12-16-2015 12:00 AM

The Rational Design and Evaluation of CK2alpha Mutants Bearing Inhibitor-Refractory Amino Acid Substitutions

Sam Reid Fess
The University of Western Ontario

Supervisor
Dr. David Litchfield
The University of Western Ontario

Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

The Rational Design and Evaluation of CK2α Mutants Bearing Inhibitor-Refractory Amino Acid Substitutions

by

Sam Reid Fess

Thesis Format: Monograph

Graduate program in Biochemistry
Faculty of Medicine and Dentistry

Submitted in partial fulfillment of the
requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
Western University
London, Ontario
November 2015

Abstract

CK2 is a ubiquitously expressed and constitutively active serine/threonine protein kinase that is implicated in many cellular functions. Previous studies have indicated that the generation of mutants that are less sensitive to inhibition can be advantageous when studying protein kinases. Importantly, studies have demonstrated that mutants of CK2 rendered less sensitive to inhibition are attainable. To extend these observations, mutants of CK2α were designed and evaluated to test their effect on the inhibition of CK2 by CX-4945 using *in vitro* enzymatic assays followed by the development of inducible cell lines. CX-4945 is a CK2 inhibitor that has demonstrated anti-tumor activity and has recently been extended into clinical trails phase II. It was demonstrated that a CK2α triple mutant (V66A/H160D/I174A) led to a reduction in the inhibition of CK2 by CX-4945. Generation of CK2 inhibitor-refractory mutants will provide valuable insight regarding the precise functions of CK2 as well as its inhibition.

Dedications

Dedicated to my loving and supportive parents

Gloria & John Fess

Acknowledgements

I would like to express my deepest gratitude to several individuals who were helpful, supportive, and encouraging throughout the completion of this thesis. Sincere thanks to Dr. David Litchfield, my supervisor, for accepting me as a part of his research team and providing me with an opportunity to contribute to the field I was particularly interested in, it was truly an invaluable experience. His continuous interest, as well as success, in the field surrounding protein kinases has inspired me to follow my dreams and goals in the discipline of Biochemistry. I greatly appreciate the constructive discussions that we had in order to help me understand and progress with my research. His investment in his students, not only to ensure they achieve their research goals, but also to ensure their success in life, will not be forgotten.

Thank you to my advisory committee members, Dr. Eric Ball and Dr. Patrick O'Donoghue for their suggestions and encouragement in the final stages of my project. I would also like to express my sincere appreciation to Dr. Laszlo Gyenis, who mentored me throughout my project and helped guide me throughout challenging aspects of my project. I would also like to acknowledge current and past members of the Litchfield lab, Stephanie Zukowski, Dr. Michelle Gabriel, Adam Rabalski, Michelle Dubinsky, Paul Desormeaux, and Dr. Jacob Turowec for all of their support and help, as well as Eddie Cruise who helped complete my immunofluorescence studies. Thank you to Dr. Karmella Haynes (Arizona State University) who kindly provided me with the Flp-In™ T-REx™ U2OS cells in order to generate the stable cell lines expressing mutants of CK2α.

A special thanks to my parents, Gloria and John Fess, my sister Kelsey and brother-in-law Kris, as well as my girlfriend, Aneta Wojcik, for all their love and support.

Table of Contents

List of Figures

List of Tables

Abbreviations

°C	degrees Celsius
µg	microgram
µL	microliter
µM	micromolar
Akt	protein kinase B
ATP	adenosine triphosphate
BCA	bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bid	BH3 interacting-domain death agonist
BSA	bovine serum albumin
CAK	cyclin-dependent kinase activating kinase
Cdk1	cyclin-dependent kinase 1
Cdk2	cyclin-dependent kinase 2
CK2	formerly casein kinase II
CK2α	CK2 alpha, catalytic subunit
CK2α'	CK2 alpha prime, catalytic subunit
CK2β	CK2 beta, regulatory subunit
CO_2	carbon dioxide
CML	chronic myelogenous leukemia
CX-4945	CK2 inhibitor, Cylene Pharmaceuticals
ddH_2O	double distilled water
DES	diethylstilbestrol
DMAT	CK2 inhibitor
DMEM	dulbecco's modified eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GAM	goat-anti-mouse antibody
GAR	goat-anti-rabbit antibody
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
G	relative centrifugal force
HA	human influenza hemagglutinin
HDAC	histone deacetylase
IAP	inhibitor of apoptosis protein
IkB	kinase of the NF-κB family
IPTG	isopropyl β-D-1-thiogalactopyranoside
IQA	inhibitor [[5-oxo-5,6-dihydro-indolo(1,2-a)quinazolin-7-yl]acetic acid]
kDa	kilodalton
Ki	inhibitor constant
Mdm2	E3 ubiquitin protein ligase
MEK	MAPK kinase

mg	miligram
mL	mililiter
mM	milimolar
Myc	c-Myc epitope tag
NF-kB	NF-kappaB
nm	nanometer
nM	nanomolar
NP-40	Nonidet P-40
p16	tumor suppressor protein 16, CDK inhibitor
p21	tumor suppressor protein 21, CDK inhibitor
p53	tumor suppressor protein 53
p65	transcription factor
PBS	phosphate buffered saline
PBST	phosphate buffered saline, with tween
PCR	polymerase chain reaction
PDB	Protein Data Bank
PKA	protein kinase A
PML	promyelocytic leukemia protein
pRB	retinoblastoma protein
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
STK	serine/threonine protein kinase
TBBt	tetrabromobenzotriazole
TBBz	tetrabromobenzimidazole
TBS	tris buffered saline
TBST	tris buffered saline
U2OS	human osteosarcoma cells
UV	ultraviolet
V	volts

1 – Introduction

1.1 – Introduction to protein kinases

It has long been recognized that one of the most important mechanisms of regulation of dynamic cellular processes is the reversible phosphorylation of proteins mediated by protein kinases[1,2]. Protein kinases are enzymes, which catalyze the transfer of a phosphoryl group, specifically from the gamma phosphate of ATP or in some cases GTP, to the hydroxyl group of serine, threonine, or tyrosine residues on a recipient substrate[2]. Importantly, the phosphorylation of substrates often results in a downstream activation cascade promoting the execution of key cellular process including but not limited to survival, proliferation, and apoptosis. To further highlight the significance of protein kinases in cells, there have been 518 genes encoding kinases that have been identified, representing approximately 1.7% of the entire human genome[3,4].

To elucidate the mechanisms that regulate protein kinases, many structural investigations have been employed to understand the general features of these enzymes, providing insight into their activation, inhibition, and substrate recognition[2,5]. Through key studies utilizing PKA and Cdk2, a major distinguishing feature of protein kinases is the conformational distinctions existing between the active vs. inactive forms of kinases[6,7]. Switching between the two states is triggered by specific signaling events, transduced via kinase regulatory domains that provide a means of activation or inactivation. With respects to substrate recognition, it has been determined that many protein kinases require certain residues located proximal to the sites that they recognize on substrates in order to achieve selectivity and specificity towards a particular substrate[8]. Typically, it is less well-ordered regions exposed to the surface of the protein substrate

that are targeted by protein kinases. This enables protein kinases to adopt an extended conformation and promote the localization of those residues that achieve specificity[8,9].

As a result of the features of protein kinases and the vast number of cellular functions in which they may coordinate, their aberrant expression and regulation can have catastrophic effects on the fate of a living cell. Second only to G-protein coupled receptors, protein kinases are emerging as one of the most popular drug targets when devising therapeutic strategies, since over 400 human diseases have been directly or indirectly associated with protein kinases[10]. As an example of a successful targeted therapy towards a protein kinase however, was the development of the drug Imatinib (Gleevec)[11–14]. This clinical compound serves to target the Bcr-Abl fusion receptor tyrosine kinase that is aberrantly expressed and constitutively active in many tumors, and most noticeably responsible for the development of chronic myelogenous leukemia (CML)[15]. By binding to the kinase in an ATP-competitive manner, Imatinib prevents ATP from gaining access to the catalytic site of Bcr-Abl, which renders the kinases inactive. This gives rise to growth inhibition and cell death through their apoptotic machinery. In addition to the production of this ATP-competitive compound, another therapeutic strategy has been the development of a chimeric monoclonal antibody, cetuximab, for the treatment of colorectal cancer was also produced. This antibody successfully binds to EGFR and competes for its natural ligand, EGF, as opposed to competing with the co- substrate, ATP[16].

One class of protein kinases gaining attention in the field of targeted therapy are serine/threonine protein kinases (STK's), as many alterations have been recently reported in a wide variety of human cancers[17]. In particular, STK's play a dramatic role in cellular

homeostasis through their ability to regulate transcriptional factors, cell cycle regulators, and an enormous number of cytoplasmic and nuclear proteins[18]. Therefore, it is undoubtedly recognized that perturbations in signaling via STK's may play a paramount role in malignant transformation. Recent efforts to identify alterations in protein kinase expression in various tumor samples have strongly implicated CK2 in a variety of human cancers[19,20]. Accordingly, the work described in this thesis has been concentrated on elucidating the precise functions it may orchestrate within cells, as well as evaluating its potential as a viable therapeutic target.

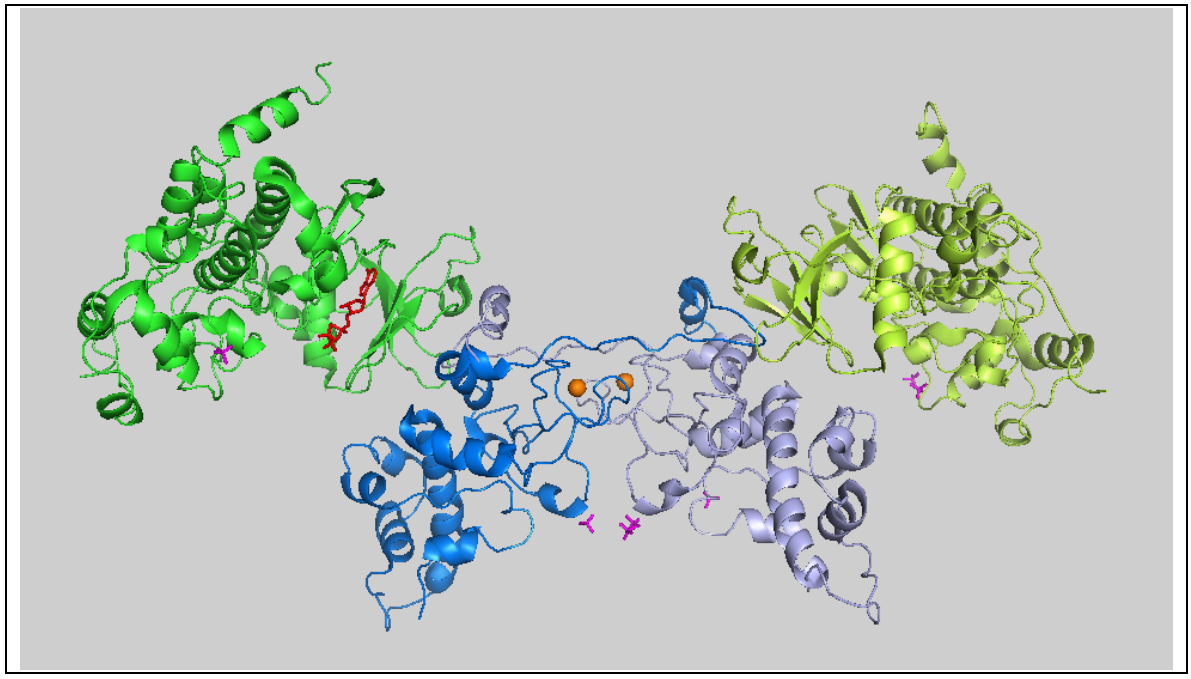
1.2 – Protein kinase CK2

Originally described by Burnett and Kennedy in 1954[21], protein kinase CK2 is a ubiquitously expressed and constitutively active protein serine/threonine kinase within eukaryotic cells. Protein kinase CK2 has been distinguished for its unique ability to phosphorylate its target substrates specifically at serine or threonine residues located proximal to acidic clusters[21,22]. Work by Pinna and colleagues have identified a minimal consensus sequence of S/T-X-X-D/E, with position n+3 being a major determinant for phosphorylation[22]. Studies investigating the interactome have identified over 100 interactors of CK2, with a large majority of these being nucleic acid synthesis proteins, as well as cellular communication and signal transduction proteins[23]. In terms of structure, CK2 is composed of two catalytic subunits (CK2α and CK2α'), as well as a regulatory subunit (CK2β), and exists in cells primarily in the form of a holoenzyme (Figure 1) ($\alpha_2\beta_2$, $\alpha'_2\beta_2$, or $\alpha\alpha'\beta_2$) although the existence of the catalytic subunits in the absence of

the regulatory subunit have also been observed[24,25]. It should be noted that the relative abundance of these isoforms within cells currently remains poorly characterized.

To date, typical phosphorylation assays have been limited in their ability to discriminate between the two catalytic isoforms of CK2 and therefore it cannot be concluded which form of CK2 is responsible for its activity in particular cellular processes[26]. What is interesting is that CK2α and CK2α' only exhibit differences in their C-termini[27]. This indicates the potential for functional compensation and specialization between the two subunits[27]. Evidence in support of this resulted from the development of tetracycline regulated stable cells lines expressing catalytically inactive forms of CK2α and CK2α'[28]. It was observed that a dramatic reduction in proliferation was detected when expressing catalytically inactive forms of CK2α', although this was not achieved with the expression of catalytic inactive CK2α[28]. To strengthen this, knockout mice models with targeted disruption of either CK2α and CK2β resulted in embryonic lethality, whereas knockouts of CK2α' resulted in viable, although sterile mice[29]. In addition, distinction between the two isoforms has also been observed when investigating isoform-specific binding partners of CK2α and CK2α' using a yeast two-hybrid approach[30]. Although evidence continues to grow, casting light onto CK2 specific interactions and additional roles within the multitude of cellular processes, much remains to be investigated to provide a thorough understanding of the functional specialization that may exist between CK2α and CK2α'.

Figure 1. Crystal structure of the CK2α holoenzyme
Using PyMol, the co-ordinates (PDB identification number 3PE1) from the high-resolution crystal structure of the CK2 tetramer[31] were used to generate a ribbon diagram representing the CK2α holoenzyme. The CK2α subunit bearing the non-hydrolysable ATP analogue adenosine 5'-[β,γ-imido] triphosphate (AMPPNP - red) within its ATP binding site is depicted in green. The other CK2α subunit is illustrated in yellow. It is worth noting that the AMPPNP analogue only binds to one catalytic subunit, however the impact of this observation still remains poorly understood[27]. The regulatory subunits, CK2β, are illustrated in blue and purple.



As we continue to advance our understanding of CK2 and its potential to maintain cellular homeostasis, one important aspect that has remained challenging to elucidate is the regulation of protein kinase CK2. For example, in the early 1990's, scientists evaluated different stimuli for their ability to modulate CK2, although no conclusive insights into the mechanisms behind CK2 regulation were identified[32]. Since then, many opposing views have been proposed including the suggestion that CK2 is indeed physiologically regulated, potentially by means of regulated CK2 assembly into tetrameric complexes[24]. As it is well known, cyclin-dependent kinases (CDK's) require the presence of cyclins to initiate their catalytic activity within cells[33], and from this many similarities have been developed between cyclins and the regulatory CK2β subunit. Through sequence analysis, it has been identified that CK2β contains a destruction box that can be subjected to ubiquitinylation, which permits degradation of CK2β via the proteasomal pathway[34]. Although CK2 levels do not change to the same extent as CDK's during the cell cycle, this observation reflects the tendency of CK2 expression to oscillate throughout the cell cycle, again reminiscent to cyclins. In the end, more work must be performed in order to provide additional insight into the regulation of CK2, as much still remains unknown.

1.3 – Cellular functions of protein kinase CK2

Both exciting and at times puzzling is the increasing literature on the multitude of potential CK2 substrates. As a result, these studies have provided insight into the broad array of cellular functions that may involve CK2. Consequently, it seems impractical to touch on all aspects of CK2 and its role within cells, and so the following will highlight

only the roles of CK2 in the context of cellular proliferation and survival as these roles are more established.

1.3.1 – CK2 and a role in proliferation

One of the better-established roles of CK2 in eukaryotic systems is its ability to monitor and maintain the cell cycle to ensure the proper production of daughter cells. In fact, CK2 has been implicated in all stages of the cell cycle[35]. For example, antisense oligonucleotides and inhibitors directed against CK2 have demonstrated the ability to completely halt the progression of cells at various stages of the cell cycle[36]. In addition, cdk-activating kinase (CAK), which is considered the master regulator of the cell cycle, is in turn regulated by CK2[37]. This was determined using various point mutations in the sequence of cyclin H, a component in CAK, which lead to the observation that CK2 phosphorylated cyclin H specifically at threonine 315[38].

With respects to the G1/S phase transition, the signal to initiate DNA replication involved hyper-phosphorylation of the retinoblastoma protein (pRB), which releases E2F transcription factor from an inhibitory state, such that it can initiate the transcription of several cell cycle genes[39]. Once the signal to begin DNA replication is transduced, CK2 has been implicated in many ways not only to serve to ensure proper cell growth, but to rather supervise the progression throughout the cell cycle to secure a successful outcome. One way in which CK2 monitors the cell cycle is through its interactions with the tumor suppressor protein, p53[35]. In brief, p53 is induced by a signal indicating that some sort of cellular stress or DNA damage has been initiated, and in the case of UV damage to DNA, will be phosphorylated at serine 392 by CK2[40]. This results in an increase of p53 binding

to DNA, as well as transcriptional activation of particular molecules such as p21 and p16[41]. In addition, it has been observed that CK2 and its interactions with p53 are mediated through the protein, Mdm2[42].

In addition to CK2 and its role with p53 as a means of monitoring the signaling through the G1/S phase transition, CK2 has also been implicated in the G2/M transition of the cell cycle. In this circumstance, Cdk1 specifically phosphorylates CK2α in a cell cycle dependent manner, typically at residues T344/T360 and S362/S370, which are located within its unique C-terminus[43,44]. In addition, it has been determined that CK2β is also phosphorylated by Cdk1, although the significance of this event is not well understood[45]. In order to elucidate the significance of cell cycle dependent phosphorylation of CK2α, phosphospecific antibodies have been generated against the four phosphorylation acceptor sites on CK2α mentioned above. Immunoblot analysis detected the presence of phosphorylated CK2α in prophase and metaphase specifically. Furthermore, expression of non-phosphorylatable mutants of CK2α led to the induction of mitotic catastrophe[45]. It is also important to highlight that this serves as an additional example of functional specialization that may exist between the two catalytic subunits of CK2, since Cdk1 does not phosphorylate CK2α' in a cell cycle dependent manner. In conclusion, the requirement for CK2 to phosphorylate, as well as be phosphorylated during various stages of the cell cycle only serve to support protein kinase CK2 as an important mitotic regulator.

1.3.2 – CK2 and a role in cellular survival and apoptosis

Another functional role of protein kinase CK2 building excitement in the field is

its implications in cellular survival and apoptosis. Protein kinase CK2 has been observed to play a fundamental role in cell survival, through maintaining cell viability and protecting cells from their apoptotic machinery. Interestingly, a reduction in viability was detected when expressing catalytically inactive forms of CK2, indicating the requirement of CK2 activity, and not just the presence of the kinase[46]. In addition, studies using genetically tractable yeast models to examine the necessity of protein kinase CK2 in maintaining viability demonstrated that down regulation of CK2 using temperature-sensitive mutants resulted in a dramatic loss of viability[47]. Interestingly, a dramatic reduction in viability was only observed following the down regulation of both CK2α and CK2α' catalytic subunits, suggesting that one may be able to functionally compensate for the other in the context of cellular viability. As previously noted, this is not however the case in mice since CK2α/ CK2β knockouts resulted in embryonic lethality, whereas knockouts of CK2α' resulted in mice that were viable but developed defects in spermatogenesis[29]. This finding suggests that CK2α can compensate for the loss of CK2α', whereas CK2α' cannot compensate for the loss of CK2α. More significantly, this observation may serve to highlight the independent regulation that may exist between the two catalytic subunits of CK2 in the process of cellular viability.

One highly studied mechanism by which CK2 functions in the protection of cells against apoptosis, is its phosphorylation of members of the apoptotic machinery. It has been observed that protein kinase CK2 can phosphorylate the pro-apoptotic protein, Bid, a member of the bcl-2 family, preventing downstream activation of mitochondrial apoptotic machinery[48,49]. Furthermore, CK2 has been implicated in the stimulation of inhibitor of apoptosis proteins (IAP's), one in particular being survivin[50]. Collectively,

these outcomes prevent the activation of caspases, which are responsible for the execution of apoptosis. As mentioned earlier, CK2 has a plethora of cellular substrates, and in fact, caspases themselves fall into this category. Caspases are synthesized in a precursor form as zymogens, where in the presence of a cellular death signal, a cascade of caspase cleavage is induced, leading to activation of executioner caspases which implement cellular apoptosis[51]. A potential direct link between CK2 and caspases was revealed by the demonstration that phosphorylation of caspases by CK2 in proximity to their cleavage sites resulted in the overall protection of caspases from cleavage, thus halting the cascade responsible for the activation of executioner caspases[51,52]. Overall, it can be seen that CK2 unquestionably plays a significant role in the protection of cells from apoptosis, thus maintaining its role as a pro-survival player within cells. However, mounting evidence has suggested that it is these very cellular processes that are often manipulated in various human cancers, and CK2 might be at the center of such oncogenic activity.

1.4 – Protein kinase CK2: Prospect for therapeutic targeting

Elevated expression of protein kinase CK2 has been detected in many different cancers, including cancers of the kidney, breast, and prostate[53–55]. Further investigating the role of CK2 in tumorigenesis, Seldin and Leder (1995) provided evidence, which implicated CK2 in transformation by targeted overexpression of CK2 in transgenic mice, resulting in the development of leukemia[56]. Likewise, in hypoxia-associated tumors, histone deacetylases (HDAC's) are activated in a CK2-dependant manner[57]. Overall, these findings ignited an exciting area of research directed at elucidating the role of CK2

in the development of tumorigenesis. Therefore, this section will highlight mechanisms identified by which CK2 may exhibit oncogenic activity and push the equilibrium towards cancer.

1.4.1 – Regulation of tumor-suppressor proteins and oncogenes

As mentioned earlier, CK2 plays central roles in cellular proliferation and survival in the context of normal cells, although it is typically these very processes that become hijacked and shift the physiological environment into one well suited for the development of cancer. In this respect, the role of CK2 in the normal regulation of the tumor suppressor protein, p53, preventing the replication of DNA at inopportune times, may often pose as a threat under cancerous conditions where CK2 levels are manipulated, and thereby establish improper control of the cell cycle. Another example of CK2 in the regulation of tumor suppressor proteins is the down regulation of the promyelocytic leukemia protein (PML), a phenomenon frequently observed in many human cancers[58]. Tumor suppressor protein, PML, is responsible for regulating pathways responsible for growth suppression, apoptosis, and senescence. Implicating CK2 in this regulation is its ability to phosphorylate PML, specifically at residue S517[59]. Furthermore, elevated CK2 activity in a lung cancer mouse model is associated with an increase in the degradation of PML via the proteasome pathway[60].

In addition to the regulation of tumor suppressor proteins, CK2 has also been implicated in the stimulation of oncogenes, which encode for proteins responsible for the uncontrollable growth of cells[61]. In fact, CK2 has been speculated to reroute signals indicative of cell death into pathways promoting cell survival, providing a so-called

"pathological rewiring" of signal transduction pathways. One mechanism by which CK2 can potentiate the elevated transcription of oncogenes is through the up regulation of the transcriptional-activator, NF-kB[62]. In general, NF-kB is found sequestered within the cytoplasm by an inhibitory protein, IkB, which becomes phosphorylated thus relieving NF-kB for activation of many genes important for cell survival and cell proliferation. With respects to regulation via CK2, it has been demonstrated that IkB is phosphorylated directly by CK2 at residue S529, providing a means of NF-kB inhibitory release that is alternative to the canonical IKK kinases pathway[63]. In addition, evidence supports that CK2 directly phosphorylates p65, a family member of NF-kB resulting in an increase in DNA-binding potential[64].

Overall, from these observations it becomes evident that CK2 may promote tumorigenesis through the manipulation of those proteins that serve as gatekeepers to prevent deleterious effects of cellular stress, as well as proteins that tend to promote the uncontrollable growth of cells. In addition, it has been considered that CK2 promotes a synergistic effect via the ability to promote increased expression of oncogenes in those very cells that also tend to lack tumor suppressor proteins, serving as an additive force to drive the equilibrium in favour of a tumorigenic environment[65].

1.4.2 – CK2 and apoptosis in cancer

Not only is cancer a consequence of uncontrollable cellular proliferation, but it also becomes an issue of cells not being able to make timely cellular decisions of life and death, ultimately choosing to promote cell survival at times when apoptosis would be beneficial and visa versa. As mentioned earlier, CK2 possesses the ability to regulate

members of the apoptotic machinery, namely those of the caspase family, resulting in the protection of downstream cleavage due to the phosphorylation of overlapping consensus sequences[51,52]. Accordingly, it can be seen that prevention of apoptosis under situations such as uncontrolled growth or intracellular damage, could be detrimental to the normal life cycle of that cell, and may help to shift the balance in support of tumorigenesis.

Recently, an additional mechanism by which CK2 plays a protective role towards cellular apoptosis is through a multiple-drug resistance phenotype. Through previous work performed by Ahmed and colleagues (1993), it was demonstrated that CK2 was dynamically regulated in terms of nuclear localization, and that this regulation was dependent on the strength of growth stimuli[66,67]. In particular, removal of androgenic growth signals from prostate epithelial cells in rats resulted in a significant reduction in levels of CK2 associated with the nuclear matrix, and could be replenished by regenerating androgenic stimuli into the cells[68]. In addition, it was recently demonstrated that reduction of CK2 from the nuclear matrix was associated with intense induction of apoptosis in an *in vivo* prostate tumor model[69]. This observation led to the hypothesis that CK2 may be imparting a protective role towards apoptosis when localized to the nuclear matrix. To test this hypothesis, chemical-inducing apoptotic compounds, etoposide and diethylstilbestrol (DES), were utilized. It was demonstrated that after treatment with both etoposide and DES, there was a significant increase in the localization of CK2 to the nuclear matrix, which ultimately led to a protective phenotype towards apoptosis[70]. This result is opposite of what is observed during receptor-mediated apoptosis, where a considerable reduction in the levels of CK2 associated with the nuclear matrix is observed[70]. Collectively, this identified a potential protective role for CK2 in drug-

induced apoptosis.

Despite the fact that the overexpression of CK2 in many cancers has been associated with a protective role towards apoptosis, recent experiments have shed light on the ability to sensitize cancer cells to apoptosis following a reduction in the levels of CK2[71]. To date, the down regulation of CK2 has commonly been achieved using RNA silencing (siRNA) techniques to selectively knock down the different subunits of CK2. Using this strategy, it was observed that there was a dramatic loss of cell viability (80%) in the ALVA-41 cancer cell line compared to the normal control (30%) following siRNA targeting of CK2[71]. This serves to reiterate the anti-apoptotic role for CK2 within cells. In addition to siRNA approaches, catalytically inactive forms of CK2 have also been engineered to determine the cellular consequences of down regulating CK2 activity in cancer cells. Similar to the effects observed from siRNA approaches, there was a significant reduction in cellular viability (75%) following transfection with catalytically inactive CK2, compared to the control, which only was slightly effected[71]. However, this strategy can often be challenging since the down regulation of CK2 via knockdown is often incomplete.

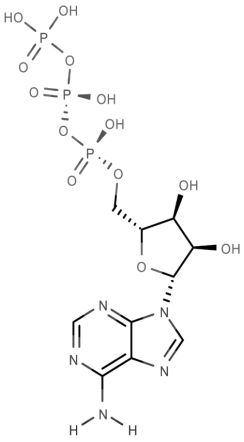
Accordingly, small molecule-inhibitors are rising as a promising tool for the inhibition of CK2, as this is predicted to inhibit all endogenous levels of CK2[72]. Overall, investigation into the inhibition of CK2 using chemical inhibitors will serve to elucidate the molecular mechanisms by which CK2 guides decisions of life and death within cells, as well as to provide insight into the future of therapeutic intervention towards those cancers in which elevated expression of CK2 is observed.

1.4.3 – Chemical inhibition of protein kinase CK2

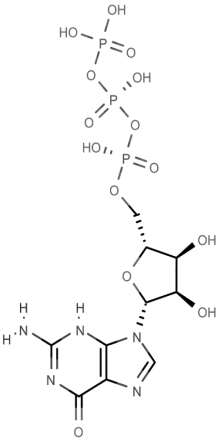
Based on its roles in cancer and the prospect for the inhibition of CK2 for therapeutic intervention, interests have been sparked in the development of chemical inhibitors of CK2. To this point, many inhibitors have been generated and their potency, as well as their selectivity has been tested in vitro[73–75] (Figure 2). Initially, inhibitors targeting CK2 were derived from an ancestral compound (5,6-dichloro-1-(b-D-ribofuranosyl)-benzimidazole) (DRB), originally described in 1986[76] that subsequently led to the development of more widely employed CK2 inhibitors tetrabromobenzotriazole (TBB) and tetrabromobenzimidazole (TBBz)[77]. Testing these compounds against a broad panel of different kinases as an indication of their selectivity, demonstrated that TBB was quite selective towards CK2[78]. The derivative, TBBz, although not as selective towards CK2 as TBB, indicated its potential at better discriminating between the various isoforms of CK2[73]. Kinetic parameters have been determined for these compounds *in vitro*, indicating a range of Ki values of 80-210 nM for TBB, and a range of Ki values of 70-510 nM for TBBz[73]. Although these compounds have for some time been recognized as common inhibitors for targeting CK2, many attempts to improve the efficacy of CK2 inhibitors have been implemented, thereby utilizing TBB has a forerunner for the development of more potent and selective inhibitors. An example is the compound, DMAT, resulting from the derivitization of the imidazole ring of TBBz that proved to be more potent than TBB when exposed to cell cultures[79].

More recently, a newly designed and first orally available ATP-competitive inhibitor of CK2, CX-4945, was developed by Cylene Pharmaceuticals[74]. The

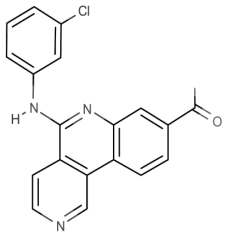
Figure 2. Structure of ATP, GTP, and current inhibitors of protein kinase CK2
Chemical structures of ATP, GTP, and characterized inhibitors of protein kinase CK2. Previously reported IC_{50} values have also been included.



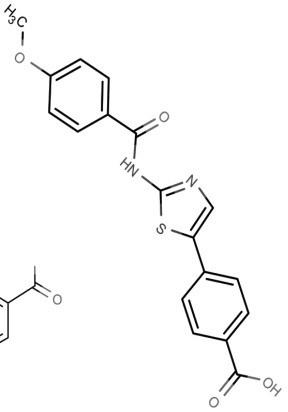
ATP



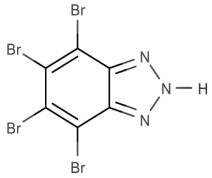
GTP



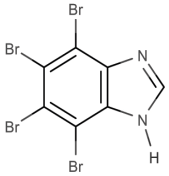
CX4945
IC_{50}=1 nM



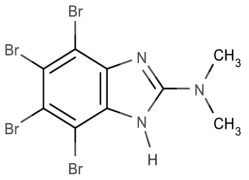
CK2 inhibitor VIII
IC_{50}=32 nM



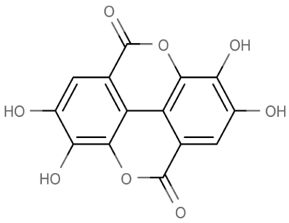
TBB
IC_{50}=500 nM



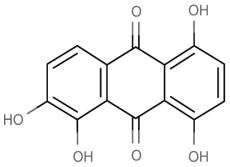
TBBz
IC_{50}=500 nM



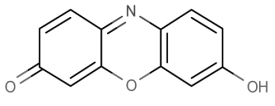
DMAT
IC_{50}=140 nM



Ellagic Acid
IC_{50}=40 nM



Quinalirazin
IC_{50}=110 nM



Resorufin
IC_{50}=1500 nM

derivatization of CX-4945 was achieved through the optimization of a PARP inhibitory compound, selected from a library due to its structural similarity to [[5-oxo-5,6-dihydro-indolo(1,2-a)quinazolin-7-yl]acetic acid] (IQA), another inhibitor of protein kinase CK2. More specifically, a series of substitutions to incorporate amino alkyl chains bearing aniline moieties and replace the existing thiophene ring with a pyridine, led to the production of the more potent CX-4945 compound[74]. Biological characterization of CX-4945 using *in vitro* enzymatic assays showed that when compared against a panel of 235 other protein kinases, CX-4945 was selective for CK2[74]. In addition, CX-4945 has been shown to be considerably more potent than other current existing inhibitors of CK2, with a reported Ki of 0.38 nM[74,80]. Elucidating the biological consequence of CK2 inhibition by CX-4945, it was determined that the pro-survival PI3'K/AKT pathway was attenuated[81]. This conclusion arose from the inability to detect the CK2-specific phosphorylation of Akt at residue S129, as well as the canonical regulatory residues, S473 and T308[81]. In addition, dephosphorylation of downstream targets of Akt such as p21 was also observed[81]. Subsequent studies have since been performed to evaluate the therapeutic potential of CX-4945 as an anti-cancer agent. Providing evidence in support of an anti-proliferative role for CX-4945 in cancer is its ability to induce cell cycle arrest and initiate apoptosis in PC3 prostate carcinoma cells[74]. Furthermore, CX-4945 has demonstrated the capacity to potentiate anti-tumor activity in a mouse xenograft model, inhibiting tumor growth by nearly 86%[74]. Although CX-4945 has primarily been described as an inhibitor of pro-survival pathways, evidence has also indicated its ability to prevent tube formation and transcription of the hypoxia-induced factor 1α (HIF-1α) in models of angiogenesis, as well as suppress the production of the inflammatory

interleukin-6 in human breast cancer cells[81]. Overall, CX-4945 is considered to be a promising therapeutic modulator of protein kinase CK2, and due to its successes in the treatment of various cancers indicating its potential as a clinical inhibitor, CX-4945 was subjected to clinical trails with recent advancement into phase II trails[82].

Although the current panel of CK2 inhibitors being designed, synthesized, and characterized is continuing to grow, and evidence behind their potency and selectivity towards CK2 has been well documented, the majority of these studies have only been performed *in vitro*[83–85]. As a result, there tends to be a lack of evaluation for these inhibitors regarding their modulation of the biological and pathological roles of CK2 within cells. Whether or not the observed effects are a direct or indirect consequence of targeting CK2 also often remains to be explored, limiting our understanding of the potential off-targets that could influence the behavior of a given inhibitor. Therefore, the development of a strategy to effectively and rapidly evaluate current and novel inhibitors of protein kinase CK2 could be instrumental to further expand our understanding of the consequences of CK2 inhibition in cells, as well as unveiling valuable information that may help advance these inhibitors for clinical applications.

1.5 – The use of inhibitor-refractory mutants for the study of protein kinases

Although the development of mutants that are rendered insensitive to inhibition by specific inhibitors may sound counterproductive and perhaps troublesome, considering the prevalence of drug resistance in the clinical setting, their use as a tactical tool in order to elucidate information regarding a protein of interest can be insightful. For example, in a recent study performed by Fujiwara and colleagues (2015), drug-resistant mutants in

combination with a specific inhibitor were utilized in order to assess isoform specificity of the Ca^{2+}/calmodulin- dependent protein kinase kinase (CaMKK)- mediated signaling pathways[86]. They were particularly interested in distinguishing whether it is CaMKKα, CaMKKβ, or both that are responsible for the phosphorylation and activation of the downstream proteins, AMPK and CAMKIV. Their findings illustrated that the suppression of the ionomycin-induced phosphorylation (Thr172) and activation of AMPK due to treatment of A549 cells with a CaMKK inhibitor (STO-609), was relieved following the expression of an inhibitor-insensitive mutant of CaMKKβ, but not CaMKKα. In contrast, they observed that the ionomycin-induced phosphorylation (Thr196) and activation of CAMKIV was no longer suppressed following the expression of inhibitor-insensitive mutants of both CaMKKα and CaMKKβ.Together, these data suggest that the utilization of inhibitor-resistant mutants of protein kinases in combination with specific inhibitors can serve to distinguish isoform-specific phosphorylation events in cells.

Studies have also demonstrated that the development of kinases that are rendered insensitive to inhibition can provide a means for evaluating the effects of current and novel inhibitors in cells. An example of this utility is displayed in the work performed by Eyers et al (1999) confirming the *in vivo* effects of an inhibitor, SB 203580, for the stress-activated protein kinase 2a/p38 (SAPK2A/P38)[87]. It was demonstrated that the phosphorylation and activation of four well established downstream targets of the stress-activated protein kinase 2a/p38 were suppressed in the presence of the SB 203580 compound. To determine if these effects were a result of the specific inhibition of the SAPK2A/P38 kinase, they generated a cell line that readily expressed a drug-resistant

mutant. Following the expression of the drug-resistant SAPK2A/P38 kinase, they observed that the downstream phosphorylation and activation events were no longer suppressed. Importantly, if the observed effects of the SB 203580 compound were due to targeting a non-specific kinase, then the expression of a drug-resistant mutant of SAPK2A/P38 should have no effect on the phosphorylation status of the four downstream targets. Therefore, the researchers were confident that the effects of the SB 203580 compound were exerted through specifically targeting the SAPK2A/P38 protein kinase.

Overall, we can begin to envisage how the creation of stable cell lines, that readily express inhibitor-insensitive mutants of the various isoforms of CK2, may pave the way for the effective and rapid separation of their catalytic activity in cells. This would be a significant achievement, as it would allow us to more precisely investigate the functional roles that CK2 may orchestrate within cells, and in particular, help to distinguish functions that may be isoform-specific from those that are compensatory. Additionally, creation of a system that allows the separation of catalytic activity exhibited by the various isoforms of CK2 will provide a platform that will enable a thorough evaluation of current and novel inhibitors, which has previously been unattainable.

To achieve the goal of engineering inhibitor-refractory mutants of CK2, our expectation is that we can use the structural basis for its inhibition to rationally identify those residues that are responsible for the tight binding of specific inhibitors to the active site of CK2[31,35,88,89]. We can also utilize kinase alignments to identify the residues that exist within other kinases at the positions of interest where key inhibitory determinants identified in CK2 reside. To generate inhibitor-refractory mutants of CK2, we will

substitute the key structural determinants for the binding of inhibitors to CK2, in order to make CK2 resemble kinases in which the inhibitors are not selective. Ultimately, we believe that cell lines expressing mutants of CK2 that are noticeably less sensitive to inhibition will serve as a tactical tool for the future study of protein kinase CK2 and evaluation of its inhibitors. Towards these objectives, this thesis describes the development and characterization of mutants of the CK2α isoform that display a reduced sensitivity to inhibition by the well-known CK2 inhibitor, CX-4945. In particular, the work described in this thesis is focused on investigations of singe, double, and triple mutants of CK2α bearing combinations of the V66A, H115L, H160D, and I174A amino-acid substitutions.

1.6 – Rationale, hypothesis, and objectives

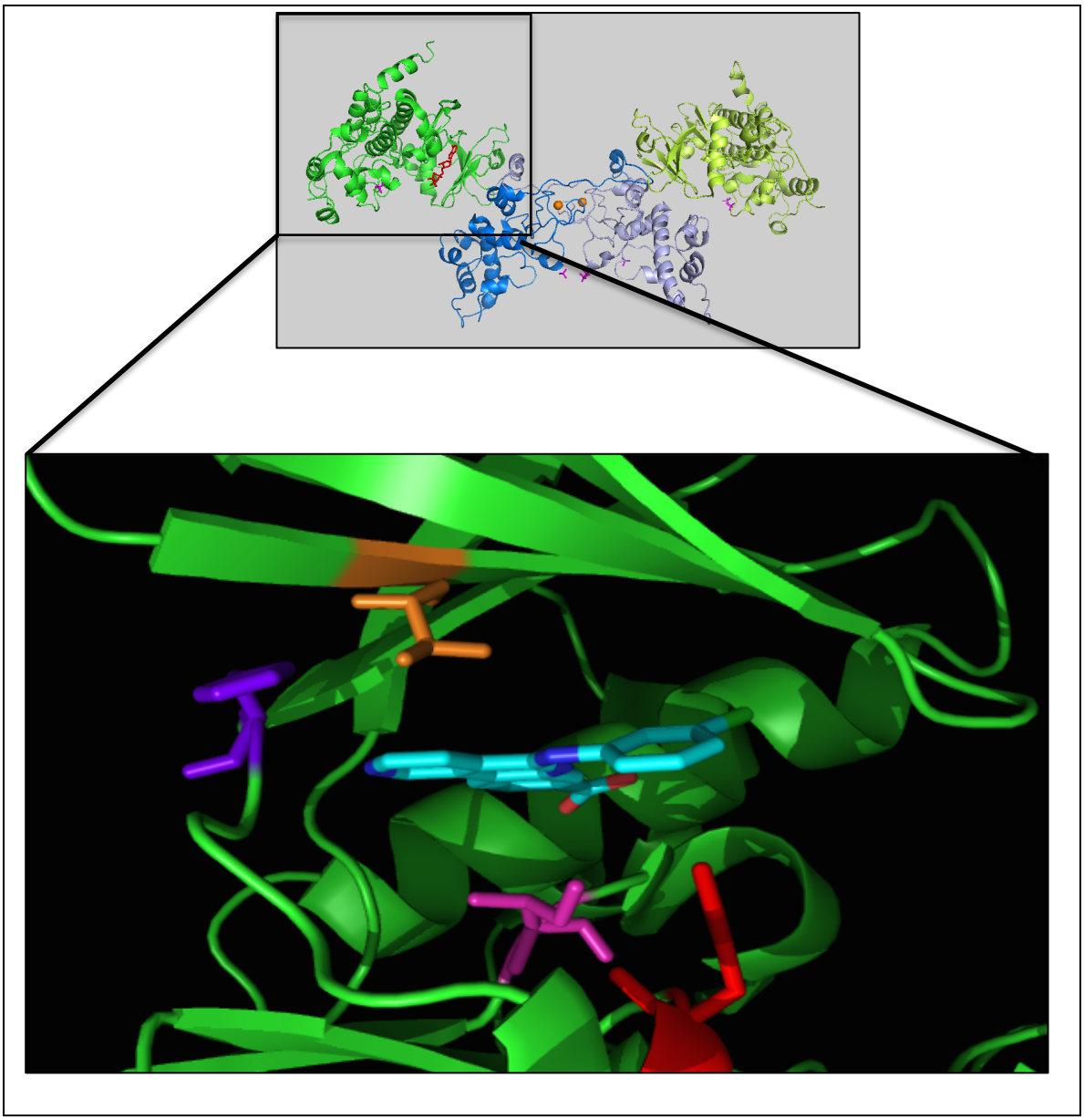
1.6.1 - Rationale

Within the last decade, several investigations into the structural basis behind the binding of inhibitors to the active site of protein kinase CK2 have been performed[25,31,89–91]. Many important observations have since been documented, most noteworthy being the repeated identification of two hydrophobic residues, valine 66 (V66) and isoleucine 174 (I174)[31,73,78,92,93]. Interestingly, these residues appear to be unique to CK2 since comparative analysis of other protein kinases revealed that the majority of protein kinases contained the much smaller amino acid, alanine[89,94]. This suggested that CK2 might contain a unique structural feature within its catalytic pocket that could be exploited for the development of CK2 selective inhibitors. Examination of the high-resolution structure of CK2α with CX-4945 (Figure 3: PDB code 3PE1, V66 in orange and I174 in pink), it is

clear that the hydrophobic residues, V66 and I174, are positioned above and below the inhibitor respectively, presumably functioning as a clamp in order to provide further stabilization for the inhibitor within the active site of CK2[31]. It was therefore predicted that by substituting these residues to alanine, in order to make CK2 resemble the majority of the remaining protein kinases, the selectivity of CK2 inhibitors for the binding of its active site could be lost, generating a mutant that is less sensitive to inhibition. In fact, mutational studies have demonstrated that substitution of these residues to smaller amino acids such as alanine, can influence the ability of CK2 inhibitors to inhibit CK2, as reflected by the dramatic change in *Ki*[31,73]. These findings are promising because they demonstrate that the generation of mutants that are catalytically active but yet display a reduced sensitivity to inhibition is feasible. To build on these observations, a central emphasis of the work described in this thesis has been directed towards the creation of tetracycline-inducible cell lines expressing a mutant of CK2α bearing the V66A and I174A mutations. Since there have been indications that additional residues may also be involved in the binding of CK2 inhibitors, I have also been evaluating the prospects that the generation of a new mutant with an increased ability to impair the inhibition of CK2 is attainable.

Although it has been considered that the hydrophobic interactions primarily established by the V66 and I174A residues are the main energetic contributors towards the binding of CK2 inhibitors, evidence suggests that electrostatic interactions and the formation of extensive hydrogen bond networks can also play a significant role[31,95]. In fact, a recent structural study investigating the selectivity of the CK2 inhibitor, CX-4945,

Figure 3. Illustration of important residues implicated in the binding of CX-4945
The co-ordinates (PDB identification number 3PE1) from the high-resolution crystal structure of the CK2 tetramer[31] were combined with PyMol to generate a ribbon diagram representing the CK2α holoenzyme. I have zoomed in on one CK2α catalytic subunit bound to the CK2 inhibitor, CX-4945, in order to highlight the residues important for its stabilization within the active site of CK2. Valine 66 (V66) is indicated in orange, histidine 115 (H115) is indicated in purple, histidine 160 (H160) is highlighted in red, and isoleucine 174 (I174) is highlighted in pink.



for the active site of protein kinase CK2 has identified a basic residue, H160, located directly below the phenyl group containing a chlorine moiety[31,90]. It is reasonable to believe that a histidine residue bearing a positive charge could be functioning to stabilize the inhibitor (CX-4945) by establishing strong electrostatic interactions with the chlorophenyl moiety. However, it was observed that when in the presence of the inhibitor (CX-4945), H160 was locked into an "upward" conformation in order to make bridging interactions to the chlorophenyl group through a conserved water molecule (W2) located within the protein matrix[31,90]. In addition, although there is no evidence to date suggesting a role in the binding of CX-4945 to the active site of CK2α, the residue H115 has been implicated in the binding of a structurally related inhibitor, CX-5279[31]. As a result of their recent implications for the binding of inhibitors into the active site of CK2, I subsequently decided to resort back to the comparative analysis of protein kinases constructed by Hanks and Hunter[94] (1999) to determine if these residues are also structurally unique to the protein kinase CK2 (as were residues V66 and I174)(Table 1: adapted from Hanks & Hunter, 1999 – CK2α is bolded and the unique residue is highlighted in red). I determined that these residues (H115 and H160) were in fact exclusive to the protein kinase CK2, suggesting another unique structural feature that may enable its selective inhibition. Accordingly, I expect that these residues would be prime candidates for substitution in order to further impair the ability of inhibitors to inhibit CK2, and therefore I now wanted to determine what residues I should consider for their replacement.

Similar to the strategy employed in order to determine the appropriate residue for the substitution of the previously established V66 and I174, I decided to identify what

residues other kinases contained at the positions of interest (H115 and H160). The rationale behind this strategy is that if the residues, H115 and H160, are exclusive to protein kinase CK2 and are responsible for the selective nature of its inhibitors, then by replacing these residues in order to make a mutant of CK2 that resembles the majority of other protein kinases, I would expect to render CK2 less sensitive to the inhibitor. It was determined that the majority of protein kinases contained leucine/tyrosine (L/Y) and aspartic acid/glutamic acid (D/E) residues at positions 115 and 160, respectively (table 1). The histidine residue at position 115 (H115) was substituted to a leucine residue (H115L), since the α' isoform of CK2 contains a tyrosine residue at that position (Y115), which would presumably maintain the selectivity of CX-4945 for CK2, since both catalytic subunits of CK2 are inhibited by CX-4945. The histidine residue at position 160 (H160) was substituted for an aspartic acid (H160D). The rationale for this decision stemmed from the previous exploration into the susceptibility of different protein kinases to inhibition by staurosporine, which highlighted the H160D amino-acid substitution in CK2α[96]. In that report, it was demonstrated that CK2α bearing a H160D mutation was less sensitive to a staurosporine derivative (CGP44171A), which is more effective than staurosporine for the inhibition of CK2.

Table 1: Protein kinase alignment highlighting non-conserved residues in CK2α. Adapted from Hanks and Hunter[97] (1995)

Kinase	2° Structure (60-80)
PKA	TGNHY**A**MKILDKQKVVKLK
PKG	ESKTF**A**MKILKKRHIVDTR
PKC	TEELY**A**IKILKKDVVIQDD
DMPK	TGQVY**A**MKIMNKWDMLKRG
CaMK2 α	AGQEY**A**AKIINTKKLSAR
Polo	TDTVF**A**GKIVSKKLMIKHN
Cdc5	SGEIF**A**AKTVAKASIKSEK
Cdk2	TGEVV**A**LKKIRLDTETEG
Erk2	NKVRV**A**IKKISPFEHQT
GSK3 α	TRELV**A**IKKVLQ
CK2 α	NNEKV**V**VKILKPV
clk	GGRHV**A**VKIVKNVDR
Ire1	QGRPV**A**VKRMLID
Cdc7	GSNYV**A**LKKIYVTS
Cot	TKKRM**A**CKLIPVD
YpkA	DKQRL**V**AKIERSIAE
MEK1	SGLVM**A**RKLIHLEIKPA
Ste7	DSKIV**A**KKTIPVEQNNST
Ste11	TGELM**A**VKQVEIKNNIG
Nek1	DGRHY**V**IKEINISRMSDK

Kinase	2° Structure (107-118)
PKA	SNLYMVME**Y**VPG
PKG	KYLYMLME**A**CLG
PKC	DRLYFVME**Y**VNG
DMPK	NYLYLVNE**Y**YVG
CaMK2 α	GHHYLIFD**L**VTG
Polo	QNIYIVLE**L**CKK
Cdc5	SNVYILLE**I**CPN
Cdk2	NKLYLVFE**F**LHQ
Erk2	KDVYIVQD**L**MET
GSK3 α	LYLNLVLE**Y**VPE
CK2 α	RTPALVFE**H**VNN
Clk	GHICIVFE**L**LGL
Ire1	RFLYIALE**L**CNL
Cdc7	DQVIAVLP**Y**YPH
Cot	ETVHLFME**A**GEG
YpkA	LMDEVDGW**R**CSD
MEK1	GEISICME**H**WDG
Ste7	NEIIILME**Y**SDC
Ste11	GNLNIFLE**Y**VPG
Nek1	GSLYIVMD**Y**CEG

Table 1: Protein kinase alignment highlighting non-conserved residues in CK2α
Adapted from Hanks and Hunter[97](1995)

Kinase	2° Structure (150-170)
PKA	DLIYRDLKP**E**NLLIDQQ
PKG	GIIYRDLKP**E**NLILDHR
PKC	GIIYRDLKL**D**NVMLDSE
DMPK	GYVHDIKP**D**NILLDRC
CaMK2 α	GVVHRDLKP**E**NLLLASKLKG
Polo	RIIHRDLKL**G**NLFLNDL
Cdc5	RVIHRDLKL**G**NIFFDSN
Cdk2	RVLHRDLKP**Q**NLLINTE
Erk2	NVLHRDLKP**S**NSSLNTT
GSK3 α	GVCHRDIKP**Q**NLLVDPDT
CK2 α	GIMHRDVKP**H**NVMIDHEH
clk	KLTHTDLKP**E**NILFVQSD
Ire1	KIIHRDLKP**Q**NILVSTSS
Cdc7	GIIHRDIKP**T**NFLFNLEL
Cot	KVIHHDIKP**S**NIVFMS
YpkA	GVVHNDIKP**G**NVVFDRAS
MEK1	KIMHRDVKP**S**NILVNSR
Ste7Ste11Nek1	KIIHRDIKP**S**NVLIVSK
	NIIHRDIKG**A**NILIDIK
	KILHRDIKS**Q**NIFLTKD

Kinase	2° Structure (169-186)
PKA	GYIQV**T**DFGFAKRVKG
PKG	GYAKL**V**DFGFAKKIGFGK
PKC	GHIKI**A**DFGMCKEHMMDGV
DMPK	GHIRL**A**DFGSCLKLRADGTV
CaMK2 α	AAVKL**A**DFGLAIEVEGEQQ
Polo	LHVKI**G**DFGLATRIEYRGE
Cdc5	YNLKI**G**DFGLAAVLANESE
Cdk2	GAIKL**A**DFGLARAFGVPVR
Erk2	CDLKI**C**DFGLARVADPDHDHTG
GSK3 α	AVLKL**C**DFGSAKQLVRGE
CK2 α	RKLRL**I**DWGLAEFYHPGQ
clk	PDIKV**V**DFGSATYDDE
Ire1	LRILI**S**DFGLCKKLDSGQS
Cdc7	GRGVL**V**DFGLAEAQMDYK
Cot	TKAVL**A**DFGLSVQNTEDVY
YpkA	GEPVV**I**DLGLHSRS
MEK1	GEIKL**C**DFGVSGQLIDS
Ste7	GQIKL**A**DFGVSKKLINS
Ste11	GCVKI**T**DFGISKKLSPLN
Nek1	GTVQL**A**DFGIARVLNSTVE

1.6.2 – Hypothesis

Therefore, in light of the evidence indicating that residuesV66, H115, H160, and I174 are exclusive to CK2α, as well as the evidence supporting that the amino acid substitutions, V66A, H160D, and I174A can influence the efficacy of inhibitors towards CK2, I hypothesize that these mutations will affect the ability of CX-4945 to inhibit CK2α. If correct, this will be reflected by changes in the *Ki*, as well as in the recovery of CK2 activity following treatment with CX-4945.

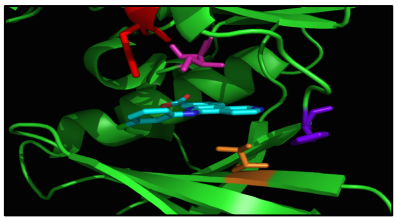
1.6.3 – Objectives

In order to accomplish the goal of the study, I have two central objectives. First, I will perform an *in vitro* evaluation of recombinant CK2α mutants to identify the mutant that demonstrates the greatest ability to affect the inhibition of CK2 by CX-4945. Second, I will generate a tetracycline-inducible stable cell line such that I can achieve regulated expression of this mutant in a cellular system. The experimental workflow is outlined in Figure 4. I will begin by performing site-directed mutagenesis in order to incorporate the H115L and H160D mutations into either wild type CK2α, or a mutant of CK2α already containing the previously established V66A and I174A amino-acid substitutions. Subsequently, I will employ *in vitro* kinase assays to assess the effects of these amino acid substitutions on the inhibition of CK2 by CX-4945. Once the mutation that demonstrates the greatest ability to affect the ability of CX-4945 to inhibit CK2 has been identified, I will utilize Flp-In™ T-REx™ technology[98] to incorporate the mutant of interest into a U2OS host cell line. Following the successful incorporation of the mutant of interest into the U2OS host cell line, a detailed characterization to examine the

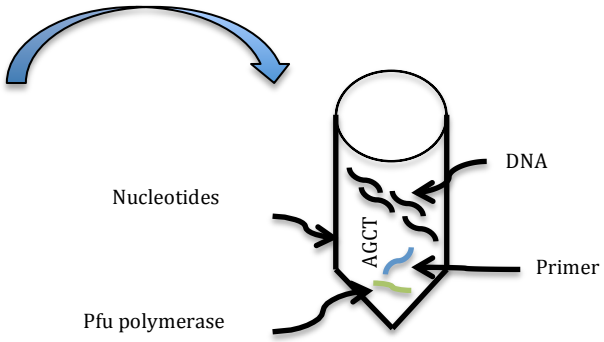
conditions required to achieve optimal expression will be performed. Lastly, if I can identify a cell line whose expression of the mutant of interest is rapid, abundant, and tightly controlled by the presence or absence of tetracycline, then I will use this cell line to examine the effect of this mutant on the ability to affect the inhibition of CK2 by CX-4945 in cells. Overall, I expect that these studies will provide new opportunities to utilize a mutant of CK2α that is noticeably less sensitive to inhibition as a tool that would surely benefit our ability to study the protein kinase CK2 in cells and to discriminate between the CK2-dependent and CK2-independent effects of the inhibitor.

Figure 4. Experimental workflow
A schematic illustration highlighting the experimental blueprint for this thesis. The experiment design consists of two central objectives. 1) *In vitro* evaluation of recombinant CK2α mutants on their ability to effect CK2 inhibition by CX-4945. 2) Generate a tetracycline-inducible stable cell line to test the effects of expressing a CK2α inhibitor-refractory mutant on the cellular response of CX-4945

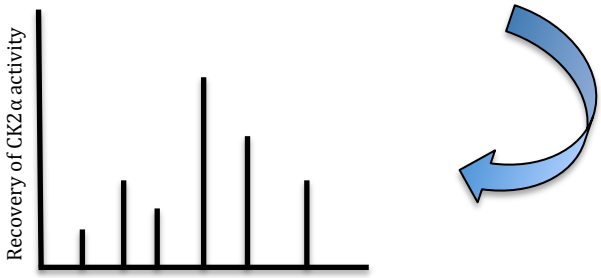
Objective 1



Identification of key residues for binding to CX-4945

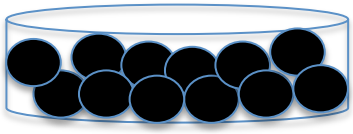


Generation of CK2α mutants via PCR

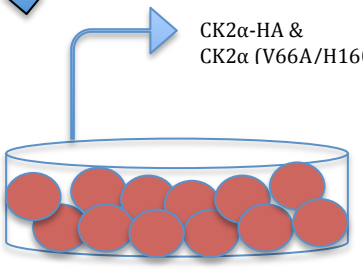


Evaluating effect of CK2α mutants on CX-4945

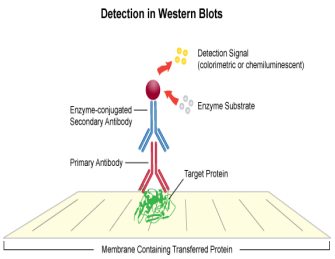
Objective 2



Development of stable cell lines inducible by tetracycline



Induction of protein expression via addition of tetracycline



Characterization of stable cell lines

2. Materials and Methods

2.1 – Plasmid constructs

2.1.1 GST-tagged mutants of CK2α

Plasmids encoding glutathione-S-transferase (GST)-tagged (a 220 amino acid fragment, originally described by Broek D, et al[99]) mutants of the CK2α catalytic subunit were generated for purification and use in *in vitro* kinase assays. Inhibitor-refractory mutants of CK2α containing H160D or H115L amino acid substitutions were generated using a site-directed mutagenesis approach via the polymerase chain reaction (PCR), the phusion DNA polymerase (Pfu, Thermo Scientific), and either a GST-CK2α or double mutant GST-CK2α (V66A/I174A) template to generate single and triple mutants. Primers utilized to introduce the various mutations into GST-CK2α are listed in Table 2. Following completion of PCR, all products were treated with restriction enzyme (Dpn1, NEB) for 30 minutes at 37°C. The amplified fragments for each mutant were verified by sequencing using the pGEX 5' primer (GGGCTGGCAAGCCACGTTTGGTG, 3' end of GST tag). Plasmids containing the mutations of interest were then subjected to purification (see section 2.2 - protein production and purification).

2.1.2 HA-tagged CK2α mutants for Flp-In cell line development

To develop stable cell lines with the tetracycline-inducible expression of CK2α-HA and CK2α (V66A/H160D/I174A)-HA, plasmid constructs were constructed for transfection into Flp-In U2OS cells. Both constructs contained the CK2α catalytic subunit with a C-terminal HA tag to help distinguish the expression of exogenous from endogenous protein. The HA-tag consists of a triple repeat of the influenza haemaglutinin

Table 2: Primers for generation of CK2α mutants

Mutation	Strand	Primers (5' to 3')
H115L	Sense	CCGCCTTGGTTTTTGAACTCGTAAACAACACAGACTT
	Anti-sense	AAGTCTGTGTTGTTTACGAGTTCAAAAACCAAGGCGG
H160D	Sense	CACAGAGATGTCAAGCCCGATAATGTCATGATTGATC
	Anti-sense	GATCAATCATGACATTATCGGGCTTGACATCTCGTG

epitope YPYDVPDY, as previously described[100]. In order to develop stable cell lines expressing CK2α-HA or CK2α (V66A/H160D/I174A)-HA, it required the transfer of the coding sequence from a pBI-vector backbone containing CK2α-HA or CK2α (V66A/I174A)-HA into the pcDNA5 Flp-In vector (Invitrogen). This was achieved by treating the pBI constructs with restriction enzymes, HindIII and ApaI for 2 hours at 37°C and 25°C respectively. Constructs were verified via sequencing (Robarts Sequencing Facility). The pcDNA5 construct containing CK2α-HA was used for transfection into Flp-In U2OS cells. The pcDNA5 construct containing the CK2α (V66A/I174A)-HA double mutant served as a template for the PCR-based site-directed mutagenesis to incorporate the H160D amino-acid substitution into the pcDNA5 Flp-In expression vector. Successful incorporation of the H160D mutation was verified via sequencing (Robarts Sequencing Facility), and the pcDNA5 construct containing the CK2α (V66A/H160D/I174A)-HA triple mutant was used for transfection into Flp-In U2OS cells. (See table 3 for primers).

2.2 – Protein production and purification

GST-CK2α, GST- CK2α (H115L), GST- CK2α (H160D), GST- CK2α (V66A/I174A), GST- CK2α (V66A/H115L/I174A), and GST- CK2α (V66A/H160D/I174A) proteins were produced in BL21 *E. coli* transformed with the respective plasmid DNA and grown in 2xYT media (16g tryptone, 10g yeast extract, 5g NaCl, 1L ddH_2O, pH 7.0) at 37°C until an optical density of 0.5 at 600 nm had been reached. Protein expression was induced by the addition of 1 mM IPTG for 16 hours at 16°C followed by centrifugation (5000 X g) for 15 minutes at 4°C. Pellets were

resuspended in 30 mL of ice-cold phosphate-buffer saline (PBS) with protease inhibitors (1 mM PMSF, 7 μg/mL PepA, 20 μg/mL leupeptin, and 2.9 μg/mL aprotinin) and a homogenizer was used to lyse the cells. Triton-X100 (10%) was added to a final concentration of 1% to the total lysate volume and the suspension was tumbled for 15 minutes at 4°C. The mixture was subsequently centrifuged at 13,000 X g for 15 minutes at 4°C to clear the supernatant. The supernatant was transferred into a 50 mL falcon tube and 2 mL of freshly prepared glutathione-agarose beads (Sigma) were added, mixed, and tumbled for 1 hour at 4°C. The mixture was centrifuged at 2000 x g for 2 minutes at 4°C in order to pellet the beads containing bound proteins. The beads were resuspended in 8 mL of cold PBS and transferred into a 10 mL gravity column. Non-specifically bound proteins were washed with 10 column volumes of cold PBS with protease inhibitors. The remaining bound proteins were eluted by washing the column with elution buffer containing increasing concentrations of glutathione (2 x 1 mL fractions of 10 mM glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl pH 8.0, 1 mM DTT) followed by 5 x l mL fractions of 30 mM glutathione elution buffer (30 mM reduced glutathione, 50 mM Tris-HCl pH 8.0, 1 mM DTT)). A 20 μL sample of all eluted fractions from each purified protein was analyzed on a 12% SDS-PAGE gel that was stained with Coomassie Blue and destained with water in order to visualize proteins. Fractions containing GST- CK2α or the GST- CK2α mutants protein were combined and dialyzed into a storage buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, and 50% glycerol for future use in *in vitro* kinase assays. A comparative analysis of each purified protein can be seen in Table 3.

2.3 – Cesium-chloride plasmid purification

In order to achieve high efficiency when transfecting plasmid DNA into mammalian cells, cesium-chloride (CsCl) purification was performed to isolate super-coiled DNA species. Briefly, pcDNA5 Flp-In (Invitrogen) constructs expressing CK2α-WT, CK2α-V66A/I174A, or CK2α-V66A/H160D/I174A were transformed into DH5α cells and grown overnight (16 hours) in LB media containing 1 mM ampicillin at 37°. The bacterial cultures were subsequently centrifuged at 13,000 x g to collect a bacterial pellet and the CsCl purification was performed as previously described[101]. After completion of the CsCl purification, 1 µL of each sample was applied to a nano-drop in order to determine DNA concentration (µg/µL). Finally, 500 ng of each sample was processed on an agarose gel to visualize the quality of the DNA.

2.4 – In vitro kinase assays

2.4.1 – Assessing activity of GST-CK2α mutants

To assess the activity of GST-CK2α and the GST-CK2α mutants, I performed *in vitro* kinase assays. Bacterially expressed GST-CK2α and GST-CK2α mutants were purified as previously described and were used as the source of kinase for each reaction. Activity assays were prepared by the addition of 4 µL of 5X kinase reaction buffer (50 mM Tris buffer, pH 7.5,10 mM $MgCl_2$, [γ-^{32}P] ATP (1 µL per 100 µL of reaction buffer), with 2 µL of RRRDDDSDDD peptide (5 mg/ml, CK2 substrate), and the appropriate amount of ddH_2O in order to bring the reaction volume to 16 µL. Each reaction was performed with increasing concentrations of cold ATP (0 - 400 uM). Finally, 200 ng of GST-CK2α or one of the GST- CK2α mutants (4 µL of kinase) were added at 15-second

intervals in order to start each reaction. Samples were incubated in a water bath at 37°C for 10 minutes and 10 μL of each reaction was spotted onto P81 phosphocellulose paper. To halt the reaction and remove any remaining ^{32}P-γATP that may not have been incorporated into substrate, the spotted P81 paper was washed 4 times in 10 mL of 1% phosphoric acid solution, once in 10 mL of ethanol, and set aside to dry under a heat lamp for 5-10 minutes. Dried P81 paper was exposed to a phosphorimaging screen (Amersham Biosciences) for one hour and analyzed using a Storm phosphorimager (Storm 860 Molecular Imager) to detect the level of ^{32}P-labelled substrate bound to the P81 paper. The amount of ^{32}P incorporated onto the RRRDDDSDDD peptide is directly proportional to the catalytic activity of CK2α.

2.4.2 – Effect of GST-CK2α mutants on inhibition by CX-4945

To determine the effect of GST- CK2α mutants on the *Ki* of CX-4945, activity assays were performed. Reaction mixtures (final volume 20 μL) were prepared by the addition of 4 μL of 5X kinase reaction buffer (50 mM Tris buffer, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, plus [γ-^{32}P] ATP (1 μL per 100 μL of reaction buffer)), 2 μL of 0.1 mM RRRDDDSDDD peptide (a CK2 substrate), and either no inhibitor (DMSO), 1 nM, or 10 nM CX-4945. Each reaction was also prepared with various concentrations of ATP (0-100 μM). The appropriate amount of ddH_2O was added to bring the reaction volume up to 16 μL. To start the kinase reaction, 180-260 ng (table 3: amount of kinase/20 μL reaction in order to normalize activity) of the appropriate form of CK2 (4 μL of either GST- CK2α or one of the GST- CK2α mutants) was added to the reaction mixture at 15-second intervals in order to test each concentration of inhibitor against each

Table 3: Comparison of purified GST-CK2α and GST-CK2α mutants

Kinase (GST-CK2α)	**Concentration (mg/mL)**	**Specific activity (pMol/min/µg)**	**Kinase (ng)/20µL reaction (Normalization)**
WT	6.06	474	194
H115L	7.25	507	184
H160D	1.42	524	176
V66A/I174A	2.20	352	260
V66A/H115L/I174A	2.45	397	230
V66A/H160D/I174A	1.39	429	215

concentration of ATP. Samples were incubated in a water bath at 37°C for 10 minutes and 10 µL of each reaction was spotted onto P81 phosphocellulose paper. To terminate the reaction and remove any remaining ^{32}P-γATP that may not have been incorporated, the spotted P81 paper was washed 4 times in 10 mL of 1% phosphoric acid solution, once in 10 mL of ethanol, and set aside to dry under a heat lamp for 5-10 minutes. Dried P81 paper was exposed to a phosphorimaging screen (Amersham Biosciences) for one hour and analyzed using a Storm phosphorimager (Storm 860 Molecular Imager) to detect the level of ^{32}P-labelled substrate bound to the P81 paper. The amount of ^{32}P incorporated onto the RRRDDDSDDD peptide is directly proportional to the catalytic activity of CK2α.

2.4.3 - Recovery of CK2 activity following treatment with CX-4945

To determine the ability of the GST- CK2α mutants to restore CK2 activity following treatment with increasing concentrations of CX-4945 (final reaction concentrations of 10, 20, 30, 40, and 50 nM CX-4945), kinase activity assays were performed. Reaction mixtures (final volume 20 µL) were prepared by the addition of 4 µL of 5X kinase reaction buffer (50 mM Tris buffer, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, plus [γ-^{32}P] ATP (1 µL per 100 µL of reaction buffer)), 2 µL of 0.1 mM RRRDDDSDDD peptide (a CK2 substrate), and 10 µM cold ATP. The appropriate amount of ddH_2O was added to bring the reaction volume up to 16 µL. In a separate set of eppendorf tubes, each purified kinase was then preincubated with either no inhibitor (DMSO), or increasing concentrations of CX-4945 (5x the final reaction concentration) for 20 minutes in order to saturate the kinase with inhibitor. To start the kinase reaction,

180-260 ng of the appropriate preincubated kinase was added to the reaction mixture at 15-second intervals in order to examine the recovery of CK2 activity at each concentration of inhibitor (final volume 20 µL, therefore inhibitor concentrations diluted from 5x to 1x and are now at the final reaction concentration of 10, 20, 30, 40, and 50 nM CX-4945). Samples were incubated in a water bath at 37°C for 10 minutes and 10 µL of each reaction was spotted onto P81 phosphocellulose paper. To halt the reaction and remove any remaining ^{32}P-γATP that may not have been incorporated, the spotted P81 paper was washed 4 times in 10 mL of 1% phosphoric acid solution, once in 10 mL of ethanol, and set aside to dry under a heat lamp for 5-10 minutes. Dried P81 paper was exposed to a phosphorimaging screen (Amersham Biosciences) for one hour and analyzed using a Storm phosphorimager (Storm 860 Molecular Imager) to detect the level of ^{32}P-labelled substrate bound to the P81 paper. Samples containing CX-4945 were compared to the no inhibitor DMSO control in order to calculate the percent (%) relative activity that was restored.

2.5 – Antibodies

The monoclonal antibody directed against the HA-epitope, biotinylated 3F10 (Roche), was prepared as previously described[28]. In order to detect the endogenous and exogenous expression of CK2α simultaneously, a polyclonal antibody raised against a GST-fusion protein containing the last 51 residues in the C-terminal region of CK2α' $^{(299-350)}$ was used (BabCO, Berkley, CA). Due to the sequence similarity between CK2α and CK2α', the GST-fusion antibody can recognize and bind both CK2 isoforms, however, the signal is typically more prominent for CK2α'. Phosphospecific antibodies were used

to recognize and bind the phosphorylated species of the known CK2 substrates, EIF2β (pS 2) and CK2β (pS 2/3/4/8). Monoclonal anti-GAPDH antibody was used as an indicator of protein loading. Goat-anti-rabbit (GAR) and goat-anti-mouse (GAM) secondary antibodies conjugated to fluorophores detectable at 680 nm and 800 nm wavelengths were purchased from (LiCOR, Mandel) to enable detection on the LiCOR Odyssey infrared scanner. Similarly, monoclonal streptavidin anti-biotin (SAV) secondary antibody conjugated to fluorophore detectable at 680 nm wavelengths was purchased from (LiCOR, Mandel).

2.6 – Development of Flp-In™ U2OS cell lines

Human osteosarcoma (U2OS) cells containing the Flp-In™ integration site were generously provided by Dr. Karmella Haynes (Arizona State University). The vector (pcDNA5) that contains identical integration sites required for insertion of the gene of interest into the host cells, as well as the Flp-recombinase capable of enabling insertion were purchased (Invitrogen). Flp-In™ U2OS cell lines expressing CK2α-HA or CK2α (V66A/H160D/I174A)-HA were generated according to the Flp-In™ T-REx™ manual provided by Invitrogen[98].

2.7 – Cell culture: Maintenance, protein induction, and harvest

U2OS cells were maintained in Dulbecco's modified Eagle's medium (Corning) supplemented with 10% fetal bovine serum (FBS – Cansera) and 1% penicillin/streptomycin (Invitrogen), at 37°C in an environment also containing 5% CO_2. The cells were also maintained with 100 μg/mL hygromycin B as a selective pressure in

order to maintain the population containing the gene of interest. It is also recommended that the confluence of cells within any dish remain above 50%. Since the Flp-In™ expression system is TET-on[102], the induction of CK2α-HA and CK2α (V66A/H160D/I174A)-HA protein expression requires the addition of tetracycline. To accomplish this, cells were plated to a confluence of 70-80% 12 hours prior to the point of protein induction. Following the 12 hours, the media was aspirated, the cells were washed 4x with PBS, and the cells were replenished with fresh medium containing 1 μg/mL of tetracycline in order to induce protein expression. The cells were then left at 37°C in an atmosphere containing 5% CO_2 for 24 hours. Following expression of CK2α-HA and CK2α (V66A/H160D/I174A)-HA for 24 hours, the media was again removed, the cells were washed 4x with PBS, and the cells were replenished with fresh medium again containing 1 μg/mL of tetracycline to maintain expression, as well as 10, 20, 30, 40, and 50 μM CX-4945 to inhibit CK2. The cells were then again left at 37°C in an atmosphere containing 5% CO_2 for an additional 24 hours. Following the 24 hour incubation with increasing concentrations of CX-4945, the cells were harvested in Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP-40, filter sterilized) supplemented with various protease and phosphatase inhibitors (protease inhibitors: 1 mM PMSF, 7 μg/mL PepA, 20 μg/mL leupeptin, and 2.9 μg/mL aprotinin - phosphatase inhibitors: 1 μM okadaic acid, 1 μM microcystin, 5 mM sodium fluoride, and 1 mM sodium orthovanadate). Cell lysates were cleared by centrifugation at 2000 x g at 4°C for 20 minutes to remove cell debris. Cell lysates were then stored at -20°C. A diagram illustrating the Flp-In™ system for the expression of CK2α-HA and CK2α (V66A/H160D/I174A)-HA can be seen in Figure 5.

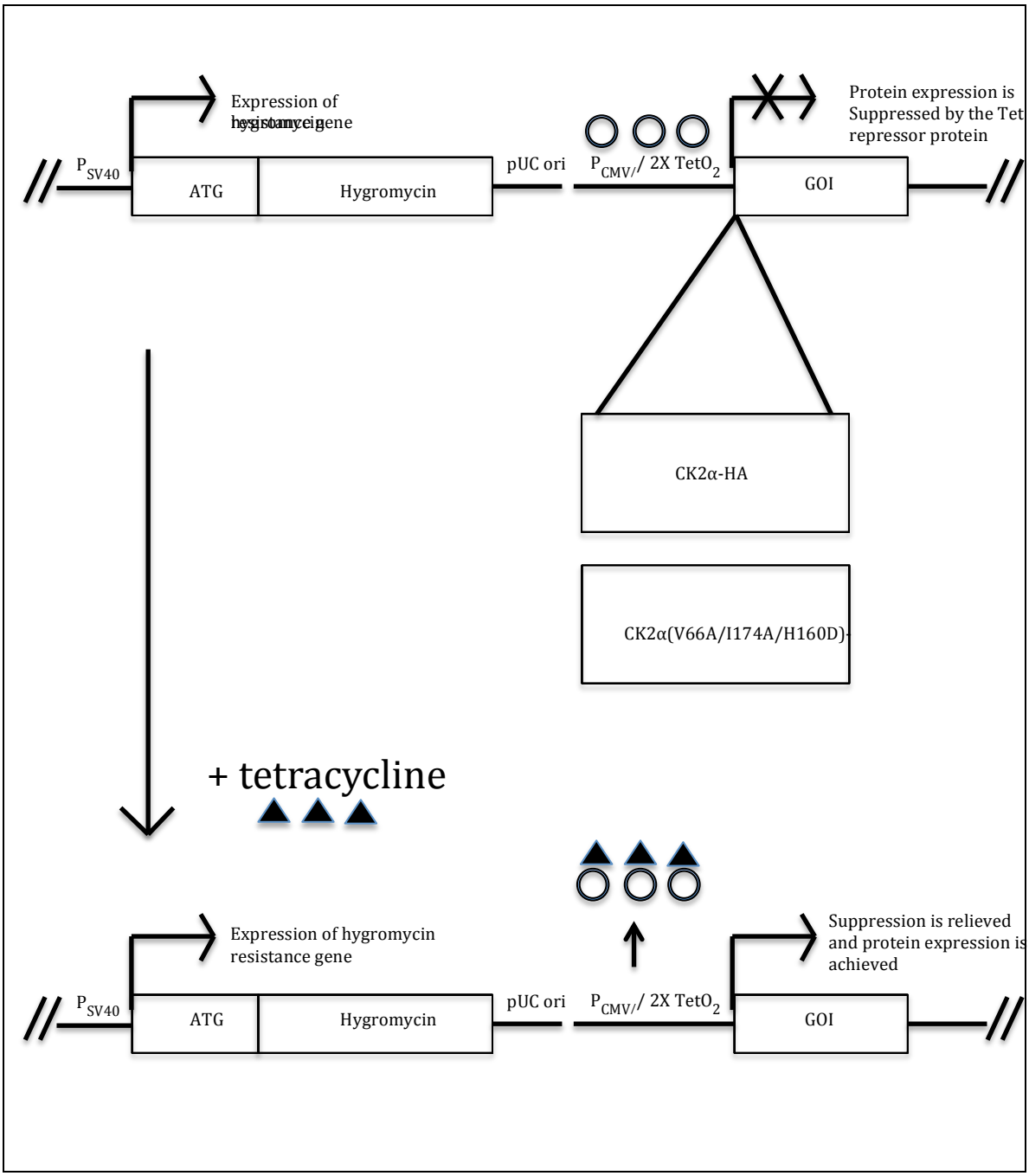
2.8 – SDS-PAGE and Western blotting

To determine protein concentration of each lysate to be processed, the BCA protein assay[103] (Pierce) was utilized. Samples were then prepared for the separation of proteins via sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis (SDS-PAGE) using the method of Laemmli[104] by adding an equal volume (relative to the volume of each sample) of 2X Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl pH 6.8, and 10% β-mercaptoethanol). Each sample was then heated at 100°C for three minutes. Gels were run in SDS-PAGE buffer (192 mM glycine, 25 mM Tris-base pH 8.0, 0.1% SDS) at 120V for 1.5 hours using the mini-protean II electrophoresis cassettes (Biorad). A BLUeye prestained protein ladder (FroggaBio) was utilized in order to provide a reference of molecular weight.

Following completion of SDS-PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher) using a wet mini trans-blot® electrophoretic transfer cell[105] (Biorad) for 1 hour at 100V in blotting buffer (20% methanol, 10 mM Tris-base pH 8.6, 767 mM glycine, and ddH_2O). Membranes were then blocked with LI-COR blocking solution (LI-COR) for 1 hour and subsequently washed three times with tris buffered saline (200 mM Tris-HCl. 500 mM NaCl, pH 7.5) (TBS) with 0.1% Tween 20 (TBST). Expression of CK2α-HA and CK2α (V66A/H160D/I174A)-HA were detected by incubating the membranes with a biotinylated anti-HA antibody (3F10) diluted 1:1000 into 3% bovine-serum albumin (BSA)/PBST for 1 hour at room temperature. The membranes were subsequently washed three times with PBST and then incubated with a biotin sensitive-streptavidin secondary antibody conjugated to a fluorophore capable of being detected at 680 nm (SAV680) for

1 hour at room temperature. To detect the CK2-dependent phosphorylation of the substrate, EIF2β, membranes were incubated with an antibody (EIF2β P S2) capable of recognizing and binding the phosphorylated serine 2 residue in the translation factor EIF2β. This antibody was diluted 1:10000 into 3% BSA/TBST and membranes were incubated for 1 hour and room temperature. Next, the membranes were washed three times with TBST and incubated with a goat-anti-rabbit secondary antibody conjugated to a fluorophore capable of being detected at 800 nm (GAR800) for 1 hour at room temperature. The antibody (CK2β (P S2/S3/S4/S8)) sensitive to the CK2-dependent phosphorylation of CK2β at up to 4 different serine residues (S2, S3, S4, and S8) was diluted 1:10000 into 3% BSA/TBST and membranes were incubated for 1 hour at room temperature. Membranes were washed three times with TBST and then were incubated with the GAR800 secondary antibody. An anti-GAPDH antibody was used to specifically serve as a protein loading control, and was diluted 1:2000 into 3% BSA/TBST and membranes were incubated for 1 hour and room temperature. Membranes were subsequently washed three times with TBST and then subjected to incubation with the GAR800 secondary antibody. Immunocomplexes were visualized using the LiCOR odyssey infrared imaging system (LiCOR).

Figure 5. Illustration of tetracycline-inducible CK2α-HA and CK2α (V66A/H160D/I174A)-HA protein expression in Flp-In U2OS cells
Adapted from the Flp-In™ T-REx™ manual[98] is an illustration of the tetracycline-inducible protein expression of CK2α-HA and CK2α (V66A/H160D/I174A)-HA. A gene encoding resistance for the selective marker, hygromycin b, is located downstream of a SV40 promoter that enables selection to maintain the population of cells containing the gene of interest (CK2α-HA or CK2α (V66A/H160D/I174A)-HA). In the absence of tetracycline (Tet), a Tet-repressor protein (TetR – represented by open black circles) will be bound to the Tet-operator (TetO) sequence, thereby suppressing the expression of GOI (CK2α-HA or CK2α (V66A/H160D/I174A)-HA). Following the addition of the appropriate concentration of tetracycline (represented by the black triangles), the Tet-repressor protein will become fully bound and the suppression of CK2α-HA or CK2α (V66A/H160D/I174A)-HA protein expression will be relieved leading to the expression of the respective proteins.


Expression of hygromycin gene
Protein expression is Suppressed by the Tet repressor protein
P_{SV40}
ATG
Hygromycin
pUC ori
P_{CMV}/ 2X $TetO_2$
GOI
CK2α-HA
CK2α(V66A/I174A/H160D)-
+ tetracycline
Expression of hygromycin resistance gene
Suppression is relieved and protein expression is achieved
P_{SV40}
ATG
Hygromycin
pUC ori
P_{CMV}/ 2X $TetO_2$
GOI

2.9 – Immunofluorescence

Cells were grown in 6-well dishes on coverslips in DMEM medium containing 1 μg/mL of tetracycline at 37°C in an atmosphere containing 5% CO_2 for 24 hours to induce CK2α-HA and CK2α (V66A/H160D/I174A)-HA protein expression. Following the 24-hour incubation, medium was aspirated and the cells were fixed in a 10% formalin solution in PBS for 15 minutes. The membranes of the cells were then permeabilized for 10 minutes with 0.1% Triton-X 100 in PBS and non-specific binding was blocked using 3% BSA/PBS for 1 hour at room temperature. To detect the expression of CK2α-HA and CK2α (V66A/H160D/I174A)-HA, the coverslips were incubated with a monoclonal anti-HA primary antibody directly conjugated to a FITC-Alexa488 fluorophore (Invitrogen) diluted 1:1000 in 3% BSA/PBS for 16-18 hours at 4°C. Cell nuclei were stained with 1 μg/mL Hoechst 33258 (Molecular Probes) for 1 minute. Coverslips were then mounted onto glass slides using Antifade Mounting Medium (Thermo Scientific) and protein expression was visually detected under a Nikon Eclipse fluorescent microscope. Coverslips were washed thoroughly (4 or 5 times) with PBS between each step.

3. Results

3.1 – *In vitro* kinase assays

3.1.1 Examining the effect of CK2α mutations on activity

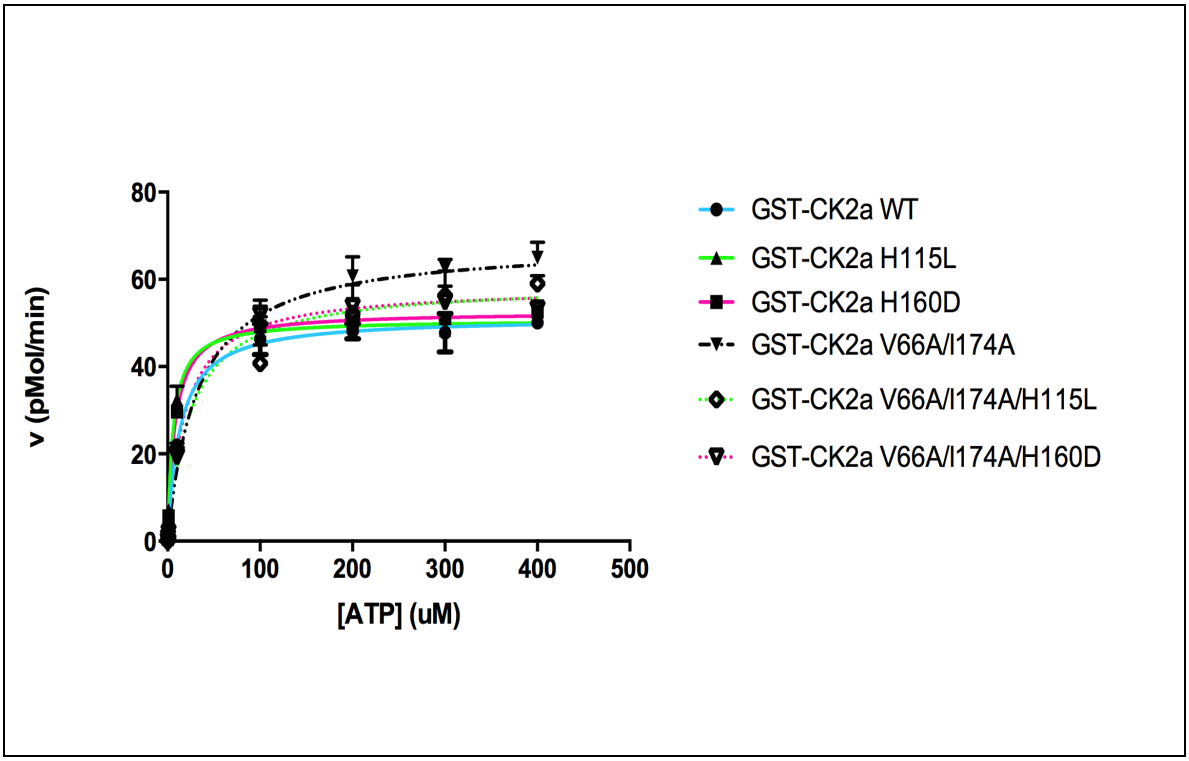
Site-directed mutagenesis via PCR was used to generate the N-terminally GST-tagged CK2α mutants containing amino acid substitutions H115L or H160D alone, as well as in combination with the previously described double mutant, CK2α-V66A/I174A[77,106,107]. To start, I wanted to determine whether these newly incorporated mutations into the active site of protein kinase CK2α influenced its activity, and to address this question, *in vitro* kinase assays were performed. From previous studies, the Km of ATP for the wild type isoform of CK2α was determined to be approximately 12 μM[108]. The kinase assays revealed that the activities of CK2α-WT, as well as the CK2α-H115L and CK2α-H160D single mutants, were not dramatically different from these reports, as I calculated a Km of 13.3 μM, 9.9 μM, and 7.8 μM respectively (Figure 6, compare CK2α-WT, CK2α-H115L, and CK2α-H160D). In contrast, it was clear that all mutants of CK2α bearing the V66A and I174A amino-acid substitutions displayed a reduction in their rate of activity (Figure 6, compare CK2α-V66A/I174, CK2α-V66A/H115L/I174A, and CK2α-V66A/H160D/I174A). Following non-linear regression, it was apparent that there was an increase in the Km, as I calculated Km values of 31.9 μM, 25.1 μM, and 18.6 μM respectively. Most importantly, it was clear that all GST-tagged active-site mutants of CK2α retained their ability to phosphorylate an optimized CK2 peptide[26,109] (RRRDDDSDDD), and therefore no mutations completely abolished activity. Collectively, these data suggest that CK2α can tolerate the introduction of

additional mutations (H115L or H160D) alone or in combination with V66A/I174A, and therefore enabling the investigation of their effects on the sensitivity of CK2 to CX-4945.

3.1.2 – Determining the effect of CK2α mutations on the Ki of CX-4945

Following our investigation into the effect of amino acid substitutions (H115L, H160D, V66A/I174A, V66A/H115L/I174A, and V66A/H160D/I174A) on the activity of CK2α, the next step was to perform an evaluation on their ability to effect the inhibition of CK2 by CX-4945. To achieve this, I continued the characterization using *in vitro* kinase assays to test various concentrations of ATP against increasing concentrations of CX-4945 (1-10 nM) (Figure 7A-F). To quantify this effect, non-linear regression was performed to calculate the constant (*Ki*) for the kinase-inhibitor complex (Figure 7G). I detected a tight binding model for the GST-tagged CK2α-WT isoform, as I calculated a *Ki* of 0.3 nM (Figure 7A). In terms of our CK2α isoforms bearing amino acid substitution, I observed that CK2α (H115L)was the most sensitive to inhibition by CX-4945, with a corresponding *Ki* value of 2.1 nM, followed by the H160D single mutant with a *Ki* value of 2.7 nM (Compare Figure 7B and 7C). This result was not surprising, as residues V66 and I174 were not altered in those isoforms. These observations are pertinent since structural studies have highlighted V66 and I174 as key hydrophobic residues that not only form the small binding pocket of CK2α, but are also critical in the binding of small molecule inhibitors[25,77,92]. In comparison, I was able to detect a notable increase in the Ki with the CK2α-V66A/I174A mutant, calculating a *Ki* of 3.9 nM (Figure 7D). Next, amino acid substitutions H115L and H160D

Figure 6. Rate (v) (pMol/min) of phosphorylation at various concentrations of ATP Phosphorylation studies using [γ-^{32}P] ATP and optimal CK2 peptide RRRDDDSDDD. The different forms of CK2 are labeled as follows: GST- CK2α (Blue), GST- CK2α H115L (Green), GST- CK2α H160D (Red), GST- CK2α V66A/I174A (dotted Black), GST- CK2α V66A/H115L/I174A (dotted Green), and GST- CK2α V66A/H160D/I174A (dotted Red). Error bars represent the variation that can be observed between replicate assays. The experiment was performed in triplicate (n=3) and each reaction was assayed in triplicate


v (pMol/min)
80
60
40
20
0
0
100
200
300
400
500
[ATP] (uM)
GST-CK2a WT
GST-CK2a H115L
GST-CK2a H160D
GST-CK2a V66A/I174A
GST-CK2a V66A/I174A/H115L
GST-CK2a V66A/I174A/H160D

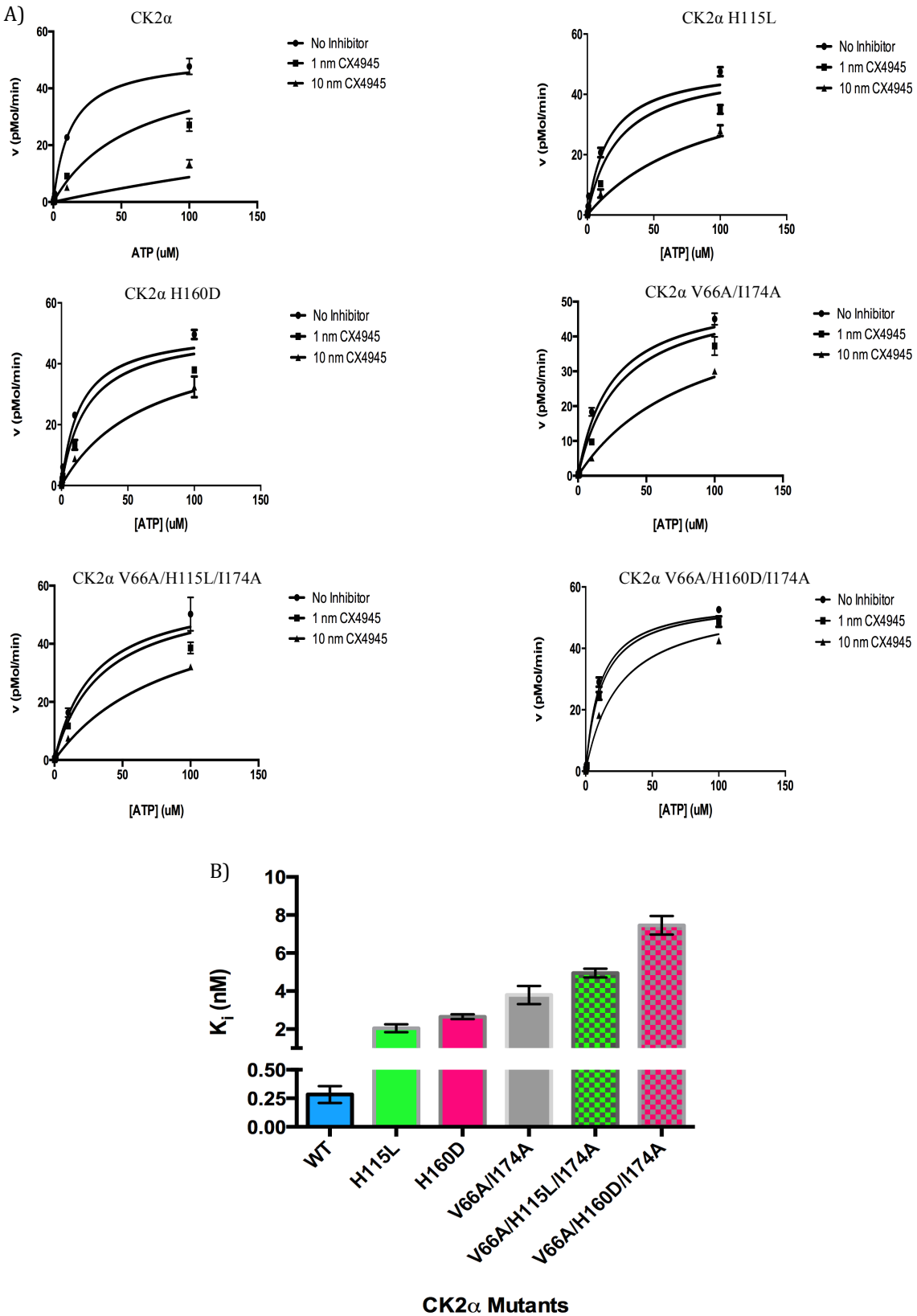
were combined with the previously characterized double mutant (V66A/I174A) to generate inhibitor-refractory triple mutants of CK2α. I observed the greatest increase in the ability to overcome inhibition by CX-4945 with the CK2α triple mutant (V66A/H160D/I174A), as I calculated a K_i of 7.4 nM (Compare Figure 7D and 7F). Pairing the H115L mutation with V66A and I174A (CK2α-V66A/H115L/I174A) also led to an increase in *Ki* of CX-4945 (*Ki*= 4.8).

3.1.3 – Assessing the ability of CK2α mutants to overcome inhibition by CX-4945

I continued the *in vitro* evaluation of the CK2α mutants by assessing the ability of the CK2α mutants to overcome inhibition by CX-4945 utilizing an enzyme reactivation strategy as previously described[110]. The expectation was that this strategy would enable the effective inhibition of wild type CK2α in order to improve the likelihood that meaningful difference would be detected between wild type and mutants of CK2α.

In fact, measuring kinase activity following the preincubation of wild type CK2α with CX-4945 demonstrated that its activity was effectively inhibited (Figure 8 – GST-CK2α). In comparison to the DMSO control, which represents the activity of the kinase in the absence of inhibitor, I observed a 10, 5, 5, 3, and 3 percent recovery (%) in wild type CK2 activity following treatment with 10, 20, 30, 40, and 50 nM CX-4945 respectively. Subsequently, I wanted to interrogate each mutant of CK2α to identify the mutant that best resists the effects by CX-4945. As a result of our *in vitro* studies investigating the changes in Ki following treatment with CX-4945, I hypothesized

Figure 7. **Effect of CK2α mutants on the inhibition of CK2 by CX-4945**
A) Phosphorylation studies using [γ-^{32}P] ATP and optimal CK2 peptide RRRDDDSDDD were performed to assess the ability of the CK2α mutants to overcome inhibition by CX-4945. B) The effect of the various mutants on the inhibition of CK2 was quantified using non-linear regression (Graphpad Prism 6) to determine the inhibitor constant (*Ki*). Error bars represent the variation that can be observed between replicate assays. The experiment was performed in triplicate (n=3) and each reaction was assayed in triplicate.


A)
CK2α
CK2α H115L
CK2α H160D
CK2α V66A/I174A
CK2α V66A/H115L/I174A
CK2α V66A/H160D/I174A
No Inhibitor
1 nm CX4945
10 nm CX4945
v (pMol/min)
ATP (uM)
[ATP] (uM)
B)
Ki (nM)
WT
H115L
H160D
V66A/I174A
V66A/H115L/I174A
V66A/H160D/I174A
CK2α Mutants

that similar trends would be observed, indicating that the single mutants are inferior to the double mutant, and the triple mutants are superior to the double mutant. In fact, our observations indicate that the CK2α-H115L single mutant was the least capable of overcoming inhibition by CX-4945, as I calculated a 15, 7, 6, 4, and 4 percent recovery (%) relative to its DMSO control. The performance of the CK2α-H160D single mutant was only marginally superior, as I observed a percent recovery (%) of 25, 9, 7, 6, and 5. Assessment of the CK2α-V66A/I174A double mutant confirmed that it could outperform the single mutants, as I attained a 31, 16, 13, 10, and 7 percent reactivation (%) of CK2α activity. In comparison, combining the single mutations with the CK2α-V66A/I174A double mutant to generate the inhibitor-refractory triple mutants, CK2α-V66A/H115L/I174A and CK2α-V66A/H160D/I174A, I was able to increase the recovery of CK2α activity beyond the previously established double mutant of CK2α. More specifically, the CK2α-V66A/H160D/I174A mutant accomplished the greatest restoration of CK2α activity, with a percent reactivation (%) of 50, 29, 24, 16, and 17 following treatment with 10, 20, 30, 40, and 50 nM of CX-4945 respectively. The CK2α mutant bearing V66A, H115L, and I174A amino acid substitutions appeared to be indistinguishable from the double mutant, with a 31, 20, 14, 9, and 7 percent reactivation (%) of CK2α activity.

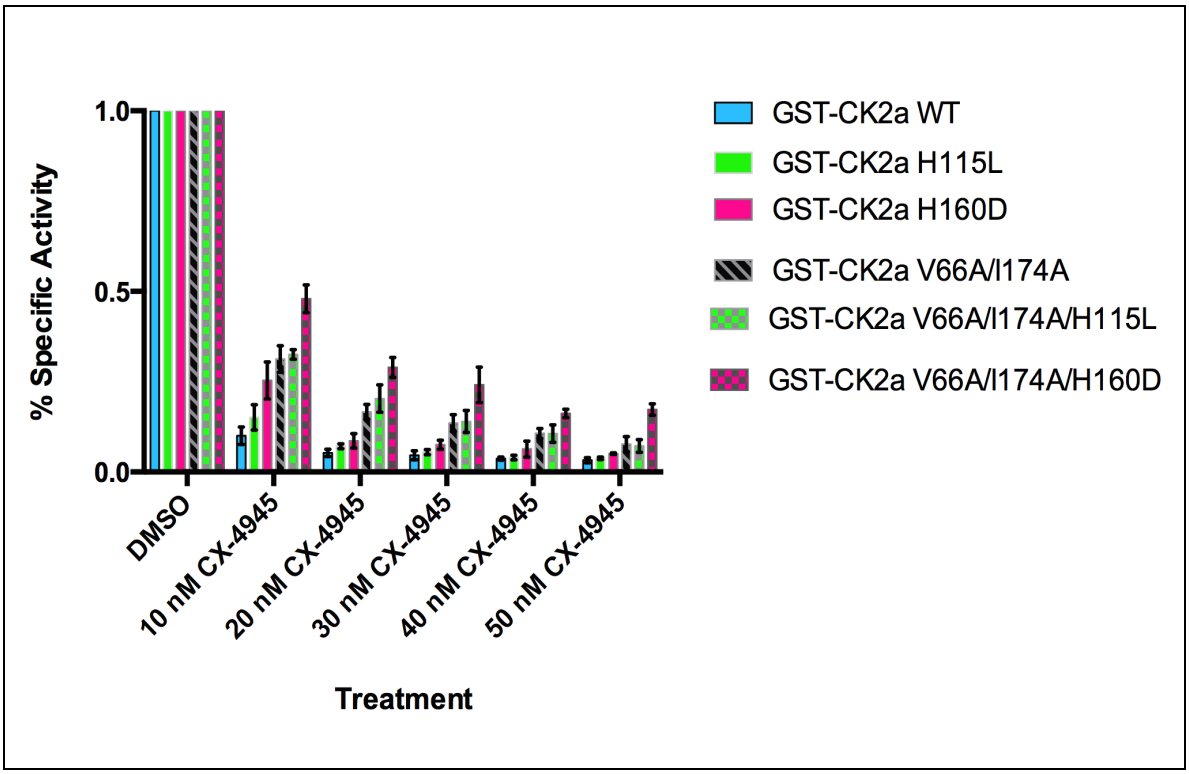
Collectively, these data have indicated that manipulation of the active site of protein kinase CK2α has generated mutants with reduced sensitivity to inhibition by the CK2 inhibitor, CX-4945. In particular, the mutant that was least sensitive to inhibition contained a new combination of amino-acid substitutions (V66A/H160D/I174A) that has not been previously examined.. To extend these findings beyond studies using purified

components, my next objective is to develop inducible cell lines expressing CK2α-WT, , or CK2α-V66A/H160D/I174A.

3.2 Characterizing the induction of CK2α-HA and CK2α (V66A/H160D/I174A)-HA in Flp-In U2OS cell lines

In order to investigate CK2 in cells, I generated tetracycline-inducible stable cell lines expressing the wild type (CK2α-HA), as well as a triple mutant (CK2α (V66A/H160D/I174A)-HA) form of the CK2α catalytic subunit without expression of the regulatory CK2β subunit. Although previous studies in the Litchfield lab have illustrated that optimal expression and stability of CK2α is achieved in the presence of the regulatory subunit, CK2β, I utilized Flp-In™ T-REx™ expression technology, which currently does not support a bidirectional system as previously described[28,98,111]. The advantage of Flp-In™ technology is its targeted approach, as Flp-In™ host cells and donor constructs contain a specific integration site such that the gene of interest can only be incorporated into a specific location within the genome[98]. To achieve titratable expression, CK2α (wild-type and triple mutant) was transfected into Flp-In™ U20S cells (generously provided by Dr. Karmella Haynes) that already contain an integration site and stably express a tetracycline transactivator fusion protein. The epitope tag (HA) fused to the C-terminus of each CK2α construct (Figure 5) allowed for the detection of exogenously expressed CK2α in the presence of endogenous CK2. Because the generation of stable cells expressing protein kinase CK2α in the Flp-In™ T-REx™ background had not previously

Figure 8. **Measuring the ability of CK2α mutants to overcome inhibition by CX-4945.** An enzyme reactivation strategy was utilized in order to assess the ability of the CK2α mutants to overcome inhibition by CX-4945. The different forms of CK2 are indicated in the legend within the Figure. Error bars represent the variation that can be observed between replicate assays. The experiment was performed in triplicate (n=3) and each reaction was also assayed in triplicate.


% Specific Activity
1.0
0.5
0.0
DMSO
10 nM CX-4945
20 nM CX-4945
30 nM CX-4945
40 nM CX-4945
50 nM CX-4945
Treatment
GST-CK2a WT
GST-CK2a H115L
GST-CK2a H160D
GST-CK2a V66A/I174A
GST-CK2a V66A/I174A/H115L
GST-CK2a V66A/I174A/H160D

been accomplished, I began with a detailed characterization to ensure I could achieve robust and equal expression of CK2α across cells lines.

To start, each cell line (CK2α-HA and CK2α (V66A/H160D/I174A)-HA) was cultured in the presence of increasing concentrations of tetracycline for 24 hours to examine modulation of CK2α expression. As demonstrated in Fig. 9 (A-B), western blot analysis using a HA-3F10 (anti-HA) antibody yielded the presence of three distinct bands. Of particular importance, is the band located at the expected molecular mass (48 kDa) for HA-tagged CK2α, as this band increased in size with increasing concentration of tetracycline[111]. The remaining two bands with molecular weights of approximately 75 kDa and 125 kDa were deemed non-specific. I detected basal levels of expression in the absence of tetracycline in both cell lines, indicating a degree of leakiness within the pCMV promoter controlling expression. Furthermore, expression of wild type and triple mutant CK2α in Flp-In U20S cells was comparable to basal levels when cultured in the presence of tetracycline concentrations up to 10 ng/mL. Following the addition of 100, 1000 or 1500 ng/mL of tetracycline, a large increase in the level of expression detected with 3F10 anti-HA antibodies can be observed. In particular, culturing cells in the presence of 1000 or 1500 ng/mL of tetracycline achieved the highest level of expression, as a dramatic difference can be observed between 100 ng/mL and 1000 or 1500 ng/mL. In contrast, no dramatic difference was observed between the levels of expression attained with 1000 or 1500 ng/mL of tetracycline (Appendix, Figure 15). Importantly, it is also worth noting that no dramatic difference in expression between cell lines expressing CK2α-HA or CK2α (V66A/H160D/I174A)-HA were observed.

Subsequently, I wanted to examine the time course of induction following the addition of tetracycline to determine when expression is detectable and reaches saturation. To accomplish this, expression was induced with 1000 ng/mL of tetracycline, and cells were harvested following 3, 6, 12, 24, and 48-hours of treatment. Under these conditions, expression of both cell lines was detectable as early as 3 hours and reached saturation at 24 hours (Figure 9. C-D). More specifically, I was able to reach a modest level of induction following 3 and 6 hours in the presence of 1000 ng/mL tetracycline, generating approximately a 5 and 10-fold increase in protein expression respectively, relative to basal levels in the absence of tetracycline. As I extended the exposure of the cell lines to 1000 ng/mL of tetracycline for 12, 24, and 48 hours, I observed an increase in the level of protein expression. In comparison to the cell lines in the absence of tetracycline, I calculated a 15-fold increase in protein expression following 12, 24 and 48 hours. Again, no dramatic difference was observed between cell lines, suggesting equal ability to respond to the presence of tetracycline in a time-dependent manner (Appendix, Figure 15 for quantifications).

Next, I was interested in comparing the expression levels of the exogenous relative to endogenous CK2α in our Flp-In U2OS cell lines. To detect both exogenously and endogenously expressed CK2α simultaneously, I used an antibody raised against the last 51 residues in the C-terminus of CK2α'$^{299\text{-}350}$. Due to the 90% sequence similarity existing between CK2α and CK2α', I am also able to detect the presence of CK2α

Figure 9. **Inducible expression of CK2α in Flp-In U2OS cell lines**
A-B) Flp-In U2OS cells (expressing CK2α-HA (**A**) or CK2α (V66A/H160D/I174A)-HA (**B**)) were cultured in the absence (0 ng/mL) or presence of increasing concentrations of tetracycline (1-1500 ng/mL) for 24 hours. C-D) Flp-In U2OS cells (expressing CK2α-HA (**C**) or CK2α (V66A/H160D/I174A)-HA (**D**)) were cultured in the presence of 1 μg/mL of tetracycline for increasing time intervals (0-48 hours). Protein expression was examined using Western blots and an Anti-HA antibody. The positions of the HA-tagged CK2α subunits are indicated and their respective molecular weights are marked. Nonspecific protein species that are detectable using the Anti-HA antibody are also indicated. An antibody used to detect the presence of GAPDH served as an indicator of protein loading (n=2).

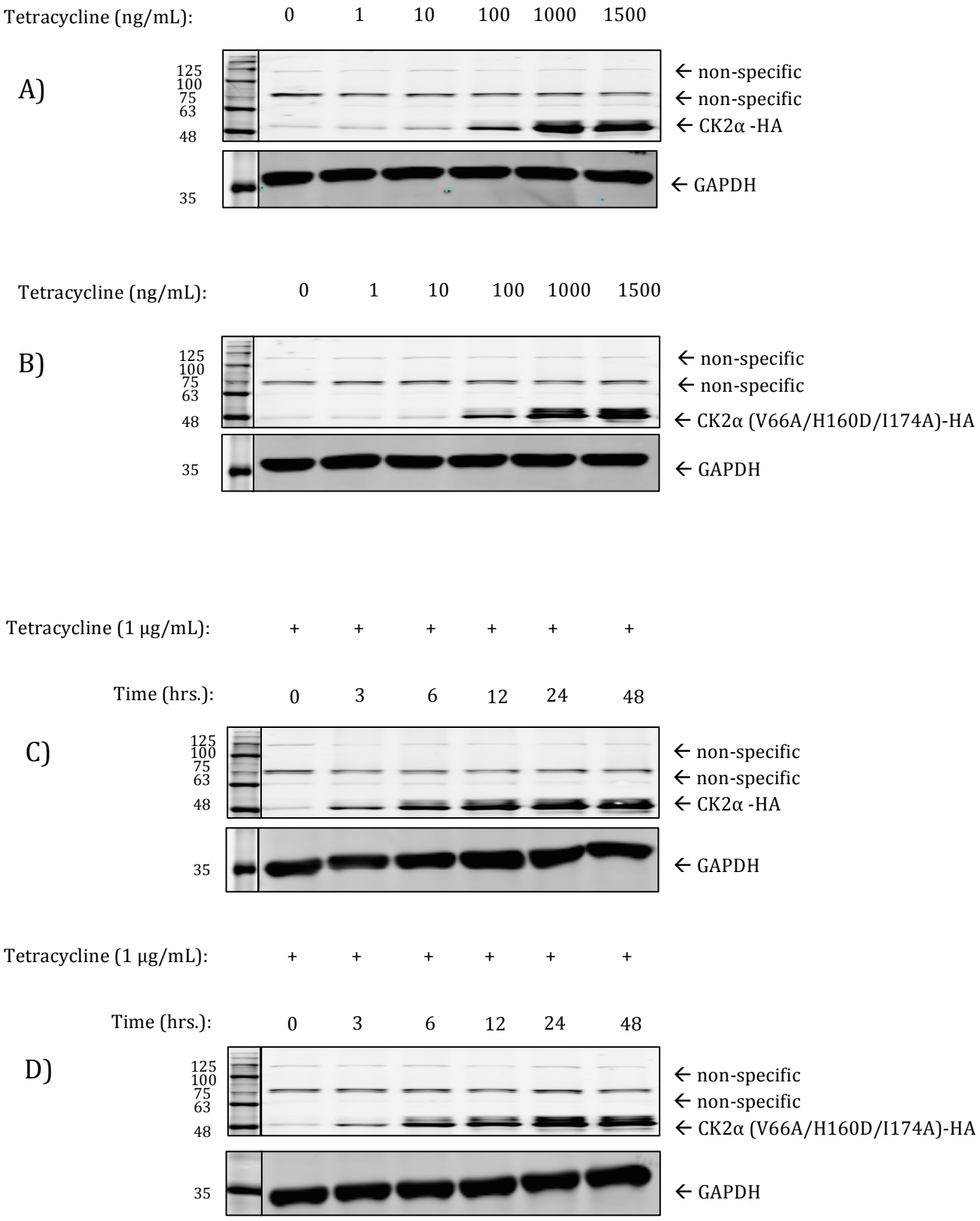
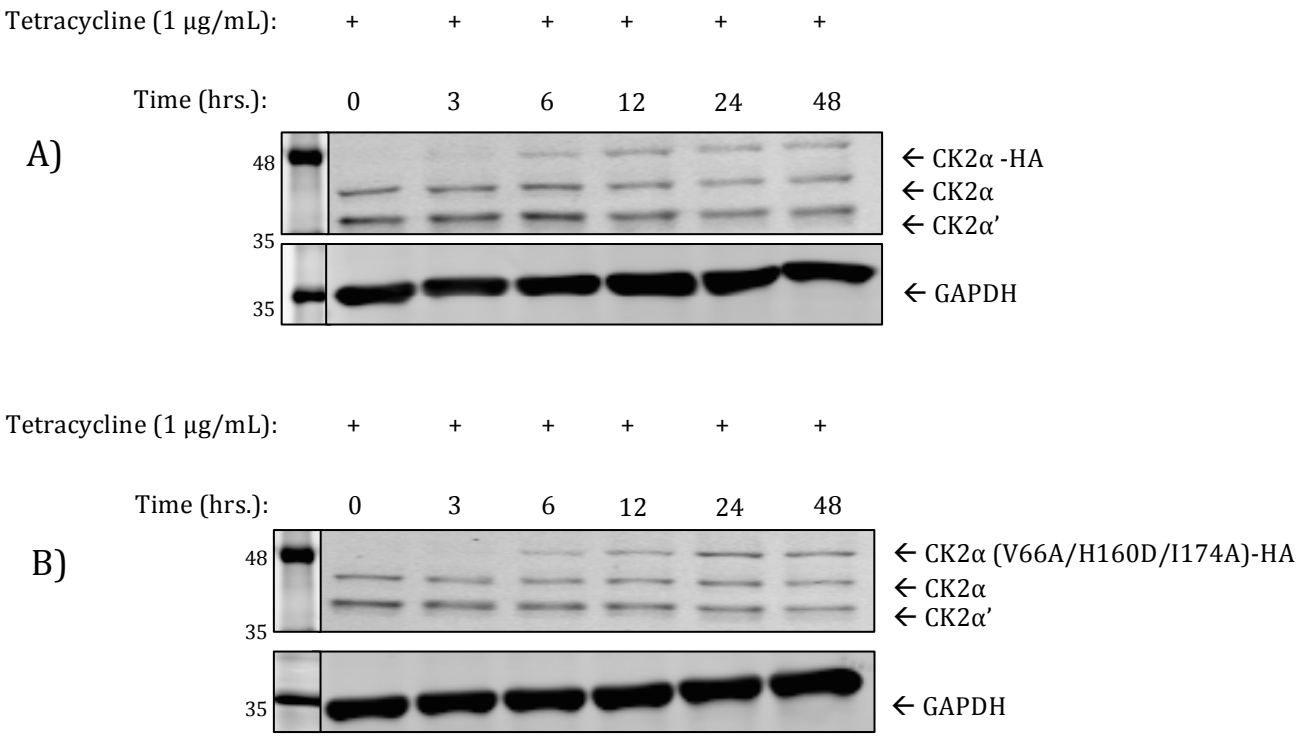

Tetracycline (ng/mL): 0 1 10 100 1000 1500
A)
125
100
75
63
48
35
← non-specific
← non-specific
← CK2α -HA
← GAPDH
Tetracycline (ng/mL): 0 1 10 100 1000 1500
B)
125
100
75
63
48
35
← non-specific
← non-specific
← CK2α (V66A/H160D/I174A)-HA
← GAPDH
Tetracycline (1 µg/mL): + + + + + +
Time (hrs.): 0 3 6 12 24 48
C)
125
100
75
63
48
35
← non-specific
← non-specific
← CK2α -HA
← GAPDH
Tetracycline (1 µg/mL): + + + + + +
Time (hrs.): 0 3 6 12 24 48
D)
125
100
75
63
48
35
← non-specific
← non-specific
← CK2α (V66A/H160D/I174A)-HA
← GAPDH

Figure 10. **Comparing expression levels of endogenous and exogenous CK2α in Flp-In U2OS cell lines**. Flp-In U2OS cell (expressing CK2α-HA (**A**) or CK2α (V66A/H160D/I174A)-HA (**B**)) were cultured in the presence of 1μg/mL of tetracycline and harvested at increasing time intervals (0-48 hours). Protein expression was assessed using Western blots and a GST-fusion antibody capable of detecting endogenous and exogenous CK2α species simultaneously. The positions of the exogenous HA-tagged and endogenous CK2α subunits have been designated and their molecular weights have been marked. An antibody used to detect the presence of GAPDH served as an indicator of protein loading (n=2).


Tetracycline (1 µg/mL): + + + + + +
Time (hrs.): 0 3 6 12 24 48
A)
48
35
35
← CK2α -HA
← CK2α
← CK2α'
← GAPDH
Tetracycline (1 µg/mL): + + + + + +
Time (hrs.): 0 3 6 12 24 48
B)
48
35
35
← CK2α (V66A/H160D/I174A)-HA
← CK2α
← CK2α'
← GAPDH

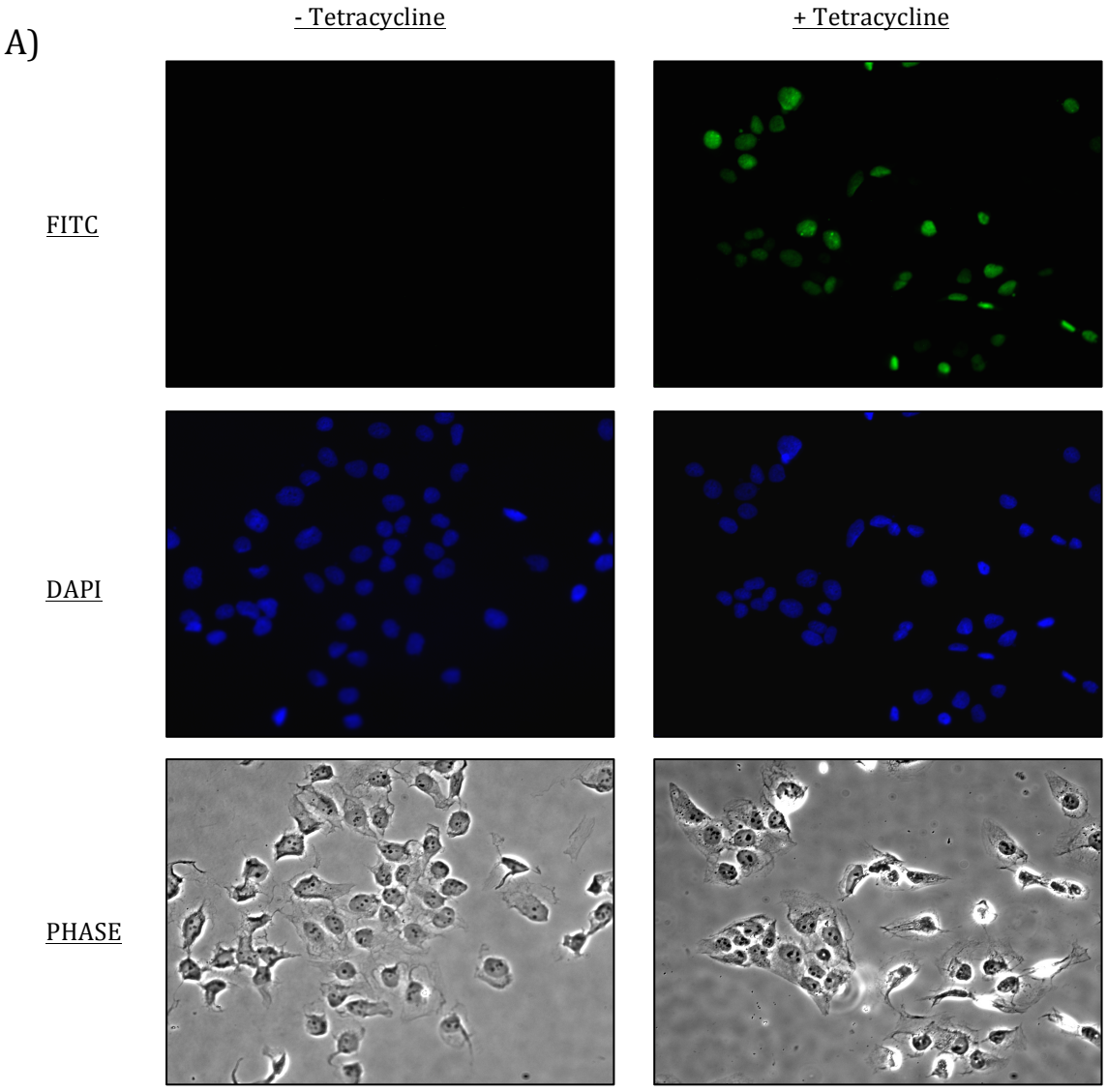
As demonstrated in Figure 10 (A-B), both cell lines are capable of achieving levels of exogenous expression below or equal, but not greater than, the endogenous levels of CK2α depending on the length of induction with 1000 ng/mL of tetracycline. More specifically, for both cell lines I detected levels of exogenously expressed CK2α below those of the endogenous CK2α following 6 and 12 hours of induction. In comparison, I observed that similar proportions (1:1) of exogenous to endogenous CK2α when cells were induced in the presence of 1000 ng/mL of tetracycline for 24 or 48 hours could be achieved.

Lastly, I wanted to determine if the majority of the population of cells were expressing the genes of interest (CK2α-HA) and (CK2α (V66A/H160D/I174A)-HA). To validate this, I performed (with help from Edward Cruise) immunofluorescence utilizing an antibody that could recognize and bind to the HA-epitope located on the C-terminus of the wild type (CK2α-HA) and triple mutant (CK2α (V66A/H160D/I174A)-HA) kinase, which was directly conjugated to a FITC-labeled secondary antibody for visual detection. As shown in Figure 11 (A-B), Flp-In U2OS cell lines demonstrate a relatively high homogeneity for the expression of both wild type and triple mutant CK2α. Additionally, it was apparent that the majority of cells stained with the FITC-conjugated primary antibody are also present with the Hoechst 33342 nuclear stain, suggesting that the majority of cells have incorporated the gene of interest. It was apparent however, that the intensity of the immunofluorescence varied from cell to cell, although it is possible that this is a product of the synchronicity of the cells as well as the amount of tetracycline that is transported into each cells.

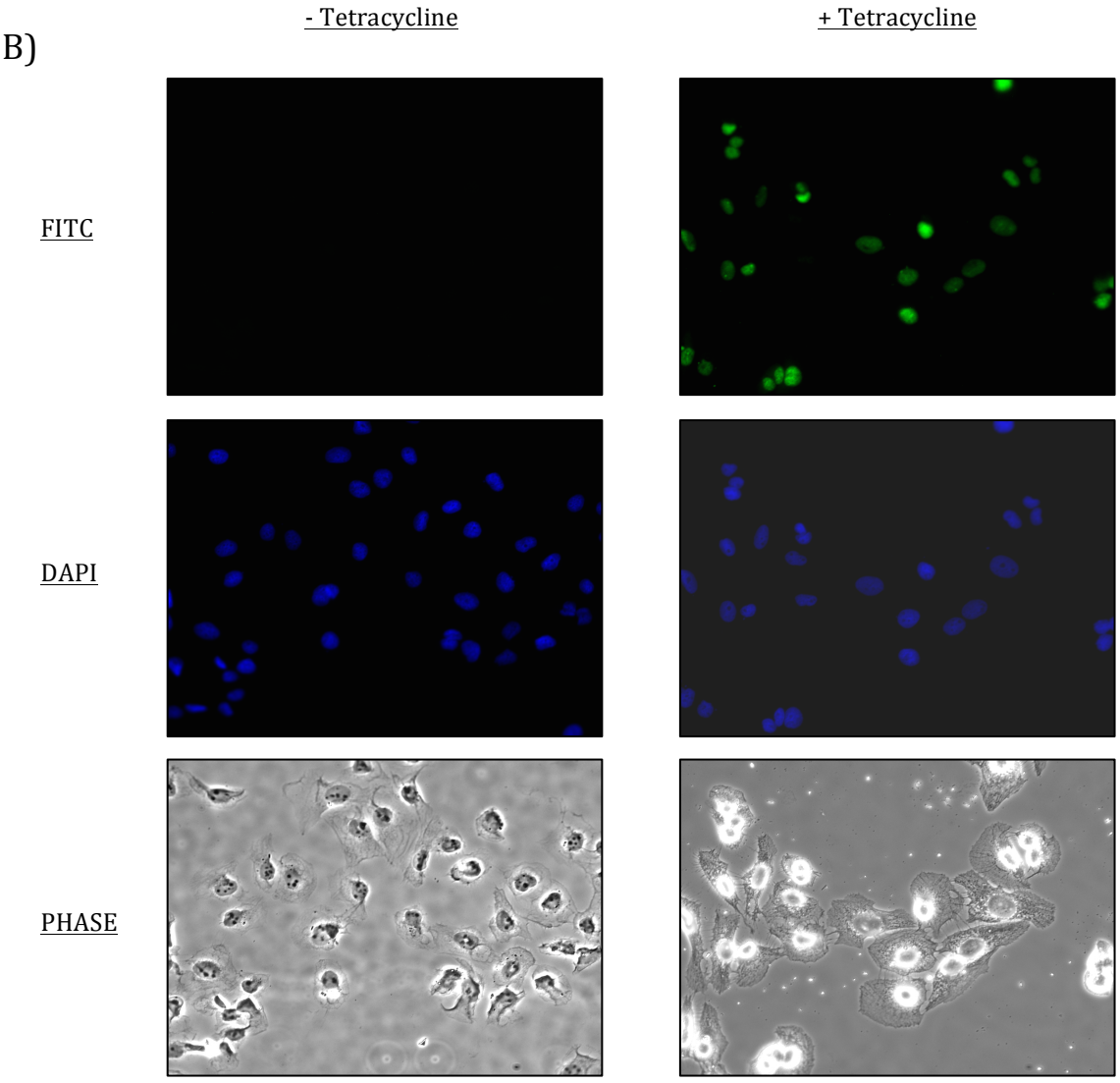
Altogether, dose response and time-course evaluations have served to demonstrate that I have successfully created stable cell-lines with the rapid, abundant, and tightly regulated expression of wild type and triple mutant CK2α (CK2α-HA and CK2α (V66A/H160D/I174A)-HA). Importantly, similar levels of endogenous and exogenous CK2α within the Flp-In U2OS can be achieved. It was also illustrated that I have generated cell lines with a large proportion of cells expressing CK2α-HA or CK2α (V66A/H160D/I174A)-HA. Therefore, because I have identified the conditions at which I can achieve the optimal expression of CK2α-HA and CK2α (V66A/H160D/I174A)-HA, I can now proceed to investigating the effect of their expression on the ability of CX-4945 to inhibit CK2. However, I first need to examine the conditions at which the inhibition of endogenous CK2 within our Flp-In U2OS cell lines can be detected.

Figure 11. **Indirect immunofluorescent detection of expressed CK2α-HA and CK2α (V66A/H160D/I174A)-HA in Flp-In U2OS cell lines**. Flp-In U2OS cells (expressing CK2α-HA (**A**) or CK2α (V66A/H160D/I174A)-HA (**B**)) were cultured in the absence (-) or presence (+) of 1 μg/mL of tetracycline for 24 hours. The cells were then fixed and stained with a FITC-conjugated Anti-HA monoclonal antibody as previously described in the materials and methods. Cells were also stained with a Hoechst 33258 DAPI stain in order to visualize cell nuclei. A phase contrast image has also been included. Identical fields were imaged in the absence (-) or presence (+) of tetracycline for both cell lines (**A-B**)(n=3).

A)



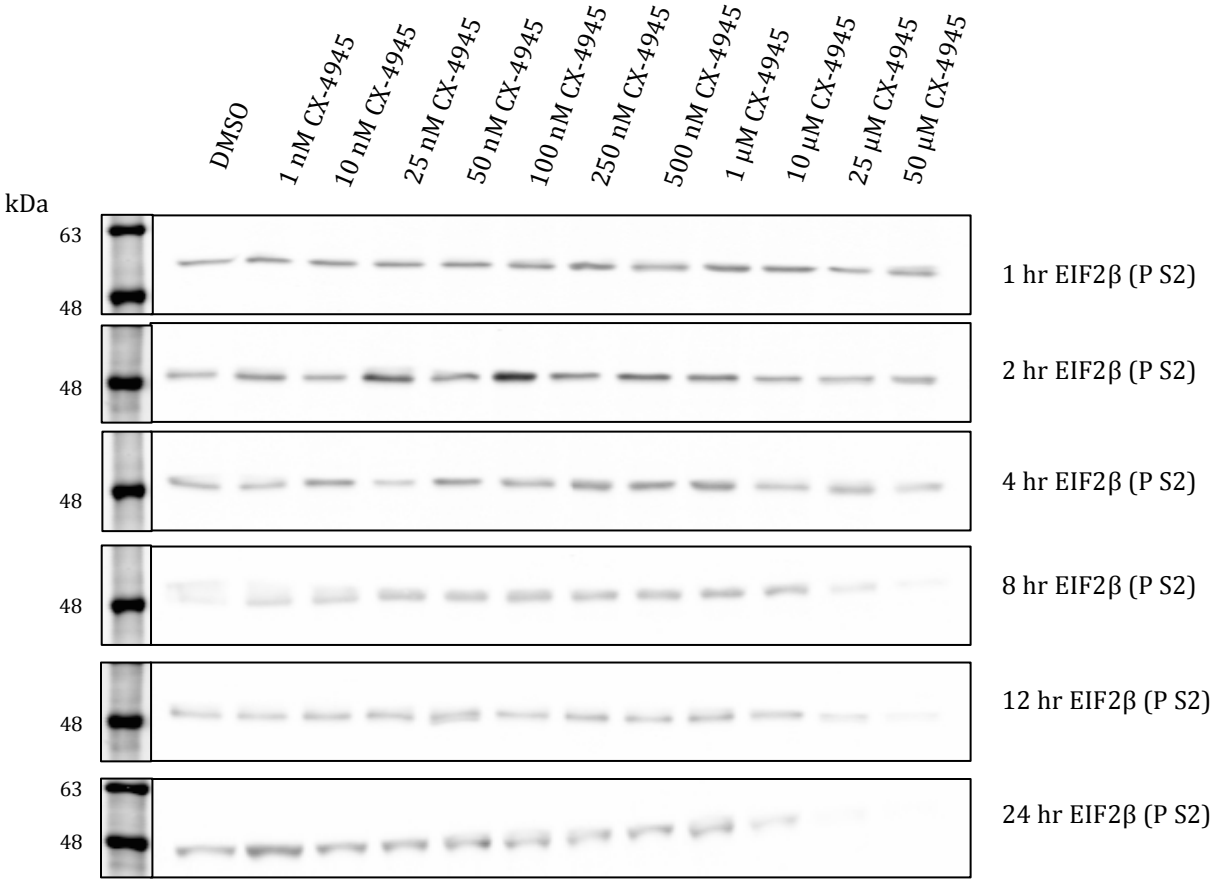
B)



3.3 Dose-response and time-course evaluation of CX-4945 on Flp-In U2OS cells

Having demonstrated that expression of the CK2a V66A/H160D/I174A mutant could be achieved in cells, I was next interested in testing whether expression of this mutant could render cells less sensitive to CX-4945. As a prelude to these experiments, I characterized the effects of CX-4945 on the Flp-In USOS cells. I employed dose-response and time-course experiments to profile the effects of increasing concentrations (1 nm – 50 μM) of CX-4945 on the activity of endogenous CK2 at various time points (1 – 24 hours). To monitor the inhibition of CK2 activity in cells, the Litchfield lab has generated an antibody that recognizes a phosphorylated serine residue located at position 2 in EIF2β (P S2), which has been characterized as a CK2-specific phosphorylation site[112–114]. Under the conditions used in this experiment paired with western-blot methodology, I was able to detect a reduction in EIF2β (p S2) as early as 8 hours (Figure 12). In respects to the objective of this experiment, I observed the greatest reduction in the EIF2β (p S2) signal following treatment of Flp-In U2OS cells with 10 – 50 μM CX-4945 for 24 hours. Overall, these data suggest that I can most effectively eliminate the EIF2β (p S2) signal, indicating inhibition of endogenous CK2α, following treatment of Flp-In U2OS cells with CX-4945 for 24 hours.

Figure 12. **Effect of CX-4945 on Flp-In U2OS cells in the absence of tetracycline** Flp-In U2OS cells were cultured in the absence (-) of tetracycline and were treated with increasing concentrations of CX-4945 (0-50 μM). Cells were then harvested at various time points as indicated and CK2α activity was analyzed via Western blots. The phosphospecific EIF2β (p S2) antibody described in the materials and methods was used to probe for the activity of CK2α (n=1).


DMSO
1 nM CX-4945
10 nM CX-4945
25 nM CX-4945
50 nM CX-4945
100 nM CX-4945
250 nM CX-4945
500 nM CX-4945
1 μM CX-4945
10 μM CX-4945
25 μM CX-4945
50 μM CX-4945
kDa
63
48
1 hr EIF2β (P S2)
2 hr EIF2β (P S2)
4 hr EIF2β (P S2)
8 hr EIF2β (P S2)
12 hr EIF2β (P S2)
24 hr EIF2β (P S2)

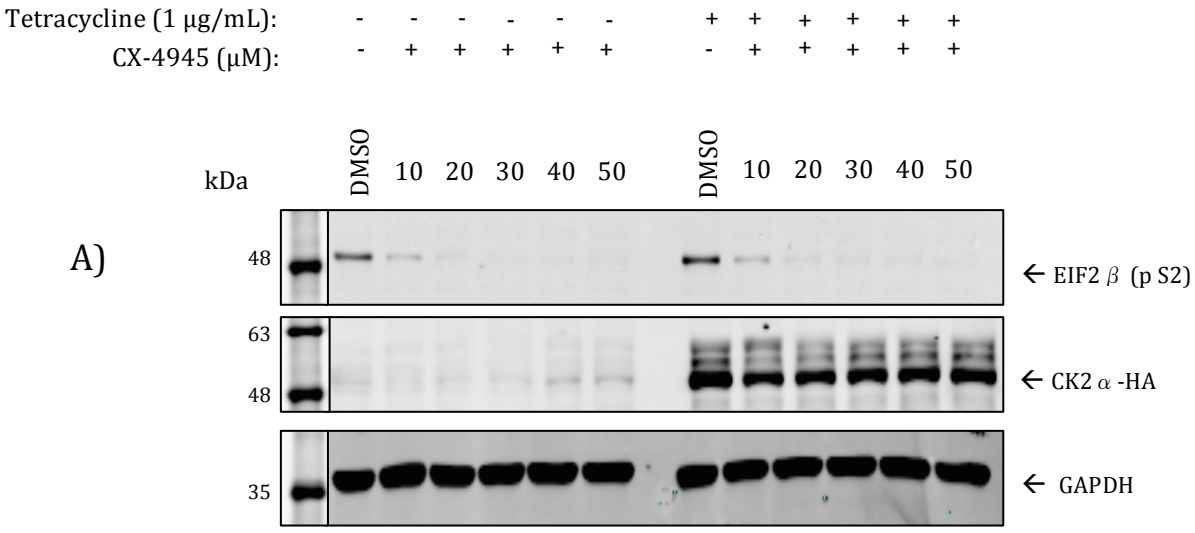
3.4 – Effect of CK2α (V66A/H160D/I174A)-HA on the inhibition of CK2 by CX-4945

Having demonstrated that I could most effectively inhibit the activity of endogenous CK2 following treatment of cell lines with CX-4945 for 24 hours, I now wanted to test the effects of expressing the CK2α triple mutant on CX-4945. Since it has been previously illustrated that the expression of inhibitor-refractory mutants can be used as a tool for the study of protein kinases[86,87,115], the implications of this study are significant. If successful, this may provide new opportunities in order to distinguish cellular functions between the various isoforms of CK2 and may set the stage for the thorough evaluation of its inhibitors. Therefore, to fulfill this objective, I will induce the expression of the CK2α (V66A/H160D/I174A)-HA triple mutant in the Flp-In U2OS cell line to determine its ability to refract the effects of CX-4945.

Demonstrated in Figure 13 (A-B, - Tet samples), in the absence of tetracycline I observed a reduction in EIF2β (P S2) following treatment with 10 μM CX-4945 for 24 hours. The EIF2β (P S2) signal was completely eliminated at concentrations of 20-50 μM CX-4945. After the addition of tetracycline to induce the expression of the wild type kinase (CK2α-HA), I observed a weak ability to recover the loss of EIF2β (P S2) (Figure 13A, + Tet samples). Quantified in Figure 14A, induction of CK2α-HA in Flp-In U2OS cells treated with 10 μM CX-4945 for 24 hours yielded a 12% recovery of EIF2β (P S2). At concentrations beyond 10 μM, restoration of phosphorylated EIF2β (P S2) was unattainable. Subsequently, I assessed the novel triple mutant (CK2α (V66A/H160D/I174A)-HA) and discovered a considerable recovery of EIF2β (P S2) following the induction of protein expression (Figure 13B, + Tet samples). Relative to its

DMSO control, I calculated an 82 %, 43 %, 18 %, 11%, and 3 % recovery in EIF2β (P S2) at inhibitor concentrations of 10, 20, 30, 40, and 50 μM respectively (Figure 14B, + Tet samples).

Figure 13. **Testing the effect of the CK2α (V66A/H160D/I174A)-HA triple mutant on CX-4945 in Flp-In U2OS cells**. Flp-In U2OS cells (expressing CK2α-HA (**A**) or CK2α (V66A/H160D/I174A)-HA (**B**)) were cultured in the absence (-) or presence (+) of 1 μg/mL of tetracycline for 24 hours. Cells were then treated for an additional 24 hours with DMSO or increasing concentrations of CX-4945 (10-50 μM). Cells were harvested and 10 μg of each cell lysate was subjected to SDS-PAGE and Western blotting to assess CK2α protein expression and inhibition. The expression of the HA-tagged CK2α subunits was detected using an Anti-HA antibody and the inhibition of CK2α was monitored using the phosphospecific EIF2β (p S2) antibody as indicated. An antibody capable of detecting GAPDH was used as an indicator of sample loading. (n=3).


Tetracycline (1 µg/mL): - - - - - - + + + + + +
CX-4945 (µM): - + + + + + - + + + + +
kDa
DMSO
10 20 30 40 50
DMSO
10 20 30 40 50
A)
48
63
48
35
← EIF2 β (p S2)
← CK2 α -HA
← GAPDH

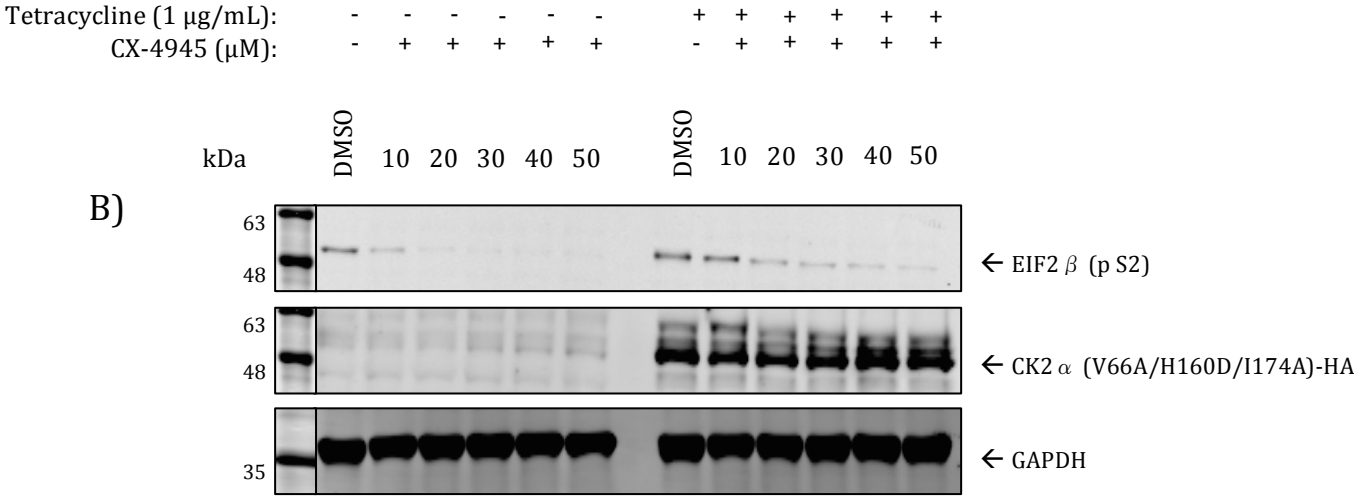
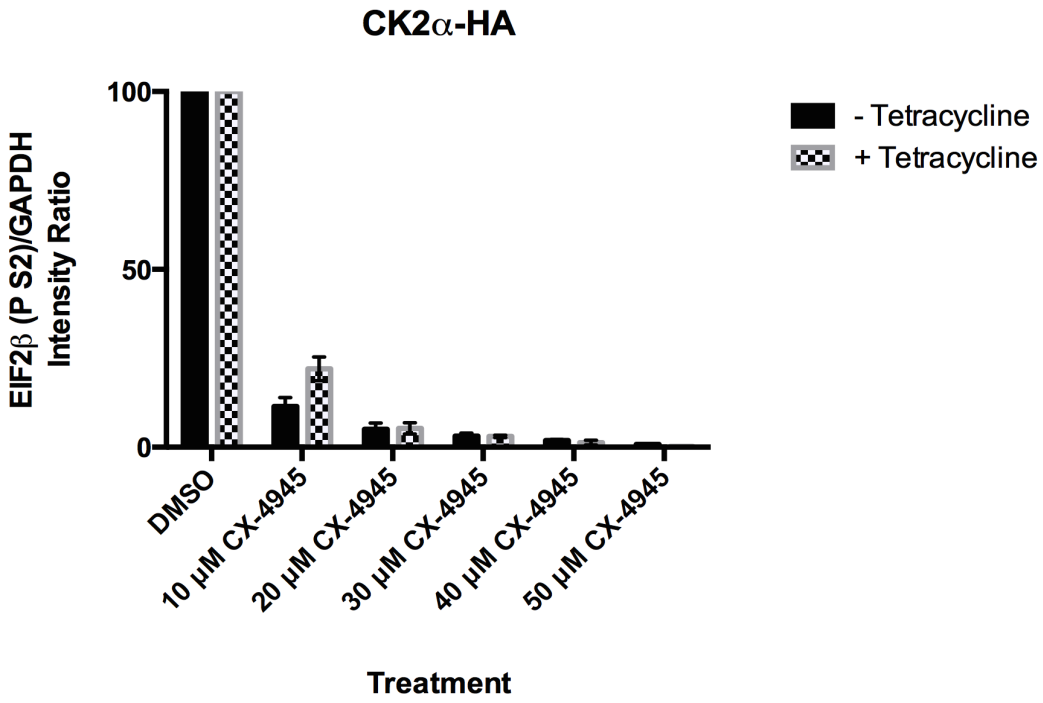
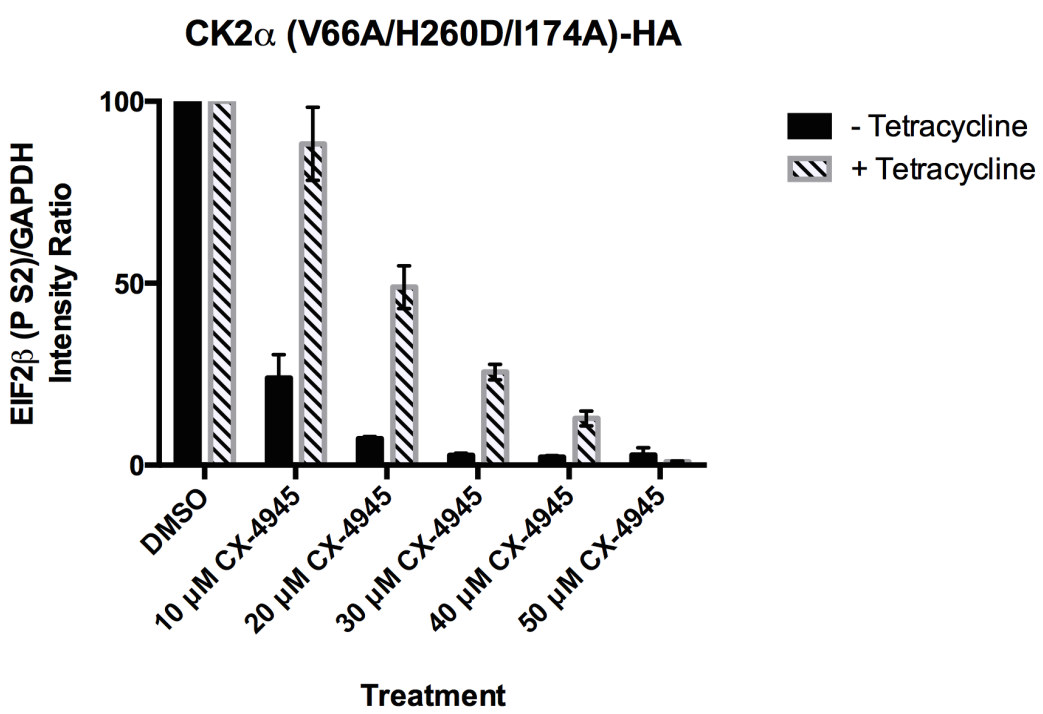

Tetracycline (1 µg/mL): - - - - - - + + + + + +
CX-4945 (µM): - + + + + + - + + + + +
kDa
DMSO
10 20 30 40 50
DMSO
10 20 30 40 50
B)
63
48
63
48
35
← EIF2 β (p S2)
← CK2 α (V66A/H160D/I174A)-HA
← GAPDH

Figure 14. Quantifying the effect of expressing CK2α-HA and CK2α (V66A/H160D/I174A)-HA on CX-4945. The effect of expressing CK2α-HA (A) and CK2α (V66A/H160D/I174A)-HA (B) on inhibition of CK2 by CX-4945 was determined by quantifying the signal intensity of the CK2-specific phosphorylation of EIF2β (P S2) using Odyssey software. The calculated EIF2β (P S2) signal for each cell lysate was compared to its GAPDH signal (EIF2β (P S2)/GAPDH signal ratio) in order to normalize for any differences in protein loading. Error bars represent the variation that can be observed between replicate cell lysates (n=3). Cell lysates in the absence of tetracycline are indicated in solid black and cell lysates in the presence of tetracycline are indicated in checkered or striped black (n=3).

A)


CK2α-HA
EIF2β (P S2)/GAPDH Intensity Ratio
100
50
0
- Tetracycline
+ Tetracycline
DMSO
10 μM CX-4945
20 μM CX-4945
30 μM CX-4945
40 μM CX-4945
50 μM CX-4945
Treatment

B)


CK2α (V66A/H260D/I174A)-HA
EIF2β (P S2)/GAPDH Intensity Ratio
100
50
0
- Tetracycline
+ Tetracycline
DMSO
10 μM CX-4945
20 μM CX-4945
30 μM CX-4945
40 μM CX-4945
50 μM CX-4945
Treatment

4. Discussion

4.1 Mutational analysis of CK2α mutants

4.1.1 – Effect of CK2α mutations on catalytic activity

Previous studies have shown that the manipulation of residues within key conserved regions of protein kinases can influence their catalytic activity[2]. Although this study was focusing on residues necessary for the binding of CX-4945 into the active site of CK2α, it was important to ensure that the ability of CK2 to bind and utilize ATP was not jeopardized. Using assay conditions previously established in the Litchfield lab for measuring the activity of GST-tagged protein kinase CK2[116] it was shown that the active site mutants of CK2α were highly comparable, with the exception of the CK2α mutants bearing V66A/I174A, V66A/H115L/I174A, or V66A/H160D/I174A amino acid substitutions. Most importantly, it was demonstrated that all isoforms of CK2α retained their ability to phosphorylate a specific substrate (RRRDDDSDDD[109]).

Studies originally describing the key structural determinants restricting access to the active site of CK2 allowing small molecule inhibitors to bind tightly have generated similar mutations[92] (V66A/I174A). They demonstrated that the activity of the CK2α (V66A/I174A) double mutant was indistinguishable from wild-type CK2α. In contrast, I observed a minor reduction in activity in all mutants bearing the V66A and I174A amino acid substitutions. However, due to the inconsistency to the observations made in the previous study[92], it remains inconclusive if these mutations truly have a negative impact on activity. It is reasonable to consider that the substitution of residues, V66 and I174, to a smaller amino acid such as alanine, does not allow ATP to remain appropriately positioned within the active site of CK2. Although it has been identified that lysine 68

(K68) is the residue necessary for ATP binding[28], altering the structure of the active site may still have an effect on the docking of ATP, as well as the alignment of the gamma (γ) phosphate with the hydroxyl group of the acceptor substrate. Presumably, this would result in a diminished rate at which the mutated kinase can phosphorylate a recipient substrate. However, more work is required to understand the effect of the V66A and I174A mutations on the activity of CK2

As a whole, amino-acid substitutions (H115L, H160D, V66A/I174A, V66A/H115L/I174A, and V66A/H160D/I174A) in the active site of CK2α did not impair the overall ability of CK2α to bind and utilize ATP to transfer the γ-phosphate group to an acceptor substrate (RRRDDDSDDD). I conclude that the minor loss in activity is not significant for achieving our objectives, as I can normalize the activity of all kinases when performing future experiments. Therefore, I moved forward to address the issue of how these mutations will effect the inhibition of CK2 by CX-4945.

4.1.2 – Examining the effect of CK2α mutations on CX-4945

It has been well documented that CX-4945 – the inhibitor of protein kinase CK2 that is of interest in the current study – behaves in an ATP-competitive manner[117,118]. As a result, I believed that I could model this competition in order to determine if the newly introduced amino-acid substitutions could alter the ability of CX-4945 to inhibit CK2α, which would be reflected by a change in Ki. Furthermore, if these mutations do impair the ability of CX-4945 to inhibit CK2α, then following preincubation with CX-4945 in order to saturate the active site with inhibitor, I should detect more CK2α activity in comparison to the wild type kinase. Presumably, this is because the inhibitor will be less

able to remain bound within the active site of our mutants. In fact, the current study reported for the first time the ability of various inhibitor-refractory mutants of CK2α to affect the inhibitory properties of CX-4945. In particular, I identified a new combination of amino-acid substitutions (V66A/H160D/I174A) that was considerably less sensitive to inhibition by CX-4945, and consequently can restore more specific activity in comparison to the previously established double mutant (V66A/I174A).

With the exception of the V66A/I174A double mutant, the CK2α mutants generated in the present study have not been previously examined for their ability to attenuate the effects of CX-4945, and there are a few important observations that should be mentioned. Notably, the GST-CK2α mutants containing single amino-acid substitutions, H115L or H160D, demonstrated that they do have the capacity to weaken the effects of CX-4945. This is a noteworthy contribution to the field because it suggests that these particular mutations may alter the internal structure of the active site of CK2α, or abolish interactions that may help to keep the inhibitor engaged within the active site. It was apparent however, that the single mutants were unable to outperform the GST-CK2α mutant containing the previously established amino-acid substitutions, V66A and I174A. It is possible that this result is simply due to the fact that two mutations may be better than one, creating more structural changes that lessen the ability of CX-4945 to bind the active of CK2α beyond what is attainable with a single mutation. Although, it is more likely that the observed differences between the single and double mutants were due to the reported function of the V66 and I174 residues. It has previously been demonstrated that residues V66 and I174 are necessary for the binding of inhibitors to CK2α, since they contribute key energy for binding and function as a clamp to stabilize

the inhibitor compound within the active site of CK2α[106]. Therefore, it may not be surprising that the mutants bearing single amino-acid substitutions (H115L and H160D) were more sensitive to inhibition by CX-4945, presumably because I maintained the existence of these critical residues (V66 and I174).

Interestingly, in a study performed by Battistutta R, et al[31] (2011) investigating the affect of the V66A and I174A mutations on the IC50 of CX-4945, only a moderate increase was observed when these substitutions were examined individually. In contrast, when combined to form the CK2α-V66A/I174A double mutant, they detected a significant increase in the IC50 of CX-4945 (15-fold). This finding was intriguing, as it suggests that certain combinations of amino-acid substitutions may have an additive effect on the ability of CK2α to resist inhibition by CX-4945. Because I have shown that the CK2α single mutants still display the capacity to impair the inhibition by CX-4945 and restore kinase activity in the presence of CX-4945, I hypothesized that I would observe an additive effect if I combined H115L or H160D with the previously established double mutant. Our results (section 4.1.2) provided support for the hypothesis that combination of these residues has an additive effect. Significantly, this served as the first time triple mutants of CK2α have exhibited an amplified ability to alter the inhibition of CX-4945, therefore recovering more activity in the presence of inhibitor than what was previously attainable[31]. The advantage to the development of such mutants is that I increase the range in which I might detect a recovery in the specific activity of CK2α. Additionally, this finding is noteworthy as the creation of protein kinases that are insensitive to inhibition hold major implications for the elucidation of their specific cellular roles, as well as the validation of their viability as a potential therapeutic target

(Discussed in detail in section 4.5 – Implications of inhibitor-insensitive mutants of protein kinase CK2).

In conclusion, I have determined that residues H115 and H160 are important for the inhibition of CK2α by CX-4945. More specifically, I have demonstrated that the replacement of these residues, in particular H160, to residues found within the majority of the remaining protein kinases, resulted in a considerable loss in sensitivity to inhibition. I do acknowledge however, that in no instance was the loss of CK2α activity fully restored, and therefore additional work may be warranted. To address these concerns, identification of additional residues that may be necessary for the binding of inhibitors to the active site of CK2α should be pursued. In addition, optimal stability and activity of protein kinase CK2 has been observed in the presence of the CK2β regulatory subunit. Therefore, future studies should consider combining CK2β with the CK2α-V66A/H160D/I174A triple mutant to determine if the level of activity restored following treatment with CX-4945 can be improved. Despite the limitations, I believe that the generation of cell lines that readily express mutants with a reduced sensitivity to inhibition will provide a platform for the investigation of CK2's roles within cells, as well as the in-cell evaluation of current CK2 inhibitors.

4.2 – Inducible expression of CK2α in Flp-In U2OS cells

Previous explorations into the expression of the various protein kinase CK2 subunits within cells have primarily relied on methods of transient transfection[78,119–121]. Even though we can often achieve robust expression via methods that introduce DNA transiently, there are several disadvantages that may hinder our ability to obtain

reproducible data[122]. Since the DNA is not incorporated into the genome of the host cell and therefore will not be replicated, expression of the transfected DNA is short-lived and will typically be diluted out of the population through cell division and common passaging of the cells[122]. This will require the need to reintroduce the DNA of interest and can result in an increase in the variability observed between experiments, because achieving consistent efficiency through various methods of transfection has proven to be challenging[122]. In addition to their short-lived nature, there is no way to control the transcriptional and translational machinery of the host cell, thereby eliminating the ability to regulate the expression of your gene of interest. To overcome these limitations, the Litchfield lab has shifted its focus into the development of tetracycline-inducible stable cell lines expressing various forms of protein kinase CK2[28,30,123,124]. Therefore, I decided to further expand our repertoire of inducible cell lines by introducing CK2 α -HA, as well as the novel CK2 α (V66A/H160D/I174A)-HA triple mutant into Flp-In™ U2OS cells using the recently developed Flp-In™ T-Rex™ recombination technology[98].

Briefly, Flp-In™ T-Rex™ recombination technology requires the use of the Flp recombinase[125–127] originally derived from the 2 um plasmid of *Saccharomyces cerevisiae* to insert fragments of DNA between short flippase recognition target (FRT) sites in host cells also containing a tetracycline-responsive element[98]. Following the generation of a host cell line containing a tetracycline-responsive element, as well as integrated FRT sites, the ability to introduce a gene of interest becomes very rapid and efficient. Furthermore, due to the site-specific integration of DNA into the genome of the host cell, Flp-In™ T-Rex™ cell lines are isogenic and consequently protein expression is equal across a population of cells[98,128]. This allows for polyclonal selection of stably expressing

cell lines rather than the isolation of individual colonies, which significantly reduces the overall time invested into the development of cell lines.

Interested in a cell-based system to achieve stable integration of a gene of interest, Milligan et al (2011) took advantage of the Flp-In™ T-Rex™ system to regulate the expression of various G protein-coupled receptors[129] (GPCR). Immunofluorescent analysis demonstrated that robust expression of the inducible GPCR cell lines was only attainable in the presence of doxycycline, although a faint detection of protein expression in the absence of doxycycline was also observed. Subsequently, they performed western-blot analysis to characterize the time it takes to detect expression following treatment with doxycycline. They were able to detect the expression of the human muscarinic M_3 GPCR as early as 6 hours, and expression was fully saturated after 14 hours of treatment with 0.5 μ g/mL doxycycline. In addition, Dr. Karmella Haynes (who generously provided us with Flp-In™ T-REx™ U2OS cells) has also utilized this technology to introduce various modular synthetic transcriptional factors (pc-TFs) into HEK293 and U2OS cells[128]. Briefly highlighted in the characterization of their Flp-In U2OS cell lines[128], western blot analysis detected robust expression in the majority of cell lines following the addition of 1 μ g/mL doxycycline. In light of the evidence provided above, I obtained the U2OS cells engineered by Haynes laboratory to introduce constructs encoding CK2 through integration via the Flp-In™ T-Rex™ recombination strategy[98], with the expectation that these cells rapid, robust, and regulated protein expression.

In general, our observations were highly comparable to those made in the previous studies mentioned above due to the universal nature of the Flp-In™ T-Rex™ system. However, there are some differences that should be noted. First, I was able to

detect changes in protein expression at earlier (3-6 hour) and later (12-24 hour) time points following treatment of the Flp-In U2OS cell lines with tetracycline. It was also demonstrated that I could achieve these differences over a broader range of tetracycline concentrations. However, this could simply be attributed to the conditions used when monitoring protein expression (different epitope tags that require the use of different antibodies for detection). Second, I observed faint protein expression in both Flp-In U2OS cell lines when in the absence of tetracycline indicating some leakiness with the promoter controlling transcription on the gene of interest, which has previously observed with tet-on systems[102,130–132]. It is plausible that sources of DMEM and FBS routinely used in cell culture contain traces of tetracycline. For this reason, all sources of media and FBS should be thoroughly examined for the presence of tetracycline, as an extra purification step could be performed to minimize the likelihood of observing leaky expression. Despite this minor limitation, on the whole, our results are in high concordance to what has previously been attainable using the Flp-In™ T-REx™ recombination technology. Importantly, I achieved our objective of creating cell lines capable of the rapid, robust, and inducible expression of CK2 α -HA and CK2 α (V66A/H160D/I174A)-HA.

Also worth noting, is the fact that I was able to achieve robust and stable expression of CK2 α -HA and CK2 α (V66A/H160D/I174A)-HA in the absence of the CK2 β regulatory subunit. Although it has been observed that the catalytic CK2 subunits (α and α') can exist and be active in the absence of CK2 β [52,119,120], earlier studies describing the expression of epitope-tagged isoforms of CK2 could only detect optimal expression and stability of the catalytic subunits when in the presence of CK2 β [119]. As a

reminder, Flp-In™ T-REx™ technology currently permits the introduction of only a single gene of interest, and does not support a bidirectional system that would be required to coordinately express both catalytic and regulatory subunits of CK2[28,133]. This is an intriguing result and therefore will warrant further investigation. To evaluate the capacity of the exogenous CK2 α subunits to form complexes with endogenous CK2 β, immunoprecipitation studies should be performed on all Flp-In™ T-REx™ U2OS cell lines following induction of protein expression. The HA-3F10 antibody that recognizes the HA-epitope on the C-terminus of the wild type and mutant CK2 α could be used to perform immunoprecipitations with immunoblots performed to detect the presence of CK2 β. Detection of CK2 β would indicate that a complex is being formed between the exogenously expressed CK2 α and the endogenous CK2 β of the Flp-In™ T-REx™ U2OS host cells, providing explanation for the ability of both cell lines to achieve, rapid, robust, stable and regulated expression of CK2.

Overall, I demonstrated that I have successfully generated a cell line that can rapidly and robustly wild type and mutant forms of CK2 α under the control of tetracycline. More specifically, I was able to achieve robust protein expression that remained stable in the absence of a requirement for increased expression of the regulatory CK2 β subunit. It is envisaged that these cell lines will be extremely advantageous for experiments investigating the specific roles of CK2 α in cellular systems, as well as providing a platform for the evaluation of Ck2 inhibitors. The implications and advantages of having inducible cell lines expressing inhibitor-resistant mutants of protein kinase CK2 will be discussed in more detail (Section 4.5 – Implications of inhibitor-insensitive mutants of protein kinase CK2).

4.3 Dose-response and time-course evaluation of CX-4945 on Flp-In U2OS cells

The selection of an appropriate biomarker is important when attempting to monitor the cellular activity of a protein kinase. Previous studies treating mammalian cells with CX-4945 have been able to observe the rapid inhibition of CK2 by monitoring its activity through the detection of its phosphorylated substrate, Akt. For example, studies investigating the use of MEK inhibitors to overcome resistance to CX-4945, as well as the preclinical characterization of CX-4945 in PC3 prostate carcinoma cells, demonstrated a rapid reduction in the CK2-specific phosphorylation of Akt (P-Akt S129) following treatment with 1 -10 μM CX-4945 for only 2-4 hours[74,134]. In some respects, these findings are not surprising, considering the role that CK2 has been shown to play in cellular processes such as proliferation and survival[27,35,52]. However, previous experience in the Litchfield lab has suggested that Akt is not a reliable indicator of CK2 activity (Appendix, Figure 16). In fact, it was demonstrated that rapid and dynamic responses to the inhibition of CK2 following treatment of HeLa and UTA6 cells with CX-4945 were detected using an antibody capable of recognizing the CK2-specific phosphorylation of EIF2β at serine 129 (P-EIF2β S2). As a result, I selected this substrate to serve as our biomarker to monitor the activity of CK2 in the Flp-In U2OS cell lines. Using this antibody (P-EIF2β S2), I demonstrated that I could most effectively inhibit the activity of endogenous CK2 following treatment of Flp-In U2OS cells with 25-50 μM CX-4945 for 24 hours. It is important to recognize however, that there is a possibility that these results may be confounded as a result of the prolonged exposure of the Flp-In U2OS cells to CX-4945 since it has been documented that inhibitors such as CX-4945 are not strictly selective to protein kinase CK2, and may target other serine/threonine protein kinases

including the PIM and DYRK families[135]. Despite the limitation, these studies revealed that inhibition of EIF2β (P S2) could be achieved using CX-4945 setting the stage for investigation of its effects on the Flp-In U2OS cell expressing wild type and the (V66A/H160D/I174A) triple mutant of CK2α.

4.4 – Testing the effects of the CK2α (V66A/H160D/I174A) triple mutant on the cellular response of CX-4945

The development of mutants that are less sensitive to inhibition can serve an instrumental purpose for the study of protein kinases. For example, the establishment of a system in which the target kinase is noticeably less sensitive to inhibition may allow for the identification of its protein substrates, which in turn can provide valuable information about the functions that are coordinated by the kinases within cells. Furthermore, mutants that are less sensitive to inhibitors may enable the establishment of experimental systems for evaluation of the effects of various inhibitors on their anticipated target. Therefore, since our *in vitro* data (refer to sections) indicated that the triple mutant of CK2α (V66A/H160D/I174A) is notably less sensitive to inhibition by CX-4945, I generated tetracycline-inducible cell lines (Flp-In U2OS) to determine how the expression of this mutant would effect the cellular response of CX-4945.

In the present study, I demonstrated that the expression of the CK2α V66A/H160D/I174A-HA triple mutant led to a considerable recovery of the CK2-specific phosphorylation of EIF2β (p S2) following treatment of Flp-In U2OS cells in the presence of 10 μM CX-4945 for 24 hours. Although there was a restoration in the loss of phosphorylated EIF2β (p S2), it is evident that phosphorylation was not completely

restored. A potential explanation for this result is that EIF2β may serve as an indicator of CK2 holoenzyme activity. In fact, it has been reported that the CK2-specific phosphorylation of EIF2β (p S2) requires the presence of both the catalytic (α) and regulatory (β) subunits[112,113]. As previously noted, the Flp-In recombination technology does not currently support a dual-expression system. Consequently, to determine if the addition of CK2β will improve the ability of the triple mutant to resist the effects of CX-4945, it would be necessary to transiently transfect CK2β into the Flp-In U2OS cell lines expressing the CK2α triple mutant. If successful, then perhaps the development of a dual-expression system capable of utilizing Flp-In recombination technology would be beneficial.

Despite the limitation that EIF2β (P S2) may not be the most appropriate biomarker for the activity of CK2α, I believe that I have now established a system that provides new opportunities in order to identify cellular substrates of CK2α, especially those substrates that do not require the presence of the regulatory CK2β subunit. This is especially important because of the complexity of protein kinase CK2. I have previously mentioned that various forms of CK2 can exist within cells, each displaying the potential to exhibit functions that are independent from one another. In addition, it remains poorly understood whether inhibitors of CK2 display uniform effects against the various forms of CK2 or if they have the ability to better target one isoform over another. Therefore, the identification of biomarkers that can serve to monitor the activity of specific CK2 isoforms will be very useful in order to expand our knowledge regarding the functions of protein kinase CK2, as well as the effects of its inhibitors.

In summary, the CK2α (V66A/H160D/I174A) triple mutant may provide valuable insight regarding the cellular behavior of CK2α when in the absence of the CK2β regulatory subunit. However, the creation of cell lines expressing inhibitor-insensitive mutants of the remaining isoforms of CK2 would enable us to conduct a more thorough investigation, which may serve to better elucidate the intricacies of CK2.

4.5 – Implications of inhibitor-refractory mutants of protein kinase CK2

The development of mutants that display a reduced sensitivity to inhibition can serve an invaluable purpose in the study of protein kinase[86,87]. Importantly, the current study has demonstrated that I have created a mutant of protein kinase CK2 (CK2α (V66A/H160D/I174A)-HA) that is noticeably less sensitive to inhibition by the CK2 inhibitor, CX-4945. Therefore, I believe that our study has now provided us with a unique advantage that may help elucidate many unknowns regarding protein kinase CK2, which may have previously been unattainable.

4.5.1 - Functional compensation vs. specialization

As a result of the recent surge in excitement surrounding protein kinase CK2, there is now ample evidence providing insight into the plethora of functions that CK2 may be responsible for orchestrating within cells. However, the precise form of CK2 responsible for its catalytic activity often remains poorly understood due to the complexity of the kinase (two catalytic subunits, CK2α and CK2α', that can be active with or without the regulatory subunit, CK2β). Consequently, I believe that the generation of the CK2α (V66A/H160D/I174A)-HA triple mutant provides new

opportunities to distinguish the catalytic activity exhibited by CK2α and CK2α'. The separation of their catalytic activity will enable us to more thoroughly examine cellular functions of protein kinase CK2, and more specifically, help distinguish functions that may be compensatory from those that may be isoform-specific. However, this will eventually require the generation of a mutant of CK2α' that is also less sensitive to inhibition by CX-4945, although this may be relatively straightforward considering the structural similarity to its CK2α counterpart.

4.5.2 - Evaluation of current and novel inhibitors of protein kinase CK2

Another challenge that has recently emerged involves the development of inhibitors for the therapeutic targeting of CK2. Due to its increasing implications in human disease, in particular cancer, the number of compounds developed to inhibit CK2 has radically increased. As previously mentioned, the majority of these compounds have only been examined *in vitro*, and even when they exhibit cellular effects it often remains unknown whether the observed effects are a result of CK2 inhibition. Following the induction of the CK2α (V66A/H160D/I174A)-HA triple mutant, the Flp-In U2OS cells can be treated with CK2 inhibitors. Using a biomarker (P-EIF2β S2) in order to specifically monitor the activity of CK2, we can then assess if the observed effects are a result of the specific inhibition of CK2. This will be reflected by the ability of the CK2α (V66A/H160D/I174A)-HA triple mutant to restore the phosphorylation of EIF2β. Importantly, I believe that the present study has now provided us with a unique opportunity that may help validate the specificity of current and novel inhibitors of CK2 in cells, which has previously been a challenge.

4.6 – Future directions

The generation of the CK2α (V66A/H160D/I174A)-HA triple mutant has provided us with a significant advantage in order to investigate protein kinase CK2 in ways that have previously been unattainable. It is envisaged that the creation of such mutants may enable identification of biomarkers that can serve to monitor the activity of a specific form of CK2. We may then be able to distinguish the catalytic activity exhibited by CK2α or CK2α' in the absence and presence of the regulatory subunit, CK2β. As a result, this may enable us to more precisely evaluate the functions of discreet forms of CK2 in cells. The creation of inhibitor-insensitive mutants of CK2 will also provide a foundation for a thorough evaluation of its inhibitors. More specifically, it will be possible to examine the ability of CK2 inhibitors to target one particular isoform over another, which may help to elucidate information that could dramatically improve the therapeutic targeting of CK2 in cancer. Overall, I believe that the generation of the CK2α (V66A/H160D/I174A) triple mutant provides evidence supporting the feasibility of creating inhibitor-insensitive mutants of CK2.

Perhaps the most noteworthy outcome of the present study was the ability of the CK2α (V66A/H160D/I174A) triple mutant to affect the ability of CX-4945 to inhibit CK2 in Flp-In U2OS cells. However, it is also a great accomplishment that I was able to achieve rapid, robust, and stable expression of the CK2α triple mutant in the absence of the regulatory subunit, CK2β. As previously mentioned, the current understanding is that both catalytic subunits (CK2α and CK2α') require the regulatory CK2β subunit in order to reach their optimal expression and stability[111]. Immunoprecipitation analysis would be

an important complement to the current study, as it would allow us to determine if the exogenously expressed CK2α-HA and CK2α (V66A/H160D/I174A)-HA can form complexes with CK2β. If not, then I have established a unique opportunity in order to identify a biomarker that can serve to monitor the activity of CK2α, in the absence of the regulatory CK2β subunit. This could be accomplished through the use of more advanced techniques such as mass spectroscopy (MS), which permits the ability to monitor hundreds to thousands of peptides or phosphopeptides simultaneously[136]. Employing a technique that has revolutionized the scale at which we can quantify proteins in cells will empower us to perform a global analysis of the phosphoproteome. This will enable us to access the consequence of inhibiting the activity of CK2α on the phosphorylation landscape of cellular proteins. Subsequently, we could perform a parallel experiment following the expression of our CK2α (V66A/H160D/I174A) triple mutant in order to identify those proteins whose phosphorylation is restored. Any protein whose phosphorylation is restored would classify that protein as cellular substrate of CK2α, since its loss of phosphorylation was a direct result of the specific inhibition of CK2α. Importantly, this may provide valuable insight regarding the cellular behavior of CK2α when in the absence of the CK2β regulatory subunit.

Although this study has demonstrated that we have achieved significant advances, we currently have only generated an inhibitor-insensitive mutant for the CK2α catalytic subunit. Therefore, the creation of a mutant of CK2α' that is also noticeably less sensitive to inhibition by CX-4945 would be a logical continuation to the present study. Due to its structural resemblance, as well as its high sequence similarity, the generation of a mutant of CK2α' should be readily attainable. In fact, CK2α' contains the same hydrophobic

residues (V67 and I175 in CK2α'), as well as the histidine residue (H161 in CK2α') that were mutated in order to create the CK2α triple mutant in the present study. Additionally, the establishment of a bidirectional expression vector capable of integration via Flp-In™ T-REx™ recombination technology should be pursued.

4.7 – General summary

The main objective of the present study was to create a mutant of CK2α that could impair the ability of CX-4945 to inhibit CK2 in cells. In order to achieve this objective our study was broken down into two parts – the rationale design, purification and *in vitro* analysis to determine which CK2α mutant displayed the greatest reduction in sensitivity to inhibition, followed by the generation of a tetracycline-inducible cell line in order to achieve its regulated expression in cells. The *in vitro* analysis lead to the discovery of a CK2α triple mutant, namely CK2α (V66A/H160D/I174), that was less sensitive to inhibition in comparison to the previously established V66A/I174A double mutant. As a result, I generated a cell line using Flp-In™ T-REx™ technology, and showed that I could achieve the rapid, robust, and stable expression of the CK2α triple mutant under tight regulation by tetracycline. This was achieved in the absence of the regulatory subunit, CK2β, which may be an unexpected observation considering previous studies have suggested that optimal expression and stability requires interaction with CK2β. Most importantly, I illustrated that expression of the CK2α triple mutant in Flp-In U2OS cells could influence the ability of CX-4945 to inhibit CK2. I acknowledge that the selected biomarker (P-EIF2β S2) may not have been ideal for monitoring the activity of the CK2α catalytic subunit alone since previous studies have demonstrated that EIF2β is

selectively phosphorylated by the CK2 holoenzyme[112,113]. Nevertheless, I have now established a system that will allow us to pursue the identification of substrates that will better serve as indicators of CK2α catalytic activity in the absence of CK2β.

Given that previous studies have revealed several cellular processes involving CK2, as well as providing information concerning its inhibition by various inhibitors, many new questions and avenues can now be explored. Accordingly, I believe that the present study has established a solid platform in which future work can be conducted. In this respect, the development of the CK2α (V66A/H160D/I174A) triple mutant that is considerably less sensitive to inhibition by CX-4945 will serve an instrumental purpose in order to broaden our understanding regarding the cellular functions of CK2, as well as its inhibition by specific inhibitors.

6 - References

1. Cohen P. The origins of protein phosphorylation. *Nat Cell Biol*. 2002;4(5):E127-E130.

2. Endicott J a., Noble MEM, Johnson LN. The Structural Basis for Control of Eukaryotic Protein Kinases. *Annu Rev Biochem*. 2012;81(1):587-613.

3. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 2002;298(5600):1912-1934.

4. Martin DM a, Miranda-Saavedra D, Barton GJ. Kinomer v. 1.0: A database of systematically classified eukaryotic protein kinases. *Nucleic Acids Res*. 2009;37(SUPPL. 1):244-250.

5. Eswaran J, Knapp S. Insights into protein kinase regulation and inhibition by large scale structural comparison. *Biochim Biophys Acta - Proteins Proteomics*. 2010;1804(3):429-432.

6. Knighton DR, Zheng JH, Ten Eyck LF, et al. Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*. 1991;253:407-414.

7. De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgan DO, Kim SH. Crystal structure of cyclin-dependent kinase 2. *Nature*. 1993;363:595-602.

8. Iakoucheva LM, Radivojac P, Brown CJ, et al. The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res*. 2004;32(3):1037-1049.

9. Lowe ED, Noble MEM, Skamnaki VT, Oikonomakos NG, Owen DJ, Johnson LN. The crystal structure of a phosphorylase kinase peptide substrate complex: kinase substrate recognition. *EMBO J*. 1997;16(22):6646-6658.

10. Cohen P. Protein kinases--the major drug targets of the twenty-first century? *Nat Rev Drug Discov*. 2002;1(4):309-315.

11. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Ab1 tyrosine kinase on the growth of Bcr-Ab1 positive cells. *Nat Med*.

1996;2:561-566.

12. Oetzel C, Jonuleit T, Götz a, et al. The tyrosine kinase inhibitor CGP 57148 (ST1 571) induces apoptosis in BCR-ABL-positive cells by down-regulating BCL-X. *Clin Cancer Res*. 2000;6(5):1958-1968.

13. Henkes M, van der Kuip H, Aulitzky WE. Therapeutic options for chronic myeloid leukemia: Focus on imatinib (Glivec, Gleevec). *Ther Clin Risk Manag*. 2008;4(1):163-187.

14. Rosee P La, O'Dwyer M, Druker B. Insights from pre-clinical studies for new combination treatment regimens with the Bcr-Abl kinase inhibitor imatinib mesylate (Gleevec/Glivec) in chronic myelogenous leukemia: a translational perspective. *Leukemia*. 2002;16:1213-1219.

15. Jain P, Kantarjian H, Cortes J. Chronic myeloid leukemia: overview of new agents and comparative analysis. *Curr Treat Options Oncol*. 2013;14(2):127-143.

16. Goetz M, Hoetker MS, Diken M, Galle PR, Kiesslich R. In vivo molecular imaging with cetuximab, an anti-EGFR antibody, for prediction of response in xenograft models of human colorectal cancer. *Endoscopy*. 2013;45(6):469-477.

17. Capra M, Nuciforo PG, Confalonieri S, et al. Frequent alterations in the expression of serine/threonine kinases in human cancers. *Cancer Res*. 2006;66(16):8147-8154.

18. Edelman a M, Blumenthal DK, Krebs EG. Protein serine/threonine kinases. *Annu Rev Biochem*. 1987;56:567-613.

19. Ruzzene M, Pinna L a. Addiction to protein kinase CK2: A common denominator of diverse cancer cells? *Biochim Biophys Acta - Proteins Proteomics*. 2010;1804(3):499-504.

20. Trembley JH, Wang G, Unger G, Slaton J, Ahmed K. CK2: A key player in cancer biology. *Cell Mol Life Sci*. 2009;66(11-12):1858-1867.

21. Burnett G, Kennedy EP. The enzymatic phosphorylation of proteins. *Cancer*. 1954:969-980.

22. Meggio F, Pinna L a. One-thousand-and-one substrates of protein kinase CK2? *FASEB J*. 2003;17(3):349-368.

23. Gyenis L, Litchfield DW. The emerging CK2 interactome: Insights into the regulation and functions of CK2. *Mol Cell Biochem*. 2008;316(1-2):5-14.

24. Graham KC, Litchfield DW. The regulatory ?? subunit of protein kinase CK2 mediates formation of tetrameric CK2 complexes. *J Biol Chem*. 2000;275(7):5003-5010.

25. Niefind K, Raaf J, Issinger OG. Protein kinase CK2: From structures to insights. *Cell Mol Life Sci*. 2009;66(11-12):1800-1816.

26. Grankowski N, Boldyreff B, Issinger OG. Isolation and characterization of recombinant human casein kinase II subunits alpha and beta from bacteria. *Eur J Biochem*. 1991;198(1):25-30.

27. Litchfield DW. Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J*. 2003;369(Pt 1):1-15.

28. Vilk G, Saulnier RB, St Pierre R, Litchfield DW. Inducible expression of protein kinase CK2 in mammalian cells. Evidence for functional specialization of CK2 isoforms. *J Biol Chem*. 1999;274(20):14406-14414.

29. Xu X, Toselli P a, Russell LD, Seldin DC. Globozoospermia in mice lacking the casein kinase II alpha' catalytic subunit. *Nat Genet*. 1999;23(1):118-121.

30. Litchfield DW, Bosc DG, Canton D a., Saulnier RB, Vilk G, Zhang C. Functional specialization of CK2 isoforms and characterization of isoform-specific binding partners. *Mol Cell Biochem*. 2001;227(1-2):21-29.

31. Battistutta R, Cozza G, Pierre F, et al. Unprecedented selectivity and structural determinants of a new class of protein kinase CK2 inhibitors in clinical trials for the treatment of cancer. *Biochemistry*. 2011;50(39):8478-8488.

32. Litchfield DW, Dobrowolska G, Krebs EG. Regulation of casein kinase II by growth factors: a reevaluation. *Cell Mol Biol Res*. 1994;40(5-6):373-381.

33. Pines J. Cyclins and cyclin-dependent kinases: a biochemical view. *Biochem J*.

1995;308(Pt 3):697-711.

34. Zhang C, Vilk G, Canton D a, Litchfield DW. Phosphorylation regulates the stability of the regulatory CK2b subunit. *Oncogene*. 2002:3754-3764.

35. St-Denis N a, Litchfield DW. Protein kinase CK2 in health and disease: From birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. *Cell Mol Life Sci*. 2009;66(11-12):1817-1829.

36. Pepperkok R, Lorenz P, Ansorge W, Pyerin W. Casein kinase II is required for transition of G0/G1, early G1, and G1/S phases of the cell cycle. *J Biol Chem*. 1994;269(9):6986-6991.

37. Lolli G, Johnson LN. CAK—Cyclin-Dependent Activating Kinase: A Key Kinase in Cell Cycle Control and a Target for Drugs? *Cell Cycle*. 2014;4(4):565-570.

38. Schneider E, Kartarius S, Schuster N, Montenarh M. The cyclin H/cdk7/Mat1 kinase activity is regulated by CK2 phosphorylation of cyclin H. *Oncogene*. 2002;21(33):5031-5037.

39. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. *Cancer Cell*. 2002;2(2):103-112.

40. Keller DM, Lu H. p53 serine 392 phosphorylation increases after UV through induction of the assembly of the CK2??hSPT16??SSRP1 complex. *J Biol Chem*. 2002;277(51):50206-50213.

41. Litchfield DW, Lüscher B, Lozeman FJ, Eisenman RN, Krebs EG. Phosphorylation of casein kinase II by p34cdc2 in vitro and at mitosis. *J Biol Chem*. 1992;267(20):13943-13951.

42. Gotz C, Kartarius S, Schwar G, Montenarh M. Phosphorylation of mdm2 at serine 269 impairs its interaction with the retinoblastoma protein. *Int J Oncol*. 2005;26(3):801-808.

43. Bosc DG, Slominski E, Sichler C, Litchfield DW. Phosphorylation of casein kinase II by p34cdc2. Identification of phosphorylation sites using phosphorylation site mutants in vitro. *J Biol Chem*. 1995;270(43):25872-25878.

44. Litchfield DW, Lozeman FJ, Cicirelli MF, et al. Phosphorylation of the beta subunit of casein kinase II in human A431 cells identification of the autophosphorylation site and a site phosphorylated by p34cdc2. *J Biol Chem*. 1991;266:20380-20389.

45. St-Denis N a, Derksen DR, Litchfield DW. Evidence for regulation of mitotic progression through temporal phosphorylation and dephosphorylation of CK2alpha. *Mol Cell Biol*. 2009;29(8):2068-2081.

46. Birnbaum MJ, Glover CVC. The Phosphotransferase Activity of Casein Kinase-Ii Is Required for Its Physiological-Function Invivo. *Biochem Biophys Res Commun*. 1991;181(2):524-528.

47. Dotan I, Ziv E, Dafni N, et al. Functional conservation between the human, nematode, and yeast CK2 cell cycle genes. *Biochem Biophys Res Commun*. 2001;288(3):603-609.

48. Olsen BB, Petersen J, Issinger O-G. BID, an interaction partner of protein kinase CK2α. *Biol Chem*. 2006;387:441-449.

49. Desagher S, Osen-Sand A, Montessuit S, et al. Phosphorylation of Bid by casein kinases I and II regulates its cleavage by caspase 8. *Mol Cell*. 2001;8(3):601-611.

50. Tapia J, Torres V, Rodriguez D, Leyton L, Quest A. Casein kinase 2 (CK2) increases survivin expression via enhanced β-catenin–T cell factor/lymphoid enhancer binding factor-dependent transcription. *Proc Natl Acad Sci USA*. 2006;103(41):15079-15084.

51. Duncan JS, Turowec JP, Duncan KE, et al. A peptide-based target screen implicates the protein kinase CK2 in the global regulation of caspase signaling. *Sci Signal*. 2011;4(172)

52. Duncan JS, Turowec JP, Vilk G, Li SSC, Gloor GB, Litchfield DW. Regulation of cell proliferation and survival: convergence of protein kinases and caspases. *Biochim Biophys Acta*. 2010;1804:505-510.

53. Stalter G, Siemer S, Becht E, Ziegler M, Remberger K, Issinger OG. Asymmetric expression of protein kinase CK2 subunits in human kidney tumors. *Biochem Biophys Res Commun*. 1994;202(1):141-147.

54. Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC. Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene*. 2001;20:3247-3257.

55. Yenice S, Davis AT, Goueli SA, Akdas A, Limas C, Ahmed K. Nuclear casein kinase 2 (CK-2) activity in human normal, benign hyperplastic, and cancerous prostate. *Prostate*. 1994;24(1):11-16.

56. Seldin DC, Leder P. Casein kinase II alpha transgene-induced murine lymphoma: relation to theileriosis in cattle. *Science*. 1995;267(5199):894-897.

57. Pluemsampant S, Safronova OS, Nakahama KI, Morita I. Protein kinase CK2 is a key activator of histone deacetylase in hypoxia-associated tumors. *Int J Cancer*. 2008;122(2):333-341.

58. Rabellino A, Scaglioni PP. PML Degradation: Multiple Ways to Eliminate PML. *Front Oncol*. 2013;3(March):60.

59. Scaglioni PP, Yung TM, Cai LF, et al. A CK2-dependent mechanism for degradation of the PML tumor suppressor. *Cell*. 2006;126(2):269-283.

60. Zhao T, Jia H, Li L, et al. Inhibition of CK2 enhances UV-triggered apoptotic cell death in lung cancer cell lines. *Oncol Rep*. 2013;30(1):377-384.

61. Hanahan D, Weinberg RA. The Hallmarks of Cancer. *Cell*. 2000;100(1):57-70.

62. Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Sonenshein GE. Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells. *Cancer Res*. 2002;62(22):6770-6778.

63. Barroga CF, Stevenson JK, Schwarz EM, Verma IM. Constitutive phosphorylation of I kappa B alpha by casein kinase II. *Proc Natl Acad Sci U S A*. 1995;92(17):7637-7641.

64. Wang D, Westerheide SD, Hanson JL, Baldwin a S. Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. *J Biol Chem*. 2000;275(42):32592-32597.

65. Duncan JS, Litchfield DW. Too much of a good thing: The role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. *Biochim Biophys Acta - Proteins Proteomics*. 2008;1784(1):33-47.

66. Ahmed K, Yenice S, Davis a, Goueli S a. Association of casein kinase 2 with nuclear chromatin in relation to androgenic regulation of rat prostate. *Proc Natl Acad Sci U S A*. 1993;90(10):4426-4430.

67. Tawfic S, Ahmed K. Growth Stimulus-Mediated Differential Translocation of Casein Kinase-2 to the Nuclear Matrix - Evidence Based on Androgen Action in the Prostate. *J Biol Chem*. 1994;269:24615-24620.

68. Guo C, Yu S, Davis AT, Ahmed K. Nuclear matrix targeting of the protein kinase CK2 signal as a common downstream response to androgen or growth factor stimulation of prostate cancer cells. *Cancer Res*. 1999;59(5):1146-1151.

69. Ahmad K a, Wang G, Slaton J, Unger G, Ahmed K. Targeting CK2 for cancer therapy. *Anticancer Drugs*. 2005;16(10):1037-1043.

70. Guo C, Yu S, Davis a T, Wang H, Green JE, Ahmed K. A potential role of nuclear matrix-associated protein kinase CK2 in protection against drug-induced apoptosis in cancer cells. *J Biol Chem*. 2001;276(8):5992-5999.

71. Wang G, Unger G, Ahmad K a, Slaton JW, Ahmed K. Downregulation of CK2 induces apoptosis in cancer cells--a potential approach to cancer therapy. *Mol Cell Biochem*. 2005;274(1-2):77-84.

72. Sarno S, Pinna L a. Protein kinase CK2 as a druggable target. *Mol Biosyst*. 2008;4(9):889-894.

73. Zien P, Duncan JS, Skierski J, Bretner M, Litchfield DW, Shugar D. Tetrabromobenzotriazole (TBBt) and tetrabromobenzimidazole (TBBz) as selective inhibitors of protein kinase CK2: evaluation of their effects on cells and different molecular forms of human CK2. *Biochim Biophys Acta*. 2005;1754(1-2):271-280.

74. Pierre F, Chua PC, O'Brien SE, et al. Pre-clinical characterization of CX-4945, a potent and selective small molecule inhibitor of CK2 for the treatment of cancer. *Mol Cell Biochem*. 2011;356(1-2):37-43.

75. Cozza G, Mazzorana M, Papinutto E, et al. Quinalizarin as a potent, selective and cell-permeable inhibitor of protein kinase CK2. *Biochem J*. 2009;421(3):387-395.

76. Zandomeni R, Zandomeni MC, Shugar D, Weinmann R. Casein kinase type II is involved in the inhibition by 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole of specific RNA polymerase II transcription. *J Biol Chem*. 1986;261(7):3414-3419.

77. Pagano M a, Andrzejewska M, Ruzzene M, et al. Optimization of protein kinase CK2 inhibitors derived from 4,5,6,7-tetrabromobenzimidazole. *J Med Chem*. 2004;47(25):6239-6247.

78. Sarno S, Reddy H, Meggio F, et al. Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). *FEBS Lett*. 2001;496(1):44-48.

79. Mishra S, Pertz V, Zhang B, et al. Treatment of P190 Bcr/Abl lymphoblastic leukemia cells with inhibitors of the serine/threonine kinase CK2. *Leukemia*. 2007;21(1):178-180.

80. Kim J, Kim SH, Kim D. Druggability of the CK2 inhibitor CX-4945 as an anticancer drug and beyond. *Arch Pharm Res*. 2012;35(8):1293-1296.

81. Siddiqui-Jain A, Drygin D, Streiner N, et al. CX-4945, an orally bioavailable selective inhibitor of protein kinase CK2, inhibits prosurvival and angiogenic signaling and exhibits antitumor efficacy. *Cancer Res*. 2010;70(24):10288-10298.

82. R. F. Marschke, M. J. Borad, R. W. McFarland, R. H. Alvarez, J. K. Lim, C. S. Padgett, D. D. Von Hoff SEO and DWN. Findings from the phase I clinical trials of CX-4945, an orally available inhibitor of CK2. *J Clin Oncol*. 2011

83. Nakanishi I, Murata K, Nagata N, et al. Identification of protein kinase CK2 inhibitors using solvent dipole ordering virtual screening. *Eur J Med Chem*. 2015;96:396-404.

84. Guerra B, Hochscherf J, Jensen NB, Issinger O-G. Identification of a novel potent, selective and cell permeable inhibitor of protein kinase CK2 from the NIH/NCI Diversity Set Library. *Mol Cell Biochem*. 2015.

85. Chekanov MO, Ostrynska O V., Synyugin AR, Bdzhola VG, Yarmoluk SM.

Design, synthesis and evaluation of 2-phenylisothiazolidin-3-one-1,1-dioxides as a new class of human protein kinase CK2 inhibitors. *J Enzyme Inhib Med Chem*. 2014;29(3):338-343.

86. Fujiwara Y, Hiraoka Y, Fujimoto T, Kanayama N, Magari M, Tokumitsu H. Analysis of Distinct Roles of CaMKK Isoforms Using STO-609-Resistant Mutants in Living Cells. *Biochemistry*. 2015;54(25):3969-3977.

87. Eyers PA, van den IjP, Quinlan RA, Goedert M, Cohen P. Use of a drug-resistant mutant of stress-activated protein kinase 2a/p38 to validate the in vivo specificity of SB 203580. *FEBS Lett*. 1999;451(2):191-196.

88. Cozza G, Pinna L a, Moro S. Kinase CK2 inhibition: an update. *Curr Med Chem*. 2013;20(5):671-693.

89. Mazzorana M, Pinna L a., Battistutta R. A structural insight into CK2 inhibition. *Mol Cell Biochem*. 2008;316(1-2):57-62.

90. Ferguson AD, Sheth PR, Basso AD, et al. Structural basis of CX-4945 binding to human protein kinase CK2. *FEBS Lett*. 2011;585(1):104-110.

91. Battistutta R. Structural bases of protein kinase CK2 inhibition. *Cell Mol Life Sci*. 2009;66(11-12):1868-1889.

92. Sarno S, Salvi M, Battistutta R, Zanotti G, Pinna L a. Features and potentials of ATP-site directed CK2 inhibitors. *Biochim Biophys Acta - Proteins Proteomics*. 2005;1754(1-2):263-270.

93. Battistutta R, De Moliner E, Sarno S, Zanotti G, Pinna L a. Structural features underlying selective inhibition of protein kinase CK2 by ATP site-directed tetrabromo-2-benzotriazole. *Protein Sci*. 2001;10(11):2200-2206.

94. Hanks S, Hunter T. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J*. 1995;9:576-596.

95. Battistutta R, Mazzorana M, Cendron L, et al. The ATP-binding site of protein kinase CK2 holds a positive electrostatic area and conserved water molecules. *ChemBioChem*. 2007;8(15):1804-1809.

96. Meggio F, Donella Deana a, Ruzzene M, et al. Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. *Eur J Biochem*. 1995;234(1):317-322.

97. Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J*. 1995;9(8):576-596.

98. Life Technologies Corporation. Flp-In™ T-REx™ Core Kit For Generating Stable, Inducible Mammalian Expression Cell Lines by Flp Recombinase-Mediated Integration. *User Guid*. 2012;(25):1-9.

99. Field J, Nikawa J, Broek D, et al. Purification of a RAS-responsive adenylyl cyclase complex from Saccharomyces cerevisiae by use of an epitope addition method. *Mol Cell Biol*. 1988;8(5):2159-2165.

100. Schembri L, Dalibart R, Tomasello F, Legembre P, Ichas F, De Giorgi F. The HA tag is cleaved and loses immunoreactivity during apoptosis. *Nat Methods*. 2007;4(2):107-108.

101. Garger SJ, Griffith OM, Grill LK. Rapid purification of plasmid DNA by a single centrifugation in a two-step cesium chloride-ethidium bromide gradient. *Biochem Biophys Res Commun*. 1983;117(3):835-842.

102. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A*. 1992;89(12):5547-5551.

103. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1985;150(1):76-85.

104. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227(5259):680-685.

105. Bio-rad laboratories. Mini-trans-blot Electrophoretic Transfer Cell. 2014.

106. Sarno S, Ruzzene M, Frascella P, et al. Development and exploitation of CK2 inhibitors. *Mol Cell Biochem*. 2005;274:69-76.

107. Sarno S, de Moliner E, Ruzzene M, et al. Biochemical and three-dimensional-structural study of the specific inhibition of protein kinase CK2 by [5-oxo-5,6-dihydroindolo-(1,2-a)quinazolin-7-yl]acetic acid (IQA). *Biochem J*. 2003;374(Pt 3):639-646.

108. St-denis N, Gabriel M, Turowec JP, et al. Systematic investigation of hierarchical phosphorylation by protein kinase CK2. *J Proteomics*. 2014:1-14.

109. Tiganis T, House CM, Kemp BE. Protein kinase CK2: biphasic kinetics with peptide substrates. *Arch Biochem Biophys*. 1996;325(2):289-294.

110. Wood ER, Truesdale AT, McDonald OB, et al. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): Relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res*. 2004;64(18):6652-6659.

111. Penner CG, Wang Z, Litchfield DW. Expression and localization of epitope-tagged protein kinase CK2. *J Cell Biochem*. 1997;64(4):525-537.

112. Llorens F, Roher N, Miró FA, et al. Eukaryotic translation-initiation factor eIF2beta binds to protein kinase CK2: effects on CK2alpha activity. *Biochem J*. 2003;375(Pt 3):623-631.

113. Llorens F, Duarri A, Sarró E, Roher N, Plana M, Itarte E. The N-terminal domain of the human eIF2beta subunit and the CK2 phosphorylation sites are required for its function. *Biochem J*. 2006;394(Pt 1):227-236.

114. Poletto G, Vilardell J, Marin O, et al. The regulatory beta subunit of protein kinase CK2 contributes to the recognition of the substrate consensus sequence. a study with an eIF2beta-derived peptide. *Biochemistry*. 2008;47(32):8317-8325.

115. Green CJ, Göransson O, Kular GS, et al. Use of Akt inhibitor and a drug-resistant mutant validates a critical role for protein kinase B/Akt in the insulin-dependent regulation of glucose and system A amino acid uptake. *J Biol Chem*. 2008;283(41):27653-27667.

116. Turowec JP, Duncan JS, French AC, et al. *Protein Kinase CK2 Is a Constitutively Active Enzyme That Promotes Cell Survival: Strategies to Identify CK2 Substrates and Manipulate Its Activity in Mammalian Cells*. Vol 484. 1st ed. Elsevier Inc.; 2010.

117. Sarno S, Papinutto E, Franchin C, et al. ATP site-directed inhibitors of protein kinase CK2: an update. *Curr Top Med Chem*. 2011;11(11):1340-1351.

118. Son YH, Song JS, Kim SH, Kim J. Pharmacokinetic characterization of CK2 inhibitor CX-4945. *Arch Pharm Res*. 2013;36(2013):840-845.

119. Penner CG, Wang Z, Litchfield DW. Expression and localization of epitope-tagged protein kinase CK2. *J Cell Biochem*. 1997;64(4):525-537.

120. Turowec JP, Vilk G, Gabriel M, Litchfield DW. Characterizing the convergence of protein kinase CK2 and caspase-3 reveals isoform-specific phosphorylation of caspase-3 by CK2α': implications for pathological roles of CK2 in promoting cancer cell survival. *Oncotarget*. 2013;4(4):560-571.

121. Di Maira G, Salvi M, Arrigoni G, et al. Protein kinase CK2 phosphorylates and upregulates Akt/PKB. *Cell Death Differ*. 2005;12(6):668-677.

122. Kim TK, Eberwine JH. Mammalian cell transfection: The present and the future. *Anal Bioanal Chem*. 2010;397(8):3173-3178.

123. Canton D a, Olsten MEK, Niederstrasser H, Cooper J a, Litchfield DW. The role of CKIP-1 in cell morphology depends on its interaction with actin-capping protein. *J Biol Chem*. 2006;281(47):36347-36359.

124. French AC, Luscher B, Litchfield DW. Development of a stabilized form of the regulatory CK2beta subunit that inhibits cell proliferation. *J Biol Chem*. 2007;282(40):29667-29677.

125. Gates C a, Cox MM. FLP recombinase is an enzyme. *Proc Natl Acad Sci U S A*. 1988;85(13):4628-4632.

126. Dymecki SM. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc Natl Acad Sci U S A*. 1996;93(12):6191-6196.

127. O'Gorman S, Fox DT, Wahl GM. Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science (80-)*. 1991;251(4999):1351-1355.

128. Haynes K a., Silver P a. Synthetic reversal of epigenetic silencing. *J Biol Chem*. 2011;286(31):27176-27182.

129. Caballero A, Marchese A. Receptor Signal Transduction Protocols. 2011;746:251-259.

130. Gossen M, Bonin a L, Freundlieb S, Bujard H. Inducible gene expression systems for higher eukaryotic cells. *Curr Opin Biotechnol*. 1994;5(5):516-520.

131. Gossen M, Freundlieb S, Bender G, Müller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science*. 1995;268(5218):1766-1769.

132. Baron U, Gossen M, Bujard H. Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Res*. 1997;25(14):2723-2729.

133. Sammarco MC, Grabczyk E. A series of bidirectional tetracycline-inducible promoters provides coordinated protein expression. *Anal Biochem*. 2005;346(2):210-216.

134. Bian Y, Han J, Kannabiran V, et al. MEK Inhibitor PD-0325901 Overcomes Resistance to CK2 Inhibitor CX-4945 and Exhibits Anti-Tumor Activity in Head and Neck Cancer. *Int J Biol Sci*. 2015;11(4):411-422.

135. Cozza G, Sarno S, Ruzzene M, et al. Exploiting the repertoire of CK2 inhibitors to target DYRK and PIM kinases. *Biochim Biophys Acta - Proteins Proteomics*. 2013;1834(7):1402-1409.

136. Liebler DC, Zimmerman LJ. Targeted quantitation of proteins by mass spectrometry. *Biochemistry*. 2013;52(22):3797-3806.

5 - Appendix

Figure 15. Quantification of protein expression in Flp-In U2OS cell lines
(A-B) CK2α-HA. (C-D) CK2α (V66A/H160D/I174A)-HA.

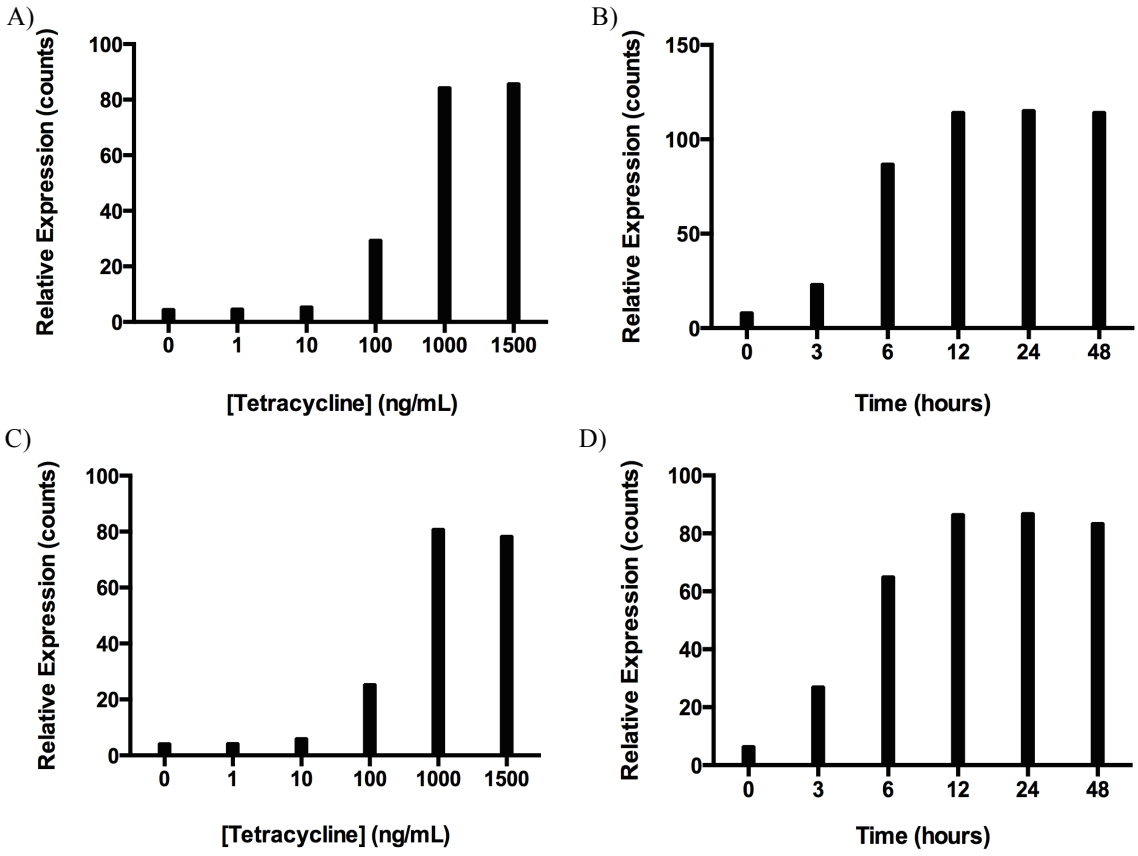
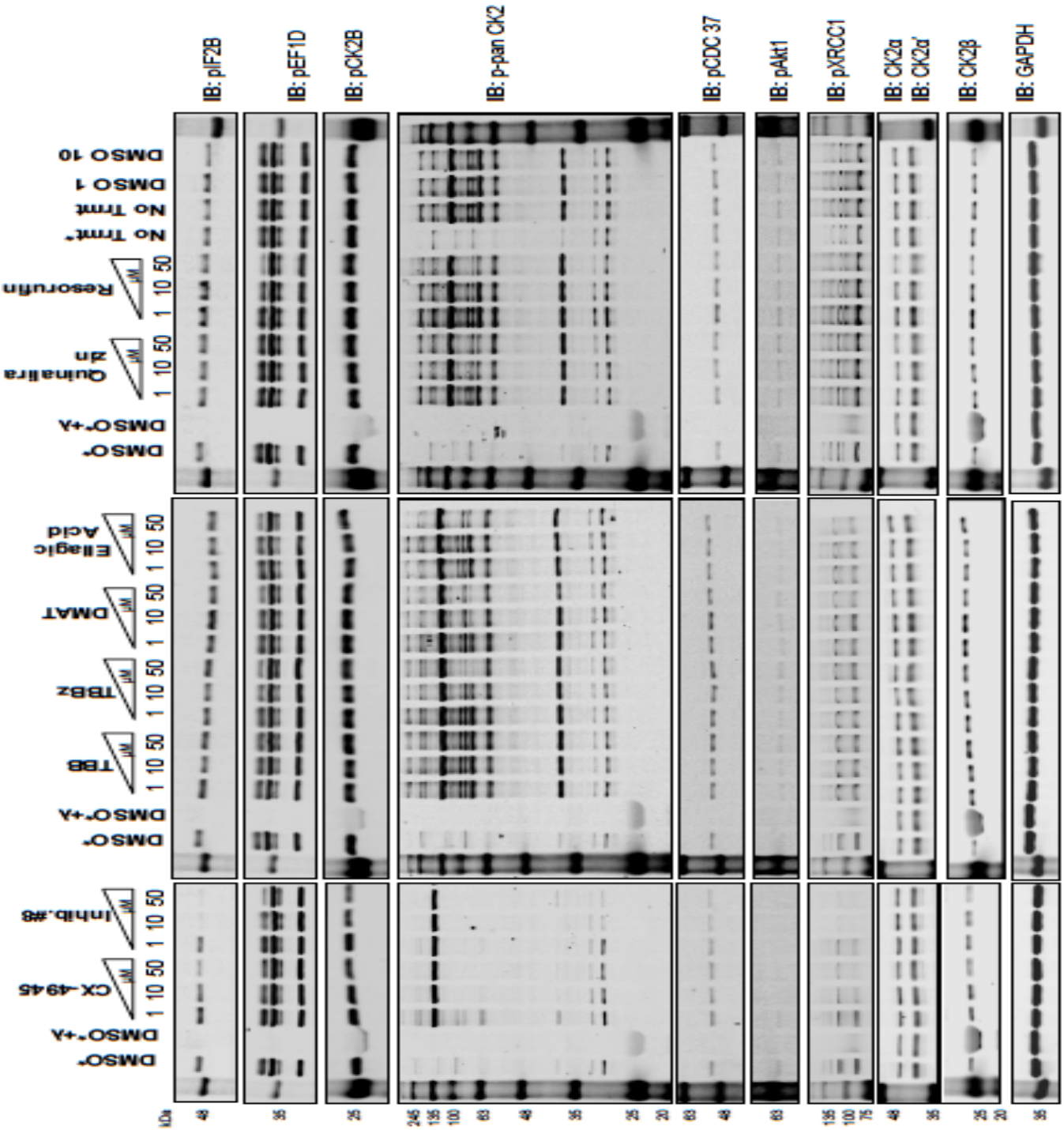


Figure 16. Characterization of CK2-phosphorylation specific antibodies



Sample calculation

Calculating specific activity (GST-CK2α)

All reactions were performed in triplicate and an average was taken, this represents a single reaction.

[GST-CK2α]= 6.06 mg/mL

2 µL of GST-CK2α (6.06 µg/µL) was initially diluted into 250 µL of CK2 dilution buffer

Therefore, (2 µL)* (6.06 µg/µL)= 12.12 µg of GST-CK2α

Next, 4 µL of the 250 µL sample was taken in order to start the kinase reaction

Therefore, (12.12 µg)*(4 µL/ 250 µL)= 0.193 µg

The reaction proceeded for 10 minutes

10 µL of the total kinase reaction (20 µL final volume) was then spotted onto P81 paper

Therefore, (0.193 µg)/2 = 0.0969 µg of GST-CK2α

The 10-minute reaction (GST-CK2α phosphorylating a RRRDDDSDDD peptide) yielded a count (CPM) of 859749.14, and the spotted standards yielded a CPM of 82236698.38

Therefore, to calculate the CPM/pMol of ATP in the standard – (82236698.38 CPM) /(10 µL spot * 100 µM ATP * 5 for the dilution)= 16447 CPM/pMol

Therefore, to calculate the specific activity of GST-CK2α:

(8597491.4 CPM) / (16447 CPM/pMol)= 522.7 pMol of ATP incorporated onto the RRRDDDSDDD peptide

(522.7 pMol) / (10 minutes)= 52.27 pMol/min

(52.27 pMol/min) / (0.0000969 mg) = **539422.1 pMol/min/mg of GST-CK2α**

Curriculum Vitae (CV) – Sam Reid Fess

Graduate Summary

Research Biochemist practiced in cell-based assay development, molecular and structural biology, with hands on experience testing the effects of various small molecule inhibitors on their intended targets

Skills

- Proficient in cell culture
- Development of cell lines and implementation of cell-based assays
- Understanding of protein separation, isolation, and purification techniques
- Experience with immunochemistry techniques (Western Blots, ELISA, fluorescent microscopy)
- Extensive work with inhibitors
- Trained in lab safety
- Laboratory work competency
- MS Office proficiency
- Outstanding interpersonal skills
- Extremely quick leaner
- Adaptive

Work History

SEPTEMBER 2014 – DECEMBER 2014

Teaching Assistant (TA) – Human Biochemistry 3385A – Dr. Bonnie Deroo, Ph.D. – Western University, London, Ontario

- Responsible for monitoring a web server to address students questions and concerns regarding course material
- Provided learning opportunities by holding tutorial sessions for exam preparation
- Proctored examinations

JANUARY 2014 – APRIL 2015

Teaching Assistant (TA) – Biochemistry Laboratory 3380G – Dr. Derek McLachlin, Ph.D. – Western University, London, Ontario

- Mentored and led a group of 8 biochemistry students through various laboratory protocols and procedures
- Demonstrated proper techniques to ensure personnel and lab safety
- Advised students on experimental design

Education

2016

Masters of Science: Biochemistry – Western University, London, Ontario

- Thesis: "The Rational Design and Evaluation of CK2α Mutants Bearing Inhibitor-Refractory Amino Acid Substitutions"

  Advisor: Dr. David Litchfield, Ph.D.

- Conducted basic and applied research on the protein kinase CK2 (previously referred to as Casein Kinase 2)
- Designed and optimized assays
- Developed and evaluated mutants of protein kinase CK2 that are less sensitive to inhibition by CK2 inhibitors
- Collected and analyzed biological data about relationships between protein kinase CK2 and its cellular environment
- Interpreted research findings, summarized data into reports, and communicated research results to individuals in the academic community and general public

2013

Bachelor of Science: Biochemistry & Cell Biology – Western University, London, Ontario

Certifications

- WHMIS
- Biosafety
- Radiation Safety Nuclear