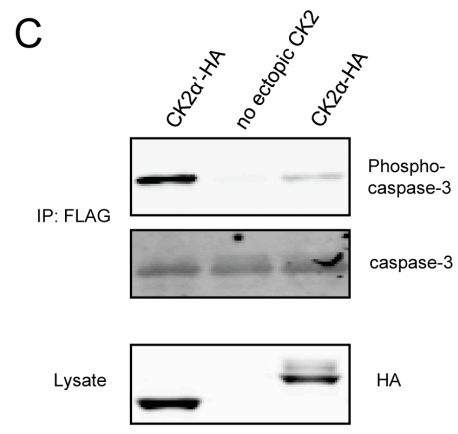
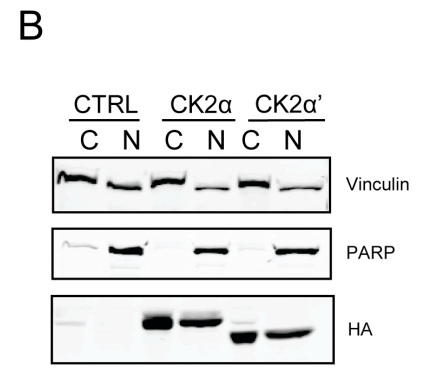
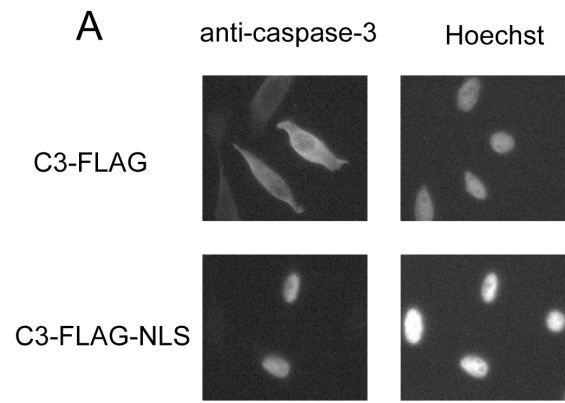
We found that endogenous CK2 was unable to generate C3-FLAG that is reactive with phospho-caspase-3 antibodies, suggesting that in HeLa cells CK2 $\alpha'$  is entirely holoenzyme (Figure 3.3A, Lane 1). CK2 holoenzyme-mediated phosphorylation of non-holoenzyme substrates can be stimulated by polyamines in *in vitro* kinase assays and by ODC1 (ornithine decarboxylase 1) – the rate limiting enzyme of spermine synthesis – over-expression in cells (39-41). However, we found no stimulation of caspase-3 phosphorylation by endogenous CK2 upon ODC1 over-expression (data not shown).

#### **3.3.4 Nuclear, endogenous CK2 $\alpha'$ is bound to CK2 $\beta$**

We were next interested in testing the effect of C3-FLAG localization on phosphorylation. The caspase-3 zymogen was previously reported to be a predominately cytoplasmic protein (42) and, indeed, was found to mostly localize outside the nucleus in our studies (Figure 3.4A, upper panels). Interestingly, multiple reports have found that



Figure 3.4 – Redirecting C3-FLAG to the nucleus does not alter the relative levels of phosphorylation. (A) Cells were transfected with C3-FLAG or C3-FLAG-NLS and then transferred to glass cover slips in 6-well dishes. Twenty-four hours after transfection, cover slips were stained using caspase-3, which was detected by Alexa-Fluor GAM-488, and Hoechst 33258, mounted on microscope slides and analyzed on an inverted fluorescence microscope. (B) Cells were transfected with CK2 $\alpha$ -HA or CK2 $\alpha'$ -HA and the cytoplasmic and nuclear fractions separated and blotted as indicated. PARP was used as a marker for nuclear separation and Vinculin for cytoplasmic. (C) Cells were co-transfected with C3-FLAG-NLS and the indicated CK2 construct, then harvested. FLAG immunoprecipitations and lysates were immunoblotted as indicated. CTRL denotes the empty vector lane.

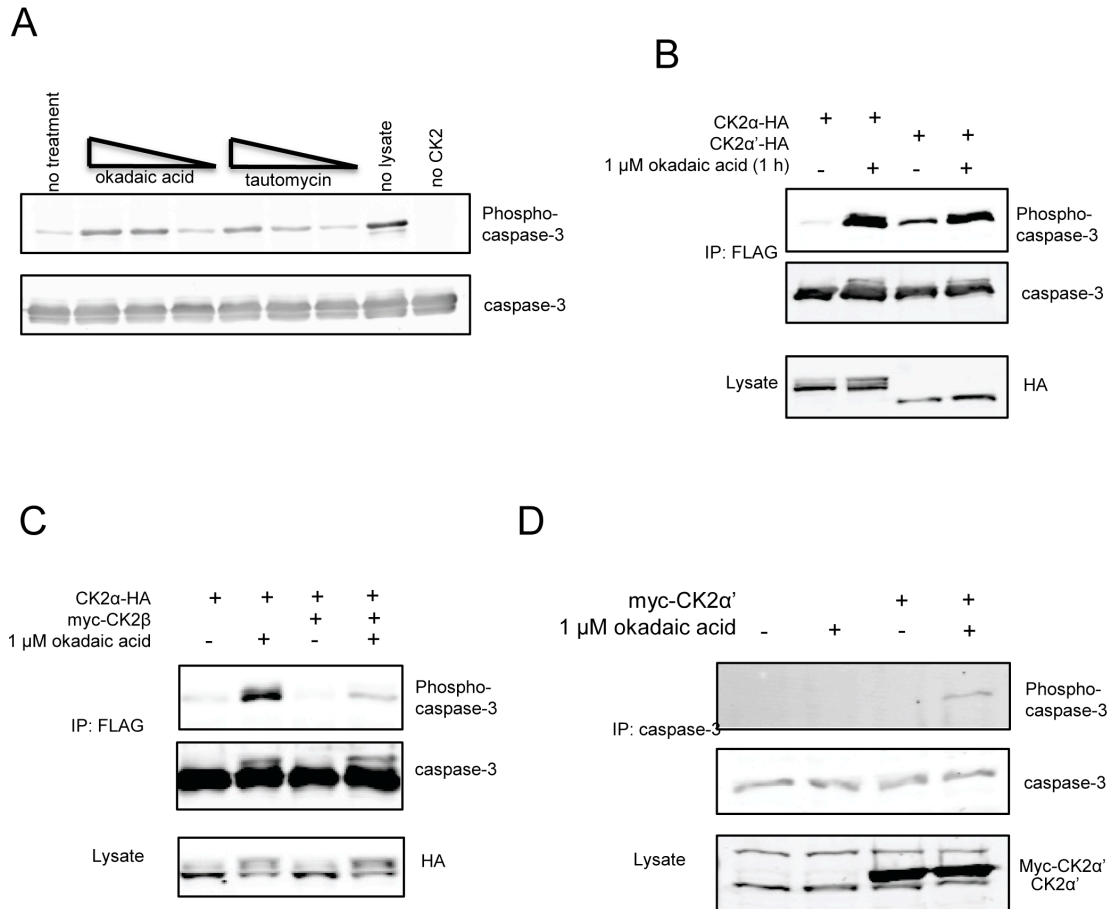


while CK2 substrates are distributed throughout many cellular compartments (reviewed in (43,44)), there is a precedent for nuclear CK2 to be the predominant form (45). We found the CK2-HA localized to both cytoplasmic and nuclear fractions in HeLa-tet off cells and predicted that C3-FLAG was only probing the cytoplasmic fraction of CK2 for the CK2 $\beta$ -free form (Figure 3.4B). Furthermore, Filhol et al. (2003) observed GFP-CK2 $\alpha$  localization as exclusively nuclear in the absence of ectopic CK2 $\beta$  and so we hypothesized that the free catalytic form of CK2 is most likely to occur in the nucleus (46). To test this hypothesis, we included a nuclear localization signal (NLS) on the C-terminal end of our C3-FLAG construct that successfully redirected caspase-3 to the nucleus (Figure 3.4A, lower panels). Upon transfecting C3-FLAG-NLS alone, or in combination with CK2 $\alpha$ - or  $\alpha'$ -HA, we found that C3-FLAG-NLS phosphorylation mirrored that observed with caspase-3-FLAG; there was no measurable phosphorylation in the absence of ectopic CK2, minimal phosphorylation by CK2 $\alpha$ -HA, and robust phosphorylation by CK2 $\alpha'$ -HA (Figure 3.4C). We concluded that these data support a model that impaired phosphorylation of C3-FLAG by CK2 $\alpha$ -HA is not dictated by nuclear versus cytoplasmic localization, and that nuclear, endogenous CK2 $\alpha'$  is predominately holoenzyme in HeLa cells.

### **3.3.5 Okadaic acid abolishes the isozymic preference of C3-FLAG phosphorylation**

The observation that endogenous CK2 was insufficient to phosphorylate both cytoplasmic and nuclear C3-FLAG led us to believe that either cellular CK2 $\alpha'$  was fully holoenzymatic, or that C3-FLAG was being actively dephosphorylated. Indeed, when recombinant phosphorylated caspase-3 was incubated with cellular extracts lacking

phosphatase inhibitors, a marked decrease in phosphorylation was observed indicating active dephosphorylation (Figure 3.5A). Furthermore, dephosphorylation was more sensitive to okadaic acid ( $K_i$  for PP2A < PP1) than tautomycin ( $K_i$  for PP2A > PP1), suggesting PP2A as the responsible phosphatase. In cells, okadaic acid was unable to induce C3-FLAG phosphorylation by endogenous kinases further suggesting that endogenous CK2 $\alpha'$  is fully bound to CK2 $\beta$  (data not shown). Interestingly, okadaic acid also completely rescued phosphorylation by CK2 $\alpha$ -HA to levels comparable to those seen with CK2 $\alpha'$ -HA (Figure 3.5B), but was blocked by myc-CK2 $\beta$  (Figure 3.5C). These data support a model by which CK2 $\alpha$ -HA is free of endogenous CK2 $\beta$  and, therefore, catalytically competent to phosphorylate C3-FLAG, but is restricted access to C3-FLAG by some mechanism that is relieved by okadaic acid. Finally, we were interested in testing if endogenous caspase-3 was phosphorylated by ectopic CK2 $\alpha'$  and found that phosphorylation occurred in the presence of okadaic acid (Figure 5D). Therefore, our results support the conclusion that caspase-3 phosphorylation could be physiologically relevant in tissues or pathologies in which non-holoenzyme CK2 $\alpha'$ , or potentially CK2 $\alpha$ , and high levels of caspase-3 are present and in the cytosol. Furthermore, we expect that transfection of C3-FLAG can be used as a tool to aid in the identification of cell culture models that have free catalytic CK2 $\alpha'$ , for which no *bona fide* experimental systems currently exist.



**Figure 3.5 – Exclusion of CK2α-HA from C3-FLAG phosphorylation is not governed by endogenous CK2β in HeLa cells.** (A) Recombinant His-tagged caspase-3-(C163A) was fully phosphorylated by GST-CK2α. Reactions were quenched by the addition of the CK2 specific inhibitor TBBz, and added to HeLa lysates harvested in the absence of phosphatase inhibitors. Okadaic acid or tautomycin at concentrations of 50, 5 or 0.5 nM was added to the reaction as indicated. (B-D) HeLa cells were transfected with C3-FLAG and the indicated constructs except in (D), where endogenous caspase-3 was being probed. After a 24 hour recovery, cells were treated with or without 1 μM okadaic. FLAG or caspase-3 immunoprecipitations and lysates were blotted as indicated.

### 3.4 DISCUSSION

Misbalanced expression of CK2 subunits in cancer has been observed, but the hypothesis that the particular CK2 form can dictate specific phenotypes through phosphorylation of different substrates remains largely unexplored prior to this work. In this study we provide direct evidence that CK2 $\alpha'$  has a specific cellular substrate that is restricted from CK2 $\alpha$  in both the cytoplasm and the nucleus. Furthermore, because C3-FLAG phosphorylation by CK2 $\alpha$ -HA could be rescued by okadaic acid, and further blocked by myc-CK2 $\beta$  (Figure 5B and C), suggests that the isozymic substrate preference is not dictated by endogenous CK2 $\beta$  in cells – an unanticipated, additional level of regulation not seen before. CK2 $\alpha'$  specific functions have previously been observed, the most notable of which is the finding that CK2 $\alpha'$  knock-out mice exhibit a hyper-apoptotic phenotype in spermatocytes (23), and that forced expression of kinase-inactive CK2 $\alpha'$  in U2-OS cells resulted in proliferation defects (24). Furthermore, Orlandini *et al.* (1999) highlighted the oncogenic activity of CK2 $\alpha'$  in experiments that demonstrated cooperativity with Ras in the transformation of rat fibroblasts (47). In a related vein, Li *et al.* (2006) showed that NKX3.1 has a decreased half-life upon CK2 $\alpha'$  knock-down, or mutation of phospho-acceptors to alanine, and that NKX3.1 phosphorylation was mediated by CK2 $\alpha'$  devoid of CK2 $\beta$  in in-gel kinase assays (48). By identifying a CK2 substrate preferentially phosphorylated by CK2 $\alpha'$ , we believe our studies reinforce the notion that CK2 $\alpha'$  has cellular functions distinct from CK2 $\alpha$ .

Reasoning that caspase-3 phosphorylation is dictated by differences in sequence between the two isozymes, we generated chimeras and observed a rescue of CK2 $\alpha$ -mediated phosphorylation of caspase-3 when constructs expressed CK2 $\alpha'$  residues 45-

300. What is striking is that a comparison of the CK2 $\alpha$  and  $\alpha'$  sequences reveals the internal catalytic portion as actually the most conserved – residues 1-45 are 74% identical, 45-300 are 88% identical and 300-350 are 56% identical. Interestingly, the catalytic domain of CK2 $\alpha$  has previously been implicated in isoform specific interactions. The Goldberg laboratory demonstrated that PP2A interacts with the HEHRKL motif within CK2 $\alpha$  – an area with a cluster of divergence from CK2 $\alpha'$  (Figure 3.1B) – though we found that swapping the HQQKKL motif of  $\alpha'$  into CK2 $\alpha$  was unable to rescue C3-FLAG phosphorylation (18). Similarly, this region has also been tested for regulating the CK2 $\alpha$  specific binding to CKIP-1, but CK2 $\alpha$ -E167Q was unable to disrupt CKIP-1 interaction, nor was CK2 $\alpha'$ -Q168E able to promote binding to CKIP-1 (49). The observation that residues 45-300 of CK2 $\alpha'$ , an area of 88% sequence identity and 93.3% sequence similarity, control C3-FLAG phosphorylation further reinforces the notion that subtle regulatory mechanisms must exist to differentially control these two isozymes. Collectively, the examples of the CKIP-1/CK2 $\alpha$  interaction and caspase-3 phosphorylation illustrate that despite extensive sequence conservation between the catalytic domain of CK2 $\alpha$  and  $\alpha'$ , significant divergence exists and can drive unique functions.

Structural variances that could impart isozymic specificity do occur in residues 45-300 in the non-holoenzyme form of the catalytic subunits, but these differences also receive contributions from the N-terminus. For example, the  $\beta$ 4/ $\beta$ 5 loop is constitutively open in CK2 $\alpha'$  and is located at residues 95-114, but stabilization is in part mediated by residues 34-37 and thought to result from a unique proline in CK2 $\alpha'$  at residue 32 (19). Interestingly, Pro<sup>92</sup> is unique to CK2 $\alpha$  and located just upstream of the  $\beta$ 4/ $\beta$ 5 loop,

though the changes that result from this divergence remain unknown. Another structural variation occurs in the interdomain region – a segment responsible for co-ordinating binding with ATP and ATP-competitive inhibitors (residues 114-122 of CK2 $\alpha'$ ). This hinge is slightly collapsed in CK2 $\alpha$ , but also correlates with a closed  $\beta$ 4/ $\beta$ 5 loop which, again, appears to receive structural contributions from the N-terminus. Tyr<sup>116</sup> and Ile<sup>117</sup> are not conserved in CK2 $\alpha$  (His<sup>115</sup>, Val<sup>116</sup>) but how these residues contribute to differences in hinge region structure remain unknown. Should the noted structural alterations be consistent with our chimeras, it is enticing to speculate that okadaic acid either governs protein-protein interactions or post-translational modifications of CK2 $\alpha$  that can resolve this divergence. With respect to the latter, a PHOSIDA search for unique post-translational modifications on  $\alpha$  and  $\alpha'$  revealed that  $\alpha'$  is acetylated on K97 (T96 on CK2 $\alpha$ ) and CK2 $\alpha$  phosphorylated on T127 (I128 on CK2 $\alpha'$ ), providing a putative mechanism that differentially controls CK2 catalytic isozymes (50). Precedence for the former has been observed with v-src as okadaic acid treatment disrupts the v-src/Hsp90 interaction (51). CK2 $\alpha$  interacts with Hsp90 (52,53), but if this interaction restricts access to CK2 substrates or is altered by okadaic acid remains speculative.

CK2 $\beta$  binds to CK2 $\alpha$  and  $\alpha'$  spontaneously in solution (54) with a low Kd (CK2 $\alpha'$  -  $45.5 \times 10^{-9}$  M, CK2 $\alpha$  -  $3.7 \times 10^{-9}$  M (19)) and will only dissociate under denaturing conditions (55). The observation that CK2-HA bound endogenous CK2 $\beta$  in immunoprecipitation assays reinforced that holoenzyme was forming in our experimental system, but seemed at odds with C3-FLAG phosphorylation in cells. We interpreted these data as support for an exclusion mechanism of catalytic CK2 from endogenous CK2 $\beta$  in cells that can be overcome by cell lysis, or over-expression of myc-CK2 $\beta$ . That



endogenous CK2 $\beta$  bound ectopic catalytic CK2 upon lysis suggests the presence of excess CK2 $\beta$  in HeLa cells, which has also been observed in Burkitt's lymphoma Ramos cells and hypothesized to occur in mouse brain and testes (56,57). Therefore, our proposed mechanism holds that CK2 form is dictated not only by relative levels of catalytic and regulatory CK2, though this is definitely a contributing factor, but appears to be regulated by exclusion upon catalytic subunit expression. Furthermore, our data suggests that the cellular form of CK2 cannot be investigated by procedures interrogating cell extracts for interaction between CK2 $\beta$  and  $\alpha$  or  $\alpha'$ . In this sense, C3-FLAG represents the first phospho-specific antibody biomarker for non-holoenzyme activity of cellular CK2 that could aid in the identification of tissue culture model systems that contain CK2 $\beta$ -free populations of CK2 $\alpha'$  and potentially CK2 $\alpha$ .

Anecdotal evidence for free catalytic CK2 functions in cells has previously been observed, but a unifying mechanism for this form of CK2 in physiology and disease remains unclear. Amongst the first proof for the CK2 $\beta$ -free form of catalytic CK2 in cells was the identification of the CK2 phosphorylation sites of calmodulin from rat liver (58). As well, NKX3.1 is phosphorylated by CK2 $\alpha'$  in in-gel kinase assays only in fractions that lacked CK2 $\beta$  and NKX3.1 protein levels are negatively correlated with both CK2 inhibitor treatment and CK2 $\alpha'$  knockdown (48). Finally, Deshiere *et al.* (2012) noted that the CK2 $\alpha'$ :CK2 $\alpha$ :CK2 $\beta$  ratio varies across breast cancer samples, and that a higher CK2 $\alpha$ :CK2 $\beta$  ratio correlates with the expression of epithelial-to-mesenchymal transition markers (9). Taken together with the demonstration that CK2 form can dictate substrate accessibility in cells, we propose that pathological function of CK2 will depend on subunit composition. Furthermore, disambiguation of free catalytic, holoenzyme and

isozyme specific functions together with the development of biomarkers for these processes should aid in identifying context specific treatments and combinatorial strategies.

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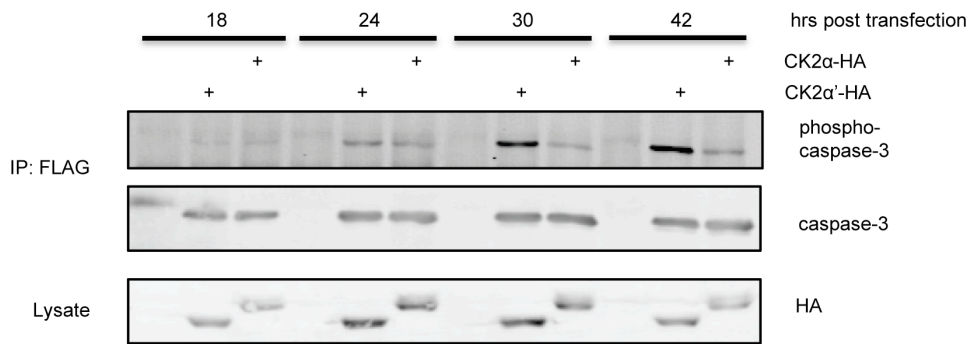
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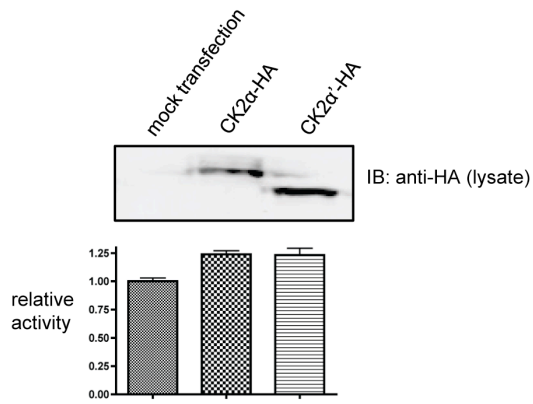
### **3.6 SUPPLEMENT**

Supplemental Figure 3.1 – Companion to Figure 3.1. (A) Cells were transfected as in Figure 1A, and washed 18 hrs post transfection. Lysates were generated at the indicated timepoints followed by FLAG immunoprecipitation and western blotting as indicated. (B) Lysates from HeLa cells exhibiting equal amounts of CK2 $\alpha$ -HA and CK2 $\alpha'$ -HA were used for kinase assays with the CK2 specific substrate peptide RRRDDDSDDD. Error bars represent the standard deviation of triplicate reactions. (C) Cells were transfected with myc-CK2 $\alpha'$  and the indicated caspase-3 construct, washed after 18 hrs and allowed to recover for 24 hrs. At this point lysates were generated, FLAG immunoprecipitations performed and samples western blotted as indicated.

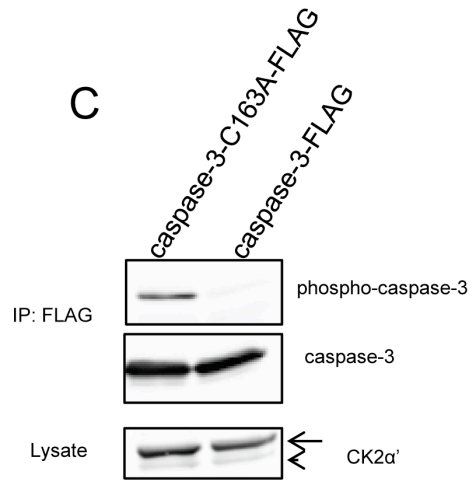
**A**



**B**



**C**



## **CHAPTER 4 – AN UNBIASED, PROTEOMIC SCREEN REVEALS NOVEL CASPASE CLEAVAGE EVENTS POSITIVELY AND NEGATIVELY REGULATED BY SUBSTRATE PHOSPHORYLATION**

### ***4.1 INTRODUCTION***

Apoptosis is a cell death program integral in various biological processes such as tissue homeostasis and development, but also misregulated in disease (1). Notably, the ability of cancer cells to evade apoptosis is considered a driving feature that imparts a cellular selective advantage and allows cells to persist inappropriately (2). Major components of apoptotic evasion in cancer involve the misregulation of two classes of enzymes – protein kinases and caspases. Kinases transfer phosphate from ATP to proteins to alter substrate function (3). Activating mutations, amplifications, gene fusions and over-expression of oncogenic kinases represent an underlying basis of disease the offers therapeutic strategies (3). In this way, inhibiting oncogenic kinases can lead to selective apoptotic induction of cancer cells. Caspases, or cysteine-containing, aspartic-acid-directed proteases, act as executioners of the apoptotic program by cleaving key structural and enzymatic proteins resulting in cellular demolition (4). Not surprisingly, constrained caspase activity, by post-translational modifications or genetic mutations or deletions, can also contribute to the malignant phenotype by blocking apoptotic progression (5,6).

Interestingly, numerous examples exist detailing the crosstalk between caspases and kinases as a major apoptotic regulatory mechanism (6,7). One intriguing mode of controlling caspase activity is through phosphorylation of caspase substrates. Anecdotal

examples exist whereby phosphorylation at P4, P2 and P1' (see Figure 4.1A for cleavage site nomenclature) can block cleavage (7). As well, there is precedent for phosphorylation sites outside the caspase motif to regulate susceptibility to proteolysis. For example, phosphorylation of Acinus-S at S422 by Akt blocks its cleavage at D355 (8). Interestingly, phospho-dependent blocking of caspase-mediated proteolytic events can actually impact cellular phenotype; inhibitory phosphorylations on Bid, Presenilin-2 and Acinus-S all obstruct caspase cleavage and apoptotic progression (8-11). In this way, phospho-dependent regulation of caspase-mediated cleavage has been hypothesized as a global regulator of apoptotic progression, especially in the context of cancer where hyper-active, oncogenic kinases may act to increase phospho-site occupancy within caspase cleavage motifs (7). Indeed, we previously tested this hypothesis using predictive, peptide match programs and identified CK2 phosphorylation sites on caspase-3 that regulated its activation by caspase-8 and -9 (12, Chapter 2).

To build on our predictive strategy, we devised an unbiased, proteomic methodology to identify novel proteins where phosphorylation regulates caspase cleavage. To establish this approach, we measured the caspase degradome in the context of a native phospho-proteome and compared it to the degradome generated when lysates were first dephosphorylated with  $\lambda$  phosphatase. The advantage of this approach is that we can concurrently monitor the effects of phosphorylation sites on cleavage that are both local and distant from the scissile bond. This latter point is important, as there is no way to predict how distant phosphorylation sites may affect caspase proteolysis. To measure and identify these events, we utilized the N-terminomic workflow TAILS (terminal amino isotopic labeling of substrates) (13). Comparative analysis of the

caspase degradomes from phosphorylated or dephosphorylated lysates revealed Yap1 and Golgin-160 as caspase substrates negatively regulated by phosphorylation. Surprisingly, we also identified a number of caspase substrates whose cleavage is promoted by phosphorylation, and validated that MST3 behaves this way.

Interestingly, during the course of our study, Dix *et al.* (2012) also found that phosphorylation at P3 can actually promote cleavage of caspase peptide substrates (14). These authors also obtained substantial evidence for phosphorylation occupancy throughout the caspase motif. Therefore, spurred by the examples of the positive effect of phosphorylation on caspase cleavage, we undertook a systematic evaluation of the role of phosphorylation throughout the entirety of the caspase cleavage motif. Along these lines, the primary amino acid sequence determinants for various caspases from P4 – P1' are well defined, but phospho-residues within this motif have only been anecdotally studied. Furthermore, residues outside this region, at P5, P2' and P3' have recently been found to alter caspase -3 and -7 recognition (15), but have unknown determinant effects when phosphorylated. To address these issues, we systematically walked phospho-Ser through the length of model caspase substrate peptides, and found that phosphorylation was generally inhibitory to caspase cleavage. Therefore, taken together with our TAILS data, these results suggest that phosphorylation of caspase substrates promotes cleavage primarily through effects not related to the primary amino acid determinants. Furthermore, our studies demonstrate that N-terminomics approaches can be tailored to identify novel, hierarchical events controlling cleavage of caspase substrates.

#### ***4.2 MATERIALS AND METHODS***

#### 4.2.1 Caspase degradome preparation

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) on 10-cm dishes. Cells were treated for 45 min with 1  $\mu$ M okadaic acid (Bioshop), followed by lysis in 0.1 % CHAPS, 20 mM PIPES (pH 7.4), 100 mM NaCl, leupeptin (10  $\mu$ g/mL), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin (10  $\mu$ g/mL), aprotinin (5  $\mu$ g/mL), 50 mM NaF, 1  $\mu$ M microcystin and 1 mM sodium orthovanadate. Cells were lysed by sonication with 2 pulses for 5 seconds each, and the samples subsequently cleared by ultracentrifugation at 13 000  $\times$  g if samples were being used for western blot analysis, or 140 000  $\times$  g for samples being used for proteomic analysis. Lysates were then exchanged into 100 mM HEPES (pH 7.0) 3  $\times$  using 3 K cut-off buffer exchange filters (Amicon) to remove phosphatase inhibitors prior to lambda phosphatase treatment, and small molecule primary amines to permit complete dimethyl labeling of primary amines on proteins. Protein concentration was assessed by a Bradford assay. After buffer exchange, the sample was split in two, and diluted in 10  $\times$  phosphatase buffer (see NEB supplier for details) and 5  $\times$  caspase buffer (see above), and then treated with or without  $\lambda$  phosphatase (10 U NEB lambda phosphatase /  $\mu$ g of lysate) for 1 h at 37°C. Next, lysates were treated with 50, 500 or 5000 nM of caspase-3 and -7 for 1 h at 37°C followed by termination of the reaction by adding 6  $\mu$ M of the irreversible caspase inhibitor z-VAD-fmk (Sigma). Finally, samples previously left phosphorylated were then treated with  $\lambda$  phosphatase as above and either further processed using TAILS for proteomic analysis, or 2  $\times$  Laemmli buffer added for western blotting.

#### 4.2.2 Sample preparation using TAILS

TAILS was performed largely as described previously (16). Briefly, caspase degradomes were diluted 1:1 in 8 M guanidine hydrochloride. Cysteines were reduced by adding 5 mM DTT and incubated at 65°C for 1 hour and then alkylated by iodoacetamide treatment for 2 h at room temperature in the dark. After quenching excess iodoacetamide with DTT, the pH was adjusted to 6.5 using 0.5 N HCl to prepare for dimethyl labeling of primary amines. Samples were dimethylated by addition of 20 mM NaBH<sub>3</sub>CN and 40 mM <sup>12</sup>CH<sub>2</sub>-formaldehyde (light) (Sigma) or <sup>13</sup>CD<sub>2</sub>-formaldehyde (heavy) by reaction at 37°C overnight, followed by quenching with 100 mM Tris (tris(hydroxymethyl)aminomethane) (pH 6.8) for 4 hours. Samples were mixed at this point and proteins were precipitated with cold methanol and acetone (8:1), washed 3× with methanol to remove excess label and amines, resuspended in 8 M guanidine hydrochloride and diluted 8-fold in 1 M HEPES (pH 8.0). Trypsin (Promega) was added (1:100 trypsin:lysate (w/w)) and incubated overnight at 37°C, followed by a fresh trypsin addition (1:200) for another 4 hours. Finally, internal tryptic peptides were reacted with HPG-ALDII polymer (Flintobox) to negatively select for protein N-termini and caspase-generated, dimethylated neo N-termini. Here, 2 mg of polymer and 20 mM NaBH<sub>3</sub>CN were added to 1 mg of lysate, the pH adjusted to 6.5 with HCl and the reaction incubated at 37°C overnight. After quenching with 100 mM Tris (pH 6.8), negative selection of N-termini was performed by filtering the polymer with a 10-kDa cutoff (the polymer is ~80kDa) Microcon spin-filter as per the manufacturers instructions. The N-terminome containing flow through was cleaned up on a C<sub>18</sub> light Waters Sep-Pak by acidifying the sample in 0.1% formic acid, applying it to the column, washing with 0.1% formic acid,

and elution in 80% ACN / 0.5% formic acid. Samples were then vacuum centrifuged and stored at -20°C until mass spectrometer analysis.

#### **4.2.3 Peptide Identification using Mass spectrometry**

The peptides are loaded onto a 2 cm long X 360  $\mu\text{m}$  o.d.  $\times$  100  $\mu\text{m}$  i.d. microcapillary fused silica precolumn packed with Magic 5  $\mu\text{m}$  C18AQ resin (Michrom Biosciences, Inc.). After sample loading, the precolumn is washed with 95% Solvent A (0.1% formic acid in water) /5% Solvent B (0.1% formic acid in Acetonitrile) for 20 min at a flow rate of 2  $\mu\text{L}/\text{min}$ . The precolumn is then connected to a 360  $\mu\text{m}$  o.d.  $\times$  75  $\mu\text{m}$  i.d. analytical column packed with 14 cm of 5  $\mu\text{m}$  C18 resin constructed with an integrated electrospray emitter tip. The peptides are eluted at a flow rate of 250  $\text{nL}/\text{min}$  by increasing the percentage of solvent B to 40% with a Nano-Acquity HPLC solvent delivery system (Waters Corp.). The LC system is directly connected through an electrospray ionization source interfaced to an LTQ Orbitrap Velos ion trap mass spectrometer (Thermo Electron Corp.). The mass spectrometer is controlled by Xcalibur software (Thermo. ver. 2.1.0.1140) and operated in the data-dependent mode in which the initial MS scan recorded the mass to charge ( $m/z$ ) ratios of ions over the range 400–2000. The 10 most abundant ions are automatically selected for subsequent collision-activated dissociation. Each sample was run in duplicate. Raw files were searched and quantified using Maxquant (17) version 1.2.2.5 using the Uniprot database (20 November, 2012). Cysteine residues were searched as a fixed modification of +57.0215 Da, oxidized methionine residues as a variable modification of +15.9949 Da and deamidated asparagine and glutamine residues as a variable modification of +0.9840. Light and heavy



dimethylation of peptide amino termini and lysine residues were set as variable modifications of +28.0313 Da and +34.0631 Da, respectively. Peptides were queried using Asp-C and Arg-C cleavage constraints with a maximum of two missed cleavages sites. The mass tolerances were 6 ppm for parent masses and 0.6 Da for fragment masses. The peptide false-discovery rate was set to 0.01. A hit was defined as appearing in at least two runs. There were 6 runs total – technical duplicates of 3 experiments. When quantitative data was present in both runs of a technical duplicate, the geometric mean of the heavy:light ratio was taken. The weblogo of P1 Asp peptides was determined as previously described (18).

#### ***4.2.4 Western Blotting***

Samples were separated on SDS-PAGE gels and transferred to PVDF membrane using standard procedures. Membranes were blocked with 5% BSA/TBST and probed overnight with primary antibodies in 1% BSA/TBST. PKC $\delta$ , MST1, MST3, Yap1 and Golgin-160 antibodies were all purchased from abcam. Presenilin-1 antibodies were from Novus Biologicals, phospho-Histone H2B from Cellular Signaling and phospho-EEF1D and phospho-EEF1B made as previously described (19).

#### ***4.2.5 Peptide Synthesis***

Peptides were synthesized on resin by standard Fmoc-based peptide chemistry with an Automated Multiple Peptide Synthesizer (Intavis). Fmoc-Lys(Dinitrophenol)-OH (Bachem) was attached to the C terminus of each peptide, and peptides were capped with Fmoc-Trp-OH and biotin at the N-terminus. Phosphorylated peptides were generated

with Fmoc-Ser [Ser[PO(OBzl)-OH]-OH (AnaSpec). Peptides were resuspended in 250 mM HEPES (pH 7.5) and the pH adjusted to 7.5 with NaOH. Peptide concentration was determined by OD 355 and identity was verified by MALDI-TOF.

#### ***4.2.6 Caspase Assays***

Recombinant caspase-3 (Addgene plasmid number 11821 (20)), -7 (Addgene plasmid number 11825 (20)) and -8 (Addgene plasmid number 11827 (20)) were purified and their activity assessed by active site titration as described previously (21). Caspase-3 assays were performed with 200 nM caspase and 100  $\mu$ M peptide at 37°C in 20 mM PIPES (pH 7.4), 100 mM NaCl, 5% sucrose, 0.1% CHAPS (w/v), and 5 mM DTT for 15 minutes. Caspase-7 assays were performed in the same buffer, but with 600 nM of caspase. Caspase-8 reactions were performed with 100 nM caspase-8 in 1 M sodium citrate and 35 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> (pH 7.4) at 37°C for 5 minutes. Reactions were quenched by the addition of the irreversible caspase inhibitor z-VAD-fmk (Sigma), diluted 1 in 4 and emission read at 355 nm after excitation at 280 nm. Fluorescence was measured on a Horiba fluorolog-3 spectrofluorometer (Supplemental figure 1A and B) or on a SpectraMax M5 fluorescence plate reader. Reactions were performed in quadruplicate and an ANOVA (analysis of variance) and Tukey's test used to compare means of phosphorylated peptides with their unphosphorylated counterparts.

### ***4.3 RESULTS***

#### **4.3.1 Unbiased proteomic evaluation of phosphorylation-dependent regulation of caspase-mediated cleavage**

In an effort to characterize the interplay between phosphorylation and caspase-mediated degradation, we applied a proteomics approach to identify *bona fide*, phospho-regulated caspase substrates. In our workflow, we first treated HeLa cells with okadaic acid to maximize phospho-site occupancy, and then treated lysates with or without  $\lambda$  phosphatase, followed by recombinant caspase-3 and -7 (Figure 4.1B). Next, we utilized TAILS (terminal amino isotopic labeling of substrates) as a means for enriching the proteome for caspase substrates (Supplemental Figure 4.1) (13). Here, caspase-degraded proteomes are isotopically labeled at N-terminal and lysine residues with heavy (+34) or light (+28) formaldehyde. Trypsin digestion reveals internal peptide N-termini whose amine groups are reactive against an aldehyde-coupled, high molecular weight resin, whereas the amine groups of protein N-termini and caspase-generated N-termini remain inert due to blockage by dimethylation. In this way, filtering the lysate through a 10K cut-off membrane that removes the reacted resin/internal tryptic peptide species allows the N-terminome to pass through enriches the N-terminome. Performing LC-MS/MS analysis on an LTQ-Orbitrap identifies N-termini and, using spectra from MS1, produces a quantitative comparison between caspase degradomes from phosphorylated or dephosphorylated lysates. Importantly, after digesting with caspases, the phosphorylated lysate was treated with phosphatase. This permits a direct quantitative comparison between caspase substrates that may have a phosphorylation site that, if occupied, would alter retention time, mass, and undermine comparative analyses.

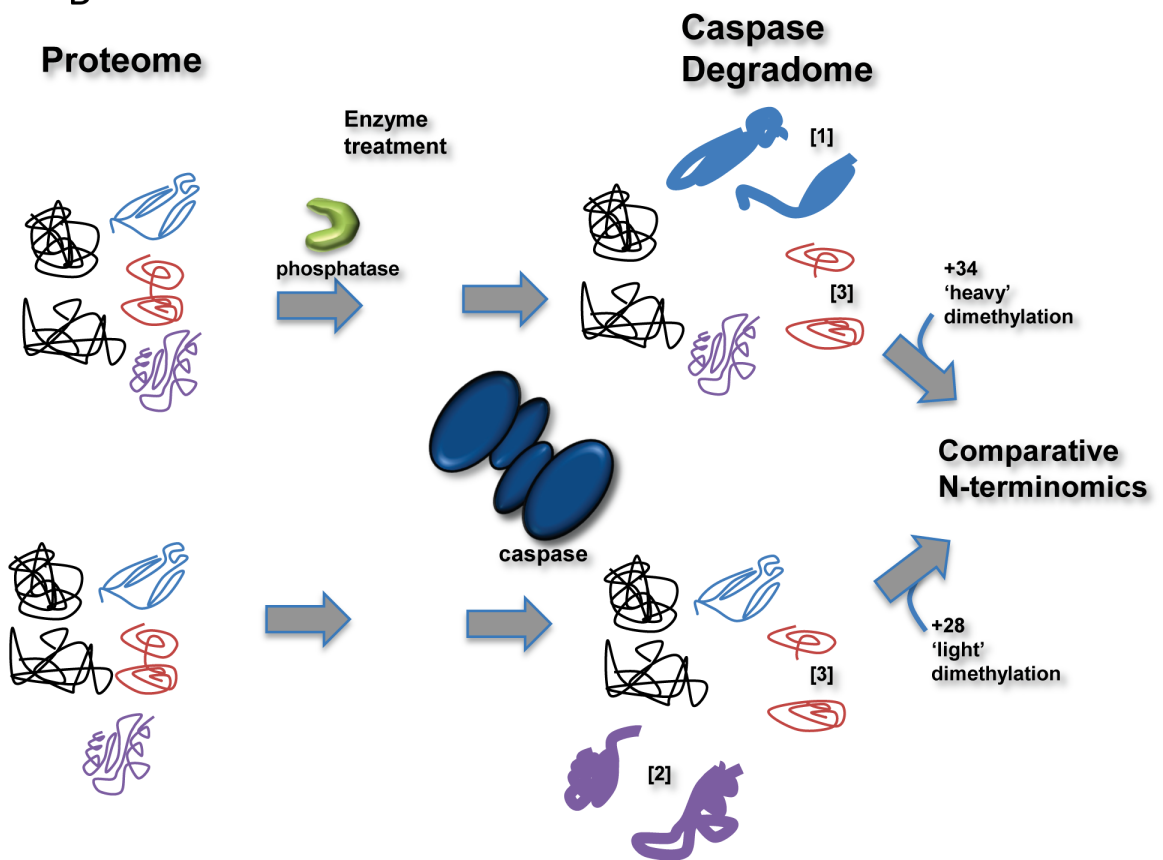
Before analyzing proteomic samples with mass spectrometry, we first tested for the efficient dephosphorylation of cell extracts and for the presence of cleaved caspase substrates. To this end, samples were analyzed at different points throughout the

Figure 4.1 – Workflow for the unbiased analysis of the integration of phosphorylation and caspase-mediated degradation. (A) Depiction of the cleavage site nomenclature for proteases. Caspases cleave the scissile bond between a P1 Asp and P1'. (B) To measure the effect of phosphorylation on caspase cleavage in an unbiased fashion, we performed caspase assays on lysates that were treated with or without  $\lambda$  phosphatase. We predict that some substrates will be cleaved more after dephosphorylation (blue, [1]), others digested only in the context of the phospho-proteome (purple, [2]), and others still unaffected by treatment with phosphatase (red, [3]). Samples are isotopically labeled using heavy or light dimethylation, followed by an N-terminomic strategy that enriches the proteome for neo-N-termini generated by caspase treatment (see Supplemental Figure 4.1 for proteomic workflow).

A



B



workflow (Supplemental Figure 4.1) for EEF1D and EEF1B phosphorylation, and caspase cleavage of the previously identified substrate MST1 (22). Lambda phosphatase treatment resulted in efficient dephosphorylation of phospho-EEF1D and phospho-EEF1B, two abundant phospho-proteins with near stoichiometric phosphorylation (Supplemental Figure 4.2A, Lanes 1 and 2) (19). Next these samples were treated with 50 or 500 nM of caspase-3 and -7, which resulted in a dose-dependent decrease in full-length MST1 (Supplemental Figure 4.2A, Lanes 3-6). Finally, adding  $\lambda$  phosphatase to previously phosphorylated lysate abolished phospho-EEF1D and phospho-EEF1B antibody reactivity (Supplemental Figure 4.2A, Lane 3 and 5). Further validating the efficiency of our enzyme treatments, we found that lysate phosphatase assays abolished reactivity of phospho-H2B antibodies when using extracts generated from nocodazole arrested cells, and increased migration speed of total Presenilin-1 levels, again indicative of complete dephosphorylation (Supplemental Figure 4.2B). We also tested PKC $\delta$ , another known caspase substrate (23), for cleavage in lysates treated with a gradient of caspase-3 and -7 concentrations (Supplemental Figure 4.2C). Proteolysis was again dose-dependent, and occurred with levels of caspases used previously in lysate cleavage assays (24).

Confident in our enzyme treatments of cell lysates, we generated TAILS samples from extracts treated with or without  $\lambda$  phosphatase and 50 or 500 nM of caspase-3 and -7. We identified and quantified 57 peptides with a P1 Asp, suggestive of caspase cleavage (Table 4.1). Only peptides identified in at least two replicates (either biological or technical) were considered hits. Weblogo analysis (18) of P1 Asp peptides generated a motif consistent with the consensus previously ascribed to caspase-3 and -7 (DEVD,

Figure 4.2A) (25). Importantly, N-terminomic analysis of untreated Jurkat cells revealed that less than 5 % of protein N-termini contained a P1 Asp (26), reinforcing the conclusion that the majority of the P1 Asp peptides we identified are caspase substrates. Interestingly, a cursory analysis of the quantified P1 Asp peptides revealed that the average peptide was cleaved  $1.2 \times$  more when lysates were not treated with phosphatase, perhaps indicating a general positive influence of phosphorylation on caspase cleavage at the proteome level. This skew is likely not due to labeling or sample bias, as P1 Asp quantitative data was normalized to the quantified protein N-termini isolated using TAILS (Supplemental Table 4.2), which should not change as a result of caspase treatment.

To validate the quantitative data returned from TAILS analysis, we repeated lysate dephosphorylation/caspase experiments, and probed for candidates using western blotting. We focused on those substrates with consistent changes in cleavage across multiple replicates, and those either previously identified as caspase substrates or whose cleavage site matched the caspase-3 and -7 consensus motif (Figure 4.2C). Using these criteria, we identified a previously reported caspase cleavage site on MST3 (AETD<sub>325</sub>) (27), and found that it was preferentially cleaved in phosphorylated lysates. Indeed, western blotting analysis revealed a modest, but consistent change in the amount of full-length MST3 that differed between  $\lambda$  phosphatase treated and untreated lysates (Figure 4.2B, upper panel). In contrast, we found that Yap1 was preferentially cleaved when lysates were dephosphorylated. Though cleavage of Yap1 at DEMD<sub>424</sub> is not a known caspase site, the primary amino acid determinants do align with the caspase-3 and -7 consensus motif, increasing our confidence that the site is a true positive. Along these

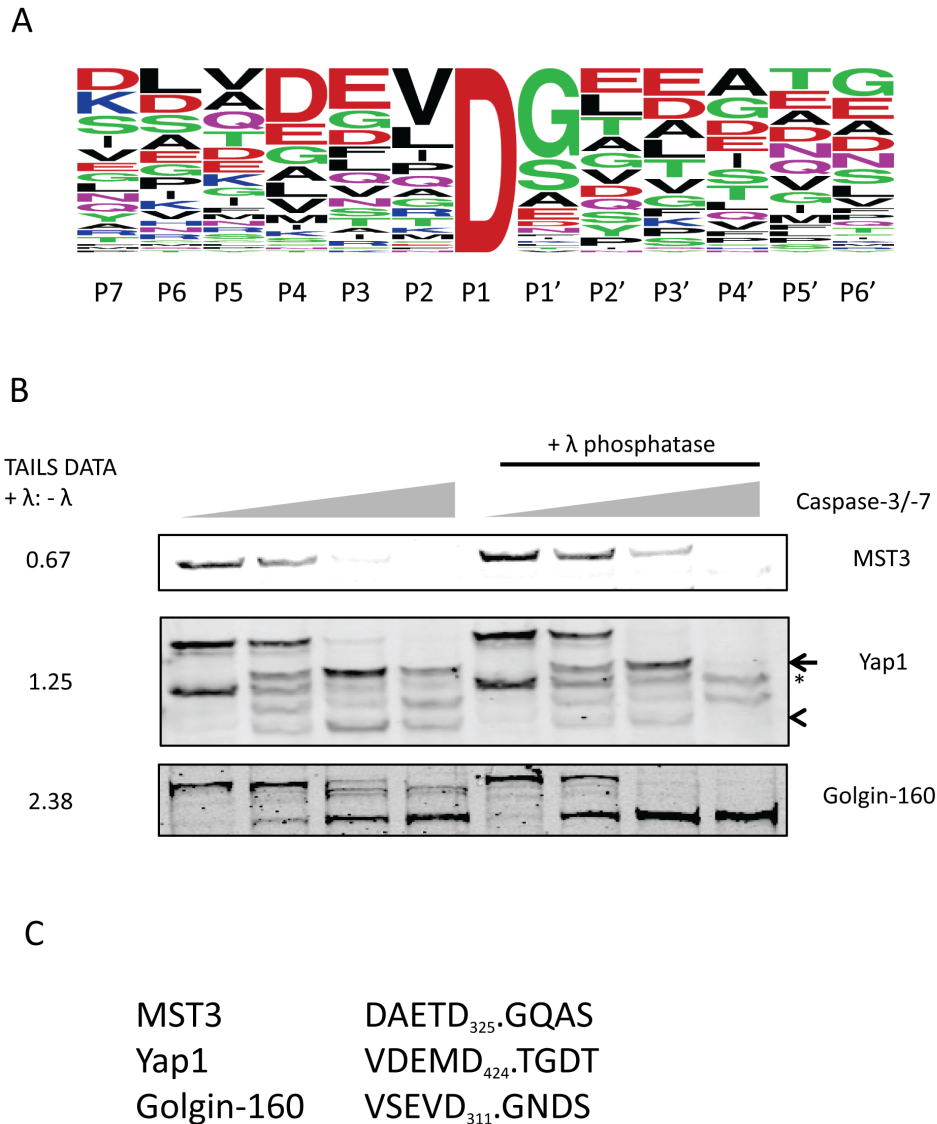


Figure 4.2 – TAILS analysis reveals MST3, Yap1 and Golgin-160 as three validated candidates whose cleavage is regulated by phosphorylation. (A) A weblogo analysis (18) of the 57 P1 Asp peptides identified by TAILS. (B) HeLa lysates were treated identically to those samples prepared for N-terminomic analysis (see materials and methods), except were utilized for western blotting with antibodies directed against MST3, Yap1 or Golgin-160 as indicated. Caspase-3 and -7 were used at concentrations of 0, 50, 500 and 5000 nM. (C) Caspase motif of TAILS hits validated in (B).



lines, treating lysates with caspase-3 and -7 resulted in a dose-dependent decrease of full-length Yap-1, as well as the generation of smaller anti-Yap1 reactive bands (Figure 4.2B, middle panel). Furthermore, full-length Yap1 was slightly more resistant to caspase cleavage when lysates were treated with  $\lambda$  phosphatase, and two degradation products were less abundant in the phosphatase treated samples which is also consistent with a protective role for phosphorylation (Figure 4.2B, arrow and arrowhead). Conversely, another Yap1 reactive band (denoted with \*) persisted only in samples treated with  $\lambda$  phosphatase, perhaps suggesting another caspase-site that is positively regulated by phosphorylation. Though the presence of multiple caspase sites on Yap1 seems apparent, our western blot validation data is consistent with the TAILS quantitation. Lastly, we found that Golgin-160 (referred to as GOLGA3 in Table 4.1) was also cleaved less in phosphorylated lysates at SEVD<sub>311</sub> – a site previously identified as being targeted by caspase-3 and -7 *in vitro* and in response to diverse apoptotic stimuli (28,29). Repeating lysate dephosphorylation and caspase assays, followed by measuring cleavage with western blotting were again consistent with the data returned from TAILS (Figure 2B, lower panel). Here, in the phosphatase treated lysates, a more rapid conversion to the fully cleaved form is observed. In contrast, an intermediate, slower migrating cleavage fragment remained even at the highest dose of caspase-3 and -7 (5  $\mu$ M) in the phosphorylated lysates. Caspase-3 and -7 can also cleave Golgin-160 at D<sub>59</sub> and D<sub>139</sub>, and so our blots are consistent with the model that intermediate cleavage fragments of Golgin-160 have been processed at these other residues, but phosphorylation blocked proteolysis at SEVD<sub>311</sub>. Ultimately, these data highlight novel mechanisms regulating the susceptibility of MST3, Yap1 and Golgin-160 to caspase cleavage.

### 4.3.2 Systematic characterization of the effect of phospho-Ser on caspase cleavage

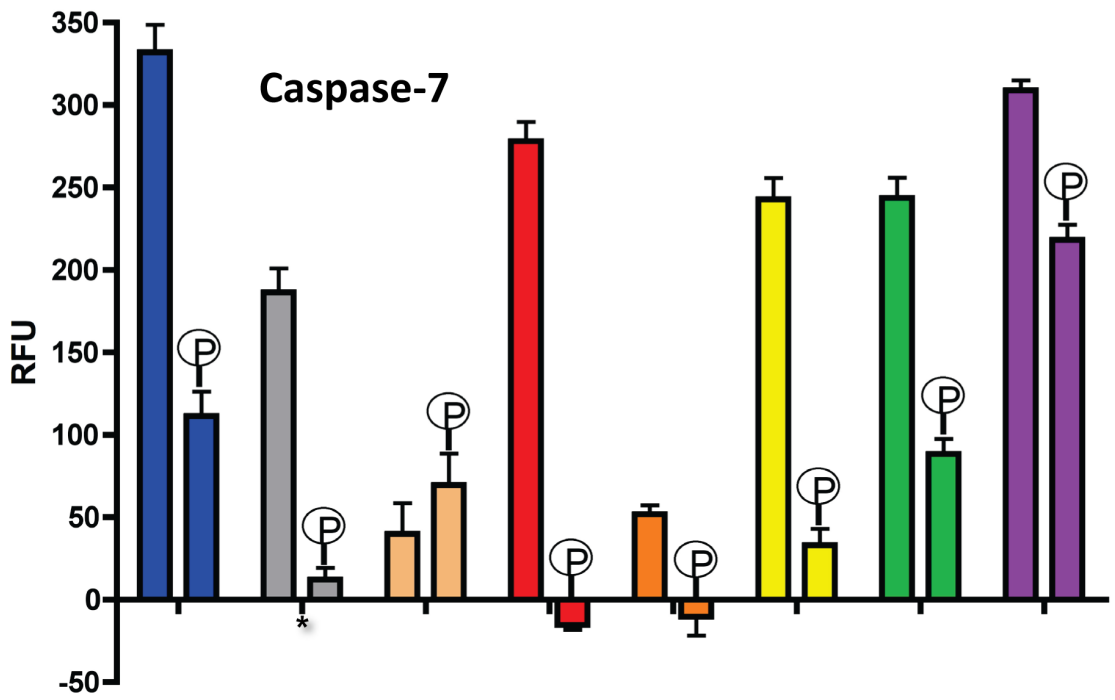
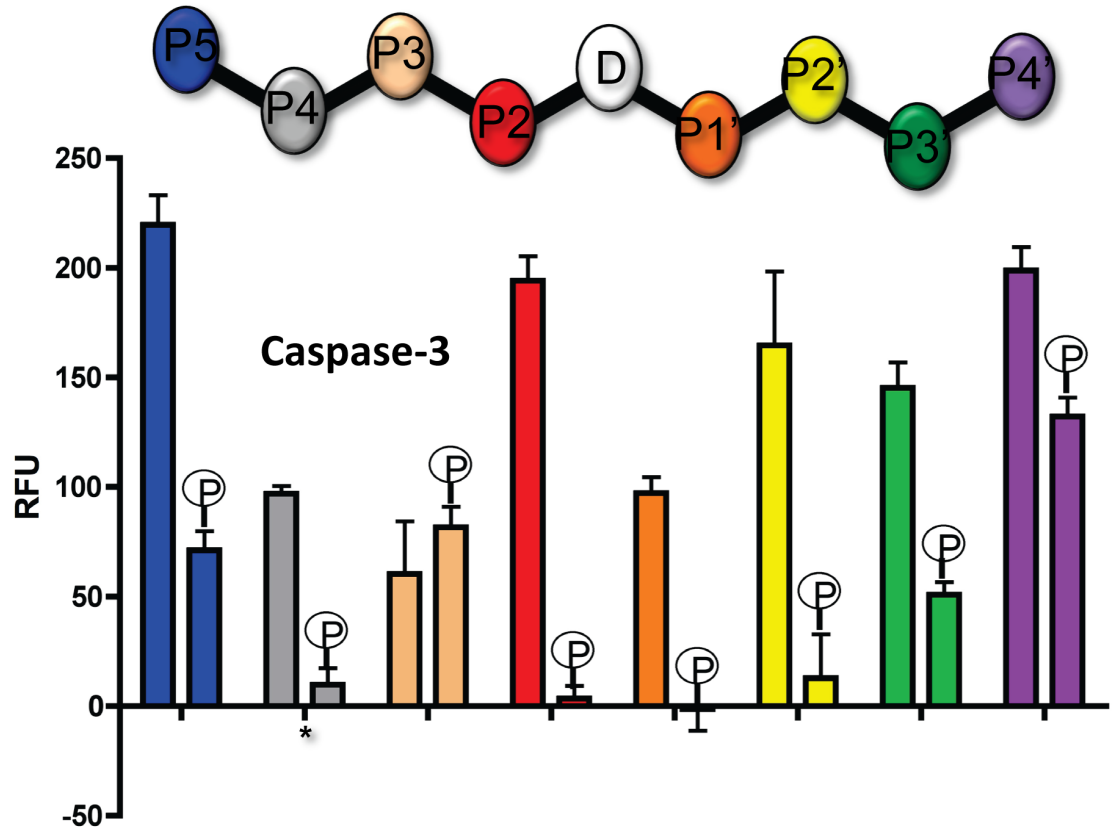
The finding that caspase cleavage of MST3, and other proteins from our TAILS analysis, were cleaved less after dephosphorylation prompted us to systematically evaluate the determinant properties of phospho-residues within the context of peptide substrates. A number of anecdotal examples of phosphorylation regulating caspase cleavage of substrate peptides exist, but the determinant properties of phospho-residues on proteolysis have not been systematically or rigorously defined. To address this question, we designed a phospho-scanning series of peptides that walked serine and phospho-serine from P5 – P4' within a caspase-3/-7 model sequence, and from P4 – P3' for a caspase-8 model sequence (Supplemental Table 4.3). A broader series was used for caspase-3 and -7 due to the observation that primary amino acid specificity can extend beyond the canonical P4 – P1' residues for these caspases (15). Peptides were synthesized with the FRET pair of tryptophan and lysine-DNP (2,4-dinitrophenol) at either termini. When the peptide is intact, emission by tryptophan at 355 nm after excitation at 280 nm is quenched by DNP, which absorbs at 355 nm. Following substrate cleavage, release of tryptophan from close proximity to DNP abolishes quenching, and permits reaction rates to be monitored by measuring tryptophan fluorescence at 355 nm. Indeed, caspase-3 and -7 reactions with a model peptide (see Supplemental Table 4.3 for sequence) maintained linearity through 15 minutes of reaction time, and were not cleaved when P1 was substituted with alanine (Supplemental Figure 4.3A, B). Caspase-8 reactions proceeded to completion more quickly, so shorter reaction times were used

below (Supplemental Figure 4.33C). As expected, caspase-8 peptides were not cleaved when P1 was mutated from Asp to alanine.

The phospho- and non-phospho walking series produced essentially identical patterns when comparing caspase-3 and -7 – perhaps not surprising given their extensive sequence and consensus motif similarity (Figure 4.3) (25,30). Analyzing the unphosphorylated series reveals that the relative effect of Ser throughout P5 – P4' is consistent with the literature. For instance, substituting Ser for Asp at P4 decreased substrate conversion by a factor of 10 (data not shown) compared to the model, but became a measurable substrate when 5× more enzyme was used (see asterisk on Figure 4.3). Stennicke *et al.* (2000) also observed a large decrease in  $K_{cat}/K_m$  when substituting P4 Asp to Ser for caspase-3 and -7 peptide substrates (31). Similarly, substituting Ser for Glu at P3 decreased catalysis, which is consistent with a previous report (25). Discrepancies were seen at the P1' position, where Ser in place of Gly reduced cleavage by about one-half – an observation previously made for caspase-7, but not -3 (31). Because different models were used between our study and Stennicke *et al.* (2000), we cannot rule out peptide length or pairwise interactions at other sites as the cause for this discrepancy. Nonetheless, our unphosphorylated peptides correlate well with the literature and validate our experimental system.

Analysis of the phospho-Ser peptides revealed an overall inhibitory effect on proteolysis (Figure 4.3). Expectedly, phosphorylation at P2 (12) and P1' (32) abolished cleavage, but upon phosphatase treatment was almost completely rescued (Supplemental Figure 4.4). As previously observed, phospho-P3 had little effect on proteolysis (32). During the course of our study, though, Dix *et al.* (2012) actually found that phospho-P3

Figure 4.3 – Relative caspase-3 and -7 activities against phospho- and nonphosphorylated model peptides. 100  $\mu$ M of internally quenched substrate peptides were reacted with 200 nM of caspase-3 or 600 nM of caspase-7 for 15 minutes at 37°C and stopped by adding an excess of the irreversible caspase inhibitor zVAD-fmk. P4 peptides, in which Ser or pSer is substituted for Asp, were not measurable at the above caspase concentrations, so 1  $\mu$ M of caspase-3 and 3  $\mu$ M of caspase-7 were used (\*). Measuring the fluorescence at 355 nm after excitation at 280 assessed activity. Error bars represent the standard deviation of 4 reactions. Means of the phosphorylated peptides and their unphosphorylated counterparts were compared using an ANOVA and Tukey's test. All pairs had P values < 0.001, except for the P3 pair, which had a P value > 0.05.



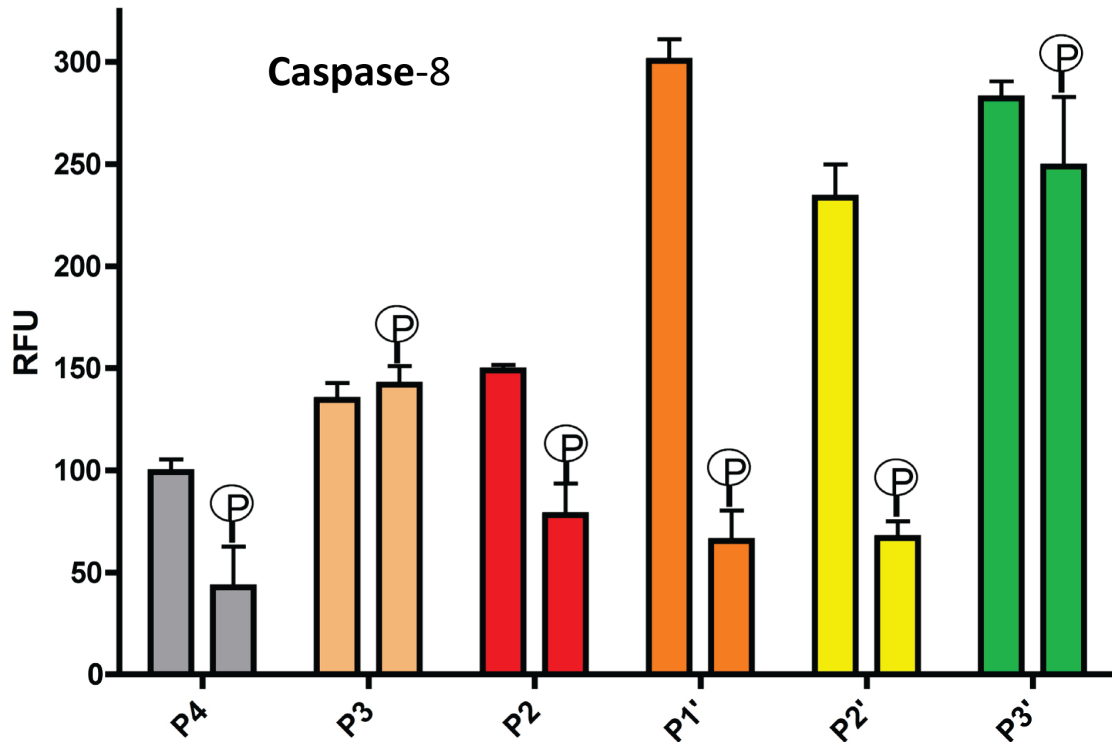
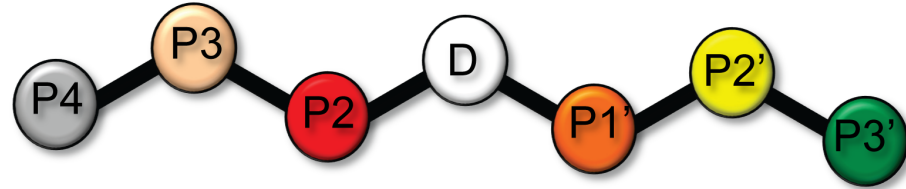


Figure 4.4 – Relative caspase-8 activity against phospho- and nonphosphorylated model peptides. 100  $\mu$ M of internally quenched substrate peptides were reacted with 100 nM of caspase-8 for 5 minutes at 37°C and stopped by adding an excess of the irreversible caspase inhibitor zVAD-fmk. Measuring the fluorescence at 355 nm after excitation at 280 assessed activity. Error bars represent the standard deviation of 4 reactions. Means of the phosphorylated peptides and their unphosphorylated counterparts were compared using an ANOVA and Tukey’s test. All pairs had P values < 0.001, except for the P3 and P3’ pair, which had P values > 0.05.

can have stimulatory or neutral effects on cleavage, depending on the peptide substrate used (14), representing a current inconsistency with part of the literature. Interestingly, phospho-P2' almost completely abolished proteolysis, phospho-Ser at P5 and P3' reduced cleavage by about 2-3 fold and phospho-P4' was slightly inhibitory. Overall, phosphorylated Ser has a negative effect on caspase-mediated cleavage, except at P3, in the context of our model peptide.

The effect of caspase-8 cleavage site phosphorylation showed similar trends to caspase-3 and -7. Proteolysis was inhibited by phospho-Ser at P4, P2, P1' and P2' whereas P3 was unaffected by phosphorylation (Figure 4.4). Again, this latter result is in contrast to a recent report by Dix *et al.* (2012), where phospho-P3 was exclusively a positive determinant for caspase-8 cleavage (14). Also, compared to caspase-3 and -7, the effect of prime side phosphorylation on cleavage is less dramatic, as no significant difference between Ser-P3' and pSer-P3' was observed. Taken together with our N-terminomic data, the generally negative determinant properties of phospho-Ser suggests that phosphorylation-dependent promotion of proteolysis is controlled by factors outside of the primary amino acid sequence of the canonical caspase motif.

#### ***4.4 DISCUSSION***

The convergence of caspase and kinase signaling has recently been described as a major mechanism in the regulation of apoptosis (6,7). One mode of kinase-mediated control of caspase signaling involves the phosphorylation of caspase substrates. To identify proteins regulated in this manner, we utilized the N-terminomic strategy TAILS to monitor alterations in the caspase degradome of HeLa extracts with a native phospho-

proteome versus lysates first treated with  $\lambda$  phosphatase. In this way, we could for the first time measure caspase-mediated proteolytic events that were both positively and negatively regulated by phosphorylation in the context of a proteome. Indeed, we identified and validated three proteins whose caspase cleavage is modulated by phosphorylation; Yap1 and Golgin-160 are degraded less when phosphorylated, whereas phosphorylation promotes cleavage of MST3.

By systematically evaluating the determinant properties of phospho-Ser within, or just proximal to, the canonical caspase consensus motif, we found that this post-translational modification primarily inhibited cleavage. Taken together with the observation that MST3 proteolysis is promoted by phosphorylation, this supports a model by which substrate phosphorylation can promote caspase motif accessibility or structure when occurring outside of the context of the motif itself. In this sense, both factors play a role in the cleavage of protein substrates by caspases; elegant substrate engineering studies by Timmer and colleagues (2009) demonstrate that increasing the size of loops that contain the scissile bond promotes cleavage by two orders of magnitude (33). These authors also made the surprising observation that caspases cleave scissile bonds located in  $\alpha$ -helices almost as much as those in extended loops. Interestingly, phosphorylation can have both stabilizing and destabilizing effects on  $\alpha$ -helices (34) which may alter scissile bond accessibility.

With respect to MST3, treating cells with the phosphatase inhibitor caliculyin A increases phosphorylation, activation, and structural reordering that alters its interaction partners (35). Because our N-terminomic strategies used lysates from cells treated with okadaic acid, a phosphatase inhibitor that like calyculin A also inhibits PP1 and 2, one



interpretation of our data supports a model whereby MST3 is preferentially cleaved in its active form (35). As well, MST3 is a pro-apoptotic kinase that exhibits increases in activity after the removal of its C-terminus by caspase cleavage (27). One function of this hypothetical hierarchical layer of regulation is that phosphorylation and cleavage could provide graded levels of MST3 activity. Precedence for promotion of cleavage by phosphorylation outside of the extended caspase motif has been observed. Phosphorylation of PKC $\delta$  at Y332 promotes cleavage at D327 (36). Indeed, Y332 is located at P5' and, based on our peptide studies, should be sufficiently far from the scissile bond to not interfere with catalysis.

Of the caspase substrates whose proteolysis is negatively regulated by phosphorylation, both Yap1 and Golgin-160 have phosphorylatable residues at positions that, when occupied with phosphate, were inhibitory towards cleavage of peptide substrates. While the P4 Ser on Golgin-160 and P1' Thr on Yap1 have not been identified as phosphorylated in phospho-proteomics studies, their occupancy would be consistent with the cleavage patterns we observed on peptide substrates and in lysate cleavage assays. At this point, we cannot rule out that remote phosphorylation sites reduce susceptibility to proteolysis much the same way that phosphorylation at S422 on Acinus-S reduces cleavage at D355 (8). Interestingly, Golgin-160, and other members of the Golgi complex involved in structure and trafficking, are dismantled via caspase cleavage early in the apoptotic response (37). Golgin-160 is cleaved at D<sub>59</sub>, D<sub>139</sub> and, D<sub>311</sub> – the site we identified – with purified caspases and in cells after diverse apoptotic stimuli (28). In our validation studies, we inferred via the differential migration of Golgin-160 cleavage products, that two of these sites (D<sub>59</sub> and D<sub>139</sub>) were cleaved in

phosphorylated lysates, but that proteolysis at SEVD<sub>311</sub> was blocked by phosphorylation. Interestingly, in response to apoptotic signals initiating from death receptors or ER stress, non-cleavable forms of Golgin-160 can actually block apoptotic progression (29), though the effect of blocking each caspase site in isolation remains unknown. Yap1 is another caspase target that we validated as having a cleavage site that is modulated by phosphorylation. While DEMD<sub>424</sub> has not previously been identified as a caspase cleavage site, ASTD<sub>111</sub> is cleaved in Jurkat cells in response to apoptotic induction by TRAIL and staurosporine, perhaps resolving why we observed multiple cleavage products in our validation studies (38). Furthermore, P3 and P2 of ASTD<sub>111</sub> have previously been identified as phosphorylated in cells (39), again clarifying results from our western blots that indicated multiple cleavage products were differentially regulated between the phosphorylated and phosphatase treated lysates. In terms of functionality, Yap1 is a transcriptional effector of the MST1 and 2 kinases (40), which themselves are also targeted by caspases and function in apoptotic progression (22,41). Interestingly, MST1 and 2 contain previously identified phosphorylation sites at P1' and P3', suggesting that caspase cleavage of multiple constituents of the same pathway are hierarchically regulated by phosphorylation (42). As well, MST1 phosphorylation by Akt at T387, a site distant from the scissile bond, blocks cleavage (43), further implicating the control of proteolytic processing of this pathway as an important signaling event.

One potential limitation of our approach relates to the high stoichiometry of caspase-site phosphorylation required for a difference in susceptibility to be measurable. In an effort to overcome this limitation, we treated cells with okadaic acid to increase

phosphorylation site occupancy. Similarly, the effect of phosphorylation on cleavage is not necessarily absolute, such as at P3', for example. To avoid complete digestion of proteins with phosphorylation sites at these residues, we used two different caspase concentrations. Nonetheless, despite these caveats, we were able to uncover a number of modulated caspase substrates, and validated Yap1, MST3 and Golgin-160 as caspase targets that experience altered degradation in response to lysate dephosphorylation. Yap1, MST3 and Golgin-160 have roles in processes pertaining to cell survival, and so it is enticing to speculate that the functionality of modular cleavage mechanisms are physiologically relevant.

As a logical extension to the unbiased investigation of the effect of phosphorylation on caspase-mediated degradation in the proteome, we were also interested in systematically evaluating the determinant properties of phosphorylated residues in the context of model substrate peptides. Given the size and charge of phosphoryl residues, we speculated that phosphorylation may have effects outside of the canonical caspase motif and so extended our analysis outside of P4 – P1'. Along these lines, even unmodified amino acids have exhibited determinant properties at P6, P5, P2' and P3' (15). Furthermore, Dix *et al.* (2012) recently catalogued a large number of caspase sites with phosphorylated residues in this extended motif, though the precise effects of phosphorylated residues at many of these positions remain unknown (14). In systematically analyzing the positional effect of phospho-Ser on caspase cleavage, we found that phosphorylated residues outside the canonical caspase motif could indeed inhibit caspase-mediated cleavage. Prime side (C-terminal of the scissile bond) determinants inhibited cleavage as far away as three residues from the scissile bond and

the inhibitory capacity was inversely proportional to distance from the catalytic machinery. This finding may be partly explained by previous observations made by Stennicke *et al.* (2000) (44). Here, the authors show that negative determinants at P1' fail to stabilize the transition state. Therefore, the bulky, highly charged nature of phospho-Ser might act similarly, but with diminishing effect as it moves away from the catalytic machinery. Evidence for an S5 binding site (that interacts with P5) on caspases has also recently become evident. P5 residues can positively influence cleavage by caspase-3 (45), and direct selectivity between caspase-3 and -7 (15), so we investigated the effect of phosphorylation at this position. Inhibition is consistent with a preference for small non-polar residues by caspase-3 (15). The determinant properties of P5 on caspase-7 are less obvious, making the specific reasons for phospho-P5 more difficult to reconcile (15).

Inhibitory effects at P4, P2 and P1' were consistent with the literature, but we did note a discrepancy at the P3 position. Dix *et al.* (2012) found that phospho-P3 on substrate peptides was strictly a positive factor for caspase-8 cleavage, and either promotes or has no effect on caspase-3 mediated degradation (14), whereas like Toszer *et al.* (2003), we observed no effect at this site for both caspase-3 and -8 (46). We cannot definitely explain this discordance, but can speculate that reaction conditions may have played a part. First, that Dix *et al.* (2012) used enzyme:substrate ratios 10-100× greater than our own, and reaction times that were 20× longer, suggests that their enzymes were less stable. As well, for caspase-8, we used the chaotropic agent sodium citrate, which promotes caspase activity by stabilizing the dimeric form (47). Collectively, it is possible that the determinant properties of phospho-P3 may depend on reaction conditions that affect enzyme stability. Another possibility includes the substrate peptides used; Dix and

colleagues (2012) tended towards longer peptides and those with sequences that differed from our model, suggesting that lengthier peptides or pairwise primary amino acid interactions could affect cleavage. At the minimum, though, our mutual findings are consistent in that phospho-P3 does not inhibit cleavage.

In accordance with the literature, phospho-P2 and phospho-P1' were also inhibitory towards proteolysis (12,46). Of note, however, was the measurable activity of caspase-8 against these peptides, whereas caspase-3 and -7 activity was not detected under the conditions used. Furthermore, baseline levels of cleavage by caspase-8 for peptides phosphorylated at P2' and P4 were also above background, perhaps suggesting higher tolerance against the inhibitory effects of caspase-8 site phosphorylation. Along these lines, the only known caspase-8 protein substrates negatively regulated by phosphorylation are phosphorylated at two sites within the cleavage motif; phosphorylation at P2 and P1' of caspase-3 blocks its cleavage by caspase-8 (12), and phospho-P2 and P2' prevent cleavage of Bid (9). In this way, phosphorylation at multiple, slightly tolerated positions may function to grade cleavage and modulate the signaling output.

In sum, we used two strategies to understand the interplay between caspase substrate phosphorylation and proteolysis. As well as systematically characterizing the primary sequence determinant properties of phospho-Ser within peptide substrates, we also utilized an unbiased, N-terminomic approach to monitor caspase substrates that were regulated by phosphorylation in the context of the proteome. Overall, we anticipate that efforts such as ours that disambiguate the hierarchical regulation of caspase substrate

phosphorylation and cleavage will improve our understanding of apoptotic signaling and undercover signaling pathways that control cell survival.

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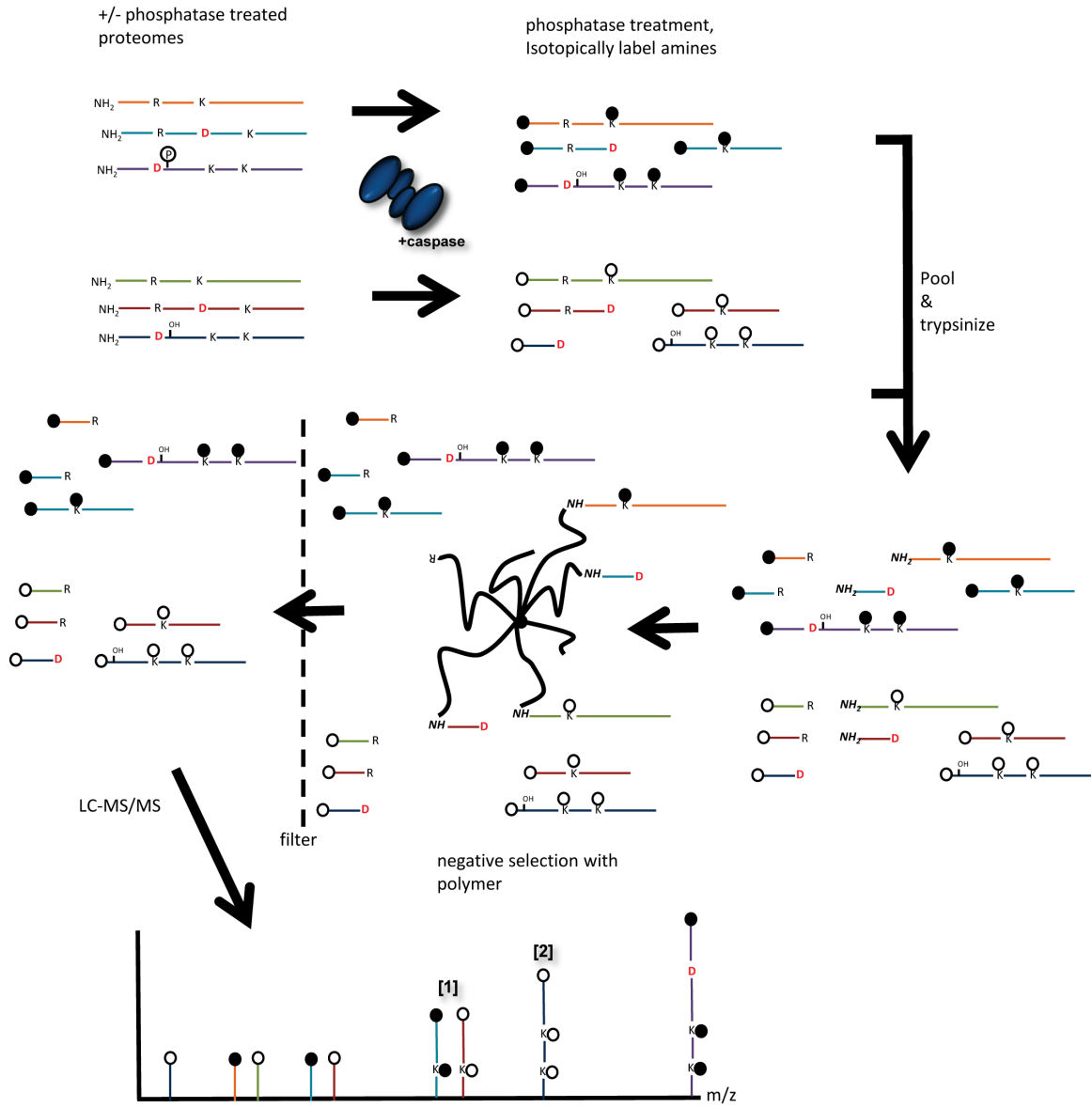
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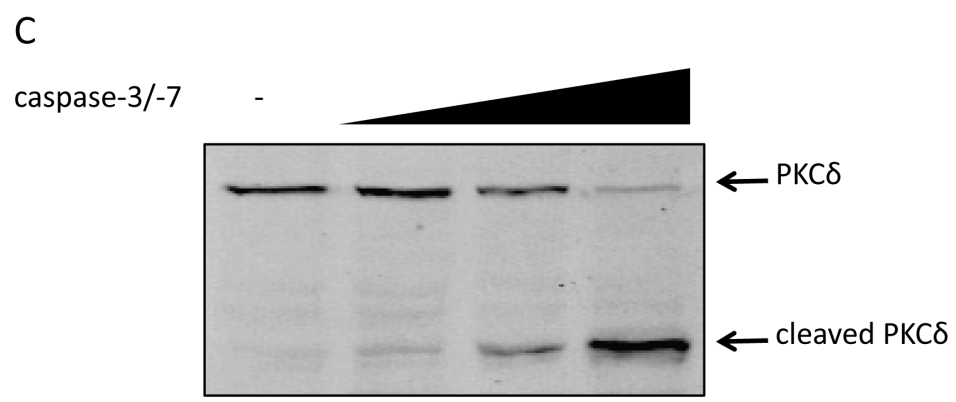
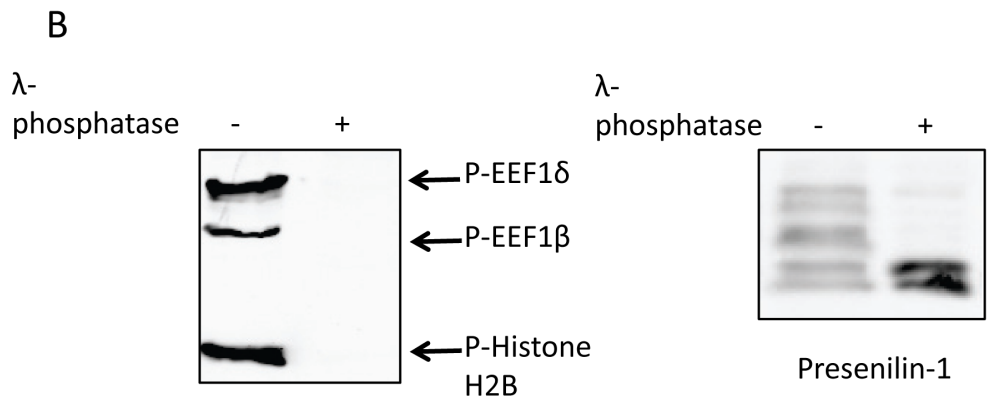
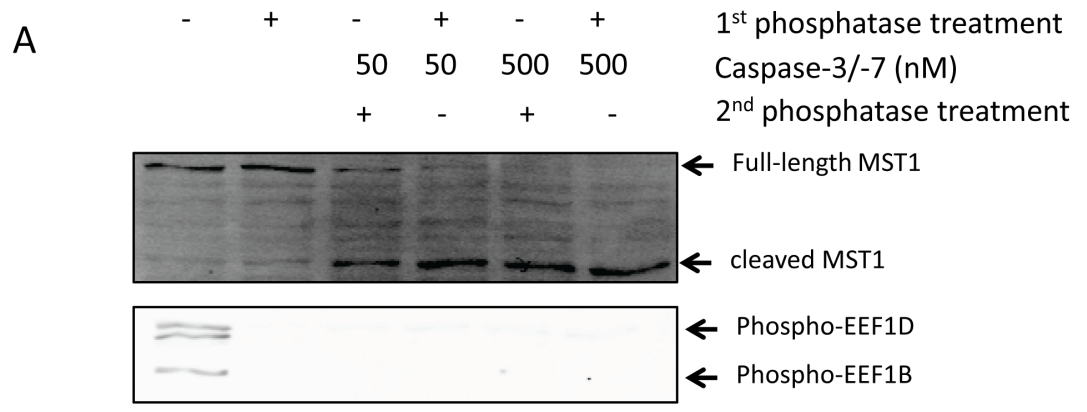
#### **4.6 SUPPLEMENTAL FIGURES**

Supplemental Figure 4.1 – N-terminome enrichment using TAILS. HeLa cell lysates were treated with or without  $\lambda$  phosphatase, followed by caspase treatment, and then dephosphorylation of the sample previously left phosphorylated. Primary amines on protein N-termini and lysine residue are dimethylated using heavy (+34, open circles) or light (+28, black circles) formaldehyde. Samples are pooled and trypsinized which exposes an amine on the N-terminus of the internal tryptic peptides. These peptides are captured by reacting with an ~80 kDa, aldehyde substituted polymer. Importantly, native protein N-termini and neo-N-termini generated by caspase cleavage are resistant to reaction with the polymer because their reactive amines have been blocked by dimethylation. Enrichment of the N-terminome then occurs by negative selection via filtering away the reacted polymer using a 10-kDa cut-off spin column. LC-MS/MS analysis of isotopically dimethylated peptides then allows comparative analysis between caspase degradomes of phosphorylated or dephosphorylated lysates. Caspase substrates will be inferred by identifying those peptides with a P1 Asp. In the event that there is no difference between caspase substrate proteolysis between phosphorylated and dephosphorylated samples, a peptide ratio of ~ 1:1 will be observed in MS1 [1]. Of interest are those peptide pairs that deviate from a 1:1 ratio [2].

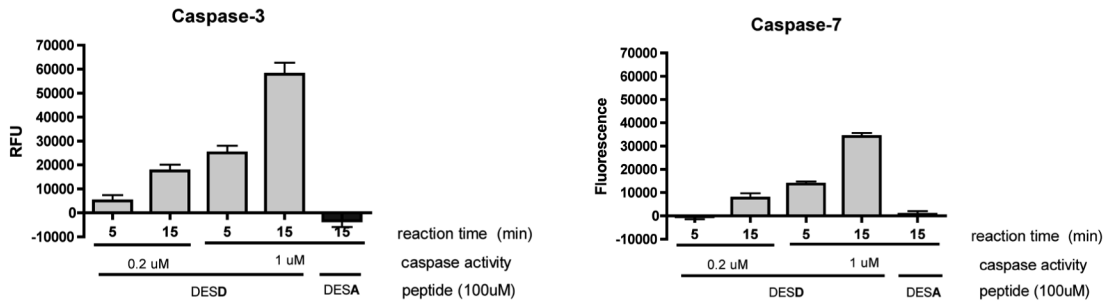


Modified TAILS workflow

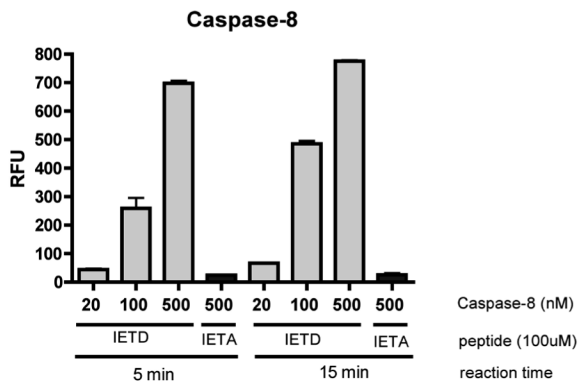
Supplemental Figure 4.2 – Proof-of-principle for phosphatase and caspase treatment of HeLa cell extracts. (A) Caspase degradomes were prepared as indicated in the materials and methods and samples taken after the first phosphatase treatment, and again after treatment with caspases and dephosphorylation of the previously ‘- phosphatase’ treated sample. Samples were western blotted and probed first with antibodies directed against MST1 and then with phospho-EEF1D and phospho-EEF1B. (B) Nocodazole-arrested HeLa cell lysates were dephosphorylated with  $\lambda$  phosphatase as indicated in materials and methods, western blotted and probed for phospho-EEF1D, phospho-EEF1B and phospho-H2B (left panel). Similarly, HeLa extracts were  $\lambda$  phosphatase treated and western blotted for Presenilin-1 (right panel). (C) HeLa lysates were treated with 0, 1, 100 or 1000 nM of caspase-3 and -7 for 1 hour at 37°C and stopped by the addition of 2 × Laemmli buffer. Reactions were western blotted with antibodies against PKC $\delta$ .



A

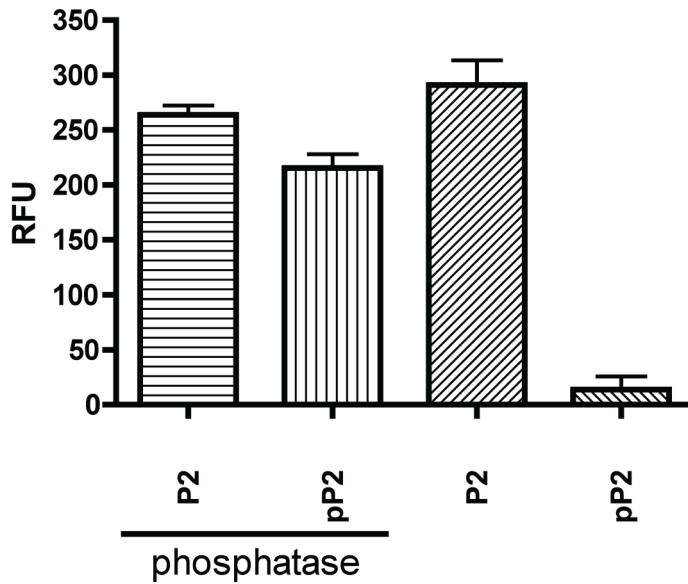


B



Supplemental Figure 4.3 – Assessing the linearity of caspase reactions using model internally quenched caspase substrate peptides. (A) The indicated amounts of caspase-3 or -7 were reacted with model (DESD) or negative control uncleavable (DESA) peptides for 5 or 15 minutes at 37°C and stopped by adding an excess of the irreversible caspase inhibitor zVAD-fmk. Tryptophan fluorescence was monitored by excitation at 280 nm and measuring emission at 355 nm on a fluorimeter. (B) Assays were performed as in (A), except fluorescence was measured using a \_ plate reader. Error bars represent the standard deviation of 4 reactions.





Supplemental Figure 4.4 – Dephosphorylation of the phospho-P2 peptide substrates increases caspase cleavage. Phosphorylated and non-phosphorylated P2 peptides were treated with or without  $\lambda$  phosphatase for 1 hour at 37°C. After dephosphorylation, 100  $\mu$ M of peptide was used in reactions with 600 nM of caspase-7 for 15 minutes at 37°C. Reactions were stopped using the irreversible caspase inhibitor zVAD-fmk, followed by measuring tryptophan fluorescence at 355 nm after exciting at 280 nm. Error bars represent the standard deviation of 4 reactions.

#### 4.7 SUPPLEMENTAL TABLES

Peptide	AA position	Gene Names	Exp1	Exp2	Exp3
			50 nM C3/7	500 nM C3/7	500 nM C3/7 #2
D.GEEQNKEALQDVEDENQ	238	YWHAE	0.76042697	0.853139912	
D.GLLAGGEKATMQNLNDR	77	KRT17		0.645234859	0.490617059
D.GEEAGAYDGPR	108	ERP57	0.706186859	0.998420266	
D.GEALSTLVLNR	279	HSP60	0.564884205	0.799471561	
D.GQPGAFTCYLDAGLAR	136	MSTP030	0.767151729	0.94784708	
D.TGDTINQSTLPSQQNR	424	YAP1	1.25342255	1.253295832	
D.GTDEYNSGVICENTCKEKGR	101	G19P1		0.279086823	1.154643734
D.GTVEEDLGKSR	28	GRP94		0.7898872	1.14751825
D.VDGTVEEDLGKSR	26	GRP94		0.756502651	
D.GVYAFEYYPSTPGR	508	FLN1L		1.138837381	
D.DEEDEEELGEEER	220	ANP32A	0.708180476		
D.NADGTQTVNYVPSR	1501	FLN	0.678917168	0.677698385	
D.GTQTVNYVPSR	1504	FLN	1.236913583	0.797100717	
D.GVTHTVPIYEGYALPHAILR	157	ACTB	0.573965852	0.82889666	0.343460067
D.SYVGDEAQSKR	51	ACTB	0.446723212	0.853228715	0.857804579
D.GQVITIGNER	244	ACTB	0.83973365	0.770662752	0.941464186
D.NGSGMCKAGFAGDDAPR	11	ACTB		0.888802517	0.936792011
D.SVEGEGEEEGEEY	438	TUBA1B	0.840066759	0.889156924	
D.DSFNTFFSETGAGKHVPR	46	TUBA1B	0.534176279	0.616769044	
D.LTEFQTNLVPYPR	251	TUBA1B	1.022727772	1.3259438	
D.EVTIVNILTNR	70	ANX2	0.898043366	0.953627141	
D.EDSLIEIICSR	142	ANX2	0.452115547	0.152048214	
D.SGPINDTDANPR	231	CASP7		0.772252503	1.298344927
D.GSGNSTIIVSR	2359	HSPC272		0.370006451	
D.SSEPVVEDER	237	ASPH	1.096267056	1.043382777	
D.KSPFNPSPPQDSPR	331	NFI			0.091363899
D.APKSQDLAKIMADIR	238	CYK18			0.621854417
D.STAAQQELR	1153	MYH9		1.020533804	0.950716508
D.IAVDGEPLGR	9	CYP4A	0.655232007	0.98551256	
D.SATAFGAQTSSR	472	C16orf84		0.890895317	
D.GADCIMLSGETAKGDYPLEAVR	354	OIP3		0.609484712	0.869139479
D.AEAAMDISEGR	767	ISOT	1.444366375	0.599292131	
D.GYETEGIR	274	MCM6		1.048528635	
D.GYLSLLQDSGEVR	96	EIF5A	0.780220749	1.01703037	
D.FETGDAGASATFPMQCSALR	6	EIF5A		0.978891496	0.832536077
D.SLKEALTYDGALLGDR	93	EIF4H		0.654161781	
D.GATQSSPAEPKSEADR	708	GCF2		0.990361312	1.026909504
D.GDLTLYQSNLILR	58	FAEES3	0.678180083	0.973817518	

D.GNDSOSSSYSSASTR	311	GOLGA3			2.37786697
D.GPEAQAEACSGER	9	HSPC286		0.654270848	
D.AGTIAGLNVLR	160	HSC70	0.70817828	0.849292298	
D.SEDLPLNISR	512	HSP90A	0.512801898	0.590617099	
D.GPAAEAEPEHSFDGLR	2771	PLEC1	1.214061449	1.305317525	
D.GQASGGSDSGDWIFTIR	325	MST3		0.67011965	
D.EQDENGAEASADLR	481	MSN	0.746966158		
D.GLVETPTGYIESLPR	57	NAP1L1	0.760847005	0.66128923	
D.GLGVARPHYGSVLDNER	8	IQGAP1		0.848174012	
D.SLLQDGEFMSMDLR	76	PFN1	1.017753873		
D.GEFMSMDLR	81	PFN1	0.853577387	0.910693462	
D.GDEDEEAESATGKR	113	PTMA	0.660056838	0.773236606	
D.GITIQAR	735	SPTAN1		0.820806464	
D.AGEFVDLYVPR	4	hCG_41768	0.837948757	1.171753589	
D.AYGPPSNFLEIDIFNPQTVGVGR	21	SNX12			1.024484234
D.GLAVTPTPVVVGSMQTR	128	U2AF2			0.501543421
D.SVLDVVR	114	TUBB2	0.821847838	0.963970257	0.987401403
D.FSLADAINTEFKNTR	85	VIM	0.7516011	0.938473968	1.064232797
D.ALKGTNESLER	331	VIM	0.978807084	0.906199105	

Supplemental Table 4.1. List of P1 Asp peptides identified using TAILS. Values in the experiment columns represent the ratio of peptide in phosphatase treated samples versus samples not treated with phosphatase.

Peptide	AA position	Gene Names	Exp1	Exp2	Exp3
			50 nM C3/7	500 nM C3/7	500 nM C3/7
M.VNFTVDQIR	1	EEF2	0.699543509	0.986643633	
.MILLEVNRR	0	ARC34	0.840853676	0.852542522	
M.VLAELYVSDR	1	ASNS	0.826466954	0.712692079	
M.GKVKVGVNGFGR	1	CDABP0047	0.810234244	0.894274269	1.138836759
M.AGGEAGVTLGQPPLSR	1	EIF2G	0.892983387	0.994338435	
M.AAEKQVPGGGGGGGSGGGGGSGGGGGSGGGR	1	SRP68	0.984748641	0.922616039	0.825511026
.MKFNPFVTSR	0	RPL26	1.016908841	0.88446624	
M.VEKEEAGGGISEEEAAQYDR	1	AOS1	1.024355292	0.887742657	1.075926056
M.AATLILEPAGR	1	ACOT1	1.14008767	0.97040373	
.MIEVVCNDR	0	UBL5	1.147738285	0.989108464	1.373512128
M.VLLESEQLTELTR	1	SRP14	1.090373158	1.098783344	1.2238283
M.GVQVETISPGDGR	1	FKBP1	1.033260802	1.005026404	1.079550496
M.VNPTVFFDIAVDGEPLGR	1	CYPA	1.084712838	1.091889859	0.979209395
M.GLLSILR	1	ARFL3	1.116298905	1.092916481	
M.VKLTAEIEQAAQYNAVR	1	SNRPA1	1.187865102		0.63157831
M.VQKESQATLEER	1	HEXC	0.414225594	0.934391966	0.985033981
M.VLDLDFR	1	RP11-352P4.2-004		1.074672348	1.133726791
M.VGVKPVGSDPDFQPELGGAGSR	1	TRP32	1.192435529	1.081319797	0.950819409
M.VIKTDELPAAPADSAR	1			1.052808496	0.850598152
.MVLESEQLTELTR	0	SRP14			0.970948742
.MVGKPVGSDPDFQPELGGAGSR	0	TRP32			0.811728567
M.GILEKISEIEKEIAR	1	DRG2			0.676888411
M.VKLFIGNLPR	1	RBM4			0.879275479
M.GIQGLAKLIADVAPSAIR	1	FEN1			1.179442974
M.APGQLALFSVSDKTGLVEFAR	1	ATIC			0.94876253
M.GGEQEEER	1	NUDC			1.186712572
.MVNPTVFFDIAVDGEPLGR	0	CYPA			1.228574637

Supplemental Table 4.2. List of protein N-terminal peptides identified using TAILS.

Values in the experiment columns represent the ratio of peptide in phosphatase treated samples versus samples not treated with phosphatase.

<b>casp3/7 substrate</b>	<b>model</b>	biotin	WGGDEVDGVAEG	K (DNP)
	<b>uncleavable</b>	biotin	WGGDEVAGVAEG	K (DNP)
<b>P5</b>	<b>ser unphos</b>	biotin	WGSDEVDGVAEG	K (DNP)
<b>P4</b>		biotin	WGGSEVDGVAEG	K (DNP)
<b>P3</b>		biotin	WGGDSVDGVAEG	K (DNP)
<b>P2</b>		biotin	WGGDES DGVAEG	K (DNP)
<b>P1'</b>		biotin	WGGDEVDSVAEG	K (DNP)
<b>P2'</b>		biotin	WGGDEV DGSAEG	K (DNP)
<b>P3'</b>		biotin	WGGDEV DG VSEG	K (DNP)
<b>P4'</b>		biotin	WGGDEV DG VASG	K (DNP)
<b>pP5</b>	<b>ser phos</b>	biotin	WGpSDEVDGVAEG	K (DNP)
<b>pP4</b>		biotin	WGGpSEVDGVAEG	K (DNP)
<b>pP3</b>		biotin	WGGDpSVDGVAEG	K (DNP)
<b>pP2</b>		biotin	WGGDEpSDGVAEG	K (DNP)
<b>pP1'</b>		biotin	WGGDEV DpSVAEG	K (DNP)
<b>pP2'</b>		biotin	WGGDEV DGpSAEG	K (DNP)
<b>pP3'</b>		biotin	WGGDEV DG VpSEG	K (DNP)
<b>pP4'</b>		biotin	WGGDEV DG VApSG	K (DNP)
<b>casp8/9 substrate</b>	<b>model</b>	biotin	WGIETDSGVG	K (DNP)
	<b>uncleavable</b>	biotin	WGIETASGVG	K (DNP)
<b>P4</b>	<b>ser unphos</b>	biotin	WGSETDSGVG	K (DNP)
<b>P3</b>		biotin	WGISTD SGVG	K (DNP)
<b>P2</b>		biotin	WGIESD SGVG	K (DNP)
<b>P1'</b>		biotin	WGIETD SGVG	K (DNP)
<b>P2'</b>		biotin	WGIETD SSVG	K (DNP)
<b>P3'</b>		biotin	WGIETD SGSG	K (DNP)
<b>pP4</b>	<b>ser phos</b>	biotin	WGpSETDSGVG	K (DNP)
<b>pP3</b>		biotin	WGIpSTD SGVG	K (DNP)
<b>pP2</b>		biotin	WGIepSD SGVG	K (DNP)
<b>pP1'</b>		biotin	WGIETDp SGVG	K (DNP)
<b>pP2'</b>		biotin	WGIETD SpSVG	K (DNP)
<b>pP3'</b>		biotin	WGIETD SpSG	K (DNP)

Supplemental Table 4.3. List of peptides used for phospho-walking studies.

## **CHAPTER 5 – DISCUSSION**

### ***5.1 GENERAL INTRODUCTION***

The balance of cell survival and death is of crucial importance to maintaining total cell number homeostasis, and, if disturbed, can lead to proliferative diseases such as cancer (1,2). Critical regulators governing these processes are two distinct families of enzymes – caspase, a family of pro-apoptotic proteases, and protein kinases, a group of phosphotransferases that transfer phosphate from ATP to hydroxyl residues on proteins. Interplay between these families is apparent, with an emerging role for the phosphorylation of caspase substrates to regulate susceptibility to proteolysis (3,4). For a number of reasons, protein kinase CK2 has emerged as the kinase with perhaps the highest degree of integration within the caspase circuitry (4). However, gaps in our knowledge still remain as to how this constitutively active kinase is regulated in disease, and what precise functions CK2 may perform when mis-regulated in cells. Given the role of CK2 in apoptosis, the goal of this thesis was to utilize targeted strategies for identifying substrates that may promote anti-apoptotic signals emanating from mis-regulated CK2.

### ***5.2 SUMMARY OF FINDINGS AND RESEARCH IMPACT***

#### **5.2.1 Predicting and characterizing caspase-3 phosphorylation by CK2**

In Chapter 2, we set out to identify CK2 substrates whose phosphorylation regulates their cleavage by caspases. In large part, our efforts were guided by three characteristics of CK2. First and foremost, CK2 is over-expressed in a number of human

cancers and contributes to tumorigenesis by, at least in part, blocking the efficient execution of apoptosis. Second, CK2 phosphorylation is promoted by acidic primary amino acid determinants in the +1 and +3 position, and more generally, anywhere from -2 to +7 (5-8). Similarly, caspases by definition cleave at aspartic acid, but cleavage is also generally promoted by Asp at the P4 position, and Glu at P3 (9). Finally, there is precedence for CK2 to regulate a number of other caspase substrates in this manner, such as Bid, Presenilin-1 and -2, and YY1 (10-14). Collectively, these characteristics suggest that the crosstalk between caspases and CK2 may be underappreciated. Furthermore, owing to the complex nature of this hierarchical regulatory mechanism, examples of phosphorylation-dependent blocking of caspase cleavage have only been found serendipitously. Therefore, we sought to develop new strategies for identifying novel proteins regulated in this manner that could function by controlling apoptotic progression. To this end, we designed a peptide match program that positions a predicted CK2 phosphorylation site adjacent to a putative caspase scissile bond, and, interestingly, identified caspase-3 itself. Caspase-3 is an executioner caspase responsible for the majority of the caspase degradome (15), and only attains catalytic activity after cleavage by upstream initiator caspases (16,17). Therefore, our guiding hypothesis was that phosphorylation by CK2 blocks activation of caspase-3, and could function, especially in the context of CK2 over-expression, to impede apoptotic progression. We found that CK2 phosphorylates caspase-3 at the predicted S176, but also at T174. These sites surround D175 – the scissile bond controlling caspase-3 activation – and when phosphorylated, efficiently blocked cleavage by upstream initiators *in vitro*.

Interestingly, caspase-3 phosphorylation was specific for the free catalytic form of CK2, as reconstitution of the holoenzyme by CK2 $\beta$  addition to kinase assays dramatically inhibited phosphorylation. Notably, of the hundreds of CK2 substrates that have been identified, only a handful are preferentially phosphorylated in the absence of the CK2 $\beta$  subunit (18,19). In some ways, this may in part be due to the fact that not every identified substrate is tested with all forms of CK2. Nevertheless, the function of free-catalytic CK2 in cells remain controversial, and so as well as characterizing the role that phospho-caspase-3 plays in apoptotic progression, the generation of phospho-caspase-3 antibodies allowed us to for the first time systematically examine the catalytic activity of non-holoenzyme CK2 in living cells.

Indeed, in chapter 3, we measured caspase-3 phosphorylation in HeLa cells by endogenous and ectopically expressed holoenzyme and free-catalytic CK2 (ie CK2 $\alpha'$ -HA, CK2 $\alpha$ -HA, CK2 $\alpha'$ -HA<sub>2</sub>/myc-CK2 $\beta$ <sub>2</sub>, and CK2 $\alpha$ -HA<sub>2</sub>/myc-CK2 $\beta$ <sub>2</sub>). By targeting caspase-3 to the nucleus with a NLS tag, or transfecting the non-NLS tagged version, which localizes to the cytoplasm, we could monitor two distinct pools of cellular CK2 for the free-catalytic form. Interestingly, in these cells, it appeared that endogenous CK2 was unable to phosphorylate caspase-3, even in the presence of okadaic acid, supporting the notion that cellular, catalytic CK2 is completely bound to CK2 $\beta$ . However, over-expression of individual catalytic subunits to levels that did not dramatically perturb total levels of CK2 activity (i.e. up to 2-fold) resulted in robust phosphorylation of caspase-3. These results also revealed a surprising preference for caspase-3 phosphorylation by CK2 $\alpha'$ , which represents the first direct evidence for a CK2 $\alpha'$  specific substrate in cells. Specificity was imparted by the catalytic domain in spite of extreme sequence identity in



this region, and sequence divergence at the N- and C-termini. Curiously, our results support a mechanism in which CK2 $\alpha'$  specificity was not the result of differential holoenzyme formation with endogenous CK2 $\beta$  in cells, and suggests that specificity is dictated by other positive or negative regulators controlling caspase-3 access to CK2 $\alpha'$  and  $\alpha$ , respectively. Furthermore, these data have implications for the formation of the holoenzyme in cells, and suggest that at least a portion of newly expressed CK2 $\alpha$  and  $\alpha'$  are not immediately packaged into the holoenzyme.

To avoid inappropriate caspase-3 activation, we used catalytically inactive caspase-3 when testing for phosphorylation in cells. In an effort to study the functional effect of caspase-3 phosphorylation, we actually found that catalytically active caspase-3 was phosphorylated much less in HeLa cells, preempting our efforts to understand the effect of phosphorylation on apoptotic progression. Moving forward, a comprehensive evaluation of other model systems to identify endogenous, phosphorylated caspase-3 will be necessary to fully understand the role this modification might play in apoptosis (see section 5.3.2).

Collectively, our results provide support for a model in which the complement of CK2 substrates phosphorylated in a given cell is dependent on the form of CK2 present. Evidence for this model is supported by the recent findings of Deshiere *et al.* (2012), as their work highlights that a shift to free-catalytic CK2 is driven by the loss of CK2 $\beta$  in various breast cancer patient samples (20). These authors propose that a distinct phenotype, namely the presence of EMT markers, mediated by increased free-catalytic CK2 is a direct consequence of reduced phosphorylation of SNAIL – a CK2 holoenzyme specific substrate – rather than increased phosphorylation of a free-catalytic specific

substrate. Therefore, our work complements this study by providing evidence for the converse, that shifting the balance of CK2 from holoenzyme to the free-catalytic can result in phosphorylation of proteins targeted by this form. Implications for these results have yet to be realized in disease models, but one obvious consequence of misbalanced CK2 expression is that the CK2-dependent phospho-proteome is modular and contingent upon the relative amount of each form of CK2 (Figure 5.1). In this way, distinct cellular phenotypes may depend on the relative abundance of the different forms of CK2.

### **5.2.2 Unbiased investigation of caspase substrate phosphorylation: identification of novel caspase targets with phospho-dependent changes in proteolysis**

As a logical extension of our peptide-match program, we devised an unbiased, N-terminomic approach to look for differences in the caspase degradome between phosphorylated and dephosphorylated lysates. N-terminomic strategies have been used extensively to identify caspase substrates in lysate cleavage assays (21), and from cells induced to undergo apoptosis (21-25), but never to directly identify substrates whose cleavage is modulated by phosphorylation. Furthermore, our experimental design did not exclusively measure the effects of phosphorylations located adjacent to the scissile bond, but also considered distant phosphorylations or phospho-dependent interaction partners that may alter scissile bond accessibility through structural changes. Finally, this approach is not limited to measuring only the effect of CK2 on the caspase degradome, but instead probes the native phospho-proteome generated by all active kinases in HeLa cells. Notably, our dataset included cleavage events such as MST3 that were positively regulated by phosphorylation, and also examples of phosphorylations inhibitory to

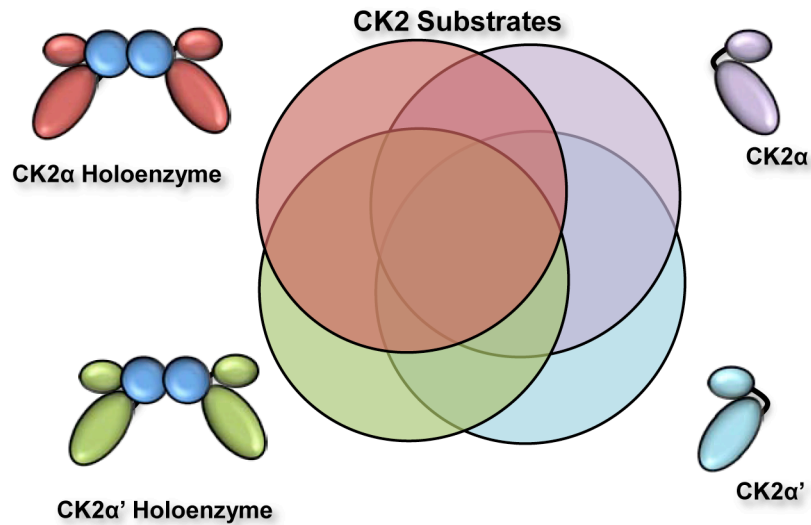


Figure 5.1 – A Hypothetical representation of the functional redundancy and uniqueness of different forms of CK2. Given the high sequence similarity between CK2 $\alpha$  and  $\alpha'$ , undoubtedly these two isozymes control a number of redundant pathways. However, our work supports a model stating that all forms of CK2 have unique cellular substrates. Misbalanced expression of these different forms in disease may lead to unique phenotypes that are dictated by the complement of available CK2 and CK2 substrates.

proteolysis, such as those on Yap1 and Golgin-160. These data validated our strategy, and reinforced that native phosphorylations can have a range of effects on caspase cleavage.

After observing that phosphorylation can both positively and negatively regulate caspase-mediated degradation, we set out to rigorously define the determinant properties of phosphorylation throughout the entire caspase motif. In this sense, positional effects for amino acids have been thoroughly studied using peptide libraries and model substrates that interrogate P4-P1', but that of phosphorylation has only been studied anecdotally. To systematically uncover the effects of phosphorylation on caspase cleavage, we designed a series of model peptide substrates that walk phospho-Ser through the entirety of an extended caspase motif. We found that phosphorylation was generally inhibitory from positions P5-P3' for caspase-3 and -7 and P4-P2' for caspase-8, but had no effect on any caspase at P3. The overall negative effect of caspase substrate phosphorylation when located near the scissile bond suggests that when phosphorylation promotes cleavage, it is likely the result of altered substrate structure that promotes scissile bond accessibility. As both caspase and phosphorylation databases continue to be populated with data from proteomics studies, we anticipate these determinant properties can be applied to guided, database mining approaches that seek to identify novel caspase substrates whereby phosphorylation regulates cleavage. Collectively, our work contributes to a fundamental understanding of the effect of phosphorylation on cleavage, and, using modified N-terminomic approaches, identifies novel substrates regulated in this manner.

### **5.3 FUTURE DIRECTIONS**

#### **5.3.1 Characterization of structural determinants that select for free-catalytic specificity**

The ability of CK2 $\beta$  to modulate substrate specificity represents a regulatory mechanism long known to control CK2 *in vitro*, but the selective factors guiding this function remain enigmatic. Primary amino acid sequence determinants surrounding the phospho-acceptor have been mapped to exosites on CK2 and found to interact with n+1, +3, +4 and +5 of the substrate (26). Therefore, it follows that holoenzyme formation is not known to dramatically alter primary sequence determinants of substrate catalysis, but instead may impart unique, higher order structural features that interact with the substrate and guide phosphorylation. Indeed, Leroy *et al.* (1997) proposed that an acidic loop of CK2 $\beta$  partially occludes the catalytic pocket of CK2 $\alpha$  and blocks access of some substrates (27); notably, structures of the holoenzyme would later reveal the acidic loop of CK2 $\beta$  to make contact near the active site of CK2 $\alpha$  (28). For substrates inhibited by CK2 $\beta$ , treating the holoenzyme with polyamines is hypothesized to stimulate activity by competing CK2 $\beta$  away from the catalytic pocket of CK2, effectively opening the active site (27,29). In spite of this, the catalytic parameters of most CK2 substrates are more favourable for the holoenzyme (18).

In chapter 2, we present evidence for the identification of caspase-3 as a CK2 substrate specific for the free-catalytic subunit. Interestingly, preliminary work from our lab shows that caspase-8 is also phosphorylated by CK2 between the small and large subunits, at a site equivalent to T174 in caspase-3, but is paradoxically more efficiently phosphorylated by the holoenzyme ( $K_{cat}/K_m$  of 0.42 versus 0.16, G. Vilks personal

communication). Possible explanations for this discrepancy lie in differences in the structural environment of the area immediately surrounding the phospho-acceptors. The most obvious variation is the length of the interdomain linker that contains the CK2 phosphorylation sites; caspase-8 is 25 amino acids long versus 16 for caspase-3. There is no structure of caspase-3 with an intact linker region, but its closest homologue caspase-7 – which has a linker of the same length – has been solved, and so certain inferences can be made (30). First, the region on caspase-7 that aligns with the CK2 phospho-acceptors is of low resolution, but, given its length, may be held closer to the catalytic groove of the enzyme than is the caspase-8 linker (Figure 5.2). Also, a nearby loop – L4 – is actually 9 amino acids longer on caspase-3 and -7, and may serve to occlude the bulkier CK2 holoenzyme from gaining access to the phosphorylation site. In contrast, the CK2 phosphorylation on caspase-8 is further away from any protruding loops, and is stationed on an outer edge of the protein (Figure 5.2) (31). Furthermore, caspase-3 exists as a dimer, whereas caspase-8 is monomeric under conditions utilized for our kinase assays (32,33); in this sense, dimeric versus monomeric form of caspases may also be responsible for selecting holoenzyme or free-catalytic CK2 specificity. Generating caspase-3 and -8 chimeras that swap interdomain linkers and the L4 loop, or contain mutations at the dimer interface that reduce association (as has been shown for caspase-8 (31)) may isolate structural determinants that select for holoenzyme versus catalytic specificity. Ultimately, we predict that phospho-acceptors shielded from the holoenzyme are done so by restrictive, structural protrusions on the substrate. In support of this model, there are no known peptide substrates preferentially phosphorylated by the

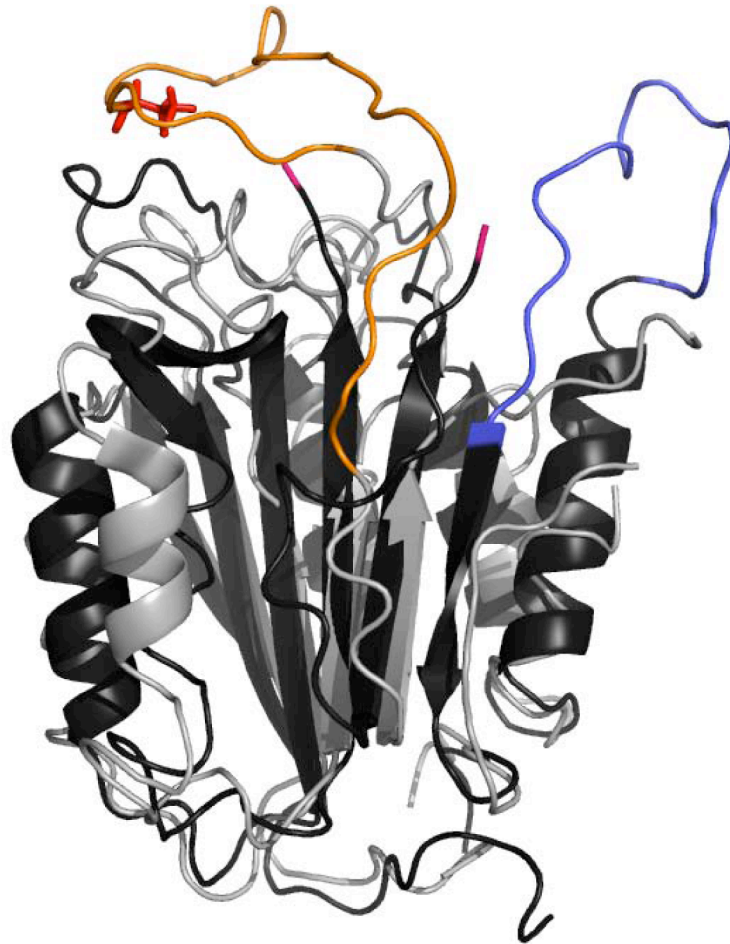


Figure 5.2 Structural alignment of caspase-7 and -8. Caspase-7 (black, PDB: 1K86 (30)), the closest relative to caspase-3, and caspase-8 (off-white, PDB: 2K7Z (53)) are shown as a structural alignment (generated using Pymol). The extended caspase-8 linker is shown in orange, with the phospho-acceptor of CK2 highlighted in red. The linker of caspase-7 is unstructured, but the ends are shown in pink. The L4 loop of caspase-7 is shown in blue, whereas the L4 loop of caspase-8 is flexible and not defined in this structure. Note: caspase-7 was crystallized as a dimer in PDB: 1K86, but the second chain was edited out for simplicity. PDB: 2K7Z represents a solution structure of monomeric caspase-8.

catalytic subunit, suggesting that only structural factors can prevent phosphorylation by the holoenzyme.

Conceptually related studies are underway in our lab, but employ engineered CK2 substrate motifs positioned between two fluorophores. This work has the added advantage of being easily translatable to cellular experiments that target biosensors to distinct cellular locations for the purpose of monitoring discrete CK2 populations in time and space. In sum, a substrate engineering approach will permit a systematic characterization of the structural determinants guiding preferential phosphorylation by the free-catalytic subunit of CK2. The closely related caspase-3 and -8 provide a unique model system with which to base these studies.

### **5.3.2 Screening for endogenous phospho-caspase-3 in tissues and disease**

The presence and function of free-catalytic CK2 in disease and physiology remains poorly understood. Interestingly, there is evidence for tremendous variation of the relative levels of CK2 subunits in tissues from patients suffering from breast and head and neck cancer (20,34). While the absolute levels in these instances remain unknown, the presence of undetectable CK2 $\beta$  and high levels of CK2 $\alpha$  or  $\alpha'$  suggests that CK2 $\beta$  may be sub-stoichiometric, which would inherently lead to cellular populations of free-catalytic CK2. Our demonstration that artificially generating free-catalytic CK2 by transfection, and the discovery that caspase-3 is phosphorylated under these conditions, suggests that the absolute and relative levels of regulatory and catalytic CK2 may control the CK2-dependent phospho-proteome. It follows that the cancer promoting functions of CK2 could also depend on the complement of CK2 forms present in a cell, but to develop



this hypothesis we need to identify cell line models with misbalanced CK2 levels. Based on its ability to discriminate between different forms of CK2, our phospho-caspase-3 antibodies may accelerate the identification of such models.

In terms of identifying caspase-3 phosphorylation in physiology, perhaps the tissue most likely exhibit this characteristic are mouse testes. Here, protein expression of CK2 $\alpha'$  was highest when compared to 7 other tissues (35). Furthermore, the observation that CK2 $\alpha'$  knockout mice experience a hyper-apoptotic phenotype in the testes suggests an anti-apoptotic role for CK2 $\alpha'$  (36), which is consistent with a functional role for caspase-3 phosphorylation. Extending beyond simply the relative levels of CK2 subunits as a key identifier for candidate tissues, there is also immunohistochemical evidence for CK2 $\alpha'$  and  $\beta$  to occupy distinct cellular locations in developing rat spermatocytes (37), suggesting that free-catalytic CK2 $\alpha'$  is theoretically present. Experiments aimed at isolating caspase-3 from mouse testes by immuno-precipitation, followed by western blotting with anti-phospho-caspase-3 antibodies, should provide a preliminary analysis of the presence of phospho-caspase-3 in this tissue. Thematically related experiments can be envisaged for cancer cell lines with misbalanced expression of CK2 subunits such as those observed in breast (20) and head and neck cancer (34). Furthermore, as repositories for cancer specimens continue to grow, misbalanced CK2 will be documented in other disease types.

An alternative approach to understanding specific functions ascribed to individual forms of CK2 is to utilize strategies that aim to identify and measure other free-catalytic and holoenzyme specific CK2 substrates in cell lines or tissue samples. To this end, we would take advantage of cell lysate kinase assays such as those described by Knight *et al.*,

where isoform specific substrates of p38 $\alpha$  and  $\beta$  were identified (38). Here, the authors treat lysates with the irreversible kinase inhibitor FSBA, which reacts with the conserved, catalytic lysine residue of protein kinases, to inactivate all endogenous kinases. Excess inhibitor is then washed away using low-molecular weight spin columns, followed by addition of the recombinant kinase of interest, in our case free-catalytic CK2 or holoenzyme, and ATP. After kinase assays, samples are trypsinized, labeled with heavy or light dimethylation as in chapter 4, the phospho-peptides enriched and then identified and quantified using mass spectrometry (Figure 5.3). Phospho-peptides unique to either treatment could then be applied to a number of downstream applications aimed at measuring the phosphorylation of these targets in different cell lines or tissues.

One approach utilizes targeted proteomics strategies such as selected reaction monitoring (SRM) to identify tissues or cell lines with specific cohorts of CK2 substrates. SRM is a LC-MS approach that relies on the synthesis of phospho-peptides, followed by protocol development that seeks to establish transitions, or precursor/fragment pairs of ions (39). By selectively measuring both the precursor and fragment, SRM is both extremely specific and sensitive. The real power of SRM, though, comes from two important characteristics. First, SRM is capable of measuring nearly hundreds of transitions in a single mass spectrometer run by toggling between the transitions of interest that occur at a predefined chromatography retention time (39). Secondly, absolute quantification is made possible by spiking isotopically labeled, synthetic phospho-peptides into the phospho-proteome being analyzed (39). Determining phosphorylation stoichiometry is even achievable when transitions of the synthesized, unphosphorylated peptides are measured (40). Furthermore, as well as monitoring

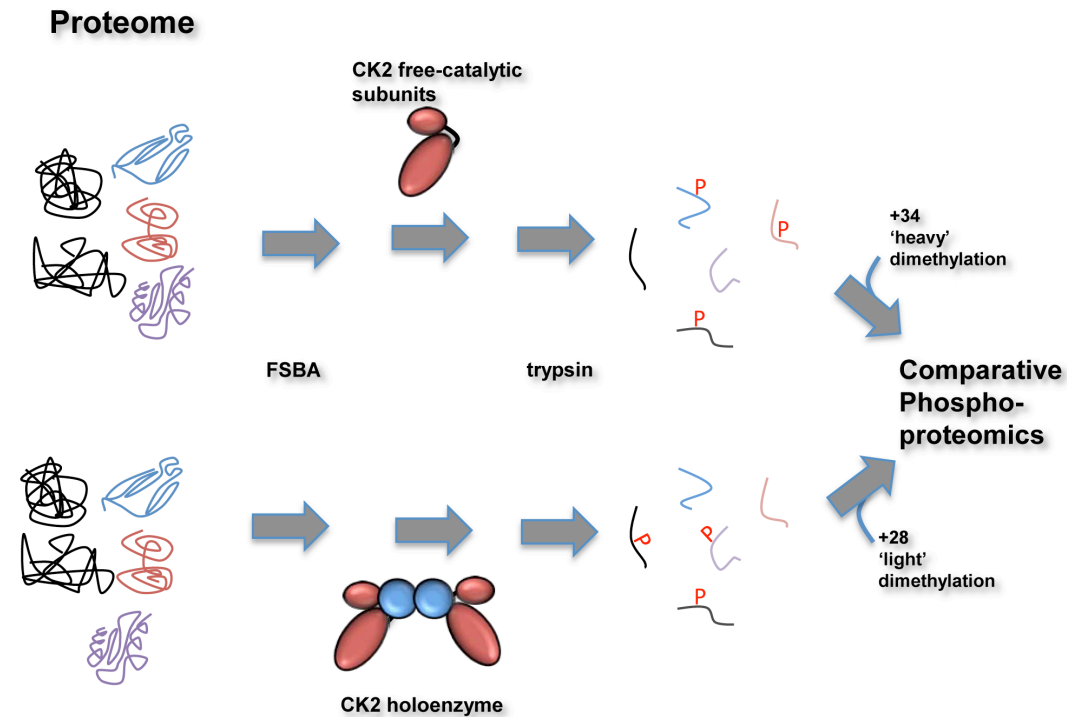


Figure 5.3 – Systematic identification CK2 substrates specific for the holoenzyme or catalytic subunits. To uncover substrates preferred by different forms of CK2, a proteomic approach that aims to identify CK2 substrates from cell lysates will be utilized. First, endogenous kinase present in the lysate will be inactivated using the irreversible kinase inhibitor FSBA, followed by its removal using low-molecular weight spin columns. Free-catalytic CK2 ( $\alpha$  and  $\alpha'$ ) or both forms of the CK2 holoenzyme are then incubated with the lysate, ATP and the kinase cofactor  $Mg^{2+}$ . Sample trypsinization, phospho-peptide enrichment and peptide dimethylation are then followed by comparative phospho-proteomics. Variations may also include the incorporation of a third label. Here, samples would be treated with phosphatase instead of CK2, to get a sense of the physiological relevance of the identified phosphorylation sites. Similarly, phosphatase treatment could preclude kinase assays to maximize the unoccupied phospho-acceptors.

phosphorylation of CK2 substrates, we could also develop SRM protocols for peptides from the CK2 subunits themselves. In this way, the ratio of catalytic:regulatory subunits of CK2 could be measured.

By determining the phosphorylation status of CK2-form specific substrates, and the levels of CK2 subunits across different cell lines or tissue types, a number of important conclusions could be drawn. First and foremost, by extending the measurement of free-catalytic CK2 beyond caspase-3, stronger conclusions about the presence and function of free-catalytic CK2 can be achieved. Secondly, the phosphorylation of free-catalytic substrates in the presence of excess CK2 $\beta$  would imply either an active, exclusion mechanism keeping the holoenzyme from forming, or the stimulation of holoenzyme by polyamines, which rescues holoenzyme inhibition of free-catalytic specific substrates. Lastly, the identification of cell lines with a spectrum of free-catalytic versus holoenzyme activity would provide a sound platform with which to study the differential functions of these different forms of CK2 in processes related to cell survival.

### **5.3.3 Determining the effect of phosphorylation on cleavage of MST3, Golgin-160 and Yap1 during apoptosis**

Golgin-160, MST3 and Yap1 all have bona fide roles in either apoptotic progression or cell survivability, suggesting that cleavage modulation may alter cellular phenotypes. Indeed, there is precedence in the literature for the inhibition of Golgin-160 cleavage to block apoptotic progression; Golgin-160 non-cleavable mutants impair apoptosis in response to extrinsic stimuli and ER stress (41). As well, MST3 is cleaved by caspases in cells, an event that activates MST3 and promotes apoptosis (42). The

putative function of regulating Yap1 cleavage in apoptosis remains difficult to assess based on its pro- and anti-apoptotic functions (43-45). However, the observation that upstream pathway constituents MST1 and 2 contain phosphorylation sites near their scissile bonds suggests that proteolytic control of this signaling pathway may be an important apoptotic checkpoint (45,46).

A logical extension from our N-terminomic screen for caspase substrates whose cleavage is modulated by phosphorylation is to identify the phosphorylation sites that regulate Yap1, MST3 and Golgin-160 cleavage. This would be performed by generating expression plasmids of the candidates that have non-phosphorylatable mutations, followed by lysate caspase cleavage assays that were treated with or without  $\lambda$  phosphatase. In the case of Yap1 and Golgin-160, the presence of phospho-acceptors at positions within the caspase motif that inhibit cleavage when phosphorylated represent strong candidates with which to initially focus mutational studies. Furthermore, the P4 Ser on Golgin-160 (SEVD) actually strongly conforms to the CK2 motif. Identifying MST3 phosphorylation sites that modulate cleavage should be more difficult because we have little basis for predicting phosphorylations that promote cleavage. One area to start may be the region just outside extended caspase motif we studied (ie P5-P4') – phospho-acceptors are present at P9-11, P6, P7' and P9'. These sites may function much the same way that P5' phosphorylation promotes PKC $\delta$  cleavage (47).

Once the phosphorylation sites responsible for altered cleavage kinetics in lysate caspase assays are identified, these mutants can be utilized to monitor kinetics after cells are induced to undergo apoptosis. Because different apoptotic stimuli can cause different degradomes (25), a variety of apoptotic inducers will be utilized, such as TNF $\alpha$ /CHX to

activate the extrinsic pathway, and staurosporine, etoposide or thapsigargin to activate the intrinsic pathway. Apoptotic time courses followed by western blotting will be used to identify differences in cleavage between wild-type and phospho-acceptor mutants.

Finally, the function of altered cleavage kinetics will be determined using a variety of readouts after expression of wild-type, non-phosphorylatable and non-cleavable mutants. Blocking Golgin-160 processing has previously been shown to reduce PARP cleavage, a caspase-3 and -7 substrate, and reduce membrane blebbing associated with apoptosis (41). Similarly, ectopic expression of the MST3 caspase cleaved fragment induces chromatin condensation, and so this readout could be used to measure altered cleavage of MST3 (42). The function of Yap1 proteolysis is difficult to predict, as its cleavage has never been studied, but a good starting point would be assessing its better-known functions after apoptotic induction. For instance, Yap1 associates with p73 to modulate apoptotic gene expression after DNA damage (43,44). As well, cytoplasmic sequestration is observed when phosphorylated by Lats1/2 or Akt (43,48), and so differences in localization between different mutants of Yap1 may be indicative of functionality in apoptosis.

#### ***5.4 PERSPECTIVES***

As highlighted in chapter 1, CK2 is a promising targeted therapeutic strategy for the treatment of various cancers. Significant evidence exists for CK2 inhibition to specifically reduce cancer cell fitness, while not affecting normal cells (49-51). However, a unifying mechanism for the function of CK2 in disease is far from complete. The simplest demonstration of this is the dependence of phenotypic output after CK2

inhibition on the genetic background of the treated cells; apoptosis (51), autophagy (52) and different stages of cell cycle arrest have all been observed (51). While other explanations are possible, these varied phenotypic responses suggest that distinct CK2 functions may be differentially hijacked depending on the genetic context. Work such as ours that attempts to define regulatory mechanisms, and context-specific phosphorylations will be crucial in instructing how to best target CK2 in disease. In this way, successful pairing of CK2 inhibitors with other therapeutic strategies may depend on the active complement of the CK2 phospho-proteome, and may well vary amongst different genetic backgrounds, and the form of CK2 present.

## ***5.5 CONCLUSIONS***

Protein phosphorylation and proteolysis are two important post-translational modifications implicated in nearly every aspect of cellular signaling. Furthermore, the ability of phosphorylation to impact proteolytic events represents a hierarchical signaling mode that affords an extra level of regulation to cellular processes such as cell death. However, the complexity of this regulatory mechanism underscored the need for novel strategies to uncover the integration of kinase and caspase networks.

Collectively, this thesis demonstrates the utility of both predictive and proteomic strategies to identify caspase substrates whose cleavage is regulated by phosphorylation. Of the identified proteins, a number have functions in cell death and proliferative processes, and so these regulatory mechanisms may be important in apoptotic progression. Of the kinases functioning within the caspase circuitry, CK2 may have the highest degree of integration. Our work highlighting non-redundant functionality of the

$\alpha'$  catalytic subunit of CK2 is an important discovery that should accelerate the understanding of the regulation of CK2, and therefore how it can be targeted in disease.



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### PUBLICATIONS

1. Gyenis L, **Turowec JP**, Bretner M, Litchfield DW. (2013) Chemical proteomics and functional proteomics strategies for protein kinase inhibitor validation and protein kinase substrate identification: Applications to protein kinase CK2. *Biochim Biophys Acta. In press*
2. Marti S, Gabriel M, **Turowec JP**, Litchfield DW, and Kraatz H-B. (2012) Versatile strategy for biochemical, electrochemical and immunoarray detection of protein phosphorylations. *J Am Chem Soc.* **134**(41) 17036-45.
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development of central nervous, skeletal, and endocrine systems. *Am J Hum Genet.* **84**(2):134-47

### **Accepted contributions to a collective work and book chapters**

1. **Turowec JP**, St-Denis NA, and Litchfield DW. (2010) Protein Kinase CK2. *Encyclopedia of Signaling Molecules* (S. Choi, ed.). *Accepted. In proof stage.*
2. **Turowec JP**, Duncan JS, French AC, Gyenis L, St-Denis NA, Vilks G, and Litchfield DW. (2010). Protein Kinase CK2 is a Constitutively Active Enzyme that Promotes Cell Survival: Strategies to Identify CK2 Substrates and Manipulate its Activity in Mammalian Cells. *Methods Enzymol* (M. Fukuda, ed.). **484**:471-493.

### **Non-refereed Contributions – Poster (International Conferences)**

1. **Turowec JP**, Duncan JS, Li SS, Gloor GB, and Litchfield DW. (2012) Convergence of protein kinase and caspase signaling: A mechanism for pathological rewiring of survival pathways by protein kinase CK2. AACR Annual Meeting. March 31 – April 4, 2012
2. **Turowec JP**, Duncan JS, Vilks G, Li SS, Gloor GB, and Litchfield DW. (2010) A peptide-based target screen of kinase/caspase overlapping substrates highlights the convergence of CK2 and caspase pathways. 6<sup>th</sup> International Conference on Protein Kinase CK2. September 7 to 10, 2010.

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<b>Banting and Best Canada Graduate Scholarship (Doctoral)</b> \$35,000	September 2011 – August 2012
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<b>Ontario Graduate Scholarship in Science and Technology</b> \$15,000	May 2008 – April 2009
<b>CIHR Strategic Training Program in Cancer Research</b> \$10,700	September 2008 – August 2009

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2008

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