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Characterization of the catalytic CK2 subunits with substitutions at residues involved in inhibitor binding

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Abstract

CK2 is a constitutively active, ubiquitously expressed and pleiotropic serine/threonine protein kinase that is implicated in many cellular functions including tumorigenesis. CK2 has two catalytic subunits, CK2α and CK2α', that carry out its function in the cell. Previous studies have indicated that inhibitor-refractory mutants have been effective in recovering residual CK2 activity, in the presence of inhibitors, when compared to wild type CK2. Based on these observations, inhibitor-refractory mutants were created for both CK2α and CK2α' and tested with various concentrations with two CK2-specific inhibitors, CX-4945 and inhibitor VIII. The CK2α triple mutant (V66A/I174A/H160D) was tested in inducible U2OS Flp-In cell lines with inhibitor VIII and CX-4945 inhibitors and was found to recover residual CK2 activity 5-fold greater than previously established CK2α-(V66A/I174A) inhibitor-refractory mutants. Seven novel mutations were made to CK2α' *in vitro* and tested with CK2 inhibitor CX-4945. The mutants that recovered the most residual CK2 activity were introduced into stable cell lines and tested with the same conditions as the CK2α catalytic subunit. Generation of inhibitor-refractory mutants for both catalytic subunits of CK2 will provide insight into the distinct functions, interactions with inhibitors and ability to recover activity of the two catalytic subunits of CK2.

Dedications

I dedicate this thesis to my mother and father for all their support throughout this entire

process.

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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
Cdk1	cyclin-dependent kinase 1
Cdk2	cyclin-dependent kinase 2
CK2	protein kinase CK2 (formerly casein kinase II)
CK2α	CK2 alpha, catalytic subunit
CK2α'	CK2 alpha prime, catalytic subunit
CK2β	CK2 beta, regulatory subunit
CK2α'-WT	CK2α'-HA
CK2α'-DM	CK2α'-V67A/I175A-HA
CK2α'-TM	CK2α'-V67A/H161E/I175A-HA
CK2α-WT	HA-CK2α
CK2α-DM	HA-CK2α-V66A/I174A
CK2α-TM	HA-CK2α-V66A/H160D/I174A
CO_2	carbon dioxide
CML	chronic myelogenous leukemia
CX-4945	CK2 inhibitor (developed by Cylene Pharmaceuticals)
ddH_2O	double distilled water
DFG	Aspartic Acid, Phenylalanine, Glycine motif
DMAT	2-Dimethylamino-4,5,6,7-Tetrabromo-1H-Benzimidazole
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulfoxide
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	epitope tag derived from human influenza hemagglutinin
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

MEK	MAPK kinase
mg	milligram
ml	milliliter
mM	millimolar
Myc	c-Myc epitope tag
NF-kB	NF-kappaB
nm	nanometer
nM	nanomolar
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
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBBt	tetrabromobenzotriazole
TBBz	tetrabromobenzimidazole
TBS	Tris buffered saline
U2OS	human osteosarcoma cells

1. Introduction

1.1 – Protein Kinases

Protein kinases are enzymes that catalyze the transfer of the gamma phosphoryl group from ATP or, in some cases, GTP to the hydroxyl group of a serine, threonine or tyrosine residue on a recipient substrate protein [1]. There are conformational differences that exist between the active and inactive forms of substrates. These changes are often controlled by the addition of a phosphate by protein kinases [2]. The phosphorylation of substrate proteins can result in activation of key cellular processes including, but not limited to, cell survival, proliferation and control over apoptosis [3].

Alterations in the natural function of protein kinases often result in a number of different diseases, including many different malignancies [4]. The dysregulation of protein kinases can result in over-expression or under-expression of key genes or proteins that can lead to uncontrolled activity with oncogenic behavior. The phosphorylation of tumor suppressor genes or oncogenic proteins is capable of regulating their activity [5]. Overactive kinases that target these key gene products can alter downstream regulation of vital cell signaling pathways including survival, proliferation and apoptosis [6].

As a result of the ability of kinases to control cellular processes, the human kinome has become an attractive drug target for the treatment of uncontrolled growth and tumorigenesis [7]. The 518 kinases of the human kinome include the targets of 30% of the inhibitors in drug development projects by pharmaceutical companies, second only to G-coupled protein receptors as the most pharmaceutically targeted protein type in the human body [8].

The first successful therapy towards a protein kinase was the use of the drug Imatinib (Gleevec). Imatinib targets the Bcr-Abl tyrosine kinase that is overactive in a number of malignancies including chronic myelogenous leukemia (CML) [9]. Imatinib is an ATP-competitive inhibitor that binds the inactive form of the Bcr-Abl kinase, where the DFG motif is pointing outwards, in order to prevent its activation. This allows for a stronger specificity of the inhibitor, as the inactive form of a kinase is less conserved throughout evolution and thus more distinct from other kinases [10].

Imatinib induced apoptosis in cells that were reliant on Abl for growth. Studies showed Imatinib exhibited little cytotoxicity, as the drug did not target cells that were not Abl-dependent. The drug showed tumor regression in leukemia after 15 weeks of treatment [11]. The success of Imatinib has provided the basis for research into the inhibition of other kinases for the treatment of uncontrolled cell growth [12]. Protein kinase CK2 (formerly casein kinase II) is one of the kinases that is being examined by researchers for its role in tumorigenesis [13].

My thesis work is focusing on protein kinase CK2 that has emerging importance in cancer research. It is overactive in a number of cancer cells and has unique features that make its regulation interesting [14]. There is also an inhibitor of CK2, namely CX-4945, that is currently in clinical trials [15].

1.2 – Protein Kinase CK2

Protein kinase CK2 differs from many kinases which are activated in response to specific stimuli, as CK2 is a constitutively active enzyme [16]. It is a highly pleiotropic protein serine/threonine kinase that can recognize hundreds of proteins as substrates and is ubiquitously expressed within the cell and throughout the body as it can be detected in

all the organs examined in a mouse embryo [17]. It is among the kinases with the most interacting partners, many of which control cell survival and growth. CK2 recognizes a consensus sequence that includes a Ser/Thr N-terminal to clusters of acidic residues with a minimum recognition requirement of: S/T-X-X-D/E [18]. Recognition of a consensus sequence of acidic residues is less common in the majority of protein kinases.

Multiple examples of CK2 dysregulation in human disease have prompted the interest in this kinase as a therapeutic target. It has been implicated in transformation and development of tumorigenesis by protecting the cells from apoptosis *via* regulation of tumor suppressor and oncogene activity [19]. However, the precise role of CK2 in tumorigenesis is still not completely understood.

1.2.1 The Structure of CK2

CK2 is a holoenzyme composed of two catalytic subunits (two CK2α, two CK2α' or CK2α and CK2α') as well as two regulatory subunits (CK2β) [16]. The kinase exists primarily in the tetramer holoenzyme form within the cell but the catalytic units remain active on their own without the need of the regulatory beta subunits. Crystallographic data demonstrated more information on the composition of the holoenzyme showing that the tetramer of the protein exists as two regulatory CK2β subunits that connect two catalytic CK2α or CK2α' subunits. The two CK2β subunits mediate the formation of the holoenzyme [20]. In lymphoid cell lines, CK2β is expressed in excess of the catalytic subunits and degraded if not immediately formed into complexes. Tetrameric complexes including two CK2α or two CK2α' show little difference in catalytic activity but the two catalytic isoforms have shown distinct functional activity when expressed on their own [21].

The constitutive activity of CK2 can be explained by its unique structure. There is a conserved DFG motif across the majority of kinases found at the N-terminus of the activation loop. "DFG-in" conformation refers to the formation of the kinase when the phenylalanine residue within DFG forms a hydrophobic spine by binding with one residue on the N-terminal lobe and one residue on the C-terminal lobe [22]. The DFG-in conformation leads to activation of the kinase. Inactivation occurs when this phenylalanine moves out of the hydrophobic pocket to alter the interaction of asparate and glycine within the DFG, in the process, blocking ATP binding to the active site [23]. This is sometimes referred to as the DFG-out conformation. In CK2, the phenyalanine residue common to most kinases is replaced by tryptophan at position 176 [24]. The presence of tryptophan allows the kinase to maintain the open active state and thus contributes to the constitutive activation of CK2. It aids in the coordination of the arrangement of the N and C terminal lobes for the proper positioning of the essential residues within the catalytic site to maintain the active state of CK2 [25]. The majority of ATP competitive inhibitors that target CK2 differ from Imatinib, which can target and stabilize the inactive state of the Bcr-Abl fusion protein.

Another unique aspect of the structure of CK2 is its small hydrophobic active site. Specifically, it contains two hydrophobic residues, V66 and I174, at positions that are occupied by alanine residues in the majority of protein kinases [26]. Additionally, due to the unique active site of CK2, it can bind both ATP and GTP, whereas most protein kinases can only bind ATP in their active sites [27]. CK2 also has a histidine residue, which has a flexible side chain, at position 160 that is at the entrance of the catalytic site. The binding of ATP alters the conformation of the histidine. When ATP is bound, its

triphosphate, protruding outside the active site, keeps the imidazole ring of histidine turned downwards, opening the mouth of the cleft in the process. When ATP is not bound, the imidazole ring is turned back to form an H-bond with the carbonyl oxygen of the Arg47 residue [28].

1.2.2 The Regulation of CK2

CK2β can have an influence over the ability of CK2 to phosphorylate targets but the catalytic subunits express similar activity towards the majority of targets regardless of whether the regulatory beta subunit is present [29]. The exact role of CK2β is not entirely known but two of its suggested functions are: (1) altering of the activity of the catalytic subunits of CK2 and (2) protecting the kinase from degradation. There are a number of mechanisms of regulation of CK2 however, there is still more research to be done to understand how the CK2β subunit contributes to the regulation and activity of the kinase [29].

CK2 appears to be regulated by a number of distinct means of regulation rather than one universal regulatory process for all CK2 proteins in an organism [30]. The regulation of CK2 is a result of its subcellular localization as well as its interaction with binding partners within the cell. The interaction partners of CK2 can coordinate the cellular location as well as its interactions with other proteins in the cell [31].

There has also been some evidence of tissue specific expression and regulation of CK2. Several studies using rats found that CK2 has the highest expression in the brain and testis and that CK2α/α' subunits were not expressed at the same level within different tissues. Specifically, CK2α was expressed at higher levels than CK2α' in the spleen, liver and lung [32, 33]. CK2 expression has also varied within neuron cells within

brain disorders such as Parkinson's disease and metabolic disorders like diabetes [34]. Overall, this contributes to the mounting evidence that tissue specific expression is a determining factor in the regulation of CK2.

CK2 has also been known to participate in hierarchical phosphorylation, which is another form of regulation. In these instances, CK2 will only phosphorylate a protein if that protein has been previously phosphorylated by another kinase. If that kinase is not constitutively active and has the ability to be turned off and on, than its regulation will in turn regulate the activity of CK2 [35]. Interestingly, phosphoserine or phosphotyrosine can be recognized as a specificity determinant within the acidic consensus sequence that was mentioned earlier. This gives further evidence for the role CK2 can play in hierarchical phosphorylation events.

Overall, while its activity is not controlled in the same fashion as the majority of kinases due to its constitutive activity, there are a number of regulatory mechanisms that the cell can use to monitor the activity of CK2. The role of the regulation and overexpression of CK2 in diseases such as tumorigenesis and cancer is justifiably the subject of much research.

1.3 – CK2 in cancer

1.3.1 – CK2 and tumorigenesis

Elevated activity of CK2 has been associated with aggressive tumor behavior in a number of cancers including lung [36], head and neck [37], prostate [38], kidney [39] and breast [40] Furthermore, overexpression of CK2 in mouse models resulted in the transformation of T-cells and mammary gland epithelium, which led to the development of tumorigenesis [41]. In addition, an increase in CK2 levels coupled with increased

levels of c-Myc or Tal-1 or with decreased expression of tumor suppressor p53 resulted in a further increase of oncogenic activity thus promoting tumorigenesis [42,43]. In addition to the role CK2 plays in interacting with oncogenes, CK2 also protects cells from apoptosis *via* interactions controlling caspase cleavage, degraded proteasome susceptibility and the altered the activity of oncogenes as well as tumor suppressors.

1.3.2 – CK2 and tumor suppressors

Tumor suppressor genes are responsible for the slowing of cell growth, the facilitation of DNA repair and they play a key role in apoptosis. The phosphorylation of tumor suppressor genes can alter their activity to increase or decrease affinity to targets. Phosphorylation also plays a role in the protection and promotion of tumor suppressor genes from degradation [44]. CK2 interacts with PML to alter susceptibility to proteasomal degradation [45]. The CK2 phosphorylation of tumor suppressor PTEN stabilizes the inactive form of the protein. CK2 also phosphorylates Tp73 to deactivate it in cell [46].

1.3.3 CK2 and Oncogenes

An oncogene is a protein that when mutated has the potential to promote cancer. They are often expressed at elevated levels in tumor cells. Elevated CK2 levels have been associated with the promotion of a number of gene products that control proliferation and apoptosis [47]. While CK2 is not itself considered an oncogene, it directly targets a number of known oncogenes including c-myc, c-myb, c-jun, B-catenin, Wnt and PI3-K [48, 49]. Overexpression of CK2 leads to elevated levels of some of these proteins potentially leading to protection from apoptosis and elevated cell growth levels [50].

1.3.4 – CK2 in apoptosis

CK2 is known to phosphorylate components of the apoptotic machinery. The pro-apoptotic protein Bid that activates mitochondrial apoptotic machinery is targeted for phosphorylation by CK2 to inhibit its activation [51]. CK2 also functions to stimulate the apoptotic inhibitor survivin *via* beta-catenin-Tcf/Lef-mediated transcription [52]. Both the deactivation of pro-apoptotic signals and the stimulation of apoptotic inhibitors function by preventing caspase activation, which is normally responsible for the execution of apoptosis. CK2 also directly phosphorylates caspases and caspase substrates proximal to caspase cleavage site to inhibit caspase-mediated cleavage [53].

Kinase negative studies, where the transfection of mutated CK2 kinases that lack key residues for the for the ability of CK2 to phosphorylate substrate proteins, and chemical inhibition studies have shown that lowering the level of CK2 in cell results in the attenuation of apoptosis. Studies performed using cells expressing mutant forms of CK2 face the issue of endogenous CK2 present within the cell population, which can confound the effect of the CK2 mutants [54]. To overcome this limitation, the development of cell permeable pharmacological inhibitors of CK2 have enabled an alternative strategy for interfering with the activity of CK2 within cells.

1.4 – The Inhibition of CK2

1.4.1 – Kinase Inhibitors

Two of the categories that kinase inhibitors can be separated into are allosteric inhibitors and competitive inhibitors. Allosteric inhibitors target outside the active site of the kinase but still inhibit the kinase's activity. They usually act by binding independent of the active site to alter the binding site so that the substrate will no longer fit, thus

keeping the kinase in the inactive state. Competitive inhibitors face the challenge of having to compete with the high cellular concentrations of ATP and therefore, in order to be effective, they must be present at intracellular concentrations that significantly exceed their *in vitro* determined K_I values [55].

Allosteric inhibitors are generally needed at lower concentrations than competitive inhibitors due to the fact that they do not need to outcompete the high intracellular concentrations of ATP. Allosteric inhibitors also tend to target kinases more specifically by binding to residues outside the active site where there is lower conservation of residues [56]. However, allosteric inhibitors are thought to be less effective due to lower affinity for their targets and therefore decreased inhibitor potency. Allosteric inhibitors also often have reduced intracellular stability, difficulty with uptake in the cell and issues with bioavailability [57].

Competitive inhibitors directly compete with the ATP substrate by mimicking the structural properties of ATP in order to efficiently bind the active site. The high level of conservation among kinases means there are only small differences within their ATP-binding pockets, which makes it difficult to develop selective inhibitors for one kinase without affecting a large number of off-target kinases. However, complete selectivity is not always necessary for a kinase inhibitor to be effective and to be accepted in clinical trials [58]. In fact, it can sometimes be seen as a benefit of a drug to bind and affect multiple pathways involved with diseases. This type of inhibitor requires a large library screen of candidate compounds, which can be time consuming and expensive. Despite this limitation, many ATP-competitive drugs are now available and are under investigation in clinical trials.

1.4.2 CK2 Inhibition

CK2 has emerged as a potential therapeutic target due to its anti-apoptotic role as well as its role in many cellular processes. One competitive inhibitor of CK2, designated CX-4945, has already entered into clinical trials. CK2 inhibitors have also been utilized in experimental studies to test the role of CK2 within cellular processes and to identify *bona fide* targets of CK2. One of the original strategies used to alter CK2 levels within cells was to transfect kinase-inactive CK2 mutants into cells. However, the long half-life, the cellular localization as well as the high endogenous expression of CK2 in cells limited the effectiveness of these studies [54]. The developments of new CK2-selective inhibitors serve as a more efficient way to study the effect of inhibiting CK2 within cells. The chemical inhibitors can penetrate and inhibit the majority of [9, 14, 15] exogenous and endogenous CK2 in the cells.

Non-competitive allosteric inhibitors would generally target the beta subunit or the interaction between alpha and beta subunits [59]. The majority of currently available CK2 inhibitors however, are ATP-competitive inhibitors that directly target the small hydrophobic active site of CK2. Researchers were able to take advantage of the unique binding pocket of CK2 to design inhibitors that made contacts with the unique hydrophobic valine and isoleucine residues within the catalytic pocket of CK2. These inhibitors are smaller than the majority of kinase inhibitors and specifically target the V66 and I174 residues. This allows them to have fewer off target reactions with other kinases that do not share these binding pocket properties [60]. Two of the first CK2-specific inhibitors that were developed were 4,5,6,7-Tetrabromobenzotriazole (TBB) ad 4,5,6,7-Tetrabromobenzimidazole (TBBz), which are the most commonly used CK2

inhibitors in cell studies to date [61]. However, comparing the selectivity of TBB and

TBBz across a panel of 80 kinases showed that they were not as selective as initially

believed. There was also evidence that the apoptosis seen in cancer cells was, at least in

part, due to off-target effects of the inhibitors [62]. This was illustrated using rescue

experiments and inhibitor-refractory mutants, as the mutants did not enable the cells to

recover from the apoptosis associated with TBB and TBBz. Additionally, an unbiased

chemoproteomics approach revealed a number of off-target interactions of the TBB and

TBBz inhibitors [63].

1.4.3 – CK2 Inhibitors: CX-4945 and Inhibitor VIII

There is a new orally available compound named CX-4945 that has shown the

efficiency and selectivity suitable to be accepted into stage two clinical trials for the

treatment of blood-based cancers [64]. The compound was synthesized from molecule: 4-

oxo-4,5- dihydrothieno [3,2-c] quinoline-7-carboxylic acid by making a number of

substitutions to incorporate alkyl chains bearing aniline moieties and replacing the

existing thiopene ring with a pyridine ring [65]. To examine the selectivity of the

compound, it was tested with *in vitro* kinase assays against a panel of 235 other kinases

where it showed great specificity for CK2. In addition to showing strong specificity for

the kinase, it was also more potent than any of the existing CK2 inhibitors. CX-4945 has

proven to be well tolerated and possesses anti-tumor activity within mouse models. In a

study using PC3 prostate cancer xenograft mice models, CX-4945 exhibited anti-tumor

activity when delivered orally twice a day over a 35-day period. Various CX-4945

concentrations showed a linear regression in tumor growth when compared to the vehicle.

The mice also tolerated the drug by showing no significant weight loss over the 35-day period. Finally, when the inhibitor was tested with PC3 prostate carcinoma cells, it showed anti-proliferation activity by inducing cell cycle arrest and initiating apoptosis [66].

Another compound was identified as a potential CK2 inhibitor from a virtual screen using a library of approximately three million compounds. The compound 2-phenyl-1,3,4-thiadizole was found and later optimized to make 4-(thiazol-5-yl)benzoic acid derivative, which was later named CK2 Inhibitor VIII. The improved potency of the drug was designed with multiple favorable interactions with CK2 binding pocket. It showed a very high level of specificity when screened against a panel of 70 kinases, only having a minor effect on two kinases distinct from the CK2 catalytic subunits [67]. It also showed heightened inhibitory effects in multiple cancer cell lines. While CX-4945 had the highest level of potency of any CK2 inhibitor tested to date [67], Inhibitor VIII had a greater efficacy on the cancer cells than did CX-4945 in terms of anti-proliferative effects on cell lines and reducing tumor size in mice models. More tests are continuing to be done to test and optimize the inhibitor with different cancer cells and tumor xenografts [67]. As more CK2-specific inhibitors come through the experimental pipeline and eventually pass to clinical trials, there is a growing need for the development of experimental strategies to test these inhibitors. Figure 1 displays the structure of two of the CK2-specific inhibitors which are the focus of this thesis and compares them to the structures of two CK2 substrates, ATP and GTP.
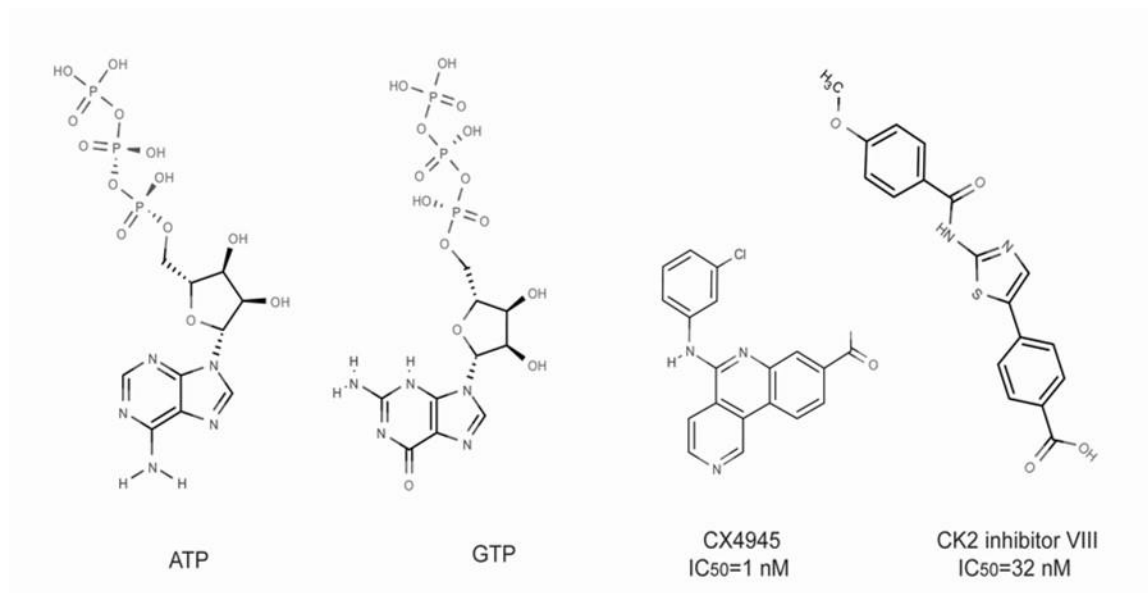
Figure 1. Structure of ATP, GTP, and current inhibitors of protein kinase CK2.
Chemical structures of ATP, GTP, and characterized inhibitors, CX-4945 and Inhibitor
VIII, of protein kinase CK2. Previously reported IC$_{50}$ values have also been included.
[28, 65, 67]

1.5 – CK2-Inhibitor refractory mutants

There have been a number of advances in devising strategies to test and identify *bona fide* CK2 targets. In order to fall under the category of a *bona fide* CK2 target, the target must meet two criteria: (1) the phosphorylation event must occur *in vivo*, and (2) the phosphorylation event must change with CK2 activity [68]. The use of CK2 inhibitors has been effective at investigating the alteration of CK2 activity in cell but there exists the potential that the off-target drug effects are responsible for the altered level of phosphorylation [63]. In order to address the potential lack of specificity of the kinase being tested, we have produced inhibitor-refractory mutants of CK2α and CK2α'. These mutant kinases provide new validation tools for CK2-specific targets as they could provide evidence that the change in phosphorylation is due to inhibition of CK2 and not an off target [69].

Inhibitor-refractory mutants were developed by examining the crystal structure of CK2 and identifying specific hydrophobic residues within its ATP binding pocket. The mutations took advantage of the unique binding properties of CK2 by mutating the hydrophobic residues, V66 and I174, in the active site to smaller and less hydrophobic alanine residues [62]. The mutant CK2 kinases can still bind ATP through a network of interactions similar to other kinases. This allows for maintained catalytic activity while resisting the effects of the inhibitors.

The goal of the inhibitor refractory mutant studies was to find a concentration of inhibitor where the wild type CK2 will be inhibited entirely but the mutant will maintain activity. This was tested with *in vitro* radioactive kinase assays and with live cell culture with the use of antibodies specific for previous established *bona fide* CK2 substrates [18].

The inhibitor refractory mutants can ultimately be used to distinguish isoform specific phosphorylation events in the cells.

Previous studies have taken advantage of a double mutant, V66A/I174A, which reveals some recovery of CK2 activity in the presence of CX-4945 *in vitro* [70]. Another study used this double mutant in cells with various concentrations of TBB inhibitor to recover the activity toward a synthetic peptide [71]. We sought to create a mutant that would achieve a higher recovery of activity than the previously established double mutant and that would achieve effective recovery in live cell culture studies.

Previous studies have shown the importance of the His160 residue in binding CX-4945. When CX-4945 binds the active site of CK2 it flips the imidazole of His160 residue downward to open up the cleft, thus locking in the inhibitor, making it more difficult for the inhibitor to reversibly bind the target. The flexibility of the residue is adjustable based on which inhibitor is bound [28].

Mutating the His160 (CK2α) / 161 (CK2α') residues into Asp/Glu residues, which lack imidazole ring that normally allow for stronger binding of the inhibitor, would alleviate the ability of CK2 to lock in CX-4945 at this position. Additionally, the Asp/Glu residues have a negative charge in place of the positive charge of the His residue, allowing for repulsion from the original binding capabilities of the His residue.

CK2α has His115 while CK2α' has a corresponding Tyr116 (with a one amino acid shift forward). Mutating the Tyr116 in CK2α' to a His residue would mimic the sequence of CK2α. The Tyr116 residue can also be mutated to a Lys residue, which has the same charge as the His residue but lacks the imidazole ring, to investigate if the charge or the imidazole ring at position 116 in CK2α' is necessary for binding the

inhibitor. We investigated if these alterations would change the activity or the recovery capacity of the kinase in the presence of inhibitors. The creation of inhibitor refractory mutants for both the CK2α and CK2α' catalytic subunits could provide a tool to investigate their independent functional roles within the cell more closely. Figure 2 shows the active site of CK2α and CK2α' with the residues of importance highlighted in different colours.
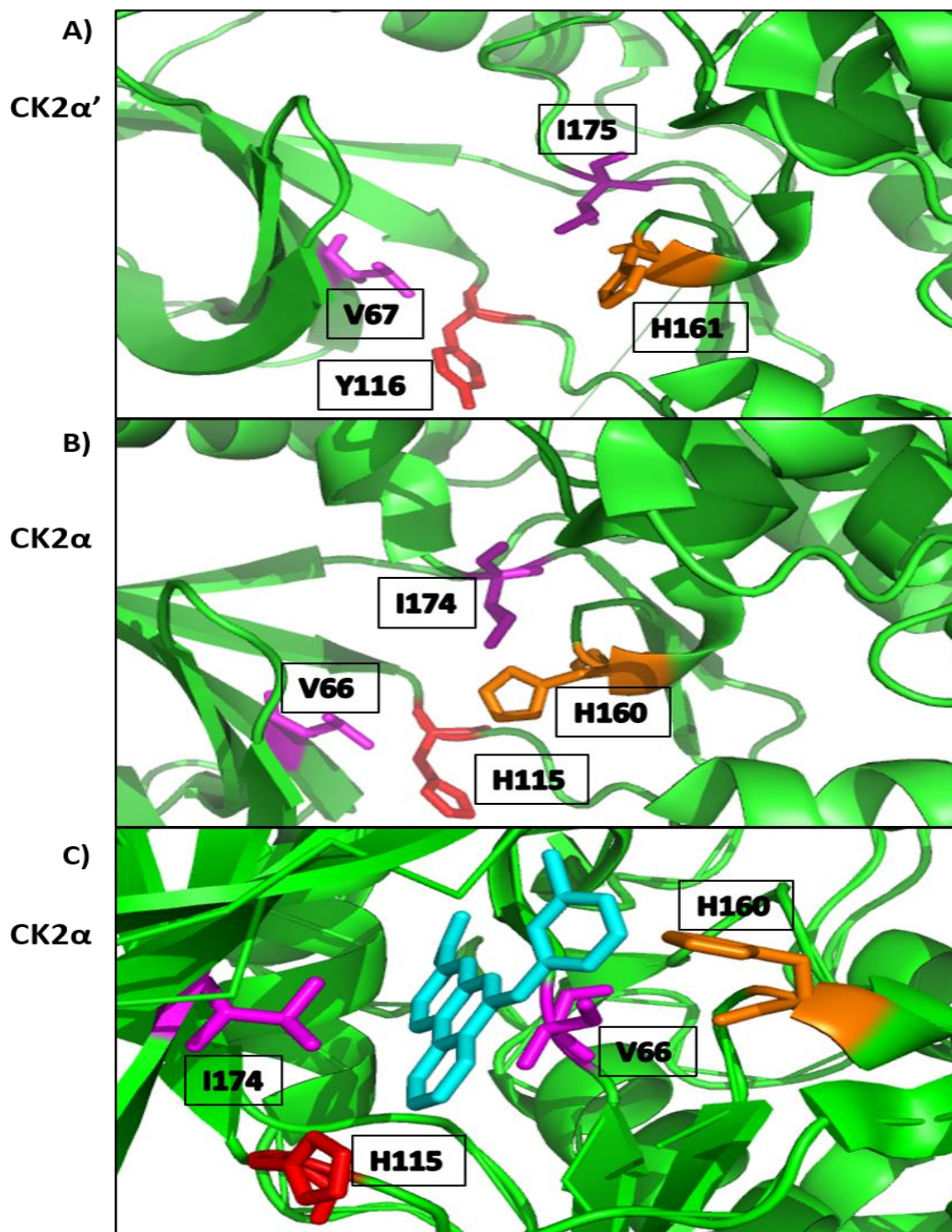
Figure 2. The crystal structure of the active site of CK2. The residues mutated in the previously established double mutant kinase are highlighted in pink. The newly mutated residue, His160, is shown in orange, residues H115/Y116 are highlighted in red and CX-4945 is shown in cyan. A) PDB ID: 3E3B. The active site with corresponding mutations in CK2α'. B) PDB ID: 1NA7. The active site of CK2α. C) PDB ID: 3PE1. The active site of CK2α with CX-4945 inhibitor bound.

1.6 – CK2α and CK2α'

  The two catalytic isoforms of CK2 are distinct genes that are located on different chromosomes [72]. They share 90% sequence identity within the N terminal 330 amino acids, and unrelated C-terminal tails [72]. While the majority of studies have shown the two isoforms to have overlapping functions within the cell, there have been some experiments that illustrate their functional specialization [73]. For example, CK2α is phosphorylated within sites specific to its unique C-terminal domain, specifically at Thr 344, Thr 360, Ser 362 and Ser 370 by Cdk1/p34^{cdc2}, while CK2α' is not phosphorylated by p34^{cdc2} [74]. Differences have also been observed when investigating isoform-specific binding partners of CK2α and CK2α'. Specifically, that CK2α binds HSP90, Pin-1, PP2A and CKIP-1 while CK2α' does not [73, 75 – 77]. Again, the C-terminal domain of CK2α has an important role in binding with some of these proteins. The C-terminal domain of CK2α has a proline rich area of phospho acceptor sites that is responsible for binding Pin-1. It also contains a unique basic lysine-rich region that is responsible for the binding of Hsp90 [74]. Figure 3 compares the sequences of CK2α and CK2α', illustrating the similarity in the N-Terminal 330 amino acids.

  Interestingly, pro-Caspase-3 was found to be the first CK2 substrate to be selectively phosphorylated by CK2α' in cells. It was shown, by manipulating the expression of the two isoforms to investigate their relationship with caspase-3, that CK2α' had a significant preference for caspase-3 phosphorylation in cells when compared to CK2α. Phosphorylation of pro-Caspase-3 by CK2 can block its cleavage by both caspase-8 and caspase-9 to prevent its activation [78].

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MSGPVP-SRARVYTDVNTHRPREYWDYESHVVEWGNQDDYQLVRKLGRGKYSEVFEAINI   59
*  **     ******    **  *  *******  **   *****************************
MPGPAAGSRARVYAEVNSLRSREYWDYEAHVPSWGNQDDYQLVRKLGRGKYSEVFEAINI   60

TNNEKVVVKILKPVKKKKIKREIKILENLRGGPNIITLADIVKDPVSRTPALVFEHVNNT   119
****  *****************  ********  ***  *  *  ******  *******    ***
TNNERVVVKILKPVKKKKIKREVKILENLRGGTNIIKLIDTVKDPVSKTPALVFEYINNT   120

DFKQLYQTLTDYDIRFYMYEILKALDYCHSMGIMHRDVKPHNVMIDHEHRKLRLIDWGLA   179
*******  ***  ********  *********  ****************         **********
DFKQLYQILTDFDIRFYMYELLKALDYCHSKGIMHRDVKPHNVMIDHQQKKLRLIDWGLA   180

EFYHPGQEYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMIFRKEPFFHGHDNY   239
*****  ****************************************************  ******   ***
EFYHPAQEYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMIFRREPFFHGQDNY   240

DQLVRIAKVLGTEDLYDYIDKYNIELDPRFNDILGRHSRKRWERFVHSENQHLVSPEALD   299
**************  **  *    **  *  ***  ******  *******  *  ****  *********
DQLVRIAKVLGTEELYGYLKKYHIDLDPHFNDILGQHSRKRWENFIHSENRHLVSPEALD   300

FLDKLLRYDHQSRLTAREAMEHPYFYTVVKDQARMGSSSMPGGSTPVSSANMMSG       354
 **********  ****  *********  ***  *          *  *          *  *
LLDKLLRYDHQQRLTAKEAMEHPYFYPVVKEQ------SQPCADNAVLSSGLTAA       349
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Figure 3. The amino acid alignment between CK2 catalytic subunits CK2α and CK2α'. The sequences are from Uniprot database (http://www.uniprot.org/) and aligned using a BLAST (http://blast.ncbi.nlm.nih.gov). Stars between the two sequences indicate identical residues on either isoform while a space indicates a mismatch between the sequences. The sequence of CK2α is on the top line and the sequence of CK2α' is shown on the bottom line. The final 40 amino acids on the C-terminal of each subunit is missing.

Several knockout studies have been performed in both yeast and rats to investigate the independent roles of the two catalytic subunits. In Arabidopsis cells, loss of CK2α results in the loss of cell polarity while loss of CK2α' results in cell cycle arrest [79]. Temperature sensitive yeast mutants illustrated that knockout of CK2α' can be compensated with CK2α. In mouse models, CK2α' knockouts resulted in sterile mice but a knockout of either CK2α or CK2β resulted in lethality of the organism [80]. Although more studies continue in this area, there remains a limited understanding of the functional specialization between the two CK2 isoforms. This functional specialization cannot however, be explained by differences in catalytic activity, as both the protein subunits have nearly identical catalytic sites.

Differences in tissue distribution have also been noted between the subunits. Nuclear CK2 activity levels were measured in different rat tissues and there were some distinctions in the activity levels between the two subunits. Western blot analysis using antibodies specific for the CK2α subunit showed that it was highly expressed in the brain, testes, spleen and liver while it maintained lower activity in the heart and kidney [81]. Within this same experiment, antibodies specific for the CK2α' subunit illustrated that it was most abundant in the brain and testes [81]. Overall, these findings suggest different roles for the CK2 isoforms in different tissues and therefore functional differences of the isoforms.

The development of double (V66A/I174A) and triple (V66A/I174A/H160D) inhibitor-refractory mutants of both isoforms of CK2 provides us with tools to investigate *bona fide* targets for the two isoforms and ultimately increase our knowledge of the unique cellular functions of the proteins. Studies that compare the inhibited activity of

CK2α and CK2α' with wild type and inhibitor-refractory mutants can be analyzed *via*

proteomics to find isoform-specific CK2 targets. The identification of these targets will

lead to a better understanding the pathways that the two isoforms participate in and how

they participate in the progression of cancer.

1.7 – Objective

A number of studies both *in vitro* and in mammalian cells have investigated the

interaction and inhibition of the catalytic subunits of CK2. Inhibitor studies have become

a promising tool in the investigation of catalytic and regulatory subunits of CK2 in many

survival pathways. The inhibitors function by taking advantage of the unique structural

properties of the CK2 binding pocket. Specifically, inhibitors targeted the two

hydrophobic residues: V66 and I174. Inhibitor-refractory mutants of wild type CK2 have

been examined in the past to seek validation for the specificity of the inhibitors being

used.

I hypothesize that the V66A/I174A/H160D inhibitor-refractory mutant will

recover more residual CK2 activity than models that have previously been documented in

the literature. Because of this, we will be able to create tools for the identification of *bona*

fide CK2 targets and the study of CK2 inhibitors unlike anything previously established. I

further hypothesize that the mutations previously created in CK2α *in vitro*, can be

replicated in CK2α' *in vitro* and that this will allow us to develop stable cell lines with

CK2α'. These new cell lines can be used to compare the efficacy of inhibitors and

identification of subunit-specific targets of CK2.

The objective of this thesis is to find conditions for the recovery of residual CK2

activity in the presence of inhibitor in mutant cell populations. When these conditions are

met, the objective is to test the effect of CX-4945 and inhibitor VIII on the phosphorylation of previously established CK2-specific phosphorylation sites as well as on the morphology of the cells. Once this is tested, the goal is to reproduce the same system with CK2α' mutants, by first producing *in vitro* mutants and then testing those same mutants in cell culture. Overall, these studies will provide a new strategy to test inhibitors, find CK2-specific substrates and compare the functions of the two catalytic subunits of CK2. Ultimately, this work and the work to follow will lead to a better understanding of an essential protein, that has been proven to be involved with many diseases and still has many questions surrounding its regulation and function.

Materials and Methods

2.1 - Development of Flp-In U2OS cell lines

Human osteosarcoma (U2OS) cells, with the Flp-In integration site, were provided by Dr. Karmella Haynes at Arizona State University [82]. The pcDNA5 vector (purchased from Invitrogen) contained the integration sites required for the insertion of the gene of interest. It also contained the Flp recombinase to enable insertion of the gene of interest into the host U2OS cells. The Flp-In U2OS cell lines expressing HA-CK2α' (CK2α'-WT), HA-CK2α'-V67A/I175A (CK2α'-DM), HA-CK2α'-V67A/H161E/I175A (CK2α-TM), CK2α-HA (CK2α'-WT), CK2α-V66A/I174A-HA (CK2α-DM) and CK2α-V66A/H160D/I174A-HA (CK2α-TM) were generated following the directions from the vendor (Invitrogen).

2.2 - Cell culture, protein induction and harvest

Briefly, cells were induced for 24 hours with 1 μg/ml tetracycline before being treated with varying concentrations of CX-4945 and inhibitor VIII to examine the effect of the inhibitors on the phosphorylation of known CK2 substrates. Cells were maintained in Dulbecco's modified Eagle's medium (Corning) with 10% fetal bovine serum (Cansera) and 1% penicillin (100 units/ml)/streptomycin (0.1 mg/ml) (Invitrogen) within an environment of 5% CO_2 at 37°C. Selection media contained 150 μg/ml hygromycin B and 15 μg/ml blasticidine. Induction of the cells to express the protein of interest relied on the addition of tetracycline. The concentration of tetracycline was titrated to find the optimal concentration of 1 μg/ml. For induction, cells were typically grown to 70-80% confluency, then washed with PBS and the addition of fresh media containing the selective hygromycine and blasticidine drugs as well as the proper tetracycline

concentration. The cells were then incubated at 5% CO_2 at 37°C for 24 or 48 hours when the media was again replaced with media containing 150 µg/ml hygromycin, 15 µg/ml blasticidine, 1 µg/ml tetracycline and varying concentrations (0 nM – 30 nM) of CX-4945 or inhibitor VIII for an additional 3, 4, 5, 6, 7 or 8 hours after induction.

The cells were harvested with Tris lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% Deoxycholic Acid) with additional protease and phosphate inhibitors (1 mM PMSF, 7 µg/ml Pepstatin A, 20 µg/ml Leupeptin, 2.9 µg/ml Aprotinin, 1 µM Okadaic acid, 1 µM Microcystin, 5 mM NaF, 1 mM sodium orthovanadate). Cell lysates were cleared by centrifugation at 2000 x g at 4°C for 15 minutes to remove cell debris. The lysates were stored at -20°C until they were run on SDS-PAGE gels.

2.3 - Antibodies

The antibody used to measure levels of exogenous and endogenous CK2 was referred to as CK2α/α' (diluted 1:2000 in 3% BSA/TBST). It was raised against the last 51 residues on the C terminal tail CK2α' protein that has some similarity with residues near the C-terminus of CK2α. Phosphospecific antibodies, pIF2B (pS2) and CK2B (pS2/3/4/8) (diluted 1:10,000 in 3% BSA/TBST), were used to assess the phosphorylation activity of CK2 on known CK2 substrates. GAPDH antibody (diluted 1:2000 in 3% BSA/PBST) was used to indicate the equal amount of protein that was loaded on the gel. The biotinylated 3F10 antibody directed against the HA epitope (diluted in 1:500 3%BSA/PBST) was used to detect the expression of the HA-tagged exogenous proteins. (Roche). The goat-anti-rabbit (GAR), goat-anti-mouse (GAM) (diluted 1:10,000 in 1% BSA/TBST) and streptavidin (SAV) (diluted 1:10,000 in 1%

BSA/PBST) secondary antibodies (LiCor) were conjugated to fluorophores that could be detected at 680 nm or 800 nm when scanned on the LiCor Odyssey.

2.4 - SDS-PAGE and Western Blotting

BCA protein assays (Pierce) were used to determine the protein concentration. Samples (10 μg of protein) were prepared for separation *via* SDS-PAGE gel first by adding an equivalent volume of 2x Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl pH 6.8, 10% β-mercaptoethanol) to each sample. The samples were heated for 3 minutes at 100°C and loaded onto the SDS-gel. They were subjected to electrophoresis in SDS-PAGE buffer (192 mM glycine, 25 mM Tris-base pH 8.0, 0.1% SDS) for 1.5 hours at 120V.

With use of a wet mini trans-blot electrophorectic transfer cell (Biorad), the samples were transferred from the gel to a polyvinylidene difluoride membrane (Thermo Fisher). The blot was transferred for 1 hour at 100 V in blotting buffer (20% methanol, 10 mM Tris-base pH 8.6, 767 mM glycine). The membrane was then blocked with LiCor Blocking buffer for 1 hour followed by washing with Tris buffered saline with Tween (TBST).

The phosphorylation state of known CK2 substrates was assessed with three antibodies, pEEF1D, pIF2B pS2 and pCK2β pS2,pS3,pS4,pS8, which were individually incubated with the blots for one hour each, washed three times with TBST then incubation with the GAR800 secondary antibody. The membranes were scanned with the Licor Odyssey scanner between each antibody. Expression of the HA-tagged proteins was detected with biotinylated anti-HA 3F10. The membrane was washed three times with PBST. Finally, the membrane was incubated SAV680 for 45 minutes. The primary

antibody used on the membranes the CK2α/α' antibody, incubated for 1 h at room temperature, washed three times with TBST and then incubated with the GAR800 secondary antibody. In order to demonstrate equal loading the final primary antibody used was GAPDH, washed 3 times with PBST and incubated with secondary antibody GAM 680.

2.5 - GST-CK2 mutants

Inhibitor-refractory mutants of CK2 α' with a point mutation at H161 or Y116 sites were generated with site-directed PCR mutagenesis. Single amino acid substitutions were made to the wild type or double mutant. After the PCR reaction was finished, samples were digested with Dpn1 for 30 minutes at 37°C. The PCR products were mixed with 90 μL of chemically competent BL21 *E. coli* cells and incubated on ice for 30 minutes. The solutions were then heat shocked for 45 seconds at 42°C, mixed with SOC media and grown up for 45 minutes before being grown on Ampicillin plates overnight.

Single colonies were picked from the LB agar ampicillin plates (100 μg/ml ampicillin), grown up in liquid media and the plasmid DNA was isolated using a plasmid DNA kit (Qiagen) following the manufacturer recommendations. The sequence was then verified by the forward sequencing primer, pGEX 5'(GGGCTGGCAAGCCACGTTTTGGTG, 3' end of GST tag). GST-CK2α', GST-CK2α' (Y116L), GST-CK2α' (H161D), GST-CK2α' (H161E), GST-CK2α' (V66A/I174A), GST-CK2α' (V66A/I174A/Y116H), GST-CK2α' (V66A/I174A/H161E), GST-CK2α' (V66A/I174A/H161D) plasmids encoding the protein of interest that were transformed into BL21 cells were grown in 2xYT media (16g tryptone, 10g yeast extract, 5g NaCl, 1L ddH$_2$0, pH7.0) at 37°C for 9 hours until the

optical density at 600 nm reached 0.6 Induction of protein expression was achieved with the addition of 1 mM IPTG overnight.

Bacterial cells were harvested by centrifugation (5000 X g) in 500ml bottles for 15min at 4°C. Media was decanted from cell pellets, the supernatant was discarded and the pellet was resuspended in 50-100ml of cold PBS cultures before centrifugation (4420 X g) for 15 minutes at 4°C. The PBS was decanted and the cell pellets were resuspended in 15ml of cold PBS (with protease inhibitors). Cells were then run through a homogenizer in order for them to be lysed. 10% Triton-X100 was then added to make a final concentration of 1% to lysate. The suspension was then rotated at 4°C for 15min and the supernatant was then cleared by centrifugation. The supernatant was mixed with glutathione-agarose beads (Sigma) in a 10 ml gravity column and allowed to flow through the column. The column was washed with 50 ml of PBS then the protein was extracted using elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl pH 8.0, 1 mM DTT).

The eluted fractions were then run on a 12% SDS-PAGE gel, stained with Coomassie blue and boiled the coomassie stained gel in water in order to destain. Fractions containing the desired protein were then dialyzed (50% glycerol, 50mM Tris-HCl, pH 7.5, 200mM NaCl, 1mM EDTA, 1mM DTT) overnight and stored at -20°C. The activity of the purified GST-CK2 was determined with a CK2 kinase assay following established protocols as described in the following section [83].

2.6 - *In vitro* kinase assays

In vitro kinase assays were performed to measure the activity of GST-CK2α'

mutants. The activity assays were prepared by mixture of 4 μL of 5X kinase reaction

buffer (10 mM $MgCl_2$, 100 mM NaCl, [γ-^{32}P] ATP, 100 μM cold ATP, 50 mM Tris

buffer), 1 μL of 100 μM RRRDDDSDDD CK2 acceptor peptide and 10 μL H_2O. Each of

the assays to determine the Km for ATP was performed by increasing the amount of cold

ATP in the kinase buffer while maintaining the same concentration of the substrate

peptide. The reactions were initiated by addition of each of the different mutant forms of

the CK2 kinase (4 μL) (diluted 1:250 in kinase buffer). The kinases were added in 20-

second intervals. The samples were then incubated in a 37°C water bath for 10 minutes,

after which 10 μL of the kinase reaction was spotted onto P81 phosphocellulose paper at

20-second intervals that were timed with the initial addition of kinase. The

phosphocellulose paper was washed 3 times with 1% phosphoric acid and once in 95%

ethanol. The phosphocellulose papers were dried with a heat lamp for 10 minutes before

being exposed to phosphorimaging screen (Amersham Biosciences) for an hour. Finally,

the Storm phosphorimager was used to detect the level of ^{32}P-labelled substrate bound to

the P81 paper. The ^{32}P incorporated into the RRRDDDSDDD peptide was measured in

order to assess the activity of each kinase reaction. The samples were quantified in

comparison to standards that were spotted onto the same P81 paper scanned by the

phosphorimager screen. The specific activity of each kinase was calculated according to

the equation used in the appendix.

2.6.1 - Inhibition of CK2α' mutants with CX-4945 inhibitor

The same *in vitro* kinase assays were prepared as described earlier this time with the addition of either DMSO, 5 nM or 15 nM of CX-4945. Each inhibitor assay was performed with 100 μM ATP, the appropriate amount of inhibitor and brought to a total volume of 16 μL with the addition of H_2O before the addition of the mutant or wild type kinase. The inhibitors were added at 20-second intervals, 10 minutes prior to the addition of the kinase solution, which was also added in 20-second intervals. Samples were processed as described above. The level of radioactivity was measured on each spot by the Storm phosphorimager. This corresponded to the activity of the reaction and thus the effectiveness of the CX-4945 inhibitor on the various mutants and concentration of drug used. From the relative intensity measured on the Storm phosphoimager, we were able to calculate the specific activity of each of the kinases at differing drug concentrations.

Results

3.1 – Short-term recovery of activity in mutant CK2α cell lines

Three constructs, CK2α-HA (Wild type, WT), CK2α-V66A/I174A-HA (double mutant, DM) and CK2α-V66A/I174A/H160D-HA (triple mutant, TM), were transfected into tetracycline-inducible U2OS Flp-In cell lines without the addition of the regulatory CK2β subunit. Because of the single integration site in Flp-In cells, the Flp-In system does not allow for bidirectional transfection and therefore the regulatory subunit cannot be transfected into the system alongside the HA-tagged CK2 kinase. Even in the absence of exogenous CK2 β, expression of the HA-tagged CK2α was detected.

A titration experiment was performed in order to evaluate the tetracycline concentration needed for optimal expression. This, collected by S. Fess, was found to be 1 µg/ml (supplemental Figure 1), which was subsequently used as the concentration for all the experiments described within this section. The expression of CK2α-HA, detected using the HA-3F10 antibody for both the CK2α-DM and CK2α-TM kinases, was consistent with the expression level of the CK2α-WT kinase. (Figure 4).
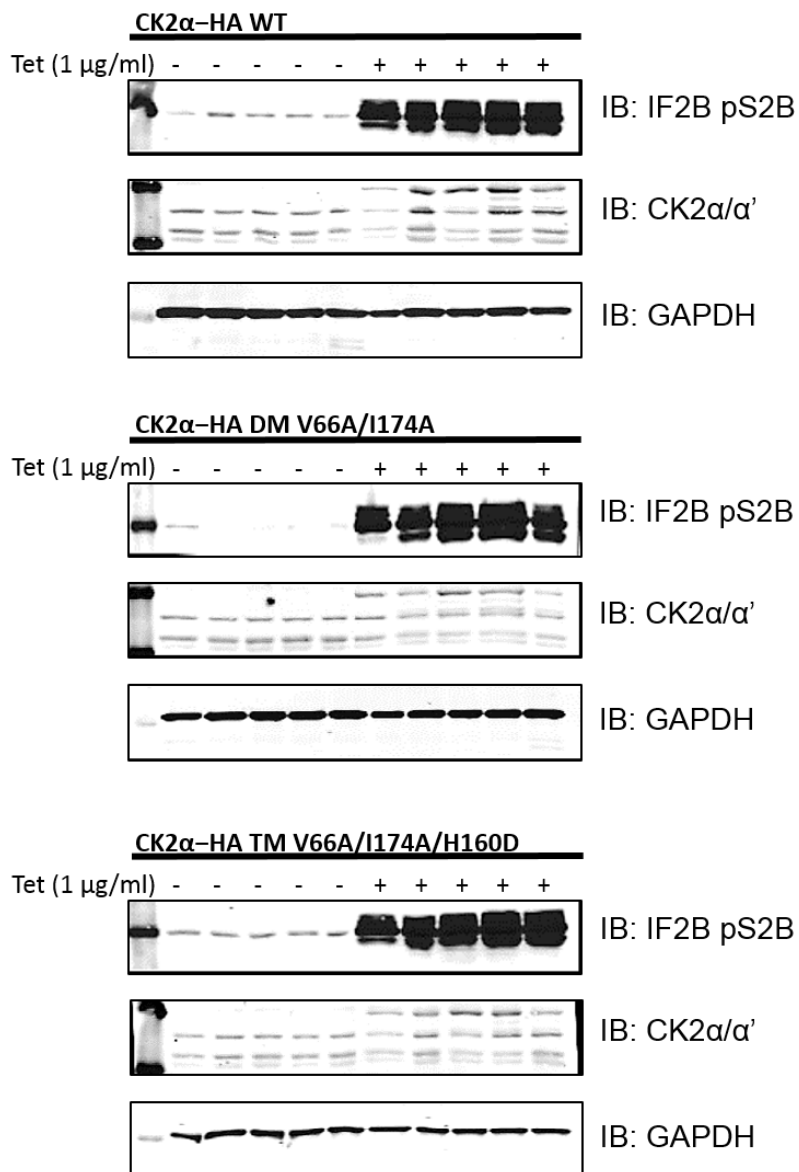
Figure 4. The expression level of CK2α-HA in CK2α-WT, CK2α-DM and CK2α-TM kinase. Cells CK2α-HA DNA construct was induced in the Flp-In U2OS cells for 24 increments over the span of 120 hours with 1 µg/ml tetracycline. The CK2α/α' antibody was used to compare the level of exogenous CK2 to the endogenous CK2α an CK2α'. The GAPDH antibody was used as a loading control. Protein lysate was harvested, blotted and incubated with HA-3F10 antibody in order to measure the expression of the protein.

After noting the consistently elevated expression level of the CK2α-WT, CK2α-DM and CK2α-TM kinases, the cells expressing different forms of the kinases were treated with a titration of two different CK2-specific inhibitors. The three different cell lines were treated with increasing concentrations (DMSO control, 15 μM and 30 μM) and time points (1, 3, 4, 5, 6, 7 and 8 hours) of the CK2-specific inhibitors CX-4945 and CK2 Inhibitor VIII. (Figure 5 and 6).

The experiment measured the ability of the different mutants to phosphorylate a known CK2 substrate in varying concentrations of CX-4945 and Inhibitor VIII. This will indicate the importance of the residue being mutated in binding inhibitors as well as indicate the ability of the mutant to overcome inhibitor treatment. Based on these experiments, the optimal condition for recovery of the CK2α-TM kinase, when compared to the activity of the CK2α-WT kinase, was found to be 7 hours at 30 μM CX-4945. The optimal condition for Inhibitor VIII was found to be 6 hours at 15 μM CK2 Inhibitor VIII; half of the inhibitor concentration needed with CX-4945 treatment. (Figure 5 and 6). Because both Figure 5 and Figure 6 were titration experiments attempting to find concentrations necessary for inhibition, they were only performed once and error bars were not included.
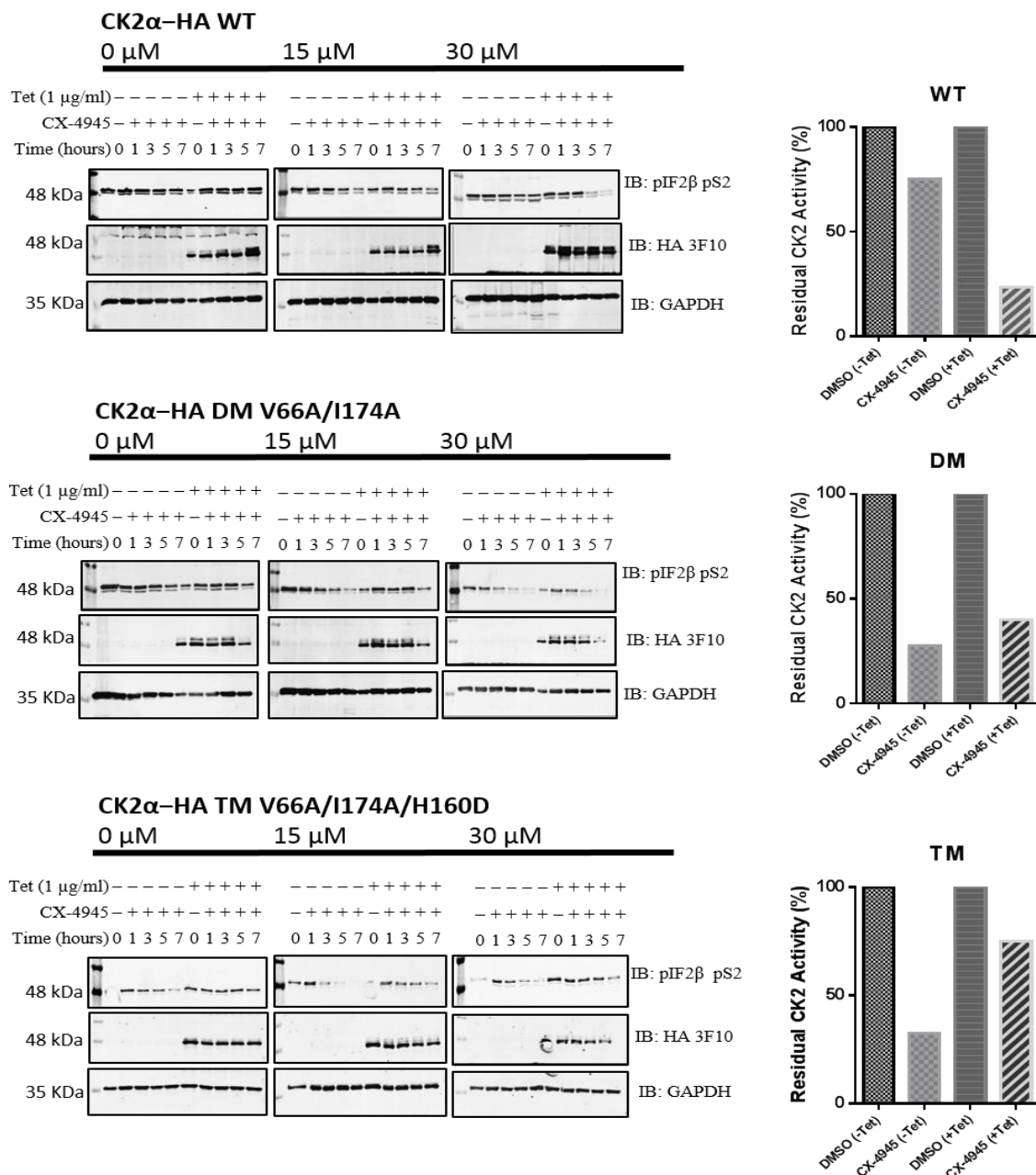
Figure 5. The titration of CX-4945. Inhibiting CK2 in cells expressing CK2 in CK2α-WT, CK2α-DM and CK2α-TM kinase. Expression of CK2α-HA was induced in U2OS Flp-In cells for 48 hours with 1 µg/ml tetracycline, then tested with various concentrations (0, 15, 30 µM) and time points (0, 1, 3 5, 7 hours) of CX-4945. The GAPDH antibody was used as a loading control. Protein lysate was harvested, blotted and incubated with pIF2β antibody to measure the inhibition of phosphorylation of pIF2β with the inhibitor-refractory mutants . The bar graphs measure the residual CK2 activity with DMSO control as 100% at 7 hours of CX-4945 treatment at a concentration of 0, 15 and 30 µM. Residual CK2 activity is measured by dividing the pIF2β signal by the GAPDH loading control and comparing that to the level of the DMSO positive control.
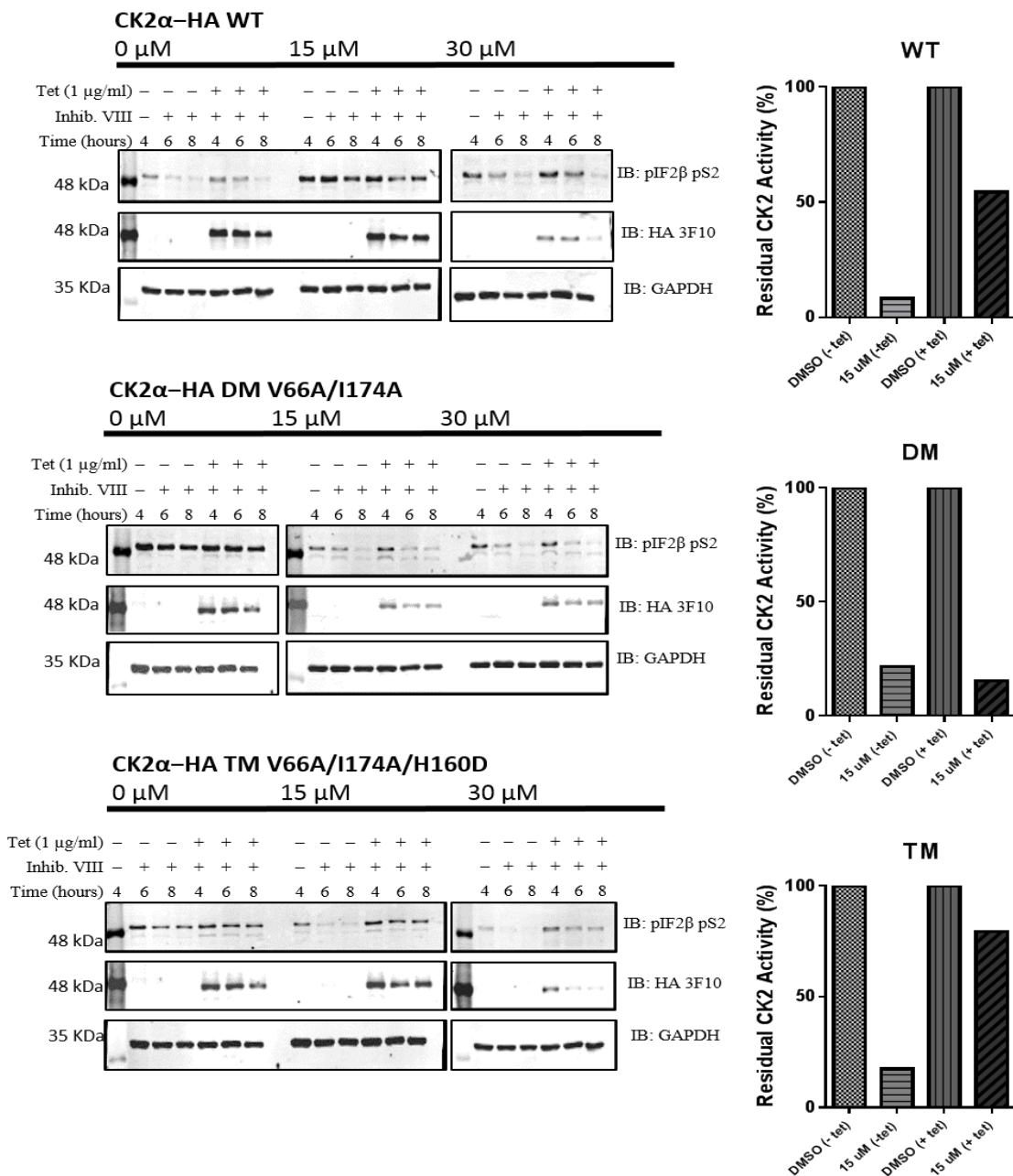
Figure 6. The titration of CK2 inhibitor VIII inhibition of cells expressing in CK2α-WT, CK2α-DM and CK2α-TM kinase. Expression of CK2α-HA was induced in U2OS Flp-In cells for 48 hours with 1 µg/ml tetracycline, then tested with increasing concentrations of inhibitor VIII from 0 to 30 µM and time points from 0 to 8 hours. The GAPDH antibody was used as a loading control. Protein lysate was harvested, blotted and incubated with pIF2β antibody to measure the inhibition of phosphorylation of pIF2β with the inhibitor-refractory mutants . The bar graphs measure the residual CK2 activity with DMSO control as 100% at 6 hours of inhibitor VIII treatment at a concentration of 0, 15 and 30 µM. Residual CK2 activity is measured by dividing the pIF2β signal by the GAPDH loading control and comparing that to the level of the DMSO positive control.

These inhibitor concentrations and incubation times were tested in quadruplicate and triplicate replicates respectively within a single experiment shown in figure 7 and 8. The CK2α/α' antibody showed the levels of exogenous CK2 in comparison to endogenous CK2α and CK2α' already present in the cell population. The exogenous level of CK2α-HA, which was stably expressed in the tetracycline-inducible cells and is shown at the 48 kDa marker, was consistently two-fold higher than that of the endogenous CK2α, which can be seen just below the exogenous CK2α-HA band. Another trend worth noting is the disappearance of the endogenous CK2α', which can be seen just below the endogenous CK2α band, after induction of exogenous CK2α and incubation with CX-4945 inhibitor. The HA-3F10 antibody shows the level of expression of the induced protein. Figure 7 illustrates that all the tetracycline-induced cell lysates were expressing a high level of exogenous CK2alpha, based on the signal near the 48 KDa band, when compared to the non-tetracycline induced negative control. (Figure 7).

Three different CK2-phosphospecific antibodies, pIF2B pS2, pEEF1D pS162 and pCK2β pS2,3,4,8, were tested with the treated cell lysates. It can be seen in Figure 7 that pIF2B showed the most dynamic phosphorylation change following inhibitor treatment, thus it was the most appropriate antibody to be used to measure the activity and inhibition of CK2 with CX-4945 and CK2 Inhibitor VIII.

Measuring the intensity from the pIF2B signal, the inhibited double mutant kinase did not recover any activity when compared to the inhibited CK2α-WT kinase. The CK2α-WT kinase displayed a relative phosphorylation signal of 15% at 7 hours of 30 μM CX-4945 when compared to the uninhibited cell lysate. The CK2α-DM kinase displayed a relative phosphorylation signal of 16% under this same condition. The CK2-

TM kinase showed 57% phosphorylation signal compared to its positive control that was

induced with tetracycline but not treated with inhibitor. This corresponded to a recovery

four-fold more than the CK2α-WT and CK2α-DM kinases. (Figure 7).

A)
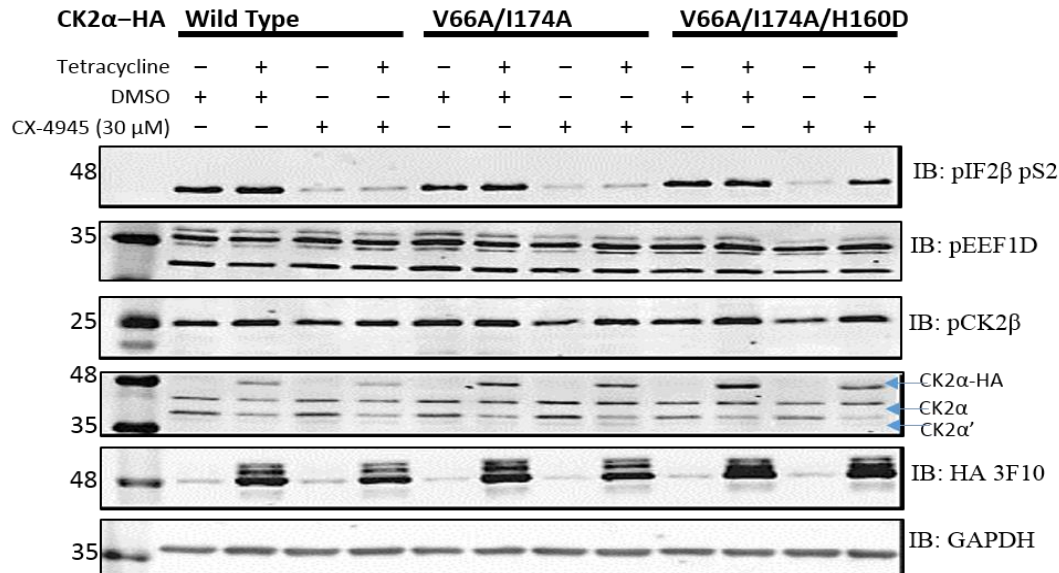
B)
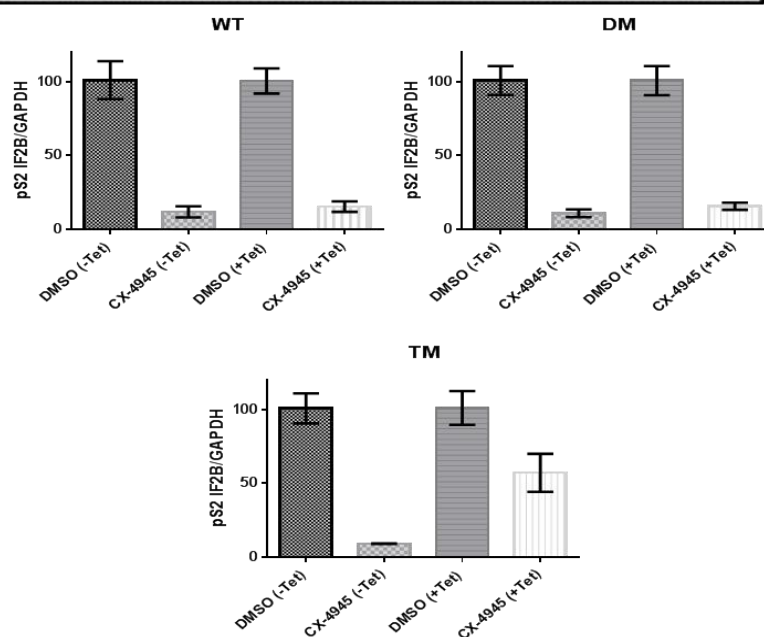
Figure 7. The measure of the ability of CK2α mutants to overcome the inhibition of CX-4945. Cells were induced for 48 hours with 1 µg/ml tetracycline, incubated with CX-4945 for 7 hours at a concentration of 30 uM. The CK2α/α' antibody was used to compare the level of exogenous CK2 to the endogenous CK2α an CK2α'. The GAPDH antibody was used as a loading control. Cells were harvested, proteins are extracted *via* lysis, blotted and incubated with pEE1D, pCK2β and pIF2β antibodies to measure the inhibition of the inhibitor-refractory. A) The blots for each antibody being used. B) The quantified values for the residual CK2 activity with DMSO control as 100% performed in quadruplicate. T-test analysis showed a p-value of 7.8×10^{-4}.

The higher potency of Inhibitor VIII over CX-4945 was the largest observable difference between the two tested inhibitors in the experiments. The CK2α-WT kinase was inhibited with Inhibitor VIII at half the concentration (15 μM) needed with CX-4945 (30 μM). The inhibitor-refractory mutants were treated with the same concentration of inhibitor as the CK2α-WT kinases. Other than that, cells inhibited with CK2 inhibitor VIII showed many of the same trends, specifically how well the CK2α-DM and CK2α-TM cell populations recovered activity compared to the CK2α-WT. CK2α-WT kinase displayed a relative activity of 17% to the uninhibited kinase at 6 hours of 15 μM CK2 inhibitor VIII. The CK2α-DM kinase displayed a 16% activity under this same metric. The CK2α-TM kinase showed 83% activity compared to the uninhibited positive control, a recovery of five-fold more than the CK2α-WT and CK2α-DM cell populations. While the overall trends between the two inhibitors were quite similar, the CK2α-TM cell line showed a higher recovery with CK2 inhibitor VIII than the same cell line with CX-4945.

Inhibitor VIII showed the exact same trends as CX-4945 concerning the CK2 antibodies used (Figure 8) and the levels of exogenous compared to endogenous CK2 expressed in the cell when induced (supplemental Figure 2).
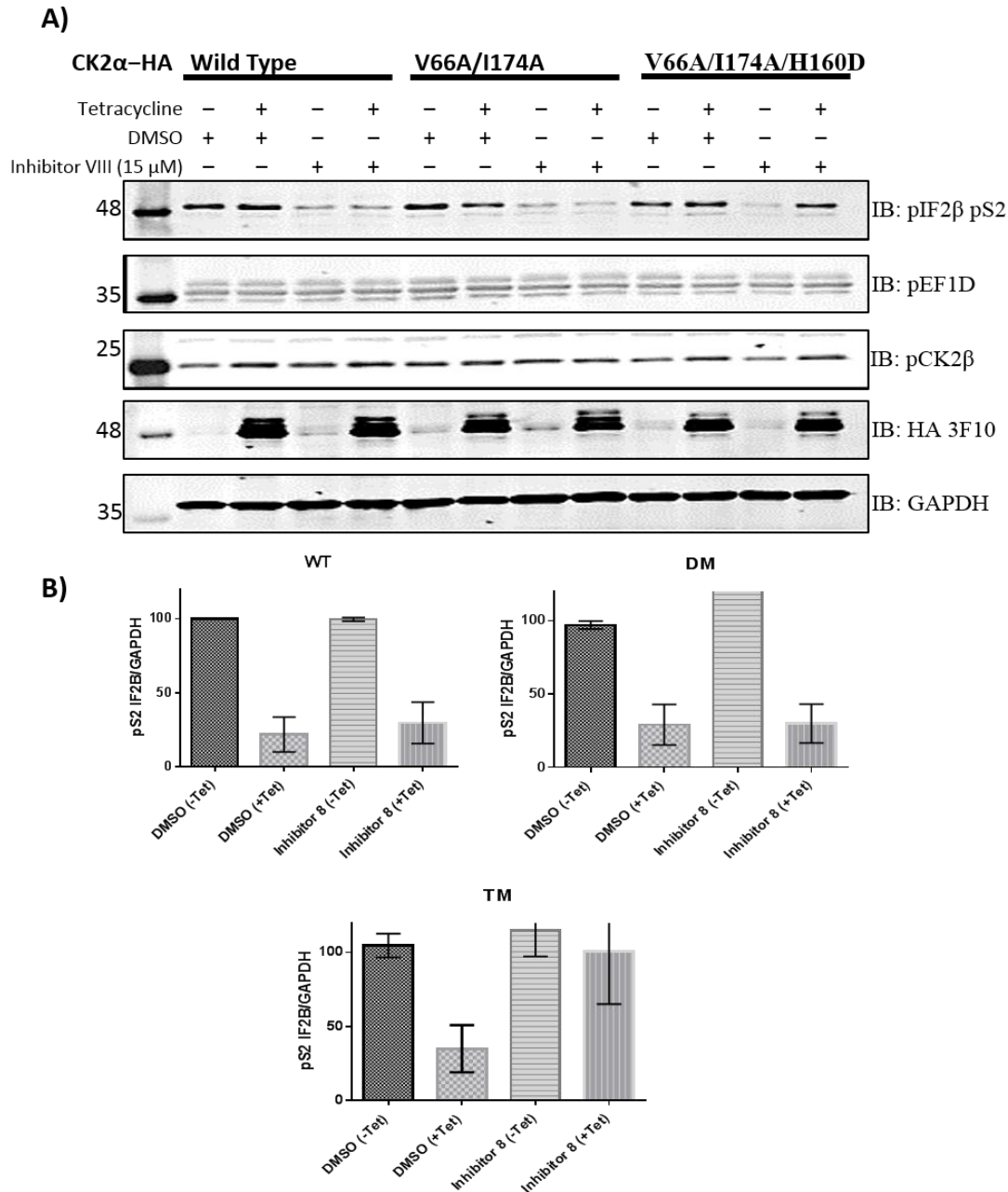
Figure 8. Measuring the ability of CK2a mutants to overcome the inhibition of Inhibitor VIII. Cells were induced for 48 hours with 1000 µg/ml tetracycline and then incubated with inhibitor 8 for 6 hours at a concentration of 15 uM. The CK2α/α' antibody was used to compare the level of exogenous CK2 to the endogenous CK2α an CK2α'. The GAPDH antibody was used as a loading control. Cells were harvested, lysed, blotted and incubated with pEE1D, pCK2β and pIF2β antibodies to measure the inhibition of the inhibitor-refractory. A) The blots for each antibody being used. B) The quantified values for residual CK2 activity with DMSO as 100%, performed in triplicate. T-test analysis showed a p-value of 3.18×10^{-2}.

3.2 – The effect of long-term CK2 inhibition on mutant recovery and cell morphology

After testing the short-term inhibition of the different mutants of CK2 within a cell population, we became interested in how the inhibitors would alter the activity of the kinases after multiple days of treatment and whether lowering the activity level of the CK2α-HA protein for prolonged periods of time would alter the morphology of the cells. The expression of CK2α-HA proteins were induced with 1 µg/ml tetracycline for 48 hours and inhibited for 24, 48 and 72 hours with 0 10 and 20 µM of CX-4945. The morphology of the cells began to change after one day. By the second day, many of the cells were floating in the media on the plate (supplemental Figure 3), indicating that they had lost the ability to adhere to the plates, likely due to the inhibitor causing cell death. By the third day, the majority of the cells were floating and likely dead. In order to test the effect of the inhibitor on all the cells, we needed to harvest both the living cells still adhering to the plate as well as the cells floating in the media. (Figure 9).

Because we saw little to no recovery of the ability of CK2 to phosphorylate pIF2β with the CK2α-DM kinase when compared to the CK2α-WT protein and the CK2α-TM kinase had recovered a significant amount, we moved forward without the CK2α-DM cells. All of the rest of the experiments on the CK2α U2OS cell lines were focused on comparing the CK2α-WT and CK2α-TM kinases.

Triple Mutant   Wild Type

DMSO

10 µM
CX-4945

20 µM
CX-4945
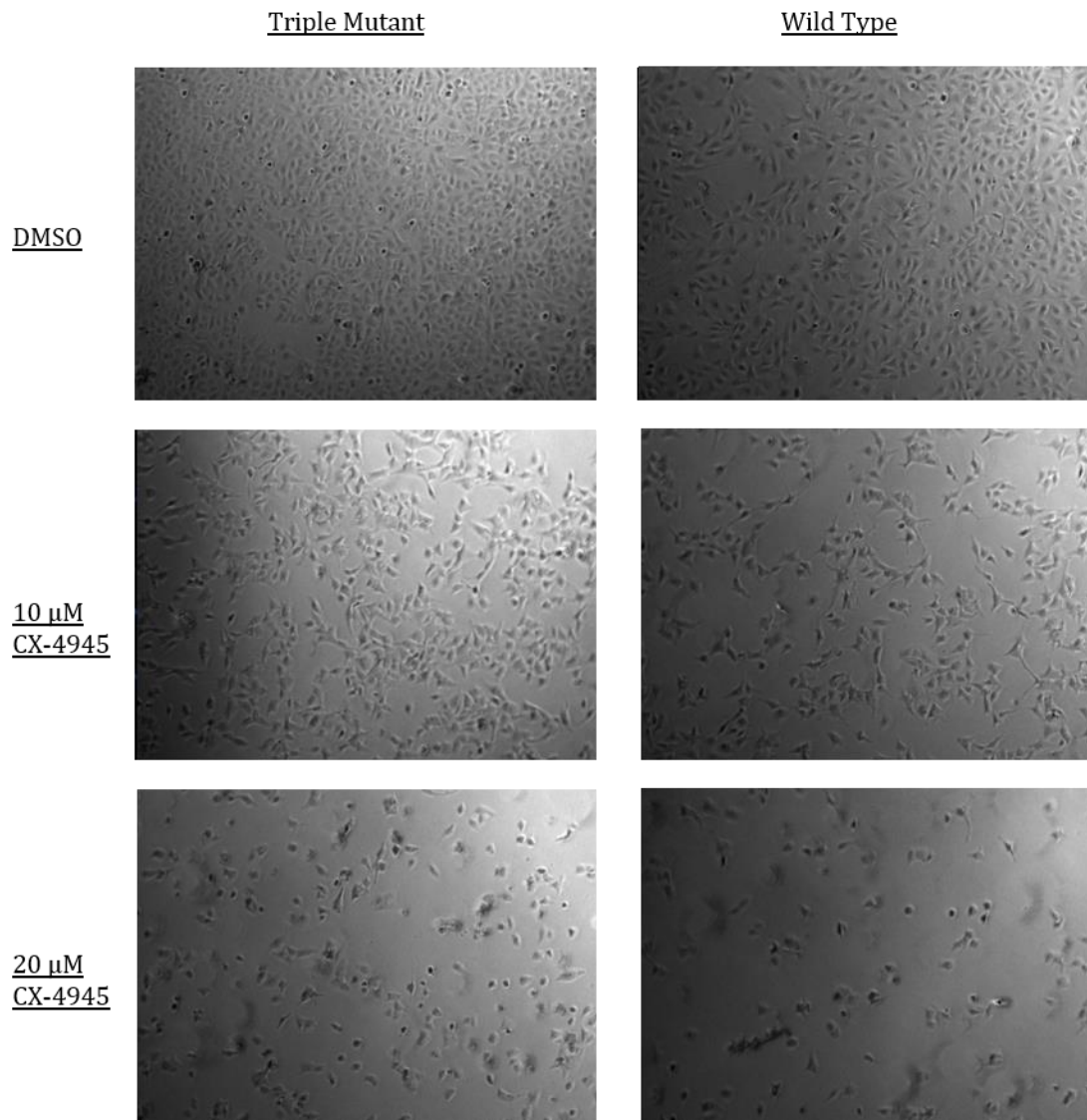
Figure 9. Brightfield images of U2OS Flp-In osteosarcoma cells taken at different inhibitor concentrations and time points. Cells were induced with tetracycline for 48 hours before being inhibited with CX-4945 for 3 days (72 hours). Pictures of the cells were taken at 10x magnification with inhibitor concentrations of DMSO control, 10 µM and 20 µM CX-4945.

After 24 hours, the CK2α-WT cells demonstrated a consistent decrease in pIF2B phosphorylation with increasing inhibitor concentration. However, there was not a linear decrease in pIF2B signal from hour 24 to 72, as the pIF2B levels did not show a significant change between hour 24, 48 and 72 (p-value = 0.37). Figure 10 shows that the activity of the CK2α-WT was 100%, 50% and 19% for DMSO, 10 μM and 20 μM CX-4945 treated cells, respectively, after day one. There was no significant change in the activity between hours 48 and 72 as there was only a 15% decrease in activity to 100%, 42% and 16% for DMSO, 10 μM and 20 μM CX-4945 treated cells (p-value = 0.71). The CK2α-TM kinase displayed no significant decrease in pIF2B activity at any of the concentrations or time points tested, the p-value for the DMSO treatment compared to the 72 hour, 20 μM CX-4945 treatment was equal to 0.16. However, there was a significant increase in the pIF2β signal between the inhibitor-refractory CK2α-TM and the CK2α-WT kinase (p-value 6.2×10^{-3}). This illustrates that the CK2α-TM kinase can maintain CK2 activity for at least 72 hours in the presence of 20 μM of CX-4945. (Figure 10).
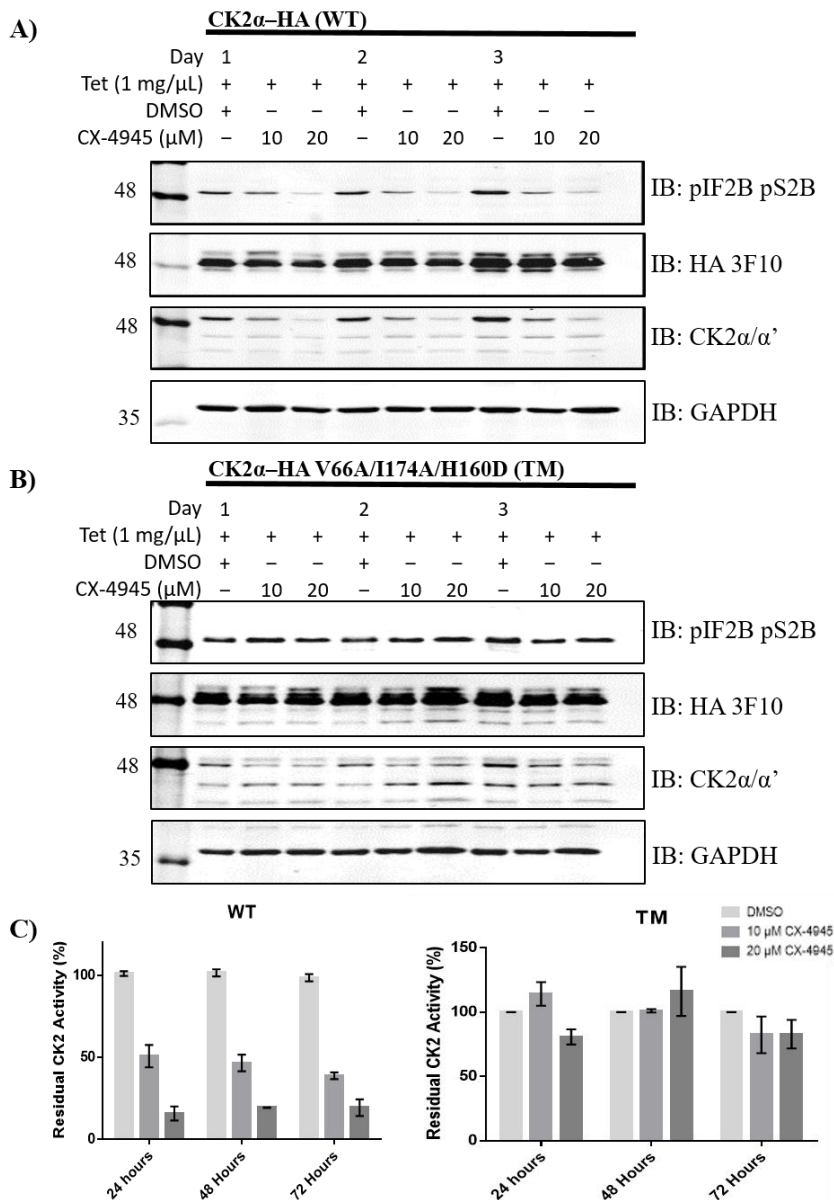
Figure 10. Measuring the ability of CK2α mutants to overcome the inhibition of CX-4945 for 1, 2 and 3 days. Cells were induced for 48 hours with 1 µg/ml tetracycline and then incubated with CX-4945 for 24, 48 and 72 hours at a concentration of 0, 10 and 20 µM. The CK2α/α' antibody was used to compare the level of exogenous CK2 to the endogenous CK2α an CK2α'. The GAPDH antibody was used as a loading control. Both live and floating cells were harvested and immune-blotted with pIF2β to measure the cellular CK2 activity. A) The immune-blots with the indicated antibodies are showing inhibition of CK2 activity when CK2α-HA (WT), B) or CK2α-HA V66A/I174A/H160D (TM) are overexpressed. C) The quantified values for the calculated residual CK2 activity with DMSO as 100%, performed in triplicate. T-test analysis showed a p-value comparing the CK2α-WT to CK2α-TM inhibition at 24 hours 20 µM CX-4945 treatment of 6.2×10^{-3}.

The cells were then tested at 0 µM, 10 µM and 20 µM concentrations of CX-4945 and 0 µM, 10 µM and 20 µM concentrations of Inhibitor VIII for 48 hours. The differences in the efficacy of inhibition are less pronounced at longer inhibition periods. Inhibitor VIII is a more effective inhibitor than CX-4945 in the CK2α-WT kinase, illustrated by the two-fold higher pIF2β signal of the CK2α-WT protein with 10 µM CX-4945 compared to that of the CK2α-WT with 10 µM Inhibitor VIII. There was no quantifiable difference between the recoveries of CK2 Inhibitor VIII compared to CX-4945 in the CK2α-TM cell line. (Figure 11). This experiment was performed only one time and thus error bars could not be included.

A)

B)
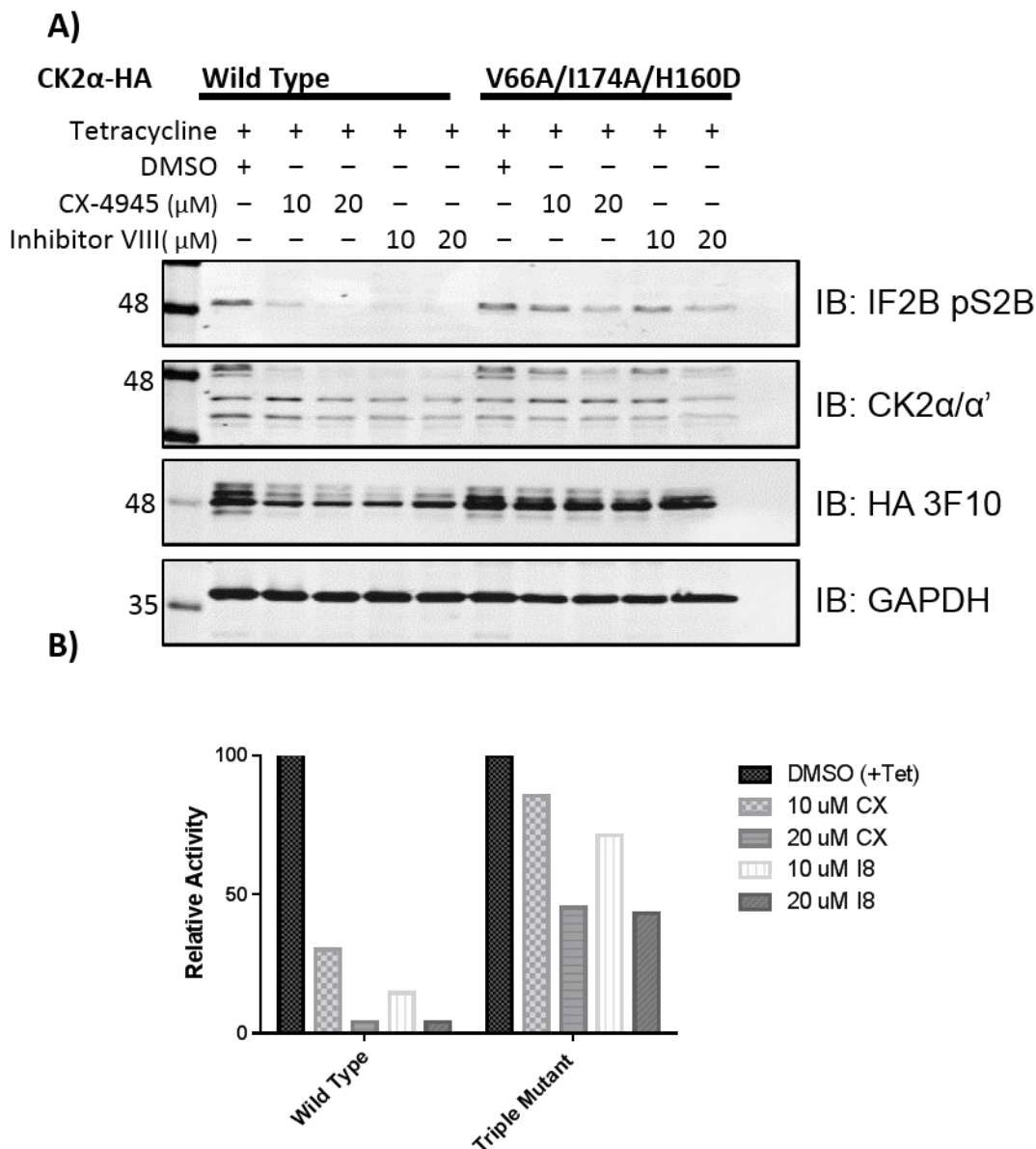
Figure 11. Comparing the ability of CK2α mutants to overcome the inhibition of CX-4945 and Inhibitor VIII for 48 hours at various concentrations. Cells were induced for 48 hours with 1 µg/ml tetracycline and subsequently incubated with CX-4945 or with Inhibitor VIII for 48 hours at a concentration of 0, 10 and 20 uM. The CK2α/α' antibody was used to compare the level of exogenous CK2 to the endogenous CK2α an CK2α'. The GAPDH antibody was used as a loading control. Both live and floating cells were harvested to show the inhibitor-refractory mutants inhibition. A) Immune-blots with the indicated antibodies are shown. B) The quantified values for the residual CK2 activity with DMSO as 100%, performed in triplicate.

3.3 - The design of CK2α' mutants to recover activity in the presence of CX-4945

After optimizing conditions for CK2α recovery in cell line models, the next step

was to design and test mutants for the CK2α' catalytic subunit. A number of mutations

were investigated *in vitro* in order to identify the mutations with the highest recovery of

CK2α' activity in response to CX-4945 treatment. This mutant kinase would eventually

be transfected into Flp-In U2OS cell lines to generate CK2α' stable cell lines. The goal

of the mutant proteins was to recover CK2α' activity in the presence of inhibitors. Many

of the same mutations that were used in the CK2α cell lines were designed in CK2α'

constructs keeping in mind the one amino acid shift difference in numbering between

CK2α vs. CK2α'.

The mutations were tested first *in vitro* and then in cells in order to make

comparisons between the two CK2 subunits. Two new residues, Y116 and H161, were

mutated in addition to previously established V67A/I175A mutations that were tested

several times in the literature. In total, 9 different kinases – 7 novel mutations - were

designed (Table 1). The goal was to design a new form of the mutant kinase that could

recover more residual CK2 activity than in the previously studied mutants. All of the

results from the recovery of the new mutant forms of CK2α' were then compared to

previous lab results with CK2α *in vitro*. (Figure 12)

The new mutations were examined to determine if the altered residues influenced

the CK2α' activity *in vitro* without any inhibitor present. We examined whether the

mutated kinases could still transfer ATP to a synthetic peptide RRRDDDSDDD, which is

an established synthetic peptide tested in previous *in vitro* studies [83]. The kinases were

then incubated with increasing amounts of ATP to measure *in vitro* K_M values. It can be

seen in supplemental table 1, that the mutant CK2α' kinases had a similar specific

activity value to the wild type CK2α'-GST kinase and also had K_M values of ATP similar

to the wild type of 12 μM (Table 1). None of the mutations eliminated activity of CK2α'

in vitro suggesting that CK2α' can tolerate these mutations. It also indicates that we can

investigate the interactions of these mutant isoforms of CK2α' with the CK2 inhibitor

CX-4945.

Table 1. The designed CK2α and CK2α' mutant kinases with their K_M values of ATP. The point mutations made to key residues both inside and outside the active site of CK2 are indicated. There is a one base-pair shift forward in the CK2α' catalytic subunit. GST-CK2α data was collected by S. Fess.

	Mutants	$K_M (\mu M)$
GST-CK2α	Wild Type	13
	H160L	9.9
	H160D	7.8
	V66A/I174A	31
	V66A/I174A/H160L	21
	V66A/I174A/H160D	18
GST-CK2α'	Wild Type	18
	H161D	13
	H161E	-
	Y116L	-
	V67A/I175A	15
	V67A/I175A/H161D	12
	V67A/I175A/H161E	10
	V67A/I175A/Y116L	-
	V67A/I175A/Y116H	-

3.4 – The recovery of phosphorylation activity of CK2α' mutants in the presence of CX-4945

The kinases were tested with and without the CK2 inhibitor CX-4945 to investigate the ability of the mutant to maintain phosphorylation while being treated with inhibitor. Each mutant kinase was tested with DMSO control, 5 nM CX-4945 and 15 nM CX-4945. The phosphate incorporation value with the DMSO control was taken to be 100% activity, while the phosphate incorporation values at each of the inhibitor concentrations was taken as a fraction of this percentage. 5 nM concentration of inhibitor was recorded because it was the concentration where the wild type kinase was lowered close to 0% in comparison to the DMSO control. Many of the tested mutants showed significant recovery at this same concentration. (Figure 12).

All of the kinases were treated with DMSO control, 5 nM and 15 nM CX-4945 to examine how well the mutants would recover activity in comparison to the wild type kinase. Table 2 shows this data along with the p-values for the percent specific activity of each mutant kinase in comparison to the percent recovered residual activity of the wild type kinase. Each mutation that was designed had an inhibition of activity that was significantly less than the wild type kinase. (Table 2).

Table 2. CK2α' mutant kinases percent specific activity compared to the specific activity of the wild type kinase. The table shows percent specific activity at 5 nM and 15 nM concentration of CX-4945 treatment. The factor higher than wild type and p-values were calculated using the percent specific activity at 5 nM for each mutant in comparison to the wild type kinase.

	Mutants	% Specific Activity - DMSO	% Specific Activity – 5 nM	% Specific Activity – 15 nM	Factor higher than wild type	P-value
GST-CK2α'	Wild Type	100	2	1	-	
	H161D	100	16	9	8x	5.0×10^{-4}
	H161E	100	21	10	10x	2.7×10^{-5}
	Y116L	100	11	7	5x	1.1×10^{-5}
	V67A/I175A	100	11	9	5x	1.1×10^{-3}
	V67A/I175A/H161D	100	28	21	14x	1.8×10^{-6}
	V67A/I175A/H161E	100	60	44	30x	1.7×10^{-4}
	V67A/I175A/Y116L	100	28	23	14x	7.2×10^{-8}
	V67A/I175A/Y116H	100	23	19	13x	-

The general observable trend was that every purified kinase that incorporated the V66A/I174A double mutation had a higher recovery than its corresponding kinase that did not contain those two mutations. Additionally, every kinase that added an additional mutation to either of the Y116 or H161 residues had a higher recovery than the corresponding kinase without that mutation. Mutations to His161 recovered better than mutations to Y116. All of the mutations made in CK2α' can be compared to similar mutations that were made in CK2α by a previous lab member (Fess 2015). (Figure 12).

The majority of trends are similar within the CK2α and CK2α' kinase mutants (Figure 10). His160/His161 is consistently the most optimal residue for mutation while mutating H115/Y116 also shows significant recovery. Overall, there are a lot of similarities within the two kinases in terms of their mutation profile *in vitro*. We were interested in seeing how these similarities translate when the live in cell model are used. The triple mutant that showed the best recovery *in vitro*, CK2α'-V66A/I174A/H161E, was therefore transfected into U2OS osteosarcoma Flp-In cell lines to be compared to the CK2α triple mutant that had previously been characterized.
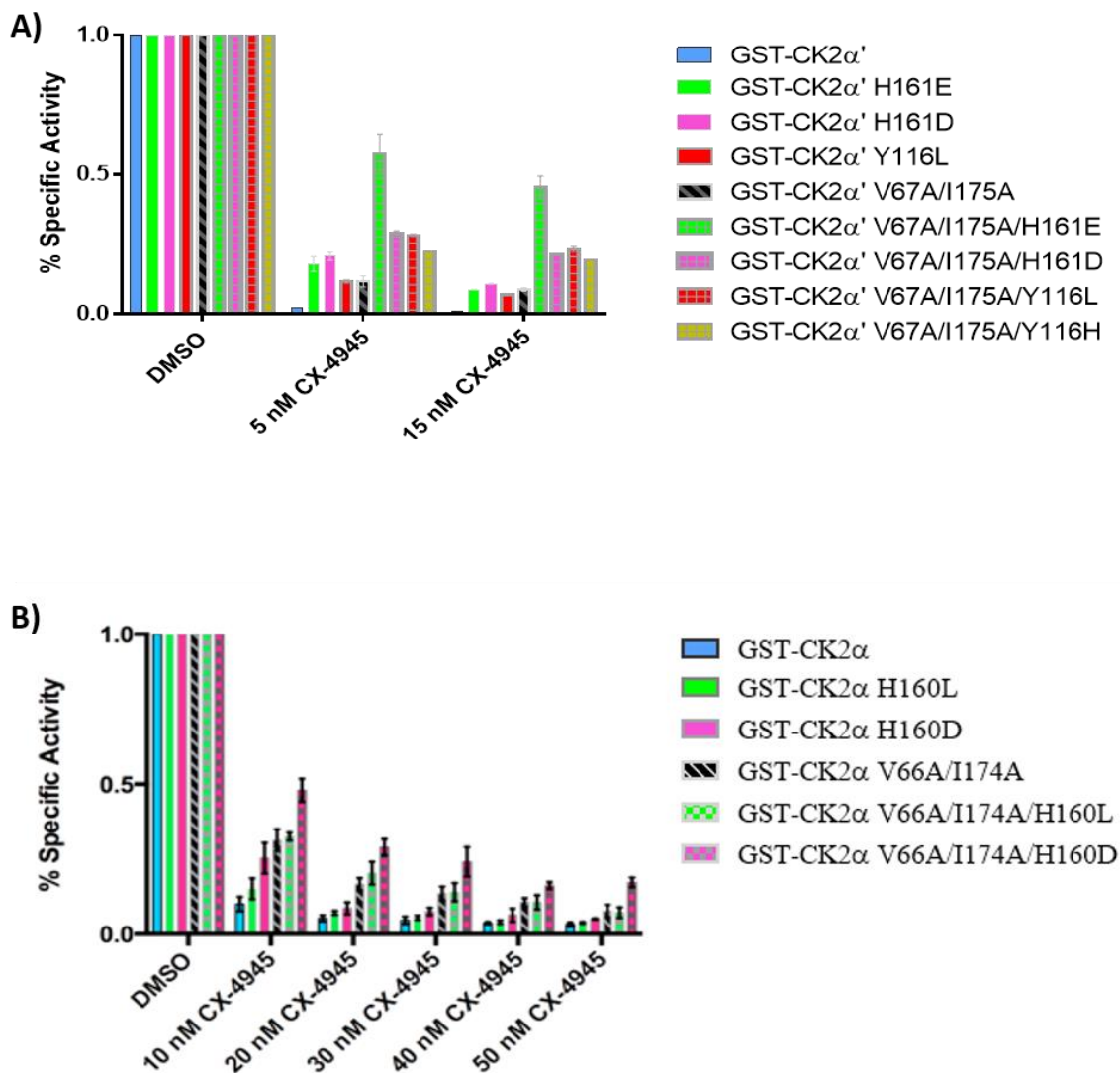
Figure 12. **Measuring the ability of CK2α and CK2α' mutants to overcome inhibition by CX-4945.** A strategy was utilized in order to assess the ability of the CK2α and CK2α' mutants to recover from inhibition by CX-4945. The different forms of CK2 are indicated in the legend. The experiment was performed in triplicate (n=3) and each reaction was also assayed in triplicate. Inhibited reaction results were compared to DMSO control. A) The residual activity of CK2α' measured at 5 and 15 nM CX-4945. B) The percent specific activity of CK2α measured at 10, 20, 30, 40, 50 nM CX-4945, this data was collected by former lab member Sam R. Fess.

3.5 - Creation and induction of CK2α' U2OS Flp-In cell lines

Three CK2α' constructs were transfected into U2OS osteosarcoma Flp-In cell lines, HA-CK2α' (WT), HA-CK2α'-V67A/I175A (DM) and HA-CK2α'-V67A/I175A/H161E (TM). The three cell lines were then titrated with different tetracycline concentrations but the HA-CK2α' (WT, DM or TM) would not express at a level comparable to the CK2α-HA constructs. CK2α-HA transfected cell lines have an expression level 8 times higher than the HA-CK2α' transfected cell lines. Through a series of transient transfections, it was discovered that the HA-CK2α' constructs required the regulatory beta subunit in order to be expressed at a level similar to the CK2α-HA constructs. (Figure 13).

While the expression of the HA-CK2α' cell lines did not produce expression levels as with CK2α-HA without co-transfection with the regulatory beta subunit, there was still a good level of expression of the HA-tagged CK2α' when compared to the negative control. (Figure 13 & 15).

The expression of exogenous HA-CK2α' was tested by immunofluorescence comparing HA-CK2α' to of CK2α-HA in the induced cell lines. The fluorescent images showed a similar trend, the CK2α' had a more pronounced signal than the negative control but its signal was consistently less than the signal produced by the CK2α cells. (Figure 14).
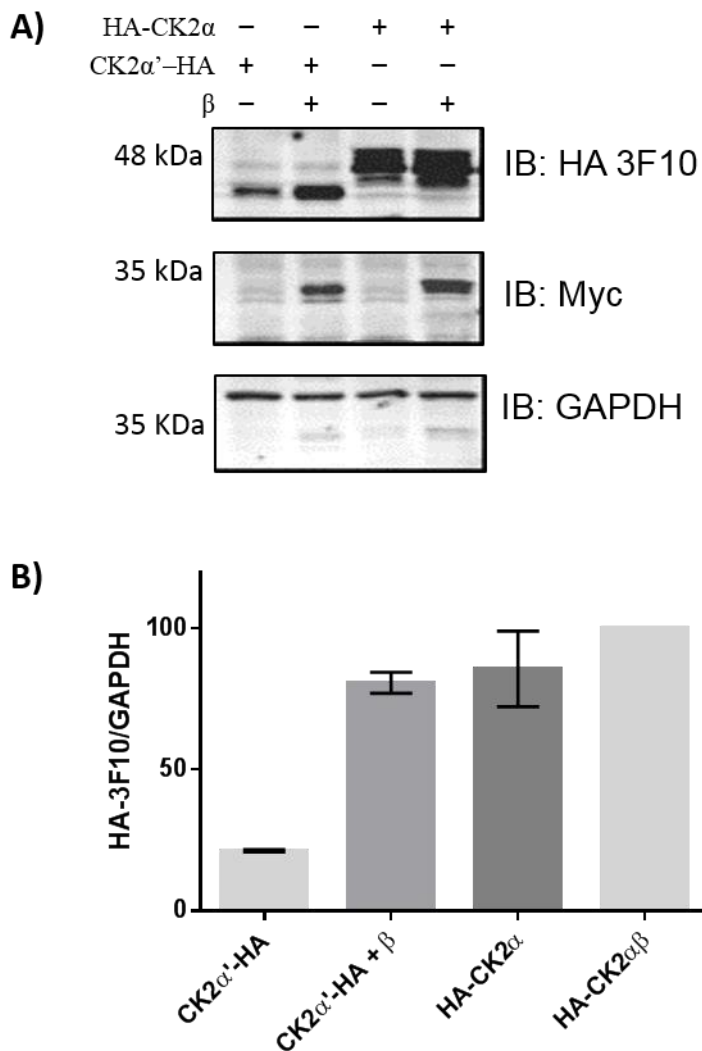
Figure 13. CK2 expression in Flp-In U2OS cell lines. The CK2α/α' antibody was used to compare the level of exogenous CK2 to the endogenous CK2α an CK2α'. The GAPDH antibody was used as a loading control. Expression level of CK2α-HA and HA-CK2α' was measured with and without the co-transfection of Myc-CK2β. Equal loading was visualized by the GAPDH antibody. A) The blots with the indicated antibodies. B) The quantified values for the fraction of HA 3F10/GAPDH.

A)

+ Tetracycline   - Tetracycline

FITC

DAPI

PHASE

B)
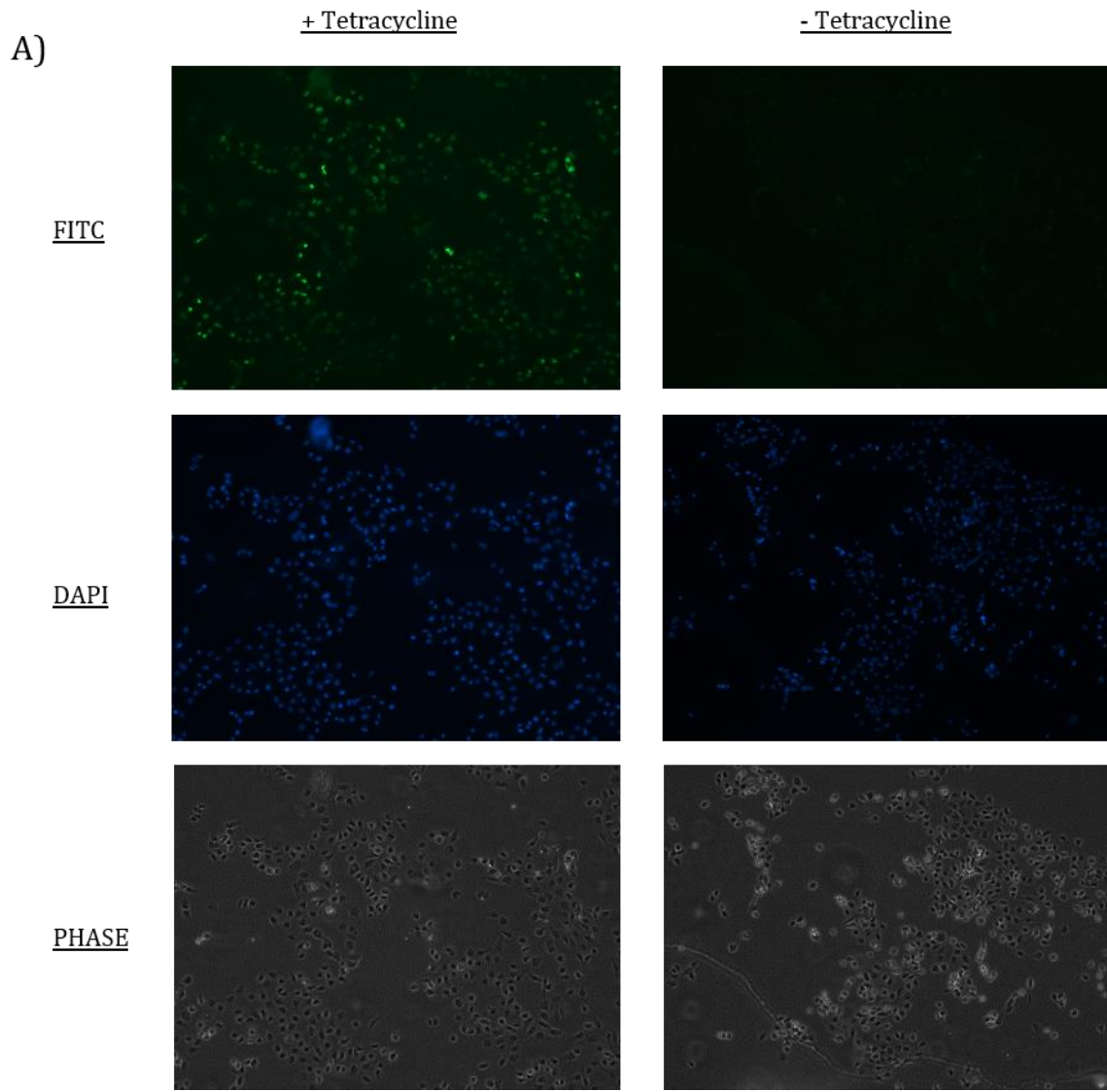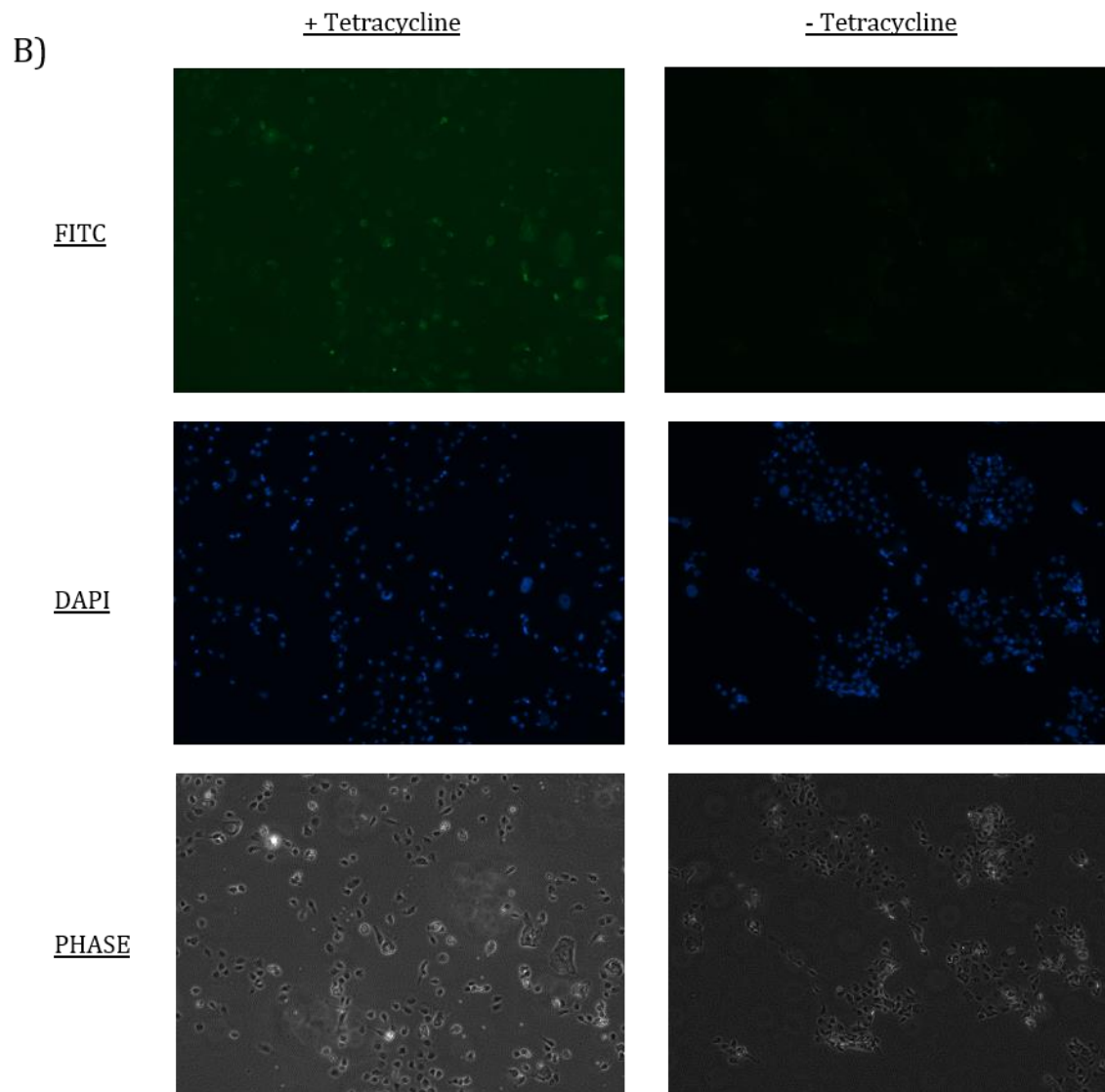
Figure 14. Indirect immunofluorescent detection of expressed CK2α-HA and HA-CK2α' in Flp-In U2OS cell lines. Flp-In U2OS cells (expressing CK2α-HA (**A**) or HA-CK2α' (**B**)) were cultured in the absence (-) or presence (+) of 1 µg/ml of tetracycline for 24 hours. The cells were then fixed and stained with a FITC-conjugated Anti-HA monoclonal antibody. Cells were also stained with a Hoechst 33258 DAPI stain in order to visualize cell nuclei. A phase contrast image has also been included. Fields were imaged in the absence (-) or presence (+) of tetracycline for both cell lines (**A-B**) (n=2).

With this level of expression there is potential to examine CK2α' specific effects such as the recovery of pIF2β phosphorylation with treatment with CX-4945 and Inhibitor VIII. The CK2α'-WT and CK2α'-DM cells expressed well but the CK2α'-TM did not express thus recovery of pIF2β phosphorylation with CX-4945 treatment was tested with only the CK2α'-WT and CK2α'-DM cells (Figure 15).

Similar to the CK2α cells, the CK2α'-DM kinase did not recover any activity when compared to the CK2α'-WT kinase. Induction of the kinase with 1 µg/ml tetracycline induction did not alter the phosphorylation level of the pIF2β antibody when treated with 10 µM and 20 µM CX-4945. Based on the experiment shown in Figure 13, the expression level of the HA-tag was lower than the expression seen with the CK2α cells. This could have an effect on the recovery of pIF2β signal but without the CK2α'-TM cell line, it is hard to make a direct comparison between the CK2α and CK2α'. Figure 15 was a titration experiment performed to find the necessary concentrations for inhibition of CK2α' cells, it was only performed once and error bars were not included in the figure.
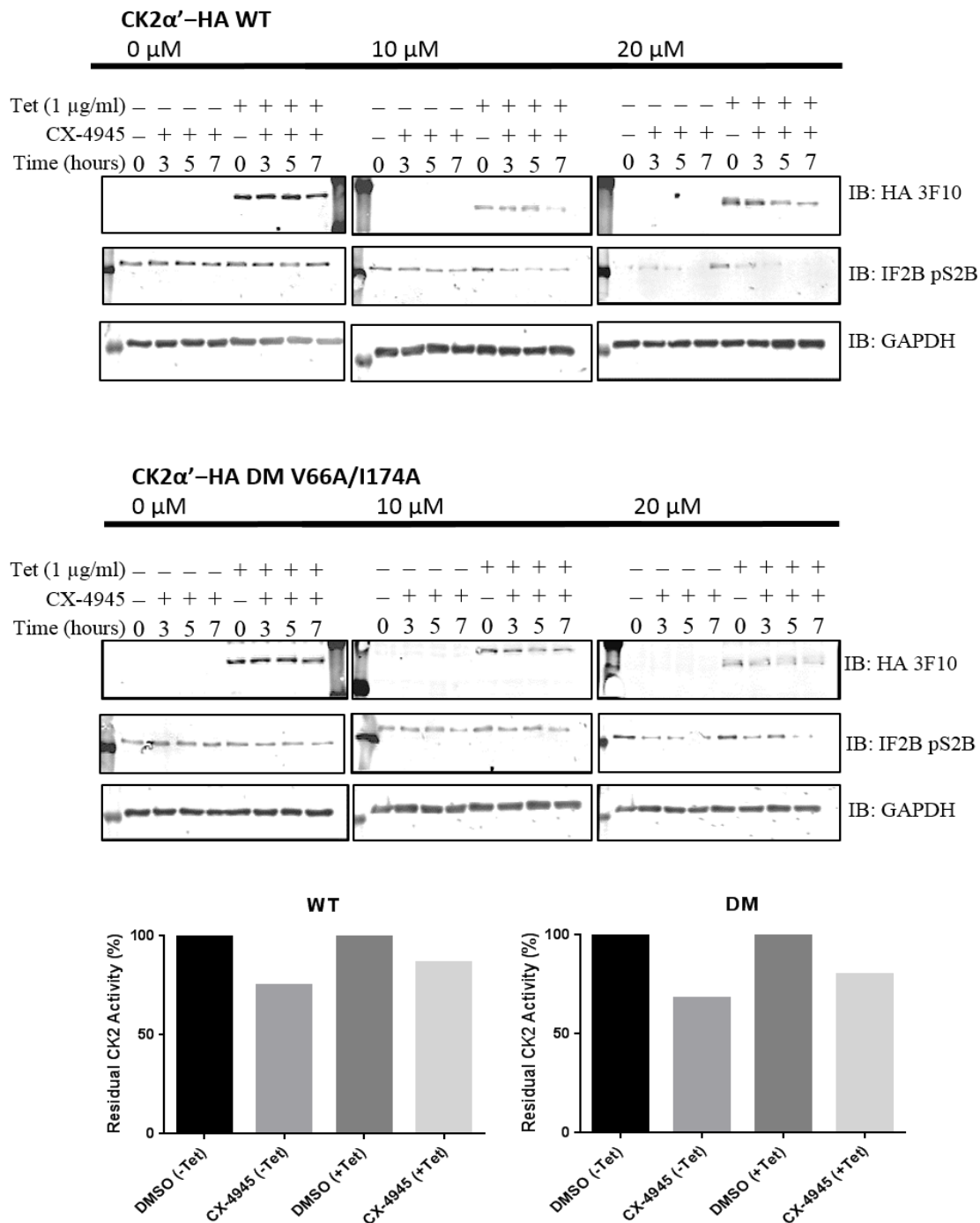
Figure 15. The titration of CX-4945 with CK2α'-WT and CK2α'-DM kinases expressed with tetracycline in U2OS cell lines. Cells were induced for 48 hours with 1 µg/ml tetracycline, then tested with increasing concentrations from 0 to 30 µM and time points from 0 to 8 hours. The GAPDH antibody was used as a loading control. Protein lysate was harvested, blotted and incubated with pIF2β antibody to measure the inhibition of the inhibitor-refractory mutants. The bar graphs measure the residual CK2 activity with DMSO control as 100%.

HA-CK2α' was only comparable to CK2α-HA expression with co-transfection of

CK2β. As a result, more CK2α' constructs were tested to determine if the location of the

HA-tag would make a difference in the expression level of the protein. Four constructs,

HA- CK2α', myc- CK2α', CK2α'-HA and CK2α-HA were transiently transfected into

Flp-In cell lines, with and without the regulatory CK2β subunit, to determine the

expression level of the HA-tagged protein.

Figure 14 shows that the constructs myc-CK2α' and CK2α'-HA constructs show

a considerably higher expression level than the HA-CK2α' construct that was previously

stably transfected into the U2OS cells. The HA-CK2α' construct was expressed when co-

transfected with the regulatory beta subunit. Without the presence of CK2β there was a

very low expression level for the HA-CK2α' construct when transiently transfected with

both 20 µg and 40 µg of DNA. Both myc-CK2α' and CK2α'-HA had significantly

higher level of expression.

The construct CK2α-HA was used as a positive control due to its high expression

level when stably transfected into Flp-In cell lines. The CK2α'-HA construct had an

expression level even higher than this positive control. EGFP was co-transfected into all

of the transiently transfected constructs to allow for monitoring of the transfection

efficiency of the cells. All of the cells were tested for their transfection efficiency before

they were harvested by *via* EGFP incorporation. The transfection efficiency was quite

low but was consistent for each of the cell populations, maintaining an efficiency rate of

approximately 20-25% for each of the populations tested. (Figure 16).

A)

HA-CK2α (µg)	–	–	–	–	–	–	–	–	–	20	40	20
HA-CK2α' (µg)	–	–	–	–	–	–	20	40	20	–	–	–
CK2α'-HA (µg)	–	–	–	20	40	20	–	–	–	–	–	–
Myc-CK2α' (µg)	20	40	20	–	–	–	–	–	–	–	–	–
β (µg)	–	–	20	–	–	20	–	–	20	–	–	20
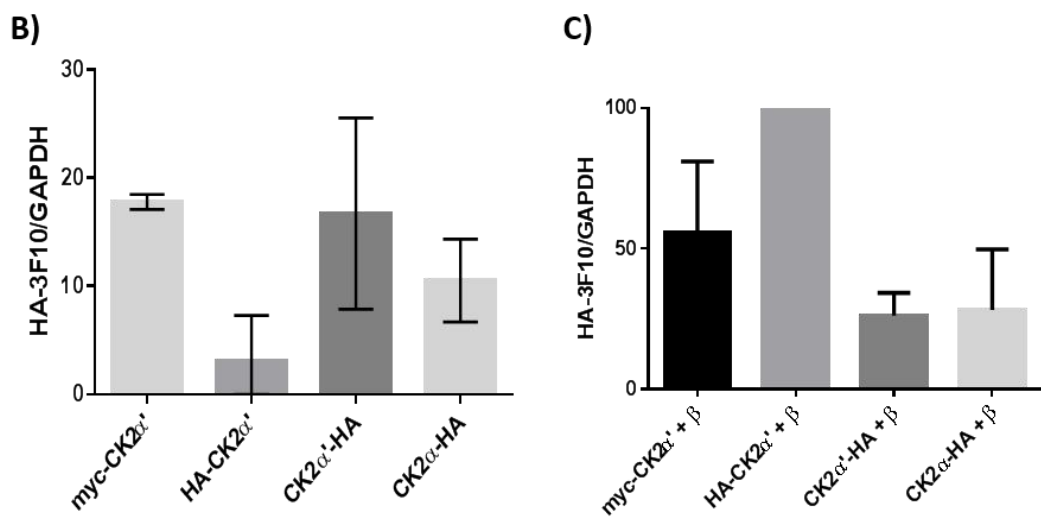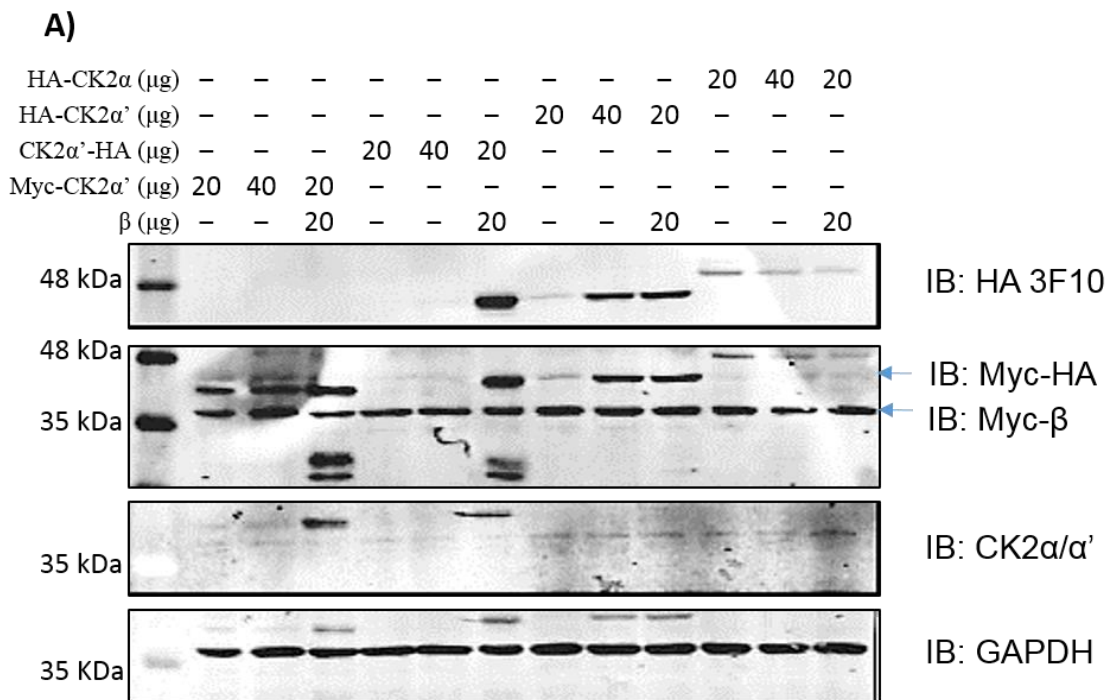
Figure 16. The measure of expression of different CK2α' constructs with and without co-transfection of the regulatory beta subunit. 20 µg of DNA of each CK2α' construct was transfected into Flp-In U2OS cell lines with and without the addition of 20 µg of beta subunit DNA. The cells grew with the transfected DNA for 48 hours before being harvested, blotted and incubated with HA-3F10 antibody to measure the expression of the different CK2 DNA constructs. A) Blots of the antibodies used. B) The quantified values for the HA 3F10/GAPDH ratio of each construct. C) The quantified values for the HA 3F10/GAPDH ratio of each construct co-transfected with Myc-CK2β. Both bar graphs with range bars are taken as a percentage of the most intense peak from the HA-CK2α' + β construct.

DISCUSSION

4.1 - Short-term recovery of mutant CK2α cell lines

Rescue experiments utilizing inhibitor-refractory mutants serve as a tool to identify *bona fide* kinase targets. Identifying protein substrates of specific kinases provides information about the pathways that the kinase is involved with and about the function of that interaction. Furthermore, inhibitor-refractory mutants can investigate the effects of different inhibitors on previously established protein substrates [84]. Based on data shown by a previous study within the Litchfield lab that tested the same CK2α-TM mutant *in vitro* that was tested in sections 3.1 and 3.2 of this thesis, I hypothesized that inhibitor conditions could be optimized to abolish the activity of the CK2α-WT kinase while maintaining the activity of the CK2α-TM kinase.

A previous study reported that the CK2α-DM kinase had a five-fold recovery with the TBB CK2-inhibitor [61]. Since this study was published, TBB has been shown to lack CK2-specificity and it has been suggested that its role in apoptosis results from off-target effects [63]. This can be explained by the fact that the previous study that reported a five-fold recovery of CK2α-DM with TBB was not performed in cellular conditions. Instead, the cells were harvested and the inhibitor was added to the cell lysate within a test tube.

Within our study, the CK2α-DM kinase showed no recovery at the inhibitor concentration tested with both CX-4945 and inhibitor VIII in cellular conditions. This provides further evidence of the difference between tests that are done *in vitro* and those that are performed in cell. It also gives validation to a concern that had previously been the subject of debate, that some studies had shown the double mutant as effective in

recovering residual CK2 activity [61] and some did not [63]. In our study, the concentration that inhibited both the CK2α-WT and CK2α-DM did not inhibit the CK2α-TM.

No previous studies have investigated the CK2α-TM kinase in cells. Thus our research provides an advancement in the field, as a mutant that can recover activity in a cell model has yet to be optimized. Optimal conditions were achieved for the recovery of the triple mutant cell line with both CX-4945 and CK2 inhibitor VIII. We found that a concentration of 30 µM for CX-4945 and of 15 µM for Inhibitor VIII eliminated pIF2β phosphorylation in the CK2α-WT cell line while maintaining pIF2β phosphorylation in the CK2α-TM cell line.

Previous *in vitro* studies have shown that CX-4945 had a IC50 of 1 nM and CK2 inhibitor VIII had an experimental IC50 found to be 32 nM [28, 67]. The concentration needed to inhibit the activity of the CK2α-WT cell line with CK2 inhibitor VIII was half of that needed for CX-4945, contradicting the previously published kinetic studies. This result is significant firstly, as it again illustrates the difference between inhibitor studies performed in *vitro* and those performed in cell models. There are a number of factors that could lead to this difference between the two models. In order for inhibitors to work within cell line models, the drugs must be able to penetrate into the cell and function properly under physiological conditions. Inhibitors can have different efficacies at different pHs, inhibitors will not be able to function properly on their cellular targets if their optimal pH does not match the pH at physiological conditions. There are also a number of phosphatases in the cell that can deactivate the inhibitor before it can bind its target [85]. Another difference between the two models is the potential for off-targets. In

the controlled environment of a test tube, it is less possible for the inhibitor to bind to anything other than the target and thus off-targets are less of an issue. Cell models are far more trustworthy and accurate in terms of predicting the efficacy of the drug within the human body [85, 86]. This result is also of significance as it reaffirms that CK2 inhibitor VIII is more effective than CX-4945 in inhibiting CK2 in cell. Previous studies have shown that inhibitor VIII does in fact slow the growth of tumors in xenograft mice models more than CX-4945 and initiates apoptosis within PC3 prostate cancer cell lines better than CX-4945 [67].

Earlier studies have shown pCK2β pS2, pS3, pS4, pS8 and pEEF1D pS162 to be proteins exhibiting CK2 inhibitor-dependent decreases in phosphorylation [87, 88]. Although there is not much information in the literature, previous studies performed in the Litchfield lab demonstrated a rapid and dynamic response of pIF2β following treatment with CX-4945. These three antibodies – pEEF1D pS162, pCK2β pS2, pS3, pS4, pS8 and pIF2β pS2 – were tested with the different mutant CK2α kinases with both CX-4945 and inhibitor VIII. When treated with CX-4945 and inhibitor VIII, pIF2β antibody showed a significant decrease in phosphorylation signal in comparison to pCK2β and pEEF1D. An earlier study investigating the effect of phosphatases on the signaling of different CK2 signaling substrates could explain this finding. Comparing PTEN negative and PTEN positive cells showed varying phosphorylation of pIF2β, indicating that the phosphatase is necessary for the lowering in signal of pIF2β. pEEF1D and pCK2β were not affected by the change in PTEN levels [89].

Overall, the inhibitor-refractory mutants served as a tool to validate CK2 targets and compare the efficacy of CK2-inhibitors and provided a comparison to inhibitor-

refractory mutants that have been tested in the past. Because of the effectiveness of the inhibitor refractory mutants when treating cells for a short period of time with the inhibitors, we moved forward to address the issue of how inhibitors would alter the recovery of the triple mutant kinase with longer treatment periods.

4.2 - Effects of long-term CK2 inhibition on mutant recovery and cell morphology

CX-4945 has been investigated in both the short term (under 24 hours) and long term (24 hours and over) in previous studies [90]. Within the present study, the cells started to lift off the plate and changed morphology after 24 and 48 hours of inhibition of both CX-4945 and CK2 inhibitor VIII. Both the cells that remained attached to the plate and the floating cells were harvested and tested for their CK2 expression and activity. It was important to harvest both the attached and floating cells after multiple days of inhibition to obtain a comprehensive analysis of what was occurring to all the cells within the population. The CK2α-TM cell line maintained the same level of pIF2β signal despite the altered morphology of the cells.

A possible explanation for the ability of the dying cells to maintain their pIF2β signal is the cell's ability to prioritize phosphorylation. It has been shown that CK2 is involved with numerous pathways, some of which are involved with cell cycle control and others that are involved with translation [91]. When the amount of CK2 is being limited due to pharmaceutical inhibitors, the cell will prioritize which processes are the most important to survival and utilize the remaining CK2 for those processes while ignoring the less important ones [92]. The osteosarcoma cells have a doubling time of 24 hours so the lessening of the ability to phosphorylate cell cycle machinery would not be

immediately necessary to the survival of the cell. pIF2β signaling is involved with the control of cellular translation, which is an immediately necessary function for the cell [93], without the proper control the cells would die much more quickly than 48 hours. The suggestion is that the cells prioritized the phosphorylation of proteins involved with pIF2β signaling over the phosphorylation of proteins controlling cell cycle progression, which led to a slow death of the cells that could maintain their pIF2β signal. This is supported by the literature in that CX-4945 has been previously proven to stop cell cycle progression at the G to M phase [66].

Our research on the change in cell morphology is based on pictures of the cells before and after inhibition in order to qualitatively assess cell morphology. Follow up studies should be performed to investigate this by performing cell counting assays to obtain a more quantitative analysis of potential cell death. PARP and caspase 3-cleavage antibodies could also be used, to investigate autophagy and apoptosis. This would test if apoptosis is being induced at different time points in both the long and short-term and provide a better understanding of the effect of the inhibitors.

Phosphorylation of pIF2β was maintained in CK2α-TM kinase even after three days of incubation with CX-4945. This illustrates the ability of the mutant to maintain activity in the long-term and it can be used as a tool moving forward to investigate both short and long-term binding partners of the inhibitor within the cell. CK2 inhibitor VIII had the more potent inhibition both in the short-term and the long-term. This is more evidence for the heightened potency of this inhibitor in comparison to what was previously considered the most potent CK2 inhibitor available. CX-4945 has gathered far more interest as a clinical inhibitor than CK2 inhibitor VIII and is currently going

through clinical trials for Multiple Myeloma cancer. The current study shows novel information on the potency of a relatively untested inhibitor. CK2 inhibitor VIII is still being optimized to improve efficiency and selectivity, therefore there is potential for a more potent CK2 inhibitor moving forward.

Future work within this section (4.2) would involve first testing to see if the change in cell morphology is indeed cell death by use of cytotoxicity testing [94]. A number of antibodies can be used to test off-targets, autophagy, apoptosis and pathway prioritization when comparing long and short-term inhibitor treatments. While there were some limitations to the experiments within this section 4.2 and there is still a great deal of work to be done to confirm any of the above mentioned theories, the research demonstrates a change in cell morphology while maintaining pIF2β phosphorylation.

4.3 - The design of CK2α' mutants to recover activity in the presence of CX-4945

In order to make an accurate comparison between the two catalytic subunits of CK2, nearly identical experiments must be performed on both CK2α and CK2α'. Therefore, once the conditions for CK2α were optimized for recovery in cell line models, the next step was to design and test mutants for the CK2α' catalytic subunit that could then also be optimized in cell lines.

Previous studies that have altered key residues within the active site of kinases have shown their influence on catalytic activity [2]. Among the most important experiments performed within this thesis was the investigation of the ability of the mutants of CK2 to phosphorylate substrates in the same way as wild type kinase. The *in vitro* tests with the mutants phosphorylating substrates with a specific activity similar to

wild type suggests that the alterations made did not affect the ability of the kinase to bind ATP and phosphorylate the RRRDDDSDDD in the absence of the inhibitor.

The activity of the previously established CK2α double mutant V66A/I174A was found to be indistinguishable from the wild type kinase in a previous study [26]. Within our results, there was a slight decrease in activity in the CK2α' double mutant V67A/I175A. The difference within these findings could be due to the different catalytic subunits tested, however previous findings have shown identical activity within the catalytic subunits when tested *in vitro* [95]. Furthermore, a previous Litchfield lab member similarly found a slight decrease in the activity of CK2α when the V66A/I174A mutations were introduced to the kinase. It is reasonable to assume that mutations to these residues into the smaller alanine residues do alter the ability of the kinase to bind ATP.

Overall, both our results and the results found by the previous Litchfield lab member show only a modest decrease in activity, not significant enough to take away from the objectives of the experiment. Furthermore, within our results, the majority of CK2α' mutants had calculated specific activities within the same general range. For the following steps, all of the activity for the mutants was normalized in order to test their ability to recover residual CK2 activity in the presence of CX-4945 inhibitor.

4.4 – The recovery of phosphorylation activity of CK2α' mutants in the presence of CX-4945

It has been well documented that CK2α and CK2α' have similar catalytic activity when tested *in vitro* [95]. Studies performed by previous Litchfield lab members have investigated the ability of point mutations, made within conserved residues in the

catalytic site of CK2α, to recover residual CK2 activity in the presence of CX-4945. However, until now no studies have been documented that have made these same point mutations within CK2α'. Such mutations could provide a new opportunity to compare the catalytic activity of the subunits *in vitro* and test whether the two truly do share the same catalytic properties *in vitro*. The work in part 3.1 of this thesis identified a new combination of amino-acid substitutions (V66A/I174A/H160D) that recovers residual CK2 activity to a level higher than previously documented. This would be the first study to investigate a similar combination of substitutions in CK2α'.

Seven novel mutations were created in the present study have not been previously examined for their ability to desensitize CK2α' to CX-4945. There are a number of trends that are worth investigating further. Firstly, every kinase that incorporated the double mutation V67A/I175A recovered more residual CK2 activity than its corresponding single mutation to residue H161 or residue Y116. This is in line with the literature that has shown that these two residues are important to the kinase-inhibitor interaction [60] and provides an interesting contribution to the field as there is no documentation that the two mutations (V67A/I175A) have ever been studied in combination with other mutations in CK2α'. The ability of the CK2α'-DM kinase to increase residual CK2 activity is consistent when it is introduced to a wild type kinase or a kinase with another amino-acid substitution. Additionally, any additional mutations to Y116 or H161 also increased recovery. This is noteworthy as neither of these two residues has previously been mutated in CK2α'. It suggests that these residues are also important to the kinase-inhibitor interaction and without those specific amino acids, interactions between the kinase and CX-4945 that help keep the inhibitor in the active site

would be abolished. The new mutations add to the recovery that was previously established with the V66A/I174A mutations.

The Y116 residue in CK2α' is a histidine residue in CK2α, which is one of the few residues that is not conserved between the two catalytic subunits within the N-terminal 330 amino acids. When this residue is mutated to a histidine residue in CK2α', there is a significant recovery of residual CK2 activity in the presence of the inhibitor. This suggests that each of these residues is necessary for binding CX-4945 in each of the catalytic subunits.

The H161 residue is responsible for locking in the inhibitor once it is bound, thus when this residue is taken away, the inhibitor can become unbound much more easily [28]. While a mutation to either residue increases the recovery of the kinase, H161D/E mutations showed higher recovery than did Y116H/L mutations. The CK2α'-V66A/I174A/H161E (CK2α'-TM) mutant showed the highest recovery *in vitro* and because of this, it was chosen to make into stable U2OS cell lines. This combination of amino-acid substitutions can be made into stable Flp-In U2OS cells to investigate the difference between the two catalytic subunits within a cell model.

The similarity between the mutation profiles of CK2α and CK2α' shows the importance of the residues for both isoforms of CK2. This is in line with the literature [72], although it is the first time anyone has investigated the differences within inhibitor-refractory mutants between the two catalytic isoforms, studies have shown that the two subunits have shown similar properties *in vitro*. The similarity of their functions *in vitro* is largely due to the 90% sequence identity within the N-terminal 330 amino acids [72]. The two isoforms also have very similar catalytic sites, which may provide another

explanation for their similar activity *in vitro*. The analysis of both CK2α and CK2α'

showed that H160/H161 was the most important residue tested in the interaction with

CX-4945. It gave further evidence that CK2α'-V66A/I174A/H161E was the optimal

kinase to be transfected into cell culture.

There is concern that none of the mutations recovered residual CK2 activity to the

level of the DMSO control. Additional amino-acid substitutions can be made, to other

conserved CK2α' residues that are necessary for inhibitor binding, to address this

concern. Despite the limitations, the trends found in the *in vitro* investigation of CK2α'

proved to be similar to a previous study performed in the Litchfield lab and provided us

with the optimal combination of amino acid substitutions that can be used as a platform

to investigate the role of CK2α' in cells. These cell lines will be compared to the studies

performed in sections 3.1 and 3.2 of this thesis.

4.5 - The creation and induction of CK2α' U2OS cell lines

Previous studies have shown that CK2α and CK2α' can be expressed at similar

levels in cells after being transfected. More specifically, CK2α' with an N-terminal HA

tag has expressed at the same level as or even higher than CK2α with N-terminal or C-

terminal HA tag [96]. Within the present study, Flp-In TRex U2OS cell lines were

created *via* transfection with CK2α'-WT, CK2α'-DM and CK2α'-TM DNA that were

tagged with the HA epitope on the N-terminal. However, all of the previous studies made

use of co-transfection with CK2β and our study was transfected with the catalytic subunit

only. This test is of significance as it is investigating whether there is any difference

between CK2α' constructs that are co-transfected with the regulatory CK2β and

constructs that are not co-transfected with CK2β. It also tests if there is any difference between transfections with CK2α and CK2α' constructs.

The lack of robust expression of HA-CK2α' comparable to CK2α-HA was somewhat surprising, possibly illustrating the importance of the regulatory CK2β subunit for optimal expression of HA-CK2a' or differences in the behaviour of CK2a and CK2a' [72]. Seemingly, CK2α-HA can be expressed at a high level without the need for the regulatory CK2β subunit while HA-CK2α' is expressed at far lower level in the absence of the regulatory CK2β subunit. To follow this up, we performed a transient transfection with CK2β into cell populations of both CK2α-HA and HA-CK2α'. There was a substantial increase in expression when CK2β was transiently transfected into the cells with the N-terminal HA tagged CK2α' protein but little change in expression when transiently transfected into the CK2α-HA. It has been shown that CK2β is able to alter substrate specificity of the catalytic subunits as well as protect the subunits from degradation [29]. Thus it is possible that in addition to stabilizing the HA-CK2α' protein, the CK2β could also change its interacting partners, which could lead to increased expression.

The expression level of the HA-CK2α' co-transfected with CK2β when compared to CK2α-HA was of a similar intensity despite its appearance on the blot. It has been proven that, during cell cycle, CK2α is phosphorylated at multiple sites on its unique C-terminal tail by the mitotic cyclin dependent kinase, Cdk1. Because CK2α' does not share this same C-terminal tail, it is not phosphorylated at all by Cdk1 [97]. The addition of 4 phosphate groups on the phosphorylation sites is responsible for the multiple

banding seen in the HA blot. This explains why CK2α-HA has a larger dark banding pattern when compared to HA-CK2α' co-transfected with CK2β.The multiple banding pattern does not necessarily imply a higher expression level of the CK2α-HA tagged protein.

While expression of the HA-CK2α' was lower than the CK2α-HA cells, there was still some level of expression of the HA-CK2α' cells when compared to the negative control. This was further exemplified within immunofluorescence studies. Because of the increase in expression with tetracycline induction, we performed a recovery experiment with the HA-CK2α' cells with similar conditions to the recovery conditions tested with the CK2α-HA cells.

Of the three cell line that were made with HA-CK2α' constructs, only the CK2α'-WT and CK2α'-DM were expressing, while CK2α'-TM was not. This may suggest that the HA-CK2α'-TM construct was not viable in cells. Thus the recovery experiment tested only the CK2α'-DM and CK2α'-WT kinase. It was shown within section 3.1 of this thesis that the CK2α-DM kinase was not recovering with the CK2α-HA cell lines. While some previous studies have seen recovery with this mutation [61], it was mentioned in section 4.1 that our research goes against the theory that the CK2α-DM kinase recovers residual CK2 activity in cell. Within our research, there was also no recovery seen with the CK2α'-DM when compared to the CK2α'-WT kinase at any of the inhibitor concentrations or time periods tested. This is in line with what we have previously seen with the CK2α-HA cells and it provides another example of this double mutant kinase not functioning in cell for CK2α or CK2α'. This experiment was limited due to the lack of expression in the CK2α'-TM kinase with CK2α' cells. Another limitation was the lack

of comparable expression between the two catalytic subunits, as a result, it was difficult to make accurate comparisons between the two. The next step, in attempt to negate these two limitations, was to test other CK2α' constructs in order to obtain similar levels of expression across the two catalytic subunits of CK2.

In prior studies in the Litchfield lab, CK2α' constructs were tested with the HA on both the N and C-terminal tag. In these experiments, the HA tag had a high level of expression on either terminal tail when co-transfected with CK2β [96]. In a separate study however, CK2α' with a C-terminal HA tag showed a high level of expression without the need for co-transfection with CK2β [98]. Based on this finding, we decided to test transient transfections with multiple epitope tags on either side of the CK2α' construct. This is noteworthy for a number of reasons. Firstly, it is the first test examining the significance of the orientation of the HA tag on the catalytic subunit of CK2α'. Secondly, it is a direct comparison with the CK2α construct. Finally, if we are able to find a construct of CK2α' that shows high levels of expression then that construct can be made into stable cell lines to be compared to the CK2α cells that were previously optimized with inhibitors.

In our study, the CK2α'-HA construct had a significantly higher expression level than the previously tested HA-CK2α'. There are limitations to comparing the expression levels with transient transfections to the expression levels in stably transfected cell lines. The limitations are mostly based on the efficiency of the transient transfection. Our study used the measures that were available to test the transfection efficiency, however these measures are not 100% accurate. It is difficult to compare cells with low transfection efficiency to cells that have stably transfected the DNA of interest into their genome.

The use of the positive control CK2α-HA construct, which was also used to make the CK2α cell lines that showed high expression levels when stably transfected in section 3.1, allowed for a proper comparison to the expression level we were hoping to achieve. The fact that, when transiently transfected, the CK2α'-HA construct has a higher expression level than the positive control CK2α-HA construct suggests that the CK2α'-HA construct will have similar expression levels in stable cell lines when compared to the CK2α-HA construct that was tested in section 3.1. The low expression levels for the transient transfection of HA-CK2α' construct explain the low expression levels for the stable HA-CK2α' cell lines tested in section 3.5.

The use of EGFP to test transfection efficiency could explain the relatively low expression of all of the constructs that were tested. 20% transfection efficiency is low and when constructs can be stably integrated into the cell's genome, they will be able to express at a much higher level. It is important to note that not all of the cells showed drastically different levels of transfection efficiency, implying that the difference in expression levels has to do with the efficacy of the constructs rather than the effectiveness of the transfection process.

Despite the limitations of the process, the transient transfections allowed us to find a new construct to be stably integrated into cell lines. It is hypothesized that these cell lines will show higher levels of expression when blotting for the HA tag and can therefore allow for a better recovery assay than the one performed within this section. The cells can then be tested in the same manner as the CK2α cells for a direct comparison of the action of CX-4945 and inhibitor VIII on the two catalytic subunits.

4.6 – Next Steps

The most important next step after this thesis is to create the stable cell lines with the CK2α'-HA constructs that induce well with tight tetracycline regulation. Once the cell lines are created and proper selection conditions are achieved, the same tetracycline titration should be done to achieve expression comparable to the alpha cell lines. The cells can be tested with the CX-4945 and CK2 inhibitor VIII to find optimal recovery conditions using the same method as performed with the CK2α cell lines in section 3.1.

The long-term treatment of CK2 overexpressed cells with CK2 inhibitors showed some very interesting trends but also raised many questions as to how cells could be changing morphology while maintaining their pIF2β signal. We have been able to suggest possible explanations as to why this occurred but the suggestions should be tested. Inhibited cells should be investigated with antibodies that indicate cells are going through apoptosis, such as PARP or caspase-cleavage antibodies, to examine at which point the cells are going through apoptosis and whether or not the inhibitor-refractory cells are able to recover from cell death. A series of other experiments can investigate whether the inhibitors are producing off-target effects within the cells.

There was a great extent of investigation in this study into the importance of orientation of epitope tags in kinase expression. While we were able to determine which orientation and which tags were optimal for expression, further studies should be done to investigate why the orientation has such a large effect. Immunoprecipitation experiments could be performed with epitope tags on either side of the kinase to investigate whether the orientation affects the ability of CK2 to bind specific binding partners.

4.7 - Conclusion

We were able to optimize mutant CK2α cell lines in terms of recovery with multiple potent CK2-specific inhibitors. CK2 inhibitor VIII and CX-4945 were compared and characterized in terms of their potency, recovery with inhibitory-refractory mutants and long-term toxicity. The inhibitor-refractory mutants were demonstrated to be important tools for the investigation of inhibitor specificity and could prove to be helpful in the identification of *bona fide* subunit-specific CK2 targets with the use of proteomics. An interesting trend was noted when osteosarcoma cells were inhibited for multiple days. Cells were changing morphology on the plates but the inhibitor refractory cells were still able to maintain their recovery. This not only shows a remarkable ability of this mutant kinase to recover activity within the cell, it also opens a series of questions on the specificity and efficacy of the inhibitors under investigation. While possible explanations for the phenomenon were suggested, further work needs to be done in order to fully understand the role of CK2 within the change in cell morphology that was observed. Seven novel mutations made to CK2α', similar to mutations made to CK2α in a previous study within the Litchfield lab, were investigated *in vitro*. The similarity in the residual CK2 activity shown in the study of both CK2α and CK2α' suggests similarity between the activity of CK2α and CK2α' *in vitro*. It was shown that in cell assays differed greatly from the *in vitro* assays, in terms of inhibitor potency and the difference between the two catalytic subunits. The orientation of HA tag was of critical importance within the CK2α' cell line, and new constructs have been made and tested to be transfected into stable cells lines. Those will be tested in future studies. The work done within this study will provide

a useful tool for the investigation of isoform specific targets of a protein kinase whose

role in tumorigenesis has been largely unknown up to this point.

5 – Appendix
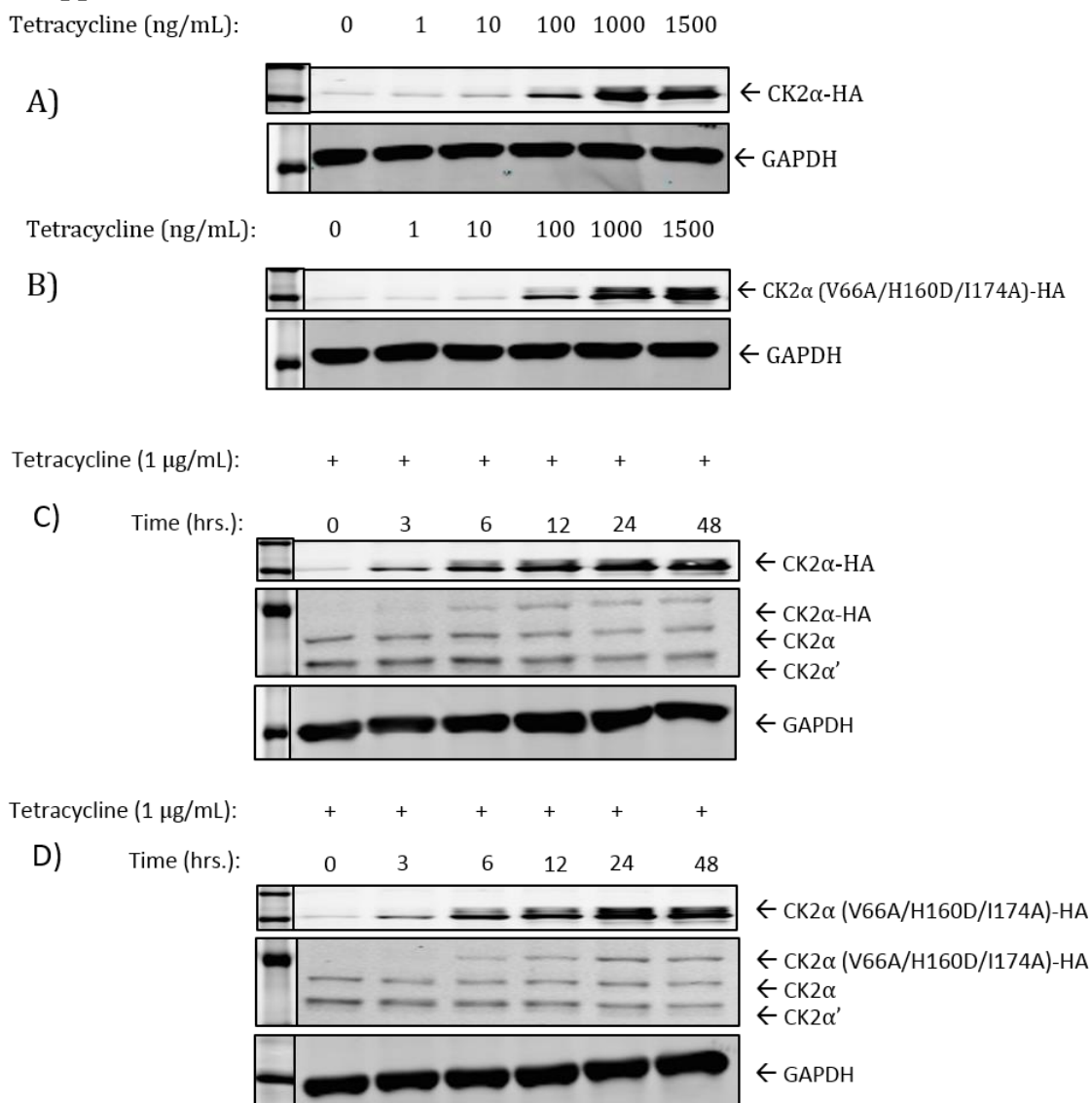
Supplemental Figure 1. Comparing expression levels of endogenous and exogenous CK2α in Flp-In U2OS cell lines. Flp-In U2OS cell (expressing CK2α-HA or CK2α (V66A/H160D/I174A)-HA were cultured in the presence of 1mg/ml of tetracycline and harvested at increasing time intervals (0-48 hours). Protein expression was assessed using Western blots and a CK2α/α' antibody capable of detecting endogenous and exogenous CK2α species simultaneously. A) Different tetracycline concentrations tested with the CK2α-WT kinase and B) CK2α-TM kinase. C) Different time points tested with the CK2α-WT kinase and D) CK2α-TM kinase.

CK2α–HA	**Wild Type**				**V66A/I174A/H160D**			
Tetracycline	–	+	–	+	–	+	–	+
DMSO	+	+	–	–	+	+	–	–
Inhibitor VIII (15 μM)	–	–	+	+	–	–	+	+

IB: CK2α/α'
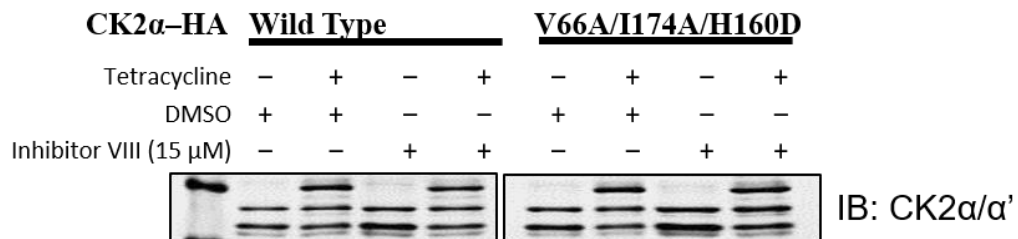
Supplemental Figure 2. Measuring the expression of exogenous levels of CK2α in the presence of inhibitor VIII. Cells were induced for 48 hours with 1000 ng/ml tetracyclin and then incubated with inhibitor VIII for 6 hours at a concentration of 15 uM. Cells were harvested, lysed, blotted and incubated with CK2α/α' antibody to measure the expression of the exogenous protein in comparison to the endogenous levels of CK2α and CK2α'.
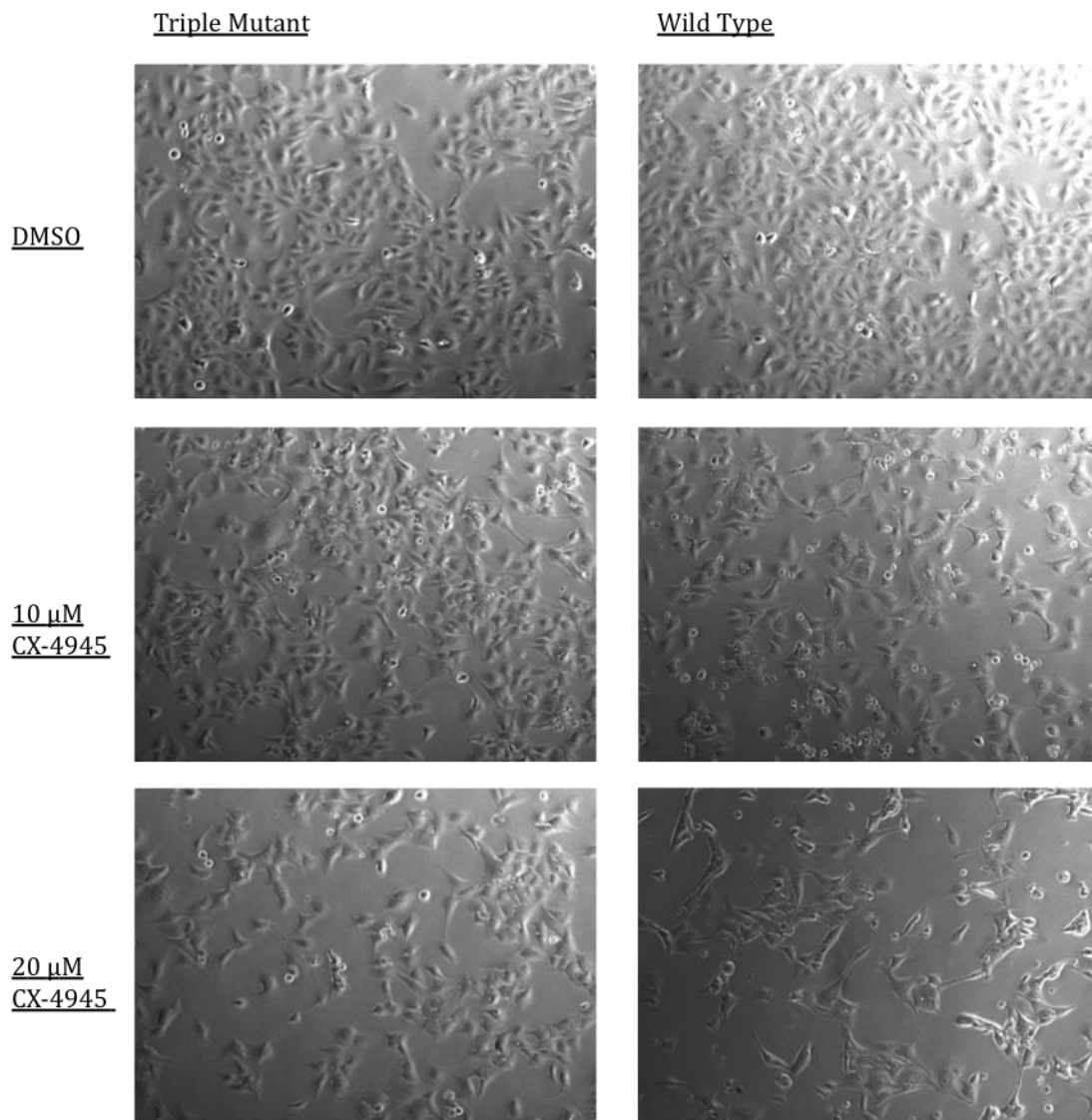
Supplemental Figure 3. Brightfield images of U2OS Flp-In osteosarcoma cells taken at different inhibitor concentrations. Cells were induced with tetracycline for 48 hours before being treated with CX-4945 for 2 days (48 hours). Pictures of the cells were taken at 20x magnification with inhibitor conditions of DMSO control, 10 μM and 20 μM CX-4945.

Supplemental Table 1. The concentration and specific activity for each of the mutants of CK2α and CK2α'.

	Mutants	Concentration (mg/mL)	Specific Activity (pMol/min/μg enzyme)
GST-CK2α	Wild Type	6.06	474
	H160L	7.25	507
	H160D	1.42	524
	V66A/I174A	2.20	352
	V66A/I174A/H160L	2.45	397
	V66A/I174A/H160D	1.39	429
GST-CK2α'	Wild Type	1.25	782
	H161D	2.85	137
	H161E	3.12	585
	Y116L	2.77	115
	V67A/I175A	1.12	528
	V67A/I175A/H161D	3.45	786
	V67A/I175A/H161E	4.11	474
	V67A/I175A/Y116L	2.72	919
	V67A/I175A/Y116H	5.88	11

<u>Sample calculation</u>

Calculating specific activity (GST-CK2α)

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

[GST-CK2α']= 1.25 mg/mL

2 µL of GST-CK2α (1.25 µg/µL) was initially diluted into 250 µL of CK2 dilution buffer
Therefore, (2 µL)* (1.25 µg/µL)= 2.5 µg of GST-CK2α
Next, 4 µL of the 250 µL sample was taken in order to start the kinase reaction
Therefore, (2.5 µg)*(4 µL/ 250 µL)= 0.04 µg
The reaction proceeded for <u>10 minutes</u>
10 µL of the total kinase reaction (20 µL final volume) was then spotted onto P81 paper
Therefore, (0.04 µg)/2 = <u>0.02 µg of GST-CK2α</u>
The 10-minute reaction (GST-CK2α phosphorylating a RRRDDDSDDD peptide) yielded a count (CPM) of <u>17000000</u>, and the spotted standards yielded a CPM of 53000000
Therefore, to calculate the CPM/pMol of ATP in the standard – (53000000 CPM) /(10 µL spot * 100 µM ATP * 5 for the dilution)= <u>11000 CPM/pMol</u>

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

(17000000 CPM) / (11000 CPM/pMol)= <u>1600 pMol</u> of ATP incorporated onto the RRRDDDSDDD peptide
(1600 pMol) / (10 minutes)= 160 pMol/min
(160 pMol/min) / (0.00002 mg) = 780000pMol/min/mg of GST-CK2α

6 – References

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Curriculum Vitae

Education

MSc, Biochemistry, Western University   Sept. 2014 – 2016
- GPA: 3.95/4.0
- Student in: Richard Ivey School of Business MBA Class - The Business of Health
- Student in: Certificate in Business and Consulting MiniMBA Seminar Series
- Member of the Graduate Management Consulting Association – Western chapter; I practice, teach and write cases

HBSc, Biochemistry, Queen's University   Sept. 2014 – Jul. 2016
- Final year GPA: 3.7/4.0
- Graduated Dean's Honour List; Queen's University Entrance Scholarship

Work Experience

Analyst, **Decision Resources Group**   Jul. 2016 – Present
- Conduct primary and secondary research to produce comprehensive market research reports
- Gather and interpret data on market conditions, business opportunities and global trends in the medical device, pharmaceutical and healthcare industries
- Work on custom consulting projects
- Company expert and written reports in gynecology, neurosurgical and neurostimulation markets

Graduate Research Assistant, **Western University**   Sept. 2014 – Jul. 2016
- Work submitted for publication to *The Journal of Science Signaling*; a well known, high impact journal
- Led an independent cancer research project focused on screening pharmaceutical drugs' efficiency in clinical trials across North America
- Work led to an improved understanding of a specific cancer pathway and how it is affected by different cancer-inhibiting drugs
- Oversaw the mentorship of 3 junior lab members, committed to helping them until graduation

Teaching Assistant, **Western University**   Jan. 2015 – May 2016
- Assisted students with laboratory techniques and scientific writing skills by providing tutoring and feedback on a daily basis; resulted in each of the 5 personally mentored students achieving a laboratory position at the Schulich School of Medicine

Consultant, **The University Consulting Group**   Jan. 2015 – May 2016
A non-for-profit consulting organization that employs graduate students and consultants working in the industry
- Worked on a team of 5 alongside fellow graduate students and industry advisers to increase membership of the non-profit organization: *Iranian Women's Organization of Ontario* by 15-20%; allowing them to increase funding as well as their organization's impact

Research Analyst, **Movement Disorders Diagnostic Technologies (MDDT) Inc.**   Apr. 2015 – May 2016
Use a novel medical device to track which muscles are involved in tremors; allows doctors to know where to target their therapies
- Led an intensive research project, assessing what countries could TremorTek and its industry partner target in the next five years
- Assisted the company's co-founder in market research, finding similar technologies on which to base our marketing and investor relations
- Conducted intensive patent search, saved the company $2,000-$3,000 in patent-lawyer fees

Research Assistant, **Queen's University**   Sept. 2013 – Jul. 2014
- Spearheaded an independent research project, using critical and analytical skills to discover a previously unknown enzyme in a pathogenic pathway; research was officially accepted for publication to *The Journal of Bacteriology* in September 2015

Community Involvement & Leadership Roles

Member of the *Schulich Graduate School Committee,* Western University

Sept. 2014 – Present

- Representative for biochemistry (76 students); worked to advocate in the interests of all Schulich graduate students
- Leader and master of ceremonies for *The Career in Academics* workshop committee for London Health Research Day 2015

Councilor for the *Society of Graduate Students*, Western University

- Act as liaison between the students, faculty and staff at Western University; provide a voice for the graduate students on many issues

Presenter for *Strong Bones, Strong Minds, Strong Muscles*, Western University

Sept. 2014 – Present

- Utilized presentation skills and communication adaptability by presenting my research and general science to a retirement home

Volunteer, Trillium Hospital,

2012 – 2014

- Helped create a musical therapy program where I connected with patients in the rehabilitation ward by playing them music

Interests and Hobbies

- Amateur musician, performed in cities: Kingston, Toronto, Mississauga, Sudbury, Winnipeg, Banff, Vancouver and Prague
- Workout enthusiast and former certified personal trainer – with experience personal training students at Queen's University
- Languages: English and French; written and spoken