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Cycle XXX

**Chemical genetic approach to identify substrates of CK2 kinase
using Mass spectrometry**

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ABSTRACT

Protein kinase CK2 plays major roles in multiple biological processes, as has been reported in literature. Phosphoproteomic and bioinformatics data suggests that CK2 is involved in many cellular pathways however there is no report published on the unbiased global validation of its *bona fine* cellular substrates and how the CK2 dependent phosphorylation is modulating these pathways. Therefore, there is a need for methods that can do this validation process. Since the kinases have similar nature to phosphorylate their substrates, it is a great challenge to find or validate new substrates of a targeted kinase. In this study, a chemical genetics approach was used when the phenylalanine bulky residues were identified in CK2 α as F113 and CK2 α' as F114. These residues were mutated to glycine to expand the ATP binding pocket, and create an analogues sensitive kinase which can utilize an ATP analogue nucleotide. The substitution did not alter the activity or specificity of the kinase. Furthermore, the analogue sensitive-CK2 (as-CK2) and wild type-CK2 (wt-CK2) α and α' SILAC labeled stable cell lines were generated, then thiophosphorylation reactions were done using N6-(2-phenylethyl) ATP γ S (2peATP γ S) to label substrates in the cell lines. The thiopeptides were identified using mass spectrometry based proteomics approach. The thiopeptides found only in as-CK2 compared to control were considered as CK2 substrates. Our results highlight the potential of using analog-sensitive CK2 to further expand our knowledge of the cellular roles of CK2 and elucidate kinase-substrate relationships.

CHAPTER ONE: INTRODUCTION

1.1 General Introduction

Protein Kinases are the enzymes which catalyze phosphorylation of cellular proteins by transfer of gamma phosphoryl group from ATP or GTP to hydroxyl group of serine, threonine or tyrosine residues of these substrates to regulate the cellular processes (Hunter, 2000; Manning et al 2002). Phosphorylation activates downstream cellular signaling pathways and, in this way, it can affect key signal transduction pathways leading to cell survival, apoptosis or cell proliferation (Cohen, 2000). Data from the literature suggest that in human proteome there might be approximately 700,000 potential phosphorylation sites (Ubersax and Ferrell 2007). Actually, this post-translational protein modification event is very common in *in vivo* cell function. The crucial role of kinases in signaling pathways is demonstrated since any alteration or dysregulation in their activity leads to the onset of several diseases and cancer (Brognard and Hunter, 2011).

1.2 Casein Kinase2 (CK2) protein kinase

CK2 protein is a pleiotropic serine/threonine kinase, which exists in a tetrameric holoenzyme form, composed of two catalytic subunits CK2 α and CK2 α' (coded by CSNK2A1 and CSNK2A2 genes, respectively) and two regulatory subunits CK2 β (coded by CSNK2B gene). The catalytic subunits have constitutive activity also in the absence of the regulatory units, the regulatory CK2 β subunits play an important role in the assembly of CK2 complexes, in enhancing the catalytic activity and stability of CK2, and in its substrate selectivity. (Poletto et al, 2008; Litchfield, 2003). The bioinformatics studies suggest that the 20% of the phosphoproteome might be the direct substrates of CK2 kinase. (Salvi et al., 2009). Based on the bioinformatic and systematic studies its known that the minimum consensus recognition motif of CK2 is Ser-Xaa-Xaa-Acidic, where the acidic residue may be Glu, Asp, pSer or pTyr (Meggio and Pinna 2003). CK2 is involved in multiple biological processes such as cell proliferation, cell survival or death (St-Denis and Litchfield, 2009), as well as it affects several biological pathways modulating protein stability and degradation (Niechi et al 2015; Scaglioni et al 2008), transcription and translation (Cabrejos et al 2004; Gandin et al 2016), and circadian rhythms (Tsuchiya et al 2009) (Figure 1). Moreover, it has been known that deregulation of CK2 could lead tumor progression in different types of cancer (Trembley et al 2009).

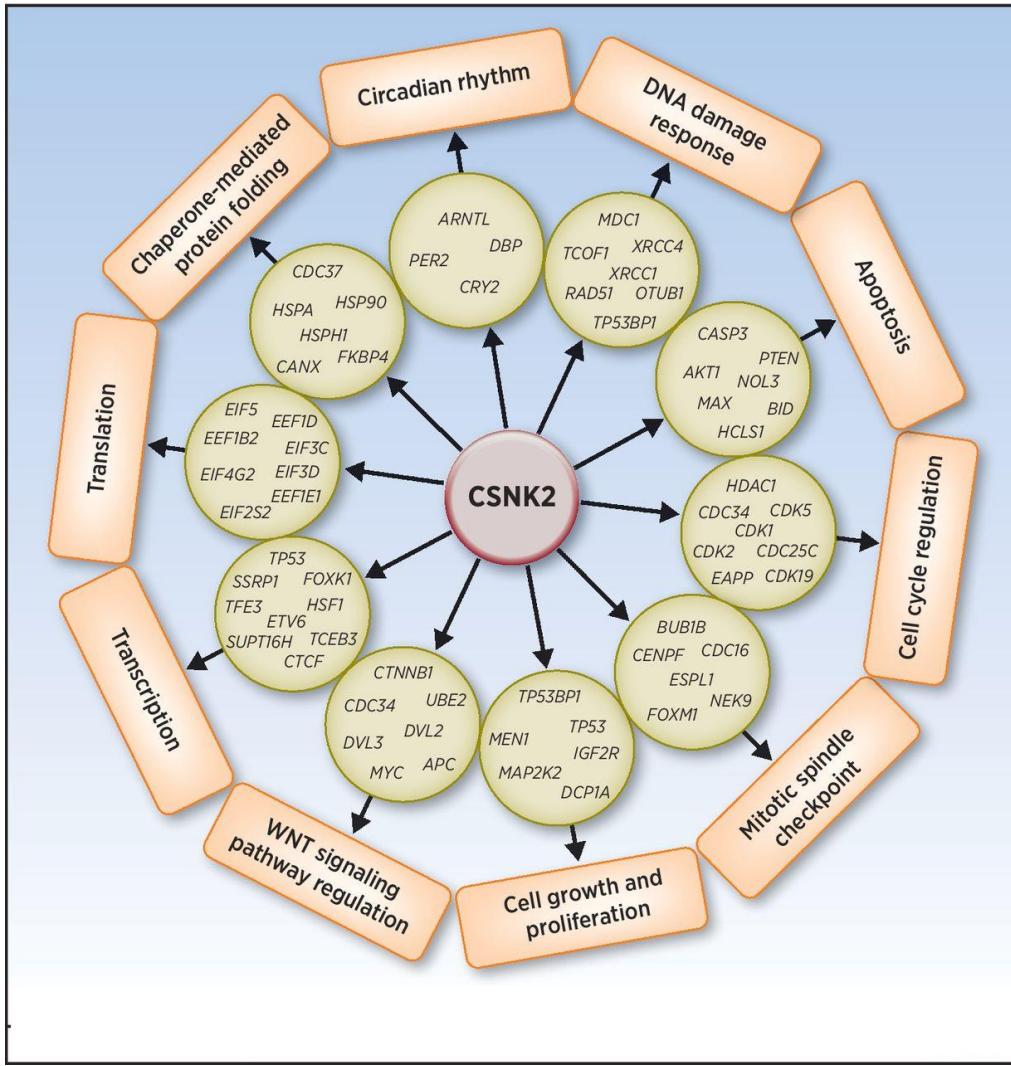


Figure 1: Involvement of CK2 in different pathways (Rabalski et al, 2016)

1.2.1 CK2 structure

As previously described, CK2 kinase is an heterotetrameric protein, consisting of two catalytic units α (42 kDa) and α' (38 kDa) and two regulatory β subunits (28 kDa) (Figure 2). Different heterotetramers, such as $\alpha_2\beta_2$, or $\alpha\alpha'\beta_2$, or $\alpha'\beta_2$ could be found (Grahem and Litchfield, 2000). The CK2 kinase has a smaller active site which is due to the presence of two bulky residues Ile74 and val66 compared to alanine residue which is present in other kinases (Sarno et al, 2005). This peculiar active site also enables the CK2 kinase to utilize ATP and as well as GTP compared to other kinases (Niefind et al, 1999). The crystal structure of tetramer reveals that one of the catalytic subunit might bind to a non-hydrolysable ATP analogue (adenosine 5 \prime -[β,γ -imido]triphosphate, AMPPNP), as well as ATP in the catalytic site (Lolli et al, 2012). CK2 β regulatory subunit consists of autophosphorylation sites, which include Ser2, Ser3 and Ser4. An additional Ser209 site is present, which is phosphorylated in cell cycle dependent manner (Litchfield et al, 1991). Furthermore, CK2 β consists of regions/domains (Figure 3), a domain similar to the sequence of destruction box (Arg47–Asp55) is believed to be involved in the stability of CK2 β (Zhang et al, 2002), whereas, CK2 β consists of acidic loop (Asp55–Asp64), which is involved in polyamine binding to regulate CK2 activity (Boldyreff et al, 1994; Leroy et al, 1997). The crystal structure of a dimeric form of the regulatory β subunit demonstrated that interaction between two CK2 β subunits is mediated by a zinc finger composed of four conserved cysteine residues: Cys109, Cys114, Cys137 and Cys140 (Canton et al, 2001). In tetrameric structure of CK2 kinase, the β subunits, without having contact with each other, form a stable dimer linking the two catalytic subunits. Anyway, each of the catalytic subunit interacts with both the C-terminal tail of the regulatory β subunits, in which the interaction occurs between the Asn181–Ala203, present in the CK2 α subunit, and the Arg186–Gln198 residues of the regulatory CK2 β domain (Niefind et al, 2001).

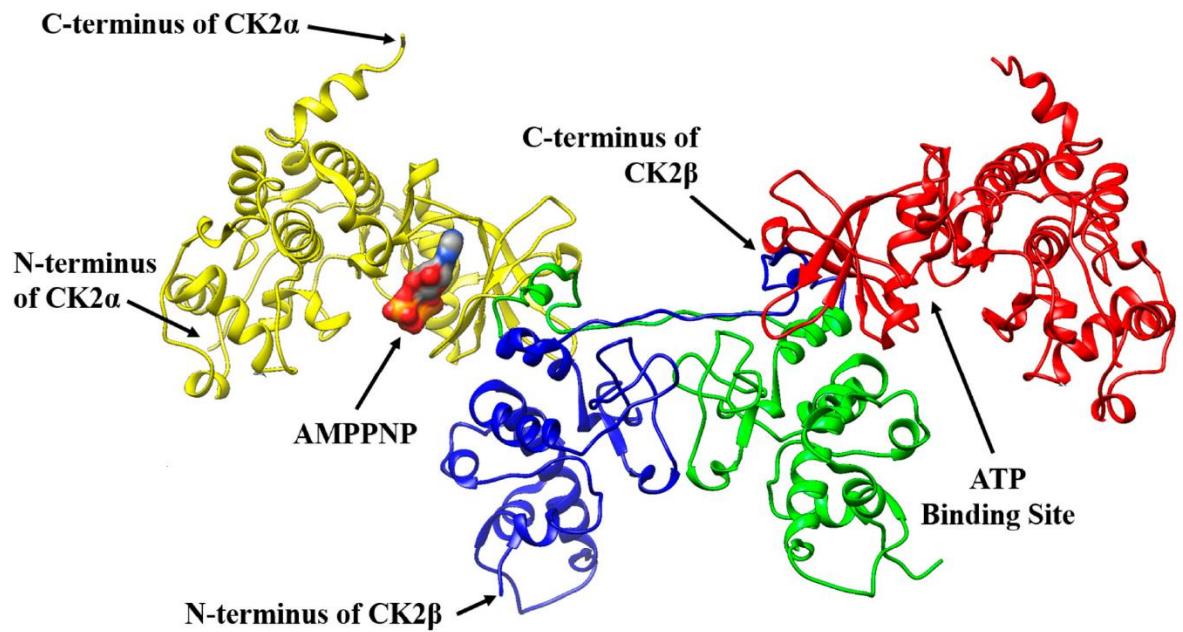


Figure 2: Ribbon diagram of tetrameric structure of CK2 kinase (Nienberg et al, 2016)

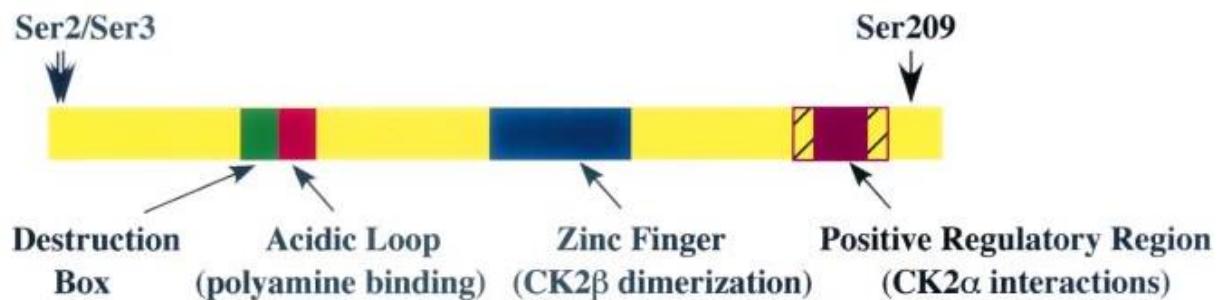


Figure 3: Sequence domains of CK2 β (Litchfield, 2003)

In majority of kinases, there is an Asp-Phe-Gly (DFG) motif in the activation loop of the N-terminus. When the kinase is active, phenylalanine of DFG forms a hydrophobic spine with N-terminal and another with C-terminal lobe residue, this conformation is called the “DFG-in” (Treiber and Shah, 2013). The inactivation of the kinases is due to the alteration of interaction of asparate and glycine with in DFG. This alteration is caused by the movement of phenylalanine out of hydrophobic pocket as a result blocking ATP binding to the active site (Treiber and Shah, 2013). CK2 is a unique kinase as in its primary sequence a tryptophan is included at position 176 instead of phenylalanine. Tryptophan causes the C and N-terminal lopes to be in an arrangement so that the essential residues in the active sites are properly positioned leading CK2 to be constitutively active (Vijayan et al, 2015).

1.3 CK2 Involvement in Cancer

Protein CK2 kinase has been shown to have elevated activity in many types of cancers such as lung (Daya-Makin et al, 1994), breast (Landesman-Bollag et al, 2001), head and neck (Faust et al, 2000) and prostate cancer (Yenice et al, 1994). CK2 is present ubiquitously in all cell types but normal cells compared to cancer cells have different localization of CK2. If in normal cells it is localized in a diffused pattern both in the nucleus and cytoplasm, in cancer cells nuclear compartment has higher level of CK2 kinase compared to cytoplasm (Faust et al, 1999; Laramas et al, 2007). In this context it should be note that CK2 kinase is involved in the regulation and stability of tumor suppresser proteins and oncoproteins, the dysregulation of these might lead to tumor formation and progression (Duncan and Litchfield, 2008).

1.3.1 CK2 regulation of Tumor suppressor proteins and oncogenic proteins

The tumor suppressor proteins are the gatekeepers of cell cycle, an important part in the cell signaling pathways. The dysregulation of activity or loss of function of the tumor suppressor protein has been associated with many types of cancers (Hanahan and Weinberg, 2000). CK2 kinase is believed to be involved in the regulation of these anti-tumoral proteins, the phosphorylation of which increases or decreases their activity and protects or promotes their degradation (Torres and Pulido, 2001; Keller and Lu, 2002).

One of the essential and well-known example of tumor suppressor protein is p53, which has been known to regulate cell cycle, differentiation and apoptosis (Oliveira et al; 2005). During DNA damage response, CK2 forms a complex with the chromatin transcriptional elongation factor, a heterodimer of both the suppressor of Ty 16 (hSPT16) and the structure specific recognition protein 1 (SSRP1). When hSPT16 and SSRP1 are complexed with CK2, they change the substrate specificity of CK2 to phosphorylate p53 over the other tested substrates. (Keller and Lu, 2002). The phosphorylation of Ser392 residue of p53 results in an increase of both DNA binding and transcriptional activity of this tumor suppressor protein (Kapoor and Lozano, 1998). CK2 kinase has shown to phosphorylate SSRP1 also, suggesting that CK2 regulates the DNA-binding ability of SSRP1 and so that this regulation may be responsive to the inhibition of transcription and replication after DNA damage (Li et al; 2005).

CK2 kinase is involved in regulation of different signaling pathways also involved in cancer, which include JAKs/STATs (Zheng et al, 2011), Wnt and NF- κ B signaling (Dominguez et al, 2009), as well as PTEN/PI3K/Akt-PKB (Miller et al, 2002) and Hedgehog signaling (Hh) (Jia et al, 2010). CK2 involvement in few pathways are discussed below.

1.3.2 CK2 involvement in Akt pathway

CK2 kinase has been to be involved in PI3K/Akt signal transduction pathway. The Akt pathway supports cell cycle progression, cellular growth and cell survival (Vara et al, 2004). Akt1 is phosphorylated at Thr308 and Ser473 residue to make it active, but an additional phosphorylation of Ser129 residue by CK2 kinase is believed to make Akt a constitutively active kinase (Di Maira et al, 2005). The phosphorylation of Ser129 residue is known to, stabilize the association of Akt with chaperone Hsp90 which protects Akt against the dephosphorylation of Thr308 done by protein phosphatase 2A (PP2A) (Sato et al, 2000; Di Maira et al 2009). It is also shown that phosphorylation at Ser129 promotes upregulation of β -catenin pathway, to increase β -catenin transcriptional activity (Ponce et al, 2011). CK2 kinase involvement in Akt pathway is also evident from its inhibitory effect on phosphatase and tensin homolog (PTEN), a well-known tumor suppressor protein. PTEN is an integral part to keep check on the Akt pathway by inhibiting the

Akt-elicited promotion of cell survival and thus prevents cell tumorigenesis (Cantley and Neel, 1999; Kim and Mak, 2006). CK2 phosphorylates PTEN at C-terminal domain to make it inactive. PTEN inactivation promotes Akt pathway thus causing oncogenesis (Vazquez et al, 2001). In summary, CK2 kinase plays a pivotal role in the induction of Akt pathway in which overexpression or high activity level of this kinase proves to be crucial in tumorigenesis (Figure 4). Indeed, in human cancer elevate Akt and lower PTEN activities compared to normal healthy cells were demonstrated (He et al, 2007).

Promyelocytic leukemia protein (PML) is another important tumor suppressor that is involved in pathways, like cellular senescence, growth suppression and apoptosis (Lallemand-Breitenbach and The, 2006). PML regulates the Akt pathway by interacting with two phosphatases, PP2A and the PH domain and leucine rich repeat protein phosphatases 2 (PHLPP2), which dephosphorylate Akt at Thr308 activation site (Trotman et al, 2006). CK2 kinase affects the function of PML tumor suppressor protein by phosphorylating it at Ser517. This phosphorylation leads PML to its degradation by proteasome pathway, resulting in avoiding of apoptosis (Scaglioni et al, 2006). In tumors where CK2 kinase is hyperactivated, the PML is degraded, which causes loss of tumor suppressor activity contributing to induce the tumorigenesis (Duncan and Litchfield, 2008).

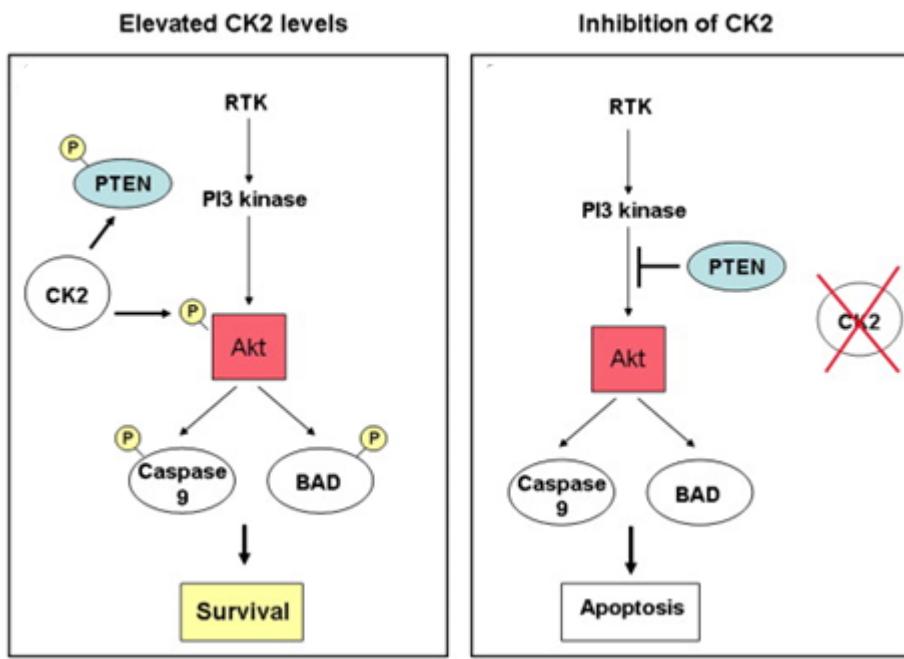


Figure 4: Effect of CK2 on PI3K/AKT pathway (Duncan and Litchfield, 2008)

1.3.3 CK2 regulation of Wnt pathway

The Wnt signaling pathway plays a crucial role during embryogenesis and is involved in cell proliferation, tissue polarity, cell survival, cell fate determination, motility and organogenesis (Miller and Moon, 1996; Komiya and Habas, 2008). Wnt signaling has essential role in embryogenesis whereas, in adult tissue the activation of Wnt pathway leads to tumorigenesis (Gordon and Nusse, 2006). CK2 phosphorylates different intermediates of Wnt pathway which include disheveled (Dvl) (Song et al, 2000), β -catenin (Song et al, 2000), adenomatous polyposis coli protein (APC) (Homma et al, 2002) and T-cell factor/lymphoid enhancer factor (TCF/LEF) (Wang and Jones, 2006).

In Wnt pathway high activity of β -catenin, a pro-transcription cofactor, causes increase in pro-survival signals such as c-Myc, cyclin D1 and c-Jun (Dominguez et al ,2004). CK2 is known to phosphorylate β -catenin to protect it from degradation by the proteasome. The mechanism consists of the dissociation of β -catenin from its destruction protein complex which consists of the scaffolding protein, Axin with its associated kinase: the glycogen synthase kinase 3 β (GSK3 β), APC and PP2A (Song et al, 2003; He et al, 2004). The complex dissociation allows the β -catenin to localize into the nucleus, where it activates transcription of pro-survival signals (Seldin et al, 2005). One of the β -catenin transcriptional target is the inhibitor of apoptosis (IAP), upregulation of which causes inactivation of caspases and block of the programmed cell death (Tapia et al, 2006). The survivin protein is a member of inhibitor of apoptosis family and it is normally expressed during embryogenesis. Mature cells do not express survivin while, in pathological conditions as in a number of cancers it is expressed through β -catenin/TCF pathway, which is the result of enhanced activity of CK2 kinase (Duncan and Litchfield, 2008). Moreover, transcription factor c-Myc is known to be involved in different types of cancer (Ponzielli, et al, 2005). Hyperactive CK2 phosphorylates C-terminal PEST domain of c-Myc which protects it from proteosomal degradation. In addition, Wnt pathway β -catenin/TCF/LEF activates c-Myc transcription which results in the promotion of the expression of cell survival genes, the high cellular proliferation rate and cancer progression. (Sasaki et al, 2003).

1.3.4 CK2 and apoptosis

A key component of normal cell turnover is programmed cell death, also known as apoptosis, and its dysregulation is known to be critical in oncogenesis (McKenzie and Kyprianou, 2006). CK2 has anti-apoptotic activity and finally increases cell survival by several indirect mechanisms, as already described (Ahmad et al, 2008.) CK2 also affects apoptosis directly by effecting the caspases activity on the caspases substrates. The consensus or recognition sequence of CK2 kinase and those of the caspases have great deal of similarity (Litchfield, 2003). When the downstream substrates of caspases are phosphorylated by CK2, at the vicinity of caspase recognition site, they are protected from cleavage by these proteases. (Krippner-Heidenreich et al, 2001; Riman et al, 2012). Bid, a Bcl-2 family protein, is phosphorylated by CK2 kinase at serine residue which the recognition site of caspase-8 preventing its cleavage (Desagher et al, 2001). Moreover, CK2 kinase has been shown to phosphorylate the activity-regulated cytoskeleton-associated protein (ARC) resulting in caspase 8 activity inhibition (Li et al, 2002). The effect of CK2 kinase on ARC and Bid are depicted in Figure 5. In response to apoptosis stimuli, Max, a transcriptional partner of c-myc oncprotein, is degraded by caspase cleavage, whereas, this cleavage is inhibited when Max is phosphorylated by CK2 (Krippner-Heidenreich et al, 2001). The other examples, in which CK2 phosphorylates caspase substrates to protect them against degradation, include the hematopoietic lineage cell-specific protein 1 (HS1) (Ruzzene et al, 2002) and gap junction proteins in the lens (Yin et al, 2001). Accordingly, studies in which CK2 kinase has been inhibited by knockdown or inhibitors demonstrated that cancer cells repurchase their sensitivity to the apoptosis stimuli (Ravi and Bedi, 2002).

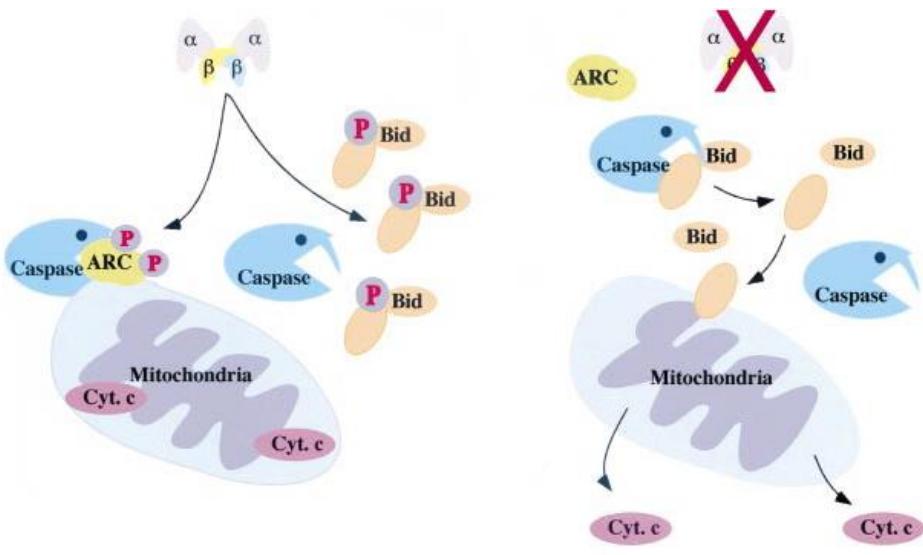


Figure 5: Effect of CK2 on substrates of caspases in apoptosis (Litchfield, 2003)

1.4 Inhibitors of CK2 kinase

Since protein kinases are involved in different cell signaling pathways, and dysregulation of protein kinases are implicated in tumorigenesis and oncogenesis, this makes them the most important pharmacological target. Different powerful inhibitors of kinases have been developed for treatment of cancer, these include as Gleevec, Irressa, and Tarceva (Klein et, 2005; Krause and Van Ette, 2005). There are two types of Kinase inhibitors, competitive and non-competitive. The competitive inhibitors are small-molecules which act as adenine mimics, competing with the ATP to the active sites on the kinase (ATP binding site) (Prudent and Cochet, 2009). The major issue with competitive inhibitors are that they bind to off targets, binding to active sites of other kinases or bind to non-kinases which have similar binding sites causing toxicities; Gleevec is a good example which causes cardiotoxicity (Kerkela et al, 2006). The other types of kinase inhibitors are those which can inhibit the activity of the kinase via allosteric inhibition. In this case, the inhibitor-binding site is on the residues away from the catalytic site, causing the change in structure and specificity of the substrate, thus inducing downregulation of the kinase activity (Garuti et al, 2010).

CK2 kinase is one of the kinases which has been shown to be a central component of different biological processes and its hyperactivity/deregulation has been shown in different cancers which makes it a pharmacological target. CX-4945, developed by Cylene Pharmaceuticals Inc., is a ATP competitive inhibitor ($K_i = 0.38$ nM for CK2 α and CK2 α' with an IC $_{50}$ of 1 nM) and it is now in second phase of clinical trials (Siddiqui-Jain et al, 2010). This compound was synthesized from an existing inhibitor of CK2: IQA, by modifying thiopene ring within a pyridine ring to incorporate alkyl chains having aniline moieties (Pierre et al, 2011). The inhibitor was tested against 238 kinases, which showed no extensive inhibitory effects suggesting its high affinity for CK2 kinase (Pierre et al, 2011). The studies conducted on PC3 prostate cancer xenograft mice model and PC3 prostate carcinoma cells showed that the inhibitor was having anti-tumor, anti-proliferative activity and initiating apoptosis (Kim et al, 2012). Other *in vitro* experiments with BT-474 breast cancer cells showed that CX-4945 caused G2/M cell arrest (Siddiqui-Jain et al, 2010). Clinic trials are being conducted in combination with gemcitabine and

cisplatin for treatment of cholangiocarcinoma (ClinicalTrials.gov Identifier NCT02128282). The FDA has given CX-4945 Orphan Drug Designation for the treatment of cholangiocarcinoma.

1.5 Mass spectrometry-based proteomics

Proteins act as building blocks of the cell and play a direct role in molecular signaling, physical interactions and enzymatic catalysis. The proteins study is most important because they are closely related to cell phenotype. At a global scale, the study of proteins is known as proteomics (Yarmush and Jayaraman, 2002). The most comprehensive and versatile tool in large-scale proteomics is the use of mass spectrometry (MS) for protein characterization (Yates et al, 2009). Mass spectrometry-based proteomics involves analysis of protein sources from complex protein samples obtained from cell, tissues of body fluids (Aebersold and Mann, 2016). The bottom down approach or shotgun approach is used often in proteomics which involves proteolytic digestion of the protein mixture and the peptides generated are separated using micro or nano-scale high performance liquid chromatography. The peptides injected are eluted by increasing gradient of organic solvents. The eluted peptides are ionized using electrospray ionization (Fenn et al, 1989) at the entry point of mass spectrometer where they are measured by mass-to-charge ratio (m/z) or by tandem mass spectrometric (MS/MS) analysis. In tandem mass spectrometry, the mass analyzer selects the precursor ions by performing survey scans (MS1). In the second stage, these selected ions are detected in the second mass analyzer (MS2) by fragmentation of ions by collision-induced dissociation (CID). This fragmentation generates b ions from amino terminus and y ions from carboxyl terminus and calculation of difference in mass observed between these fragment ions results in the identification of amino acid and of peptide sequence (Steen and Mann, 2004).

PTM's play a key role in cell signaling pathways by influencing the properties of proteins such as interaction, turnover and localization (Larance and Lamond, 2015). The most common PTM's include phosphorylation (Ser, Thr and Tyr residues), ubiquitylation (Lys residue) and acetylation. These modifications provide crucial insights into the regulation of protein properties during cell signaling pathways (Olsen

et al, 2010). The identification and quantification of PTM's has been evolved with the use of mass spectrometry. The advanced separation techniques, better ionization techniques, sensitive analyzers and improved fragmentation methods in tandem mass spectrometry have made the mass spectrometry an ideal approach to study PTM's (Silva et al, 2013). These advances allow to identify PTM sites in single experiments. Different enrichment strategies could be adopted to capture specifically modified peptides to study the global effect of that modification within the proteome. These capture strategies include, antibody based enrichment of phospho Ser, Thr, Tyr residues (Doll and Burlingame, 2014), Ionic interaction based enrichment, which includes immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO_2) to capture phosphopeptides (Silva et al, 2013).

1.6 Aim and proceeding of the project

The findings in the literature suggest that the majority of substrates of CK2 kinase have not been studied and therefore the CK2 dependent cellular signaling events are not fully characterized yet. Since CK2 has constitutive catalytic activity, the large scale global cellular validation of bona fide substrates is a highly challenging task. In order to overcome this limitation, Kevin Shokat and his colleagues developed chemical genetic approach in which a gatekeeper residue (bulky residue) is mutated to a smaller residue in ATP binding pocket to expand the binding site so that different analogues of ATP can be utilized by the analogue sensitive kinases. In this study, the CK2 α and CK2 α' gatekeeper residues were identified by sequence and structural alignment to other kinases to identifying the bulky residue in order to create the analogue sensitive CK2 kinase (as). The residues of F113 in CK2 α and F114 in CK2 α' were mutated to glycine (a smaller amino acid) to open up the space in the ATP binding pocket. We tested different commercially available ATP-analogues *in vitro* by testing the activity of these analogues with the recombinant wt and as CK2 α and CK2 α' . The results showed N6-(2-phenylethyl) ATP γ S (2peATP γ S) can be utilized more efficiently by the as-CK2 α and as-CK2 α' compared to the wild type.

Stable cell lines expressing as-CK2 α and α' were developed and characterized. Since the ATP-analogue used is not cell permeable, the cells were treated first with digitonin (mild detergent) to make the ATP-analogue available, in which cell were added with ATP-analogue for thiophosphorylation reaction to take place. Thiophosphorylated serine, threonine or tyrosine residues were alkylated by PNBM so that the as-CK2 dependent phosphorylation could be detected with anti-thiophosphate ester antibody by western to compare utilization of the ATP-analogue by wt and as-CK2 kinases. The mass spectrometric proteomic approach was used further to ID the thiophosphorylated protein in the lysates. The proteins were digested by trypsin and thiophosphorylated peptides were captured using Iodoaceyl beads. The captured peptides were run on mass spectrometer and were analyzed using Peaks8, the thiophosphorylated protein found only in as-CK2 were considered to be the substrates of CK2 kinase. The rationale was to optimize a workflow which would help in future to develop a strategy to validate the bona fide substrates of CK2.

Chapter TWO

2.1 Material and Methods

2.1.1 Sequence alignment and modelling

Sequence alignments were performed using NCBI COBALT to compare the protein sequence of human CK2 α (NP_001886) and CK2 α' (NP_001887) with Cdk1 (NP_001777), Cdk4 (NP_000066), Cdk6 (NP_001250), Cdk7 (NP_001790), and c-Src (NP_005408). After Identifying the gatekeeper residue, modelling was done by using Pymol. (The alignment experiments were conducted by Michael Gabriel at Litchfield lab)

2.1.2 Site-directed mutagenesis and cloning

Site-directed mutagenesis (Stratagene) was used to substitute CK2 α F113 and CK2 α' F114 to a glycine residue with the following primers, respectively:

5'-TGTTGTTACGTGTTACCAACCAAGGCAGGGGGTCTCGTG-3'

5'-CACGAACCCCCCGCCTGGTGGTGAACACGTAAACAACA-3' and

5'-CTGTATTATTGATATATTCACCTACCAAAGCTGGTGTCTTGACACGGGG-3'

5'-CCCCGTGTCAAAGACACCAGCTTGGTAGGTGAATATATCAATAATACAG-3'

The vector pGEX having cDNA insert coding for CK2 α and CK2 α' with GST tag at their N-terminal were used as template for site-directed mutagenesis. The mutagenesis was performed using high fidelity *Pfu* polymerase with the following parameters on Biorad thermocycler.

1. Initial denaturation at 98 °C for 2 min
2. Denaturation at 98 °C for 40 seconds
3. Annealing at 58 °C for 30 seconds
4. Extension at 72 °C for 3.5 min
5. Final extension at 72 °C for 5 min

In total 20 cycles were run from step 2 to 4. After the mutation and amplification, the reaction was treated with Dpn I enzyme for 2 hours, the enzyme was inactivated by heating at 80 °C. The plasmid DNA was then added into 100 µl of DH5 α chemical competent cells in the Eppendorf tube. The mixture was mixed gently and was kept on ice for cold shock for 25 min. Then, the mixture was placed into a water bath at 42 °C for 1 min and on ice for 3 min. The mixture was then added with 500 µl of LB media and kept at 37 °C in the incubator with agitation for 45 min. Finally, the culture was seeded on LB agar plates with ampicillin (100 µg/ml) and the plates were incubated at 37 °C overnight. The colonies were picked in, placed in LB media with ampicillin (100 µg/ml) and placed at 37 °C with agitation overnight. Next day the Miniprep kit (GeneJetTM) was used to isolate the plasmid DNA from the cultures. The Plasmid DNA was sent for sequencing to confirm the site-directed mutagenesis.

2.1.3 Protein purifications

The pGEX-wt-CK2 α or pGEX-as-CK2 α and pGEX-wtCK2 α' or pGEX-as-CK2 α' having GST tag on the N-terminal were transformed in BL21 *E. coli* cells using the method as previously mentioned. The colonies were picked and grown in 15 ml 2XYT media (16g tryptone, 10g yeast extract, 5g NaCl, 1L ddH₂O, pH7.0) for each construct as pre-cultures. The pre-cultures were grown in 1 liter 2XYT media containing ampicillin (100 µg/ml) at 37 °C in the shaker incubator. The cells were induced with 0.5mM IPTG at OD₆₀₀ 0.8. After induction, the cultures were grown overnight at 16 °C in a shaker incubator. Next day, the culture was centrifuged at 4000 RPM and the cells were re-suspended in PBS to remove residual media, centrifuged again and supernatant was discarded. The resulting cell pellet was re-suspended in resuspension buffer (PBS with 30 mg/ml aprotinin, 20 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The cells were passed through the homogenizer for cell lysis in the presence of 1% of Triton X-100 to ensure the complete lysis of the cells. The cells were tumbled for 15 min. The lysate was centrifuged at 10,000 RPM for 20 min at 4 °C. The supernatant was added with 2ml slurry of 1:1 Glutatione-agarose: PBS beads. The beads with supernatant were tumbled for 1 hour at 4 °C. The beads along with the supernatant were added to the gravity columns in

which the supernatant was passed through the column and collected. The beads were washed 3 times with PBS. After the washes, the beads were added with 1 ml of elution buffer 1 (10 mM reduced glutathione, 50 mM Tris-Cl (pH 8.0), 1 mM DTT) and incubated for 5 min at 4 °C. After the incubation, the flow, was collected. The elution was done 4 times using elution buffer 1. The elution was also done two times using elution buffer 2 (30 mM reduced glutathione, 50 mM Tris (pH 8.0), 1 mM DTT). The eluted fractions were run on SDS-PAGE gels for confirmations of the presence of proteins in the eluted fractions. The fractions 2 to 6 showing presence of the GST-CK2 were pooled together, concentrated and the buffer was changed with the Storage buffer (15 mM Mops (pH 7.0), 0.75 mM DTT, 300 mM (NH₄)₂SO₄, 25% glycerol).

2.1.4 In vitro kinase assay

Recombinant wt-CK2α, as-CK2α and wt-CK2α', as-CK2α' kinases were diluted to 50 ng/μl in CK2 dilution buffer (5 mM MOPS pH 7.0, 200 mM NaCl, 1 mg/ml BSA) immediately before use in kinase assays. 500 ng of recombinant His-elongation factor 1-delta (EEF1D), as a substrate, was phosphorylated by CK2 (100 ng) in CK2 kinase assay buffer (final concentration of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT) supplemented with 100 μM ATPγS (Sigma) or N⁶-modified ATP analogs: N⁶-2phenylethyl-adenosine-5'-O-3thiotriphosphate (2peATγP), N⁶-Furfuryladenosine-5'-O-3-thiotriphosphate (6-Fu-ATP-γ-S) and N⁶- Benzyladenosine-5'-O-3-thiotriphosphate (6-Bn-ATP-γ-S) (Biolog).

Assays were performed at 30°C for 30 min with slight mixing. After 30 min, all the reactions were stopped by adding EDTA at final concentration of 50mM. The samples, after the thiophosphorylation reactions using 100 μM ATPγS or N⁶-modified ATPγS, were alkylated by treatment with 2.5 mM *p*-nitrobenzylmesylate (PNBM, Epitomics) for 2 hours at room temperature. The reactions were stopped by adding 2X Laemmli buffer and the samples were boiled for 5 min. The samples were run on 10% SDS-PAGE gels, blotted and the membrane was hybridized with thiophosphate ester antibody (Abcam).

2.1.5 Competition assays

Competition assays were done using 100 μ M 2peATP γ S, ATP or GTP in different combinations. After the kinase reactions were done, as described in the previous section, and the alkylation obtained by incubation with 2.5 mM PNBM for 2 hours, the samples were analyzed on Western blots using thiophosphoester antibody to detect thiophosphorylated proteins and phospho-S162 EEF1D antibody to detect phosphorylated substrates.

2.1.6 Cell Culturing

U2OS (human bone osteosarcoma cell line) cells were grown in 10 cm or 6 well dishes in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-Invitrogen) medium containing 10% FBS (Invitrogen) and antibiotics (penicillin, 100 units/ml and streptomycin, 0.1 mg/ml). The cells were grown at 37 °C with 5 % CO₂. The cells were allowed to grow up to 70 to 80% confluence before proceeding to experiments.

2.1.7 Optimizing the Digitonin concentration

The analogues of ATP are not cell permeable, so the optimum amount of digitonin to be used for the thiophosphorylation experiments were determined by titrating with different concentration of digitonin. U2OS cells were grown in 6 cm plates, as described above, to 80 % confluence. The media was aspirated and cells were washed 3 times with PBS and treated with different concentrations of digitonin (Millipore) for 25-30 min. Propidium iodide (PI) (Thermo), a nucleic acid stain, was used to observe the permeabilization of the cells. After the treatment with digitonin, cells were washed with PBS and then washed again with 2XSSC buffer (0.3M NaCl, Sodium Citrate pH7) to remove non-adherent cells. The cells were added with SSC buffer having 0.5 mM of PI for around 2 min. The cells were washed with 3XSSC buffer. The adherent cells in the 6 well plates were observed under Nikon Microscope using TRITC filter.

2.1.8 Testing Thiophosphorylation and as-CK2 expression in cells

U2OS cells were grown in 10 cm plates to 80 % confluence. 20 µg of pRC/CMV-as-CK2 α -HA and α' -HA and pRC/CMV wt-CK2 α -HA and α' -HA were transiently transfected into the cells by using calcium phosphate method (Sambrook et al, 1989). 20 µg of each pRC/CMV construct was brought a volume of 45 µl with distilled water. After the suspension of each plasmid 5 µl of 3M NaOAc (sodium acetate) and 150 µl of ice cold ethanol were added. The suspensions were incubated on ice for 10 min and the plasmid-DNAs were precipitated by centrifugation at 13000 RPM. The supernatants were aspirated and the pellets were allowed to dry in the flow hood. The pellets of each plasmids were re-suspended in 450 µl of 0.1x TE. After complete re-suspension 500 µl of 2X HBS (pH 7.05) were mixed and all suspensions were kept at room temperature for 5 min. 50 µl of 2.5 M CaCl₂ were placed on the side of each tube and tubes were quickly vortexed for 15 sec to avoid precipitation formation. 1 ml of the total solution (450 µl TE+ 500 µl HBS + 50 µl CaCl₂) was added to the 10 cm plates and the medium was gently mixed by swirling and all dishes were kept in the incubator for 12 hours. After 12 hours, the media were aspirated, the cells were washed 3 times with PBS and supplemented with fresh media. Separately, in a 10cm plate, U2OS cells were also transfected by 5 µg of eGFP C2 (Clontech) construct using the above-mentioned protocol to check the efficiency of the transfection by this method. The cells in the plates were observed under Evos Fl Auto (Life technologies) microscope using GFP filter.

After 24 hours, the transfected cells were 3 times washed with PBS and added with thiophosphorylation buffer (final concentration: 20 mM HEPES pH 7.5, 5 mM NaOAc, 100 mM KOAc, 2mM MgOAc₂, 1 mM EGTA, 10 mM MgCl₂, 0.5 mM DTT, 5mM creatine phosphate (Calbiochem), 57 µg/ml creatine kinase (Calbiochem), 5 mM GTP (Sigma), 0.1 mM ATP (Sigma), Pepstatin A 2 µg/mL, Aprotinin 10 µg/mL, Leupeptin 1 mM, PMSF 1 mM, NaF 1 mM Na₂VO₄ 1mM). The buffer was also added with 0.1 mM 2peATP γ S and 30 µg/ml of digitonin to permeabilize the cells. After, the plates were placed on the rocker for 40 min at room temperature. The thiophosphorylation buffer was aspirated and cells were lysed using Tris lysis buffer (50 mM Tris pH 7.5, 150 mM Nacl,

50mM EDTA, 1% NP-40, 0.1 Deoxycholic Acid, Pepstatin A 2 µg/ml, Aprotinin 10 µg/ml, Leupeptin 1 mM, PMSF 1 mM, NaF 1 mM, Na₂VO₄ 1mM). The samples were centrifugated at 15000 rpm for 30 min at 4°C. The samples were moved to a new tube and added with 2.5 mM PNBM for 2 hours for alkylation. The reaction was stopped by adding 2X Laemmli buffer, the samples were run for Immunoblotting and the membranes were hybridized with thiophosphate ester antibody, to detect thiophosphorylated proteins, and anti-HA 3F10 (Roche) antibody, to detect CK2 constructs expression.

2.2.9 Inducible stable cell lines development

The human osteosarcoma U2OS cell line containing a gene integration site regulated by a Tetracycline responsive element (FT-U2OS) were provided by Dr. Karmella Haynes at Arizona State University (Haynes and Silver, 2011). Stable cell lines expressing wild type CK2α and α' and analogue sensitive CK2α with c-term HA affinity tag (wt-CK2α, as-CK2α and as-CK2α') were generated according to the manual of Flp-In T-REx, ThermoFisher Scientific (www.thermofisher.com). wt-CK2α' stable cell lines were already established in Litchfield lab. FT-U2OS cells were cultured in DMEM (Wisent) supplemented with 10% FBS (Thermo Fisher Scientific) and antibiotics (0.1 mg/ml streptomycin and 100 units/ml penicillin, Gibco). The cell lines were transfected with of plasmid DNA of wt-CK2α-HA, as-CK2α-HA or as-CK2α'-HA insert cloned into pcDNATM5/FRT/TO vector and pOG44 (expression of recombinase) in the ratio 1:9 (both from ThermoFisher Scientific). After the transfection, cells were grown in selective media containing 150 µg/ml hygromycin B (Wisent) and 15 µg/ml blasticidin (Wisent). The surviving cells were grown until confluence in the selectable media. The cells were induced by different concentrations of tetracycline (from 0.2 to 1 µg/ml) or at the highest concentration (1 µg/ml) at different time points (from 15 min to 24 hours). The cells were harvested using TLB lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% deoxycholic acid) with additional protease and phosphatase inhibitors (1 mM PMSF, 7 µg/ml PepstatinA, 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 centrifuged at

2000 x g at 4°C for 15 minutes and were immunoblotted with the HA-3F10 antibody to check the expression of the different exogenous proteins.

2.1.10 Immunoprecipitation

Stable U2OS Flp-in wt-CK2 α , as-CK2 α and as-CK2 α' cell lines were grown in DMEM media with 150 μ g/ml hygromycin B and 15 μ g/ml blasticidin, as described before. After reaching confluence, the cells were induced with 1 μ g/ml of tetracycline for 24 hours. The cells were lysed in TLB buffer (with protease and phosphatase inhibitors) and protein concentration was determined by BCA assay (Thermo Fisher Scientific). Total of 300 μ g of lysate was used for immunoprecipitation. The lysates were precleared by adding 30 μ l of PGS bead slurry. The slurry was tumbled on a rotator for 30 min at 4 °C. The lysate and beads were centrifuged at 13000 rpm for 2 min and the supernatant was transferred to a new tube. The lysates were added each with 0.5 μ l of HA12CA5 (Roche) antibody and tumbled on rotator for 1 hour at 4 °C. After one hour, lysates were added with 30 μ l of PGS bead slurry and additionally tumbled for another hour. The suspensions were then centrifuged at 13000 rpm for 5 min, the supernatants were aspirated and beads were washed 3 times with TLB buffer. After washing, Laemmli buffer was added to the beads of each sample and the suspensions were heated at 95 °C for 3 min. The beads were centrifuged and the supernatants were analyzed on the blot.

2.1.11 Immunofluorescence

Stable U2OS Flp-in wt-CK2 α , as-CK2 α and as-CK2 α' cell lines were grown on coverslips in 6 cm plates with DMEM media in the presence of selective markers, as previously described. After reaching confluence, cells were washed 3 times with cold PBS. The cells were fixed on the cover slips using 10% formalin for 15 min. After fixing, the cells were washed 3 times with PBS. The cells were then permeabilized using 0.1% Triton-X 100. Permeabilization was followed by 3 washes of PBS and blocking with 3% BSA in PBS for 1 hour. The cells were incubated with 50 μ l per well of HA-Alexa Fluor® 488 conjugated antibody (1:400 in 3% BSA PBST, Invitrogen) for 1 hour at room temperature for hybridization. After, the cells were washed with PBS and added with 50 μ l of 1 μ g/ml

of Hoescht 33258 nuclei stain (Thermo Fisher Scientific). Glass cover slips were washed 3 times with cold PBS and mounted on glass slide using mounting media. Next day the images were acquired on Nikon Eclipse microscope at 20X magnification using FITC filter for HA-conjugated antibody and DAPI for Hoescht staining of nuclei.

2.1.12 SILAC (Stable Isotopic Labeling of Amino Acids in Cell Culture) labeling and incorporation

The stable cell lines were grown in the growth medium (DMEM, Wisent) supplemented with stable isotope labels. The media were supplemented with unlabeled L-lysine and L-arginine (Silantes GmbH) at concentrations of 83.1 mg/l and 60 mg/l for "Light media" (Lys0; Arg0), isotopically labeled L-lysine-²HCl 4,4,5,5-D4 and L-arginine-HCl¹³C6 at concentrations of 86.2 mg/l and 61.14 mg/l for "Medium" media (Lys4; Arg6), and isotopically labeled L-lysine-²HCl¹³C6¹⁵N2 and L-arginine-HCl¹³C6¹⁵N4 at concentrations of 52.4 mg/L and 87.7 mg/L for "Heavy" media (Lys8; Arg10). Unlabeled L-proline (Cambridge Isotope Laboratories Inc) was supplemented at 400 mg/l in all the SILAC label media. The SILAC were also supplemented with 10% dialyzed FBS (Corning) and antibiotics (0.1 mg/ml streptomycin and 100 units/ml penicillin, 150 µg/ml hygromycin B and 15 µg/ml blasticidin).

FT-U2OS stable cells were adapted in their respective media as-CK2α and α' (heavy media) (Lys8; Arg10), wt-CK2α and α' (medium media) (Lys4; Arg6) and U2OS parental cell lines in light media (Lys0; Arg0) over a period of 7 passages. The cells were then lysed, as mentioned before, and precipitated using methanol/chloroform method. The proteins were digested using Trypsin (Pierce) (1:100) (refer to section 2.1.14 for precipitation and digestion method). The peptides were run on Orbitrap Elite mass spectrometer. The raw data obtained from the run was analyzed by using MaxQuant v1.5.3.8 (Cox and Mann, 2008) and searched against Swissprot-Uniprot database (*homo sapiens* taxon, accessed on December 26, 2015). The search on MaxQuant was set to multiplicity of 2, with heavy labels selected as Arg10 and Lys8. Mass tolerances were set to 4.5 ppm for the parent ion mass (MS) and 0.5 Da for the fragment ion mass (MS/MS). FDR was set to 1% and trypsin was selected with maximum of 3 missed cleavages. The variable modifications selected were, methionine oxidation (+15.9949), protein N-

terminus acetylation (+42.0105), asparagine and glutamine deamidation (+0.9840Da). Cysteine carbamidomethylation (+ 57.0215) was set as a fixed modification and Re-Quantify option was turned on. The data acquired from MaxQuant was then loaded into Perseus version 1.5.2.6 (Tyanova et al, 2016). The “evidence.txt” file was used to extract information about peptides that were quantified in MS1 and identified in MS2. The heavy/light (H/L) ratios were calculated using heavy and light intensity values from MS1 quantitation. The calculation used to calculate incorporation was: H/L ratios larger than 1 were calculated with $(100 - (1/\text{ratio}) \times 100)$, and ratios less than 1 were calculated with $(\text{ratio} \times 100)$. The median of all incorporation values for each individual SILAC pair was calculated and represents the incorporation efficiency for the labeled amino acids.

2.1.13 Thiophosphorylation reaction

FT-U2OS stable cell lines expressing as-CK2 α and α' were grown in heavy media (Lys8; Arg10), wt-CK2 α and α' in medium media (Lys4; Arg6), and U2OS parental cell lines in light media (Lys0; Arg0) SILAC media. These stable cell lines were grown in labelling media for seven cell divisions. The cells were tested for label incorporation of >95 percent. After checking the incorporation, stable cell lines were grown in 15 cm dishes to reach 80% confluence in their respective labelled media and were induced for 24 hours with 1 $\mu\text{g}/\text{ml}$ tetracycline. After 24 hours, cells were harvested in NP-40 Lysis Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 $\mu\text{g}/\text{ml}$ Pepstatin A, 2 $\mu\text{g}/\text{ml}$ Aprotinin, 10 $\mu\text{g}/\text{ml}$ Leupeptin, 1 mM PMSF, 1 mM NaF, 1 mM Na₂VO₄), and lysed by sonicating for 3 x 15 sec on ice. Kinase assays were performed by supplementing 1 mg of lysate with CK2 kinase assay buffer (final concentration: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂ with 0.3 mM GTP, 100 μM ATP and 100 μM 2peATP γ S) and incubating for 30 min at 30 °C with gentle shaking. The reaction was stopped with addition of 50mM EDTA (final concentration). 10 μg of lysate from each reaction was taken and alkylated in solution by the addition of PNBM (2.5 mM) for 2 hours, then they were analyzed by western blot analysis using thioester antibody. The thiophosphorylation reaction for each label was done in nine biological replicates.

2.1.14 Thiophospho peptide capture for mass spectrometric analysis

After the kinase reaction, the lysates from all 3 labels were mixed at 1:1:1 ratio and the proteins were precipitated using a methanol/chloroform method (Rappsilber et al, 2007). Briefly, the lysate volume was brought up to 200 µl with mass spec grade water (Pierce). Each sample was also added with 800 µl of methanol and 200 µl of chloroform and it was vortexed briefly. Then, it was added with 600 µl of water and vortexed again. Each sample was centrifuged for 10 min at 130000 rpm. The supernatant was aspirated and additional wash was done using 800 µl of methanol. After washing, it was centrifuged for 10 min at 130000 rpm and the supernatant was aspirated. The pellet obtained was re-suspended in 50mM Tris pH 8 and 10 mM TCEP. Proteins were digested with the addition of Trypsin/LysC (Promega) at 1:100 (w/w) ratio for 4 hours at 37 °C. Additional Trypsin (Pierce) (1:100) was added and the sample was incubated overnight at 37 °C.

Ultralink™ Iodoacetyl beads were washed with 50mM Tris pH 8 for 3 times. 200 µl of beads were added to each mg of protein used. The tubes with peptides and beads were rotated in dark overnight. Next day, the beads were washed with 1 ml of, in the following sequence: H₂O, 5M NaCl, 50% ACN, and 5% formic acid. Following the washes, beads were treated with 500µl of Oxone solution (1mg/ml) for 30 min to elute the thiophosphorylated peptides from the beads. The eluted peptides were concentrated to ~200µl using a SpeedVac Concentrator and desalting using C18 Stage Tips (Empore) according to a protocol of Rappsilber et al, 2007. The concentrated peptides were quantified using NanoDrop™ (Thermo Fisher Scientific). 0.8 µg of peptides were loaded on the mass spectrometer.

2.1.15 Mass Spectrometry

Protein digests were analyzed using an Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer coupled with an M-class nanoAquity UHPLC system (Waters) via a *NanoFlex* (*Thermo Electron Corp., Waltham, MA*) *nanospray ionization*. The Buffers used were Buffer A (Water/0.1% formic acid (FA)) and Buffer B (Acetonitrile (ACN)/0.1% FA). Peptides, re-suspended in 0.1% FA, were trapped for 4 min at a flow

rate of 10 μ l/min at 99% of A/1% B using an ACQUITY UPLC M-Class Symmetry C18 Trap Column (5 μ m, 180 μ m x 20 mm). After trapping, peptides were separated on an ACQUITY UPLC M-Class Peptide BEH C18 Column, 130 \AA , 1.7 μ m, 75 μ m x 250 mm operating at a flow rate of 300 nl/min at 35°C using a 130 min non-linear gradient: 5% B over 1 min, 7.5% B over 59 min, 25% B over 14 min, 32.5% B over 6 min, 60% B over 5 min, 98% B over 10 min, 2% B over 10 min, 98% B over 10 min, and 5% B over 15 min.

The mass spectrometer was controlled by Xcalibur software (Thermo, v. 2.7.0) and operated in the data-dependent mode using an FT/IT/CID Top 20 scheme. The MS scan recorded the mass-to-charge ratios (m/z) of ions over the range of 400–1450 in FT (resolution of 120000 at m/z 400), positive ion, profile, full MS mode using a lock mass (445.120025 m/z). The 20 most abundant multiply charged ions were automatically selected for subsequent collisional induced dissociation in ion trap mode (IT/CID) with an isolation width of 2.00 Da, rapid scan rate, centroid mode, with charge state filtering allowing only ions of +2, +3, and +4 charged states. Normalized Collision energy was 35, and precursor ions were then excluded from further CID for 30 s.

2.1.16 Peaks analysis

The raw data were analyzed using PEAKS Studio 8.0 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Precursor mass error tolerance was set to 4.5 ppm and fragment ion mass error tolerance was set to 0.5 Da. Trypsin was selected as an enzyme and up to three missed cleavages and one non-specific cleavage were allowed. Variable modifications were selected as follows: phosphorylation of Serine and Threonine, oxidation of Methionine, deamidation of Asparagine and Glutamine, acetylation of protein N terminus, (4,4,5,5)-deuterated Lysine and $^{13}\text{C}(6)$ Arginine (Medium SILAC label), and $^{13}\text{C}(6)^{15}\text{N}(2)$ Lysine and $^{13}\text{C}(6)^{15}\text{N}(4)$ Arginine (Heavy SILAC label). The maximum number of variable modifications was set to 4. The false discovery rate was set to 1%. Following de novo sequencing, data were searched against the UniProt sequence database (homo sapiens taxonomy, accessed on February 19, 2017). The unique phosphopeptides found only in a Heavy label based on the identification were considered as CK2 substrates. The unique phosphopeptides were identified and filtered using a

peptide confidence level of $\geq 99\%$ ($-10\log P$ value of ≥ 20) and a phosphorylation site localization confidence of $\geq 99\%$ (AScore value of ≥ 20) as per PEAKS scoring. The list of phosphopeptide/proteins were analyzed through KinomeXplorer (Horn et al, 2014) and Phosphositeplus (Hornbeck et al, 2012) for finding putative substrates of CK2.

2.1.17 Gel electrophoresis and immunoblotting

Protein concentration was determined by BCA using BSA standards and absorbance measured on a Victor3 V 1420 multilabel counter (Perkin Elmer). Proteins were separated by 12% SDS-PAGE, and transferred to PVDF membrane (Millipore). The electrophoresis was done in running buffer (Tris 25mM PH 8.3, Glycine 200 mM and SDS 0.1%) at 120 V initially to allow the proteins to pass through stalking gel, then the voltage was increased to 160 V when protein reached the running gel. The transfer was done on ice in transfer buffer (Tris 25mM PH 8.3, Glycine 200 mM, methanol 10 %) at 400 mA for 1 hour. Membranes were blocked in 5% BSA/TBST for 1 hour at room temperature with rocking. Thiophosphorylation was analyzed by immunoblotting with anti-thiophosphate ester antibody (1:2,000 in 5% milk/TBST, Epitomics) and phosphorylation was analyzed by anti-phospho-S162 EEF1D antibody (1:20,000 in 3% BSA/TBST). Expression of transiently transfected HA-tagged proteins was observed with anti-HA-3F10 antibody (1:500 in 3% BSA/PBST, Roche). Membranes were incubated with primary antibodies at 4°C overnight with rocking. Membranes were incubated with secondary antibody (1:10,000 GAR800 or 1:10,000 GAM680 in 5% BSA/TBST, Mandel) for 45 min at room temperature with rocking. The immunoblots were scanned and quantified using the LiCor Odyssey System (LI-COR Biosciences).

2.2 Results and Discussion

2.2.1 Identification of the gatekeeper residue in CK2

The chemical genetic approach pioneered by Shokat and colleagues exploits the highly conserved structure of the nucleotide-binding pocket among protein kinases. Within this site, there is a strictly conserved bulky residue, called the “gatekeeper” residue. The mutation of this residue in a number of protein kinases generates analog-sensitive kinases (as-kinases) with altered nucleotide requirements as the gatekeeper residue acts as a natural steric barrier to bulky ATP analogs.

The mammalian CK2 family is composed of two catalytic subunits, CK2 α and CK2 α' , and two regulatory subunits, CK2 β . To adapt the gatekeeper strategy to CK2 and engineer an as-CK2 kinase, the protein sequences of both CK2 α and CK2 α' were aligned with closely related cyclin-dependent kinases (Cdks) Cdk1, Cdk4, Cdk6, and Cdk7 as well as c-Src (Figure 1a). The sequence alignment identified a phenylalanine, F113 and F114 in CK2 α and CK2 α' , respectively, that aligns with conserved bulky threonine and phenylalanine residues in c-Src and Cdks. To further confirm this phenylalanine as the gatekeeper residue in CK2, the available structures of c-Src, Cdk6, and Cdk7 were aligned with those of CK2 α (Figure 1b). The gatekeeper residues are highlighted and clearly demonstrate that these residues are superimposed on one another. In CK2 α , F113 is located in the back of the nucleotide-binding pocket (Figure 1c). The mutation of this residue to a glycine enlarged the pocket as illustrated by an *in silico* substitution (Figure 1d).

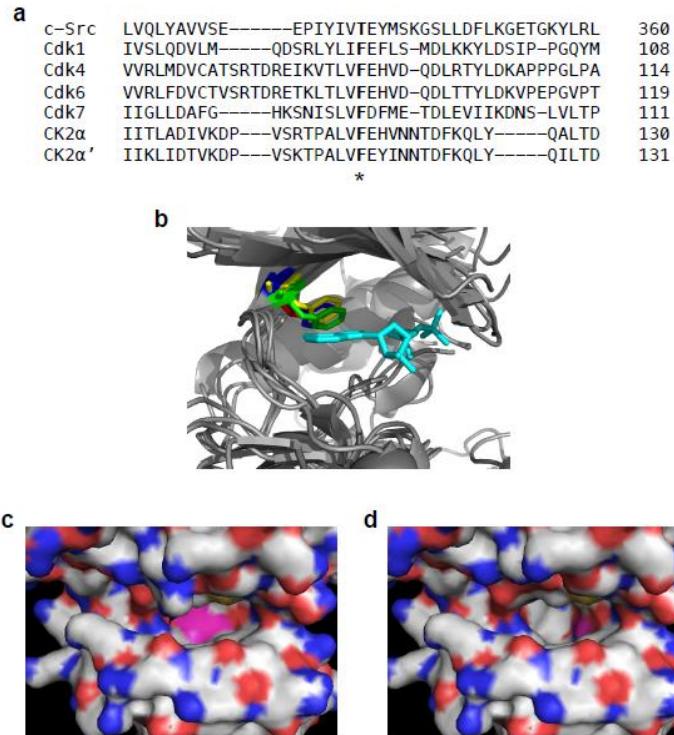
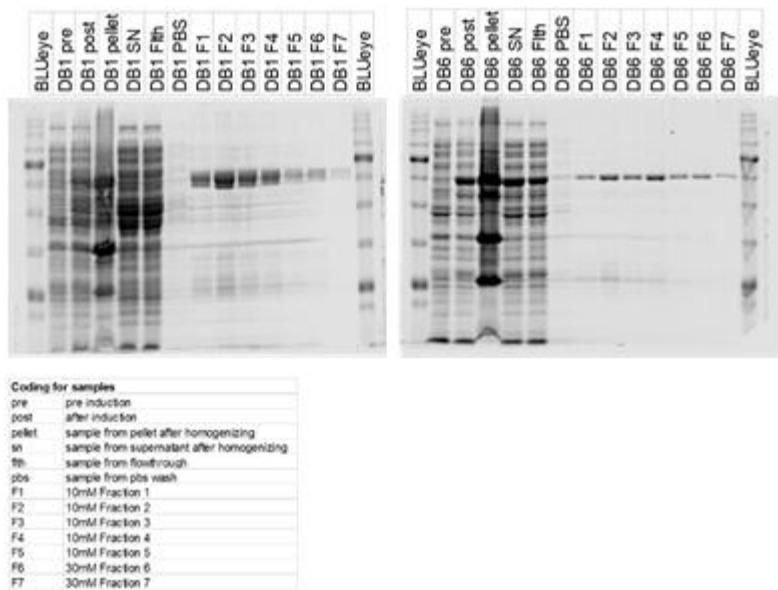
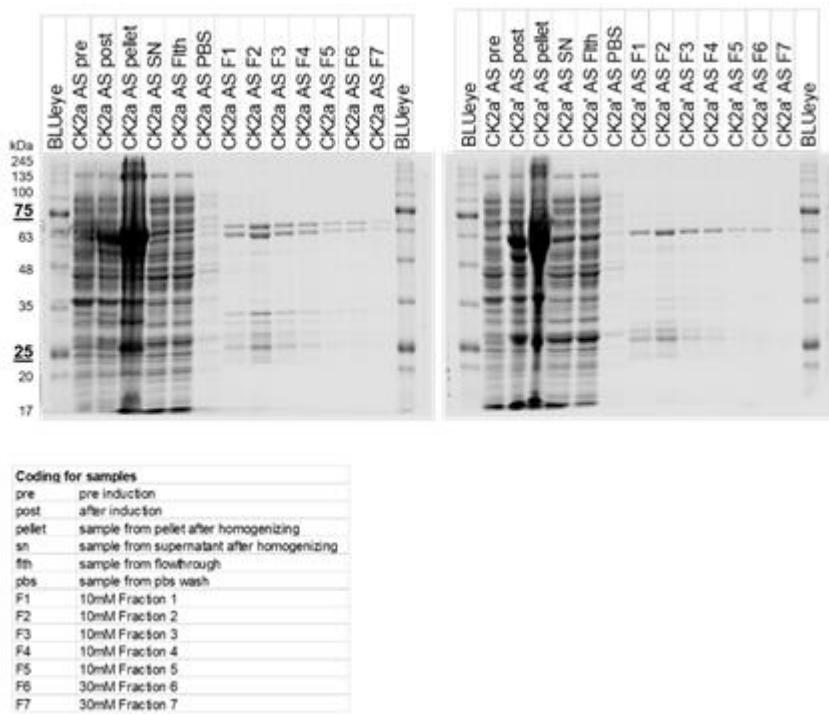


Figure 1. Identification of the gatekeeper residue in CK2. (a) A sequence alignment of the catalytic subunits CK2 α and CK2 α' with c-Src, Cdk1, Cdk2, Cdk4, Cdk6, and Cdk7 identifies a conserved phenylalanine, F113 and F114 in CK2 α and CK2 α' , respectively (the gatekeeper residue is bolded and denoted by *). (b) A structural alignment highlighting the gatekeeper residues in CK2 α (yellow) with c-Src (red), Cdk6 (blue), and Cdk7 (green). ATP is coloured in cyan. An *in silico* substitution of the gatekeeper residue in CK2 α (c) with a glycine in as-CK2 α (d), illustrates the enlargement of the nucleotide-binding pocket. The gatekeeper residue is coloured in magenta. The substitution was introduced using Swiss PDB Viewer and images were created in PyMol. PDB: CK2 α , 1JWH; c-Src, 3EL7; Cdk6, 4EZ5; Cdk7, IUA2.

2.2.2 Purification of GST tagged proteins

pGEX-wt-CK2 α or pGEX-as-CK2 α and pGEX-wt-CK2 α' or pGEX-as-CK2 α' , which were GST tagged proteins, were purified using Glutathione-agarose beads. The elution of the proteins was done in 4 fractions of 1 ml each with 10mM Glutathione and 2 fractions of 1 ml each with 30 mM Glutathione. All the fractions were run on SDS-PAGE gel (Figure 2A and 2B). The fractions 3 to 6 showed less contaminants, so these fractions were pooled together. After pooling, the fractions were dialyzed and concentrated. The concentration was determined by using Bradford assay. The concentrated samples were loaded on SDS-PAGE, to see if some contaminants are present or not (Figure 2C), together with the known concentrations of BSA protein for comparison. The as-CK2 α and α' show little more contaminants when compared with the wild type ones. This could be due to the partial degradation of the as-kinases because the mutation is reported to cause kinase instability and degradation.

Moreover, single bands were observed on SDS-PAGE profile of as-CK2 α' as well as wt-CK2 α' , while, two bands are observed on SDS-PAGE profile of both as-CK2 α and wt-CK2 α . These double bands might be due to the proteolytic cleavage on the C-terminal of the α subunit of the kinase which has been reported by Grasselli et al., 2003. It is worth mentioning that previous data in the literature have demonstrated that this cleavage does not alter the activity of the kinase since a robust activity has been documented even if the part of C-terminal domain is cleavaged (Turowec et al, 2010).

A**B**

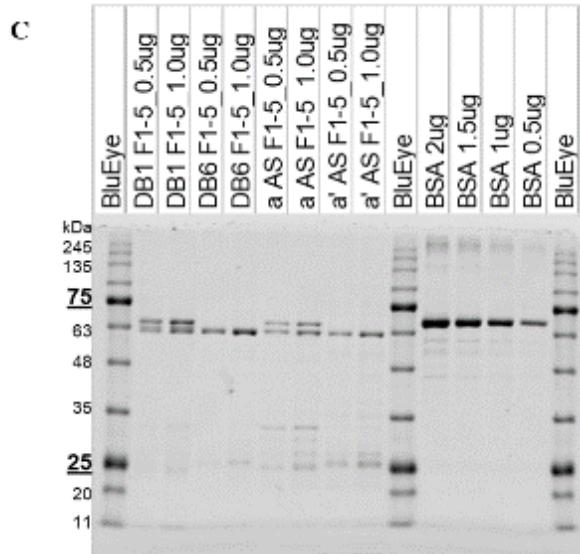


Figure 2: Purification of recombinant wt and as-CK2 α and α' . (A) SDS gel profile of DB1 (wt-CK2 α) and DB6 (wt-CK2 α'). The fractions of un-purified and purified GST tag proteins were loaded on the gel to visualize their degree of purity. The treatment with different concentrations of glutathione shows the elution of specific proteins. The eluted samples were compared to these washed just with PBS. The detail of coding for sample is presented in the figure. (B) SDS gel profile of as-CK2 α and as-CK2 α' . The fractions of un-purified and purified GST tag proteins were loaded on the gel to visualize their degree of purity. The treatment with different concentrations of glutathione shows the elution of specific proteins from the beads. The eluted samples were compared to these washed just with PBS. The detail of coding for sample is presented in the figure. (C) After dialyzing and concentration, proteins were quantified using BCA assay and loaded onto the gel together with well-known concentration of BSA for comparison.

2.2.3 In vitro kinase assay using different ATP analogues

Previous work of Grankowski et al. reported that the catalytic subunits of CK2 kinase tend to have robust activities without the presence of their regulatory subunits when they are expressed in bacterial system. This indicates that CK2 α and α' , in this system, are constitutively actives (Grankowski et al, 1991) and do not require any external stimuli for activation. This makes it possible to just use the catalytic subunits for the *in vitro* assays also because the GST tag on the N-terminal of the proteins does not alter the activity or specificity of each catalytic subunits (Turowec et al, 2010).

Kinase assays with recombinant wt-CK2 α , as-CK2 α and α' kinases were performed using EEF1D as a substrate to screen three commercially available N⁶-modified ATP analogs: 2peATP- γ -S, Fu-ATP- γ -S and Bn-ATP- γ -S (Figure 3A and 3B). EEF1D was used as it is a well-known and documented substrate of CK2 kinase which is readily phosphorylated at residue S162 (Gyenis et al, 2011). The kinase reactions were started with the addition of ATP-analogues. After the reaction, the samples were treated with PNBM, an alkylating reagent, which adds the alkyl group to form thioester bonds, that could be detected by anti-thioester antibody (Allen et al, 2007). In the presence of wt-CK2 α and α' , robust phosphorylation of the EEF1D was observed with ATP γ S while with the ATP analogs, wt-CK2 α and α' also had weak activity with Fu-ATP- γ -S and Bn-ATP- γ -S and with 2peATP- γ -S analog (Figure 3b). In contrast, as-CK2 α and α' , showed marginal activity with ATP γ S but increased thiophosphorylation efficiency of EEF1D with 2peATP- γ -S. Minimal phosphorylation was detected using the other N⁶-modified ATP analogs. This screen identified efficient utilization of 2peATP- γ -S by as-CK2 in contrast to the low activity level observed for wt-CK2.

In principle, the gatekeeper residue in the ATP binding pocket is hydrophobic and is known to act as hydrophobic spine which promotes enzymatic activity, indeed the mutation of this residue causes opening of the ATP binding pocket and may reduce activity or make the kinase completely inactive (Azam et al, 2008). In that case this problem could be overcome by introducing another mutation in β -sheet the N-terminal lobe of the kinase to branched aliphatic residues e.g. isoleucine or valine, which can

increase its activity (Bishop et al, 2000). This is not our case since the *in vitro* kinase assay suggests that the mutation did not affect the kinase activity but only changes the selectively for the co-substrate ATP as as-CK2 α and α' are able to use the ATP-analogue (2peATP- γ -S) better than ATP γ S.

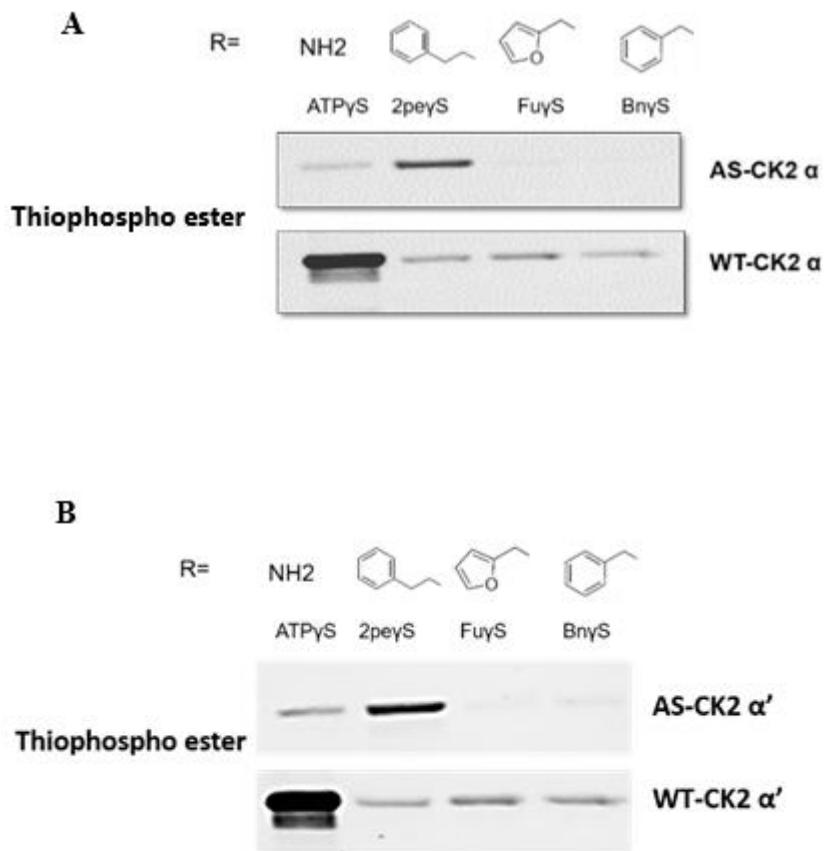


Figure 3. Screening catalytic activity of CK2 with N⁶-substituted ATP analogs reveals utilization of 2peATP by as-CK2. (A) wt and as-CK2 α (B) wt and as-CK2 α' . The chemical structure of ATP illustrating the addition of bulky chemical groups at the N⁶-position on the adenine ring as denoted by R. The catalytic activity of wt- and as-CK2 was assessed with EEF1D using N⁶-substituted ATP analogs, (2peAT γ P), (6-Fu-ATP- γ -S) (6-Bn-ATP- γ -S). Phosphorylation was detected by immunoblotting with Thiophospho ester antibody after treatment with PNBM.

2.2.4 as-CK2 utilizes 2peATP- γ -S more efficiently

In an effort to further investigate the observation that as-CK2 has a higher affinity for 2peATP γ -S, competition experiments were performed. Phosphorylation of EEF1D substrate by wt-CK2 α and α' with ATP and GTP was unaffected by the addition of same concentrations of 2peATP γ S as no change in the utilization of ATP and GTP was observed (Figure 4 A and B). However, the utilization of 2peATP γ S was minimal by wt-CK2 when it was added with ATP or GTP. These results show that ATP and GTP are preferred to be used by wt-CK2. In the similar way, for as-CK2, thiophosphorylation with 2peATP γ S was not affected by the addition of ATP or GTP. This result indicates that ATP cannot efficiently compete with 2peATP for binding to the active site of as-CK2, further reinforcing the observation that as-CK2 preferentially uses 2peATP.

The goal of the competition assays were to confirm that the utilization of the 2peATP γ S would be preferred by the analogue mutants in the cellular environment where ATP and GTP are present. These results had formed bases to make transition from *in vitro* assays to complexity of *in vivo* cell line experiments in order to investigate direct substrates of CK2 kinase.

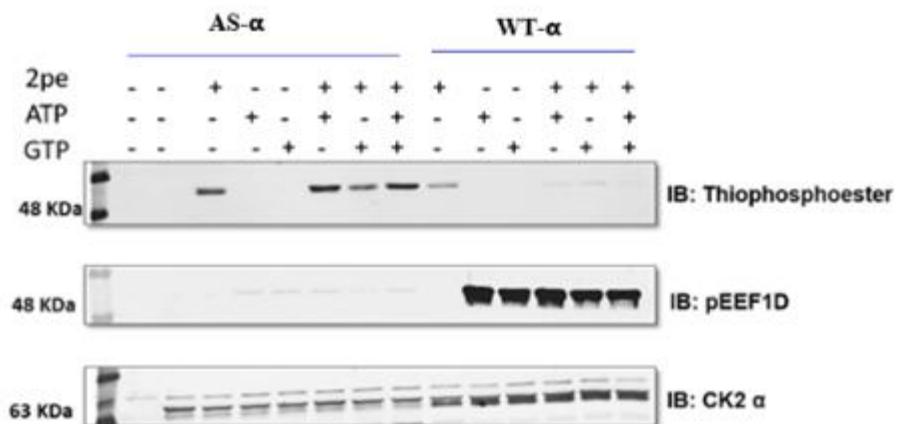
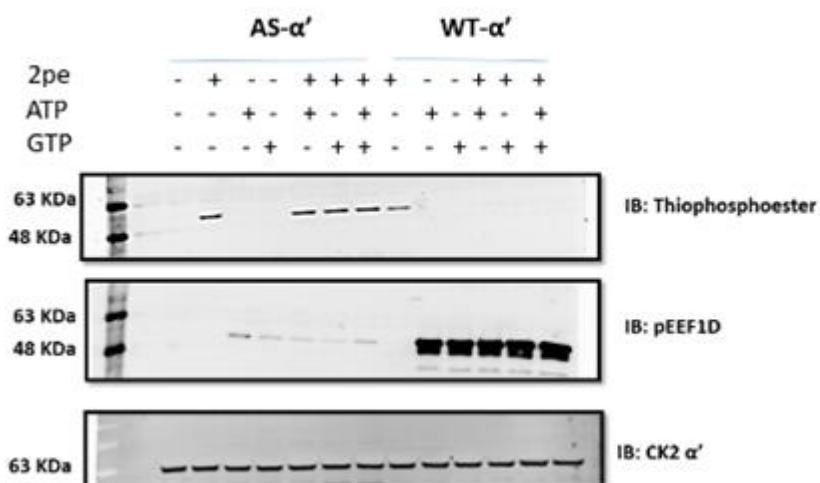
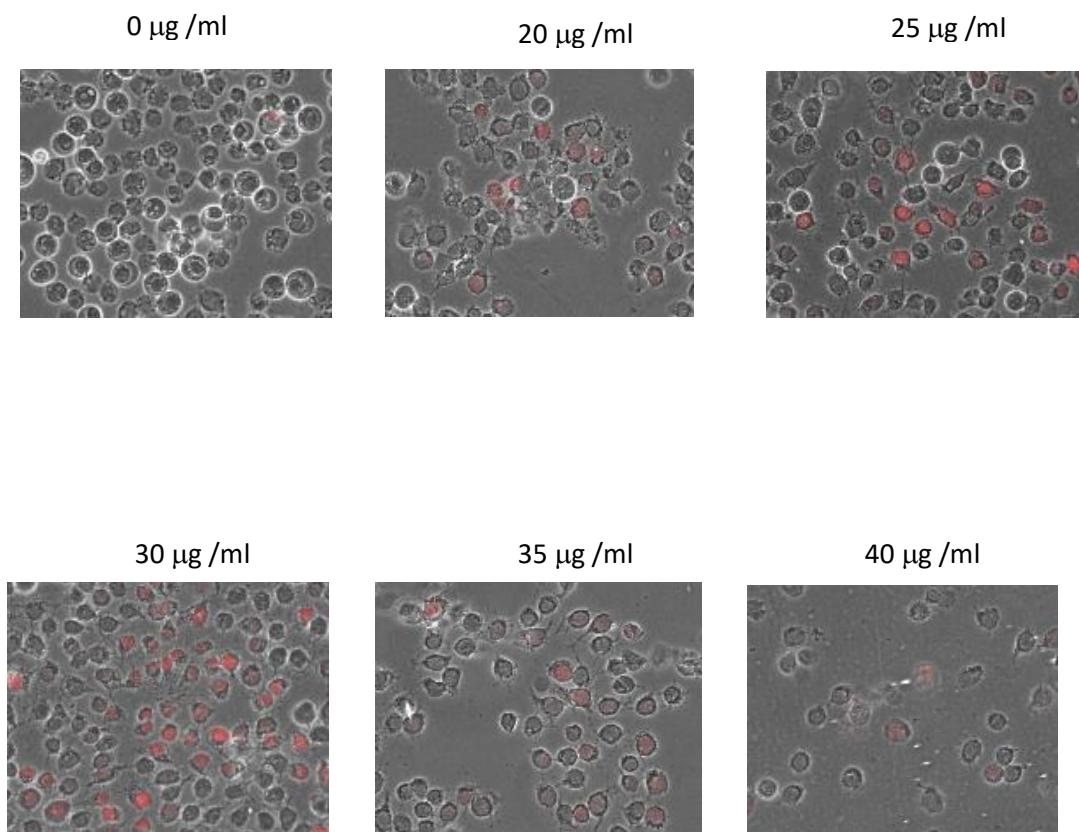
A**B**

Figure 4. As-CK2 preferentially uses 2peATP over ATP and GTP. (A) wt and as-CK2 α ; (B) wt and as-CK2 α' . Kinase assays were performed to evaluate the preferential use of 2peATP γ S, ATP and GTP. (A) Recombinant His-EEF1D was phosphorylated by wt-CK2 and as-CK2 using 2peATP γ S, ATP ad GTP in different combinations. Thiophosphorylation was detected by anti-thiophosphoester antibody and EEF1D S162 phosphorylation by anti-phospho antibody. Total CK-2 kinases were detected by anti-CK2 α or α' antibodies.

2.2.5 Optimizing Digitonin concentrations

N^6 -ATP-analogues are not permeable through the cell membrane. In order to make ATP-analogues to be available for the cell, digitonin was used, which is a mild non-ionic detergent able to permeabilizes cellular membranes. Digitonin treatment on unfixed cell has been used in the literature (Tissera et al; 2010) where it has been reported that cellular components stay intact under the permeabilization condition (Chaudhary et al; 2002). In particular, DNA functions, actin structures, PDGFR, and membrane-integrated molecules remain responsive even after permeabilization (Chaudhary et al; 2002). It is known that digitonin treated cells have been able to successfully reverse permeabilization (Miyamoto et al, 2008) suggesting that the cells were not completely damaged when treated with digitonin. This shows that permeabilization could be utilized to allow the bulky ATP-analogue to enter into the cells without affecting its cellular machinery and kinase activity. Thus, the permeabilizing property of digitonin was utilized to make 2peATP γ S to enter the cell and to be used by as-CK2 α and α' . Titration of digitonin was done in cells to know its optimum concentrations. PI stain, which is a red fluorescent nuclear stain, was used to detect if the cells were permeabilized. The Nikon images observed with PI stain shows that the cells were adherent to plates but were not permeabilized at low concentration of digitonin. 30 $\mu\text{g}/\text{ml}$ of digitonin seemed optimum concentration at which cells were permeable and still adherent to plate (Figure 5A). Digitonin concentration higher than 30 $\mu\text{g}/\text{ml}$ killed most of the cells, since the number of cells after treatment was very low in comparison to the number of cells initially seeded on the plates (Figure 5B). So, for further experiments, 30 $\mu\text{g}/\text{ml}$ of digitonin was used for cells permeabilization.

A



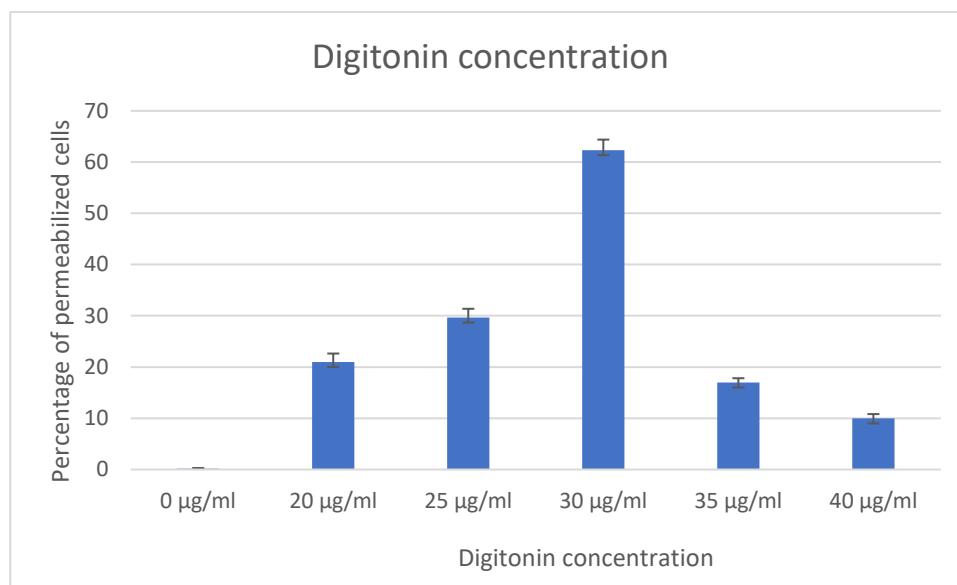
B

Figure 5: Setup of different concentrations of digitonin for cell permeabilization

U2OS cells were treated for 25 min with different concentration (from 20 to 40 µg /ml) of digitonin to permeabilize cells. The cells were observed at 20x magnification under Evos microscope using FITC filter (A). The histogram represents the percentage of permeabilized cells at different concentrations of digitonin with respect to the total number of cells initially seeded on the plates. Mean ± standard error of at least three separate experiments. (B).

2.2.6 as-CK2 expression in cells and its utilization of 2peATP γ S for thiophosphorylation

The pRC/CMV as-CK2 α -HA-C-terminal, wt-CK2 α -HA-C-terminal, as-CK2 α' -HA-C-terminal, as well as as-CK2-HA- α' N-terminal and wt-CK2-HA- α' C-terminal, were transiently transfected in U20S cells. Transient transfections of constructs in the cells were done to check both, the level of expression between the wild type and mutant subunits, and the utilization of 2peATP γ S by wt-CK2 α and the analogue mutants. The additional construct of as-CK2 HA- α' N-terminal was utilized to check whether the tag is influencing the expression of the protein or not (the expression of wtCK2 α' with HA tag on N-terminal had previously shown in Litchfield lab to be expressed less, data not shown). The transfection efficiency was checked by transfecting eGFP construct in the cells as well. After 24 hours, approximately, 50 % of the cells were expressing the construct (Figure 6) suggesting that the overall transfection efficiency was appropriate using this method. Immunoblotting with HA-3F10 antibody was done to check the as-CK2 α and α' expression. Thiophosphate-ester antibody was used to verify if the as-CK2 α and α' were also active in the cells. The blot showed that both pRC/CMV as-CK2 α -HA and pRC/CMV as-CK2 α' -HA were expressed equally when compared with the wt-CK2 α -HA and wt-CK2-HA- α' (Figure 7). The expression of as-CK2-HA- α' N-terminal tag was lower when compared to other constructs with HA tag on the C-terminal, as expected from previous experiments (data not shown).

The thiophosphorylation reaction was done on the cells transiently expressing wild type or mutant catalytic subunits. The permeabilization of the cells was done with a buffer containing digitonin and a cocktail of 2peATP γ S, ATP and GTP. After 30 min of incubation to perform the thiophosphorylation reaction, the cells were lysed and treated with PMBM. The lysates were blotted and the membrane was hybridized with anti-thiophosphate ester antibody. The recombinant competition assays, described in the previous section, indicated that as-CK2 can utilize 2peATP γ S efficiently in the presence of ATP and GTP, whereas, wt-CK2 catalytic subunit prefer ATP and GTP instead of 2peATP γ S. These results were confirmed in the *in vivo* experiments since the blots (Figure 5) show that both as-CK2 α and α' (C-terminal HA tag) show more intensity of the

bands with thiophosphate ester antibody compared to the bands of wt-CK2 α and α' . In conclusion, in the *in vivo* system, our results show that:

- a) the expression of analogues sensitive CK2 α and α' kinases are not affected by the mutation in the ATP binding pocket, which may cause degradation of the proteins; b) the expressed mutants are active in using 2peATP γ S.

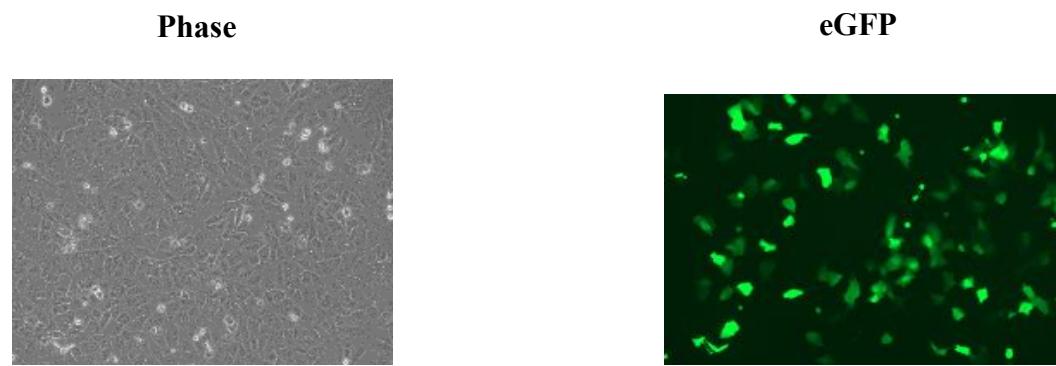


Figure 6 Transfection efficiency using CaCl_2 : The eGFP construct was transfected using CaCl_2 method, after 24 hours the GFP emission was observed at 20x magnification under Evos microscope using eGFP filter. The transfection efficiency was observed to be around 50 %.

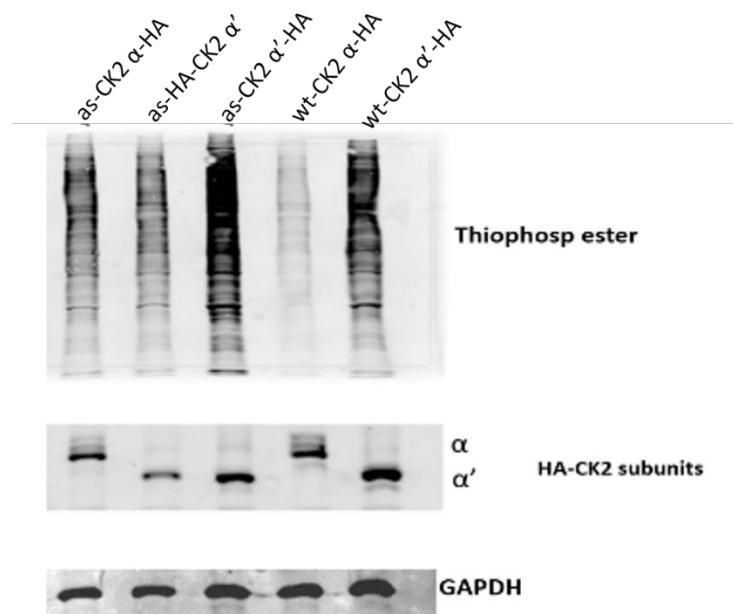


Figure 7 : Transient expression of as-CK2 α and α' and thiophosphorylation. U2OS cells were transfected with wt and as-CK2 α and α' with HA tag on C-terminal and additional as-CK2 α' with HA tag on N-terminal. The cells were treated with 30 μ g/ml digitonin, and cells were added with 2peATP γ S and alkylated. Thiophosphorylation was detected by anti-thiophosphate ester antibody and expression of all protein constructs were detected by HA-3F10 antibody in immunoblot analysis. The amount of thiophosphorylation was calculated by the densitometric analysis of the bands on the blots detected by anti-thiophosphate ester antibody in immunoblot analysis for three different experiments. The amount of thiophosphorylation, in each experiment, is normalized due to the different level of expression within all protein constructs.

2.2.7 Stable cell line characterization

Flp-in system is efficient in generating inducible cell lines and allows polyclonal selection. The U2OS Flp-in cells consists of integrated FRT (pFRT/lacZeo vector) site, where the integration of gene of interest takes place through pCDNA5 (cDNA construct) and pOG44. The pOG44 construct expresses Flp recombinase so that the integration of pCDNA5 construct could take place and produce hygromycin resistance. The stable expression is under tetracycline control, regulated by CMV promoter. The U2OS stable cell lines expressing as-CK2 α and α' and wt-CK2 α were generated using Flp-in Trex system. The stable cell line expressing wt-CK2 α' were previously established in Litchfield Lab (data not shown). To determine the optimum condition to obtain maximum expression of exogenous proteins the experiments were performed at different time points. Moreover, after 24 hours of treatment, the expression of the exogenous proteins in all three stable cell lines, after induction with concentration of tetracycline (from 0.2 to 1 μ g/ml), were measured by western blotting (HA3F10 antibody). The expression of wt-CK2 α (Figure 8A), and as-CK2 α (Figure 8B) were increased with the doses of tetracycline. In fact, both as-CK2 α and wt-CK2 α showed maximum expression at 1 μ g/ml. The as-CK2 α' (Figure 8C) showed similar expression level at all concentration although the expression at the dose of 0.2 μ g/ml seems slightly higher. It should be noted that, at all doses tested, the expression level of as-CK2 α' is lower in comparison to those of wt-CK2 α and as-CK2 α . The expression of the total Ck2 kinase was detected by CK2 α antibody. Further, time course analysis was performed to determine at which time point maximum expression could be attained. Thus, all cell lines were induced with 1 μ g/ml of tetracycline and harvested at different time points. In all cases the maximum amount of expression of exogenous proteins was observed at 24 hours after tetracycline induction. (Figure 9A and B). The results, once again, showed that the as-CK2 α' shows less expression when compared to the other two cell lines.

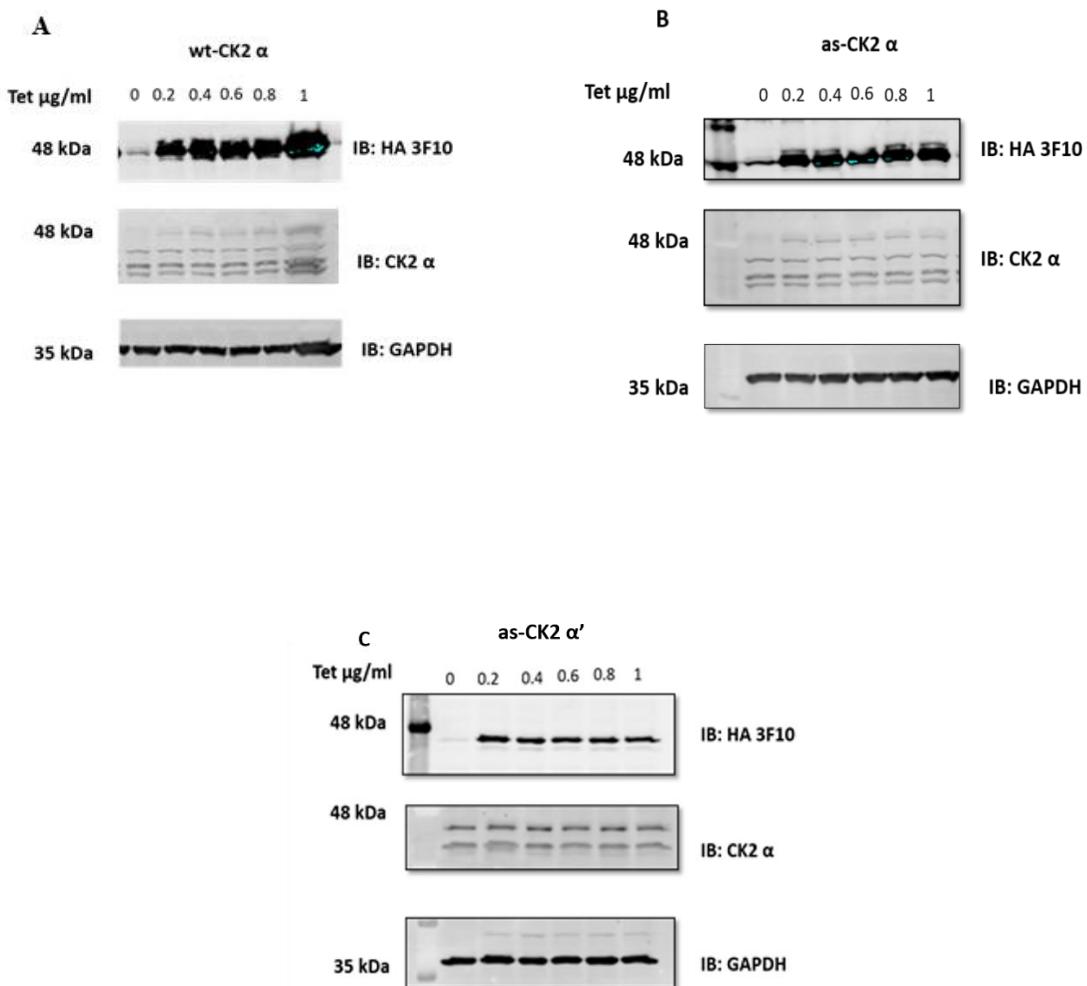


Figure 8: Flp-In U2OS stable cell lines expression using different concentrations of tetracycline: Stable Flp-In U2OS cell lines were induced to express the exogenous proteins with different concentrations of tetracycline for 24 hours. Stable cell line expressing wt-CK2 α (A), as-CK2 α (B) and as-CK2 α' (C) were shown. Expression levels of stable cell line were measured using HA3f10, CK2 α and GAPDH antibodies without tetracycline or after induction with different concentration of tetracycline for 24 hours.

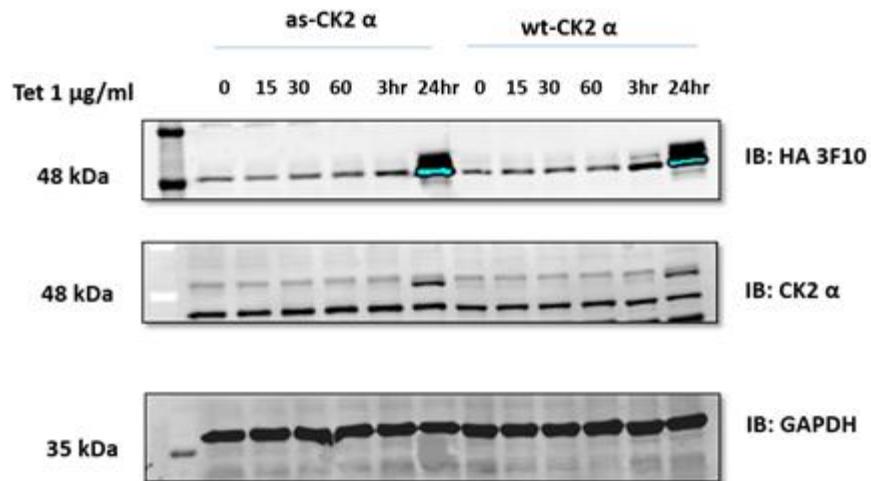
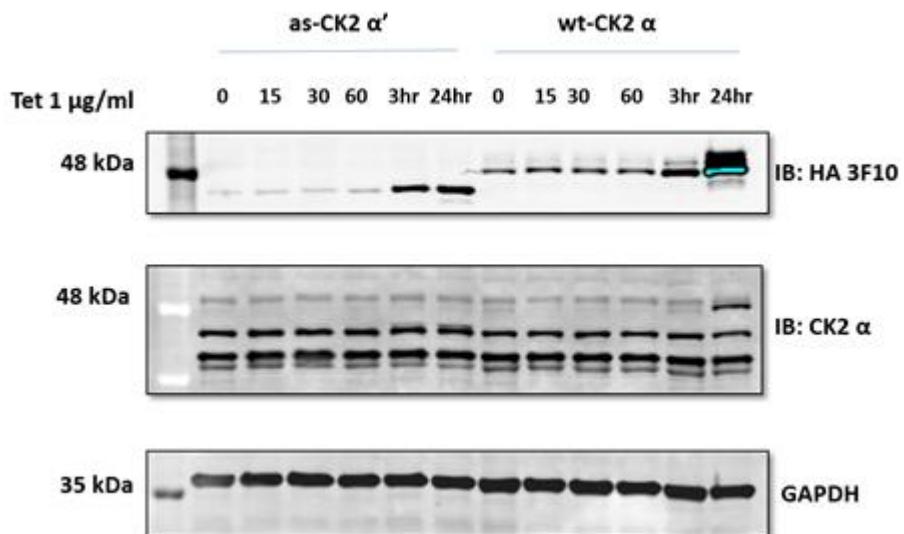
A**B**

Figure 9: Time course analysis: comparison of time course induction by 1 μ g/ml of tetracycline between wt-CK2 α and as-CK2 α (A), and between wt-CK2 α and as-CK2 α' (B). The stable Flp-In U2OS cell lines, induced to express the exogenous proteins with 1 μ g/ml tetracycline, were harvested at different time points. The blots were hybridized with HA3F10, CK2 α and GAPDH antibodies.

In order to further characterize the stable cell lines, immunoblotting and immunofluorescence were done to compare the level of expression of as-CK2 α and α' versus those of wt-CK2 α and to determine if the expression in the cells is homogeneous. The subsequent experiments were performed using 1 μ g/ml of tetracycline for 24 hours to acquire maximum expression.

The Immunoprecipitation (IP) experiments were done by using PGS beads (conjugated with HA3F10 antibody) to pull down the expressed proteins. The lysates and IP pull down fractions from induced and non-induced cells, were analyzed by western blots (Figure 10). The IP captures showed the same profile as the respective lysates suggesting that the IP's were successful and confirming that the as-CK2 α' expression is less compared to other proteins.

Immunofluorescence assays were done to demonstrated if the expression of exogenous proteins in the three cells lines were homogeneous. The expression of wt-CK2 α -HA (Figure 11A), as-CK2 α -HA (Figure 11B) and as-CK2 α' -HA (Figure 11C) is evident in the microscopic images only after the induction with tetracycline. wt-CK2 α -HA and as-CK2 α -HA show similar expression profile, as-CK2 α' -HA shows comparatively less expression. The not homogenous expression in all the cells could be due to polyclonal rather than monoclonal method adopted to grow the stable cell lines.

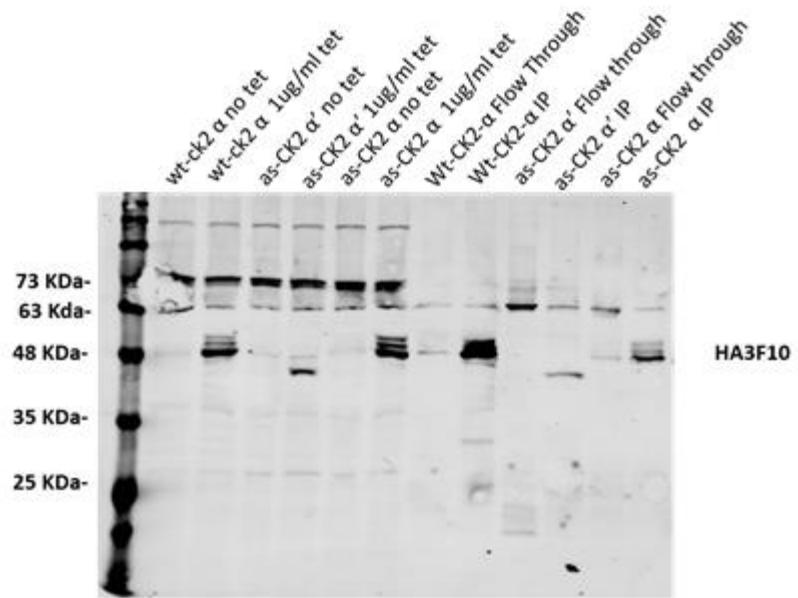
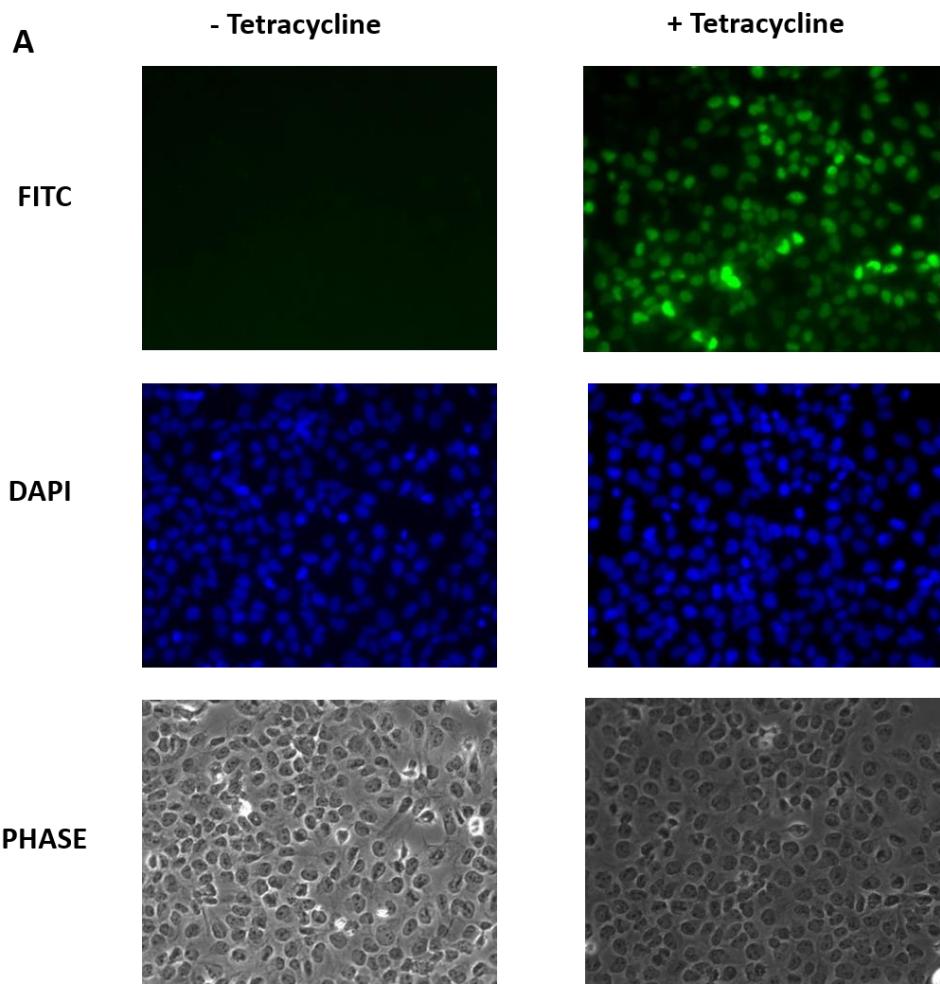
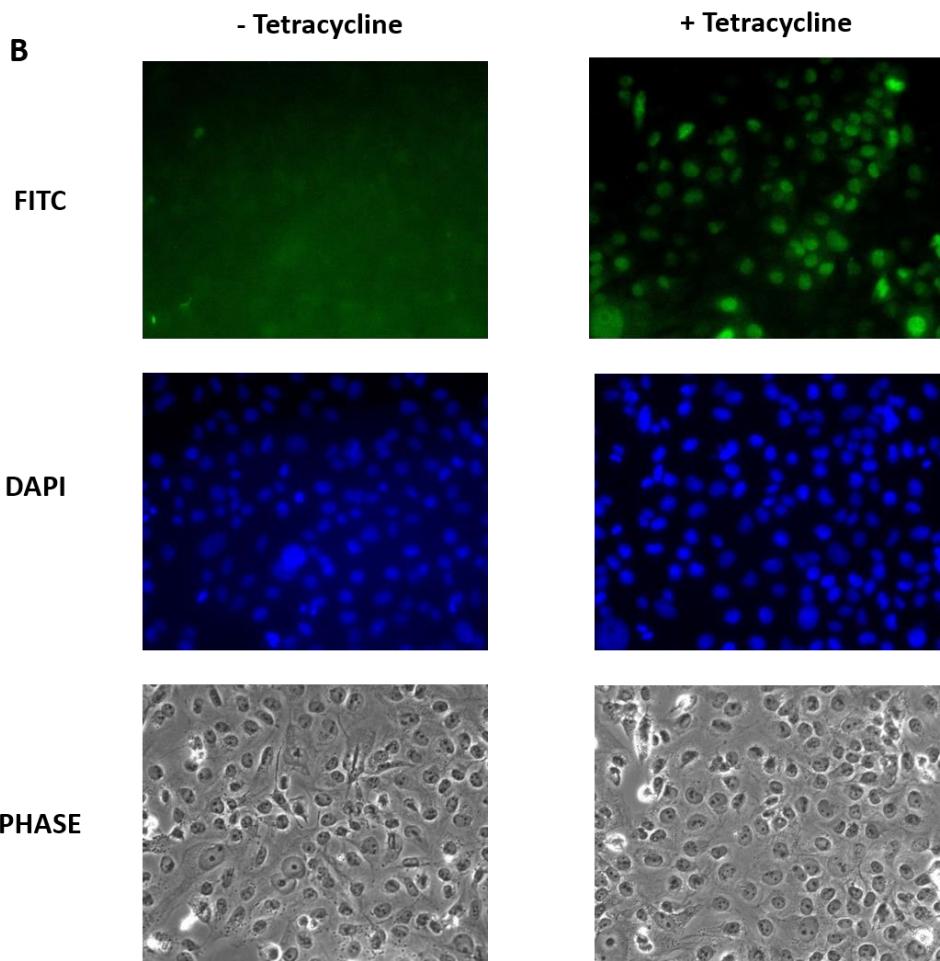


Figure 10: Immunoprecipitation of induced wt-CK2 α , as-CK2 α and as-CK2 α' stable Flp-In U2OS cell lines. All three cell lines were induced with and without 1 μ g/ml of tetracycline for 24 hours. The cells were harvested and incubated with PGC/antibody conjugate beads. The lysates, flow through and IP pull downs were analyzed with HA3F10 antibody.





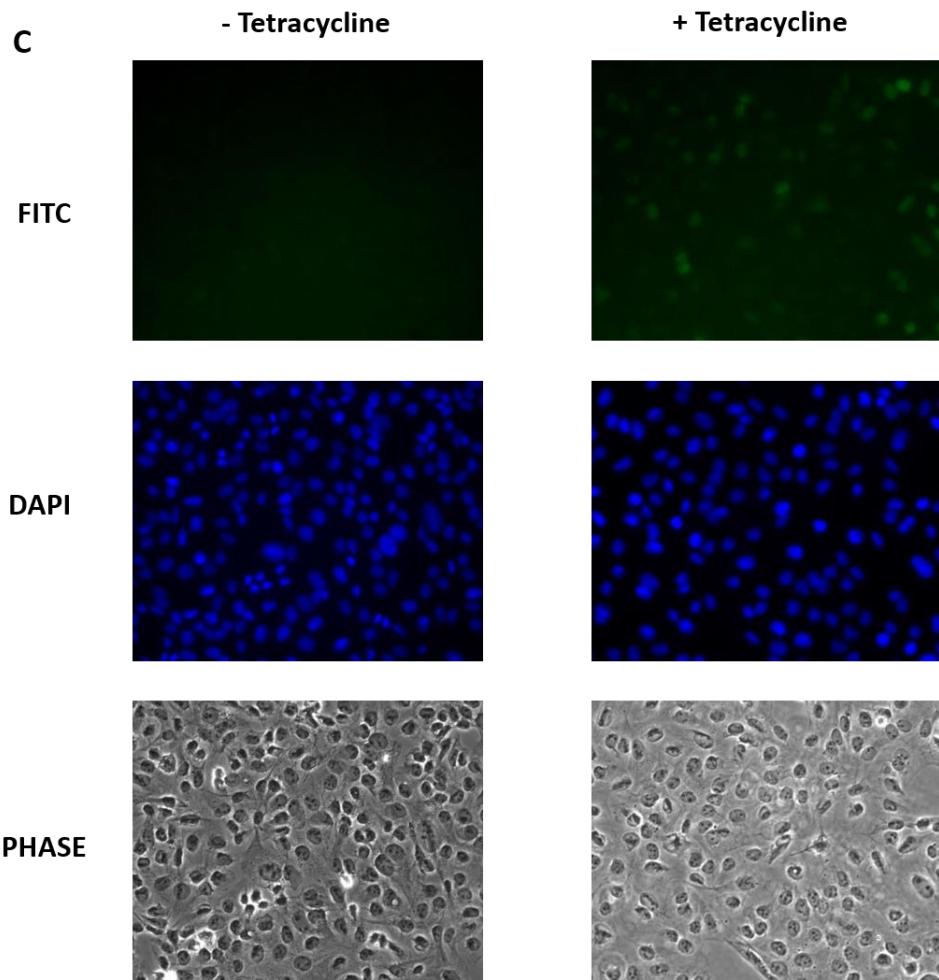


Figure 11. Immunofluorescent detection of exogenous proteins expressed in Flp-In U2OS cell lines. Flp-In U2OS cells expressing wt-CK2 α -HA (**A**), as-CK2 α -HA (**B**) and as-CK2 α -HA (**C**) were cultured in the absence (-) or presence (+) of 1 μ g/mL of tetracycline for 24 hours. The cells were then fixed and stained with a FITC-conjugated anti-HA monoclonal antibody, as previously described in the materials and methods. Cells were also stained with a Hoechst 33258 DAPI stain in order to visualize cell nuclei. A phase contrast image has also been included. The images were obtained on Nikon Eclipse microscope at 20X magnification. Identical fields were imaged in the absence (-) or presence (+) of tetracycline for each cell line.

2.2.8 Assessment of the ability of as-CK2 to phosphorylate cellular substrates in cell lysate

Mass spectrometry-based proteomics can be used to give the quantitative state of a proteome, and thereby provides details of the biochemical state of the relevant cell and tissue in differential biological conditions (Schubert et al, 2017). SILAC labelling is one the strategies used for quantitative proteomics. SILAC labelling involves the labeling of arginine and lysine amino acids with heavy ¹³C or ¹⁵N stable isotopes (Ong and Mann, 2006). SILAC labelling strategy allows differentially treated biological conditions to be mixed in 1:1 ratio and digested together, as the trypsin cleave after C-terminus of arginine and lysine. During LC-MS/MS analysis the heavy and light peptides elute together during chromatographic separation. These eluted peptides are distinguishable only due to differences in their mass because of the heavy label of arginine or lysine.

The stable cell lines were adopted in SILAC media for 7 passages, so that the proteins were isotopically labelled to greater than 95%, as it is the standard conditions required in SILAC labelling (Ong and Mann, 2006). Proline was supplemented in the media to avoid the metabolic conversation of Arginine to Proline. The incorporation efficiency calculated was 96%. To assess the utility of as-CK2 to identify direct substrates of CK2 within the complexity of the cellular environment, we used SILAC labelling approach for proteomic analysis of the substrates thio-phosphorylated by the as-CK2 α and α' . The Stable cell lines, as-CK2 α (Lys8; Arg10), wt-CK2 α (Lys4; Arg6) and U2OS-Flp-In parental cells (Lys0; Arg0) were induced with tetracycline for 24 hours in their respective SILAC media. The lysates were supplemented with ATP and 2peATP γ S in 1:1 ratio. The workflow is depicted in Figure 12. After performing the thiophosphorylation reaction small amount of sample from each reaction was alkylated by PNBM and analyzed by western blot analysis (Figure 13). An additional control kinase reaction was done in U2OS-parental cells without addition of 2peATP γ S. The thiophosphorylation observed in the sample without 2peATP γ S addition was considered as background. The U2OS-Flp-In parental cells and wt-CK2 α showed similar intensities as background showing that the 2peATP γ S utilization is minimal in these samples. In contrast, as-CK2 α thiophosphorylated more proteins and utilized 2peATP γ S more efficiently as seen on the

blots using thiophosho-ester antibody (Figure 13A, last lane). A comparison between the expression levels of as-CK2 α and wt-CK2 α was detected using HA3F10 and CK2 α antibody (Figure 13A). On the other hand, as-CK2 α' showed similar utilization of 2peATP γ S to the wt-CK2 α' and the U2OS-parental cells, as seen on the blots using thiophosho-ester antibody (Figure 13B). The expression level of both as-CK2 α' and wt-CK2 α' was really low and all the data suggests that this utilization is similar to the background.

After the kinase reaction with 2peATP γ S, all three labelled cell lines were mixed in 1:1:1 ratio, precipitated (methanol/chloroform method), digested with trypsin and the thiopeptides were captured using Iodoacetyl beads. To elute thiopeptides, beads were treated with oxone. The oxone treatment elutes these thiopeptides in the form of phosphopeptides, since the thiophosphoryl sulfur atom is replaced with oxygen after hydrolysis of sulfur-phosphorus bond (Han et al, 2016). The experiments were performed in nine biological replicates. The samples were analyzed using nano LC-MS/MS and the identification of the peptides were done using PEAKS 8.

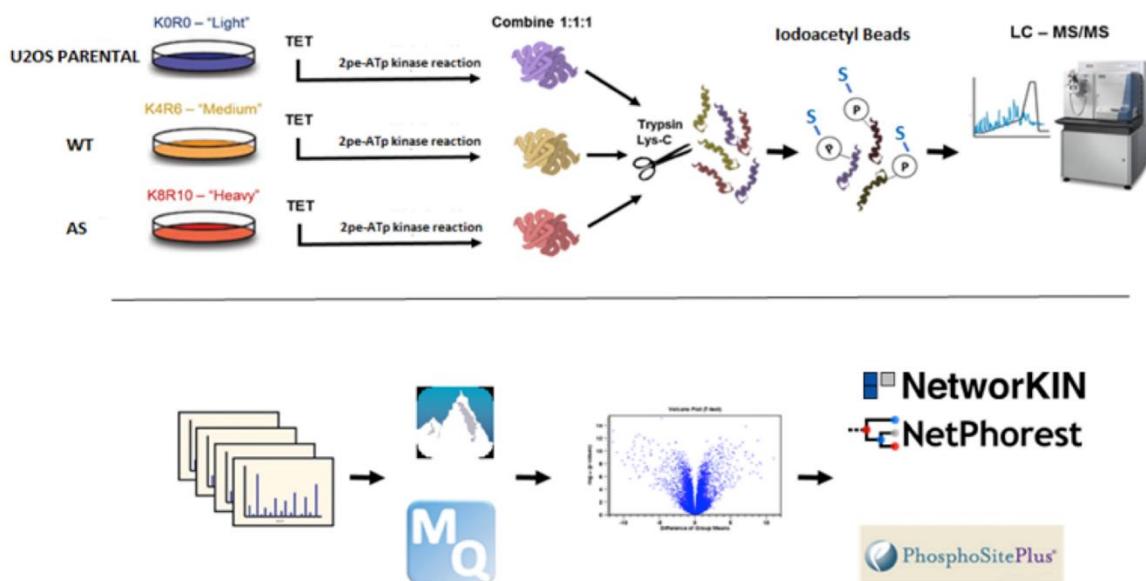
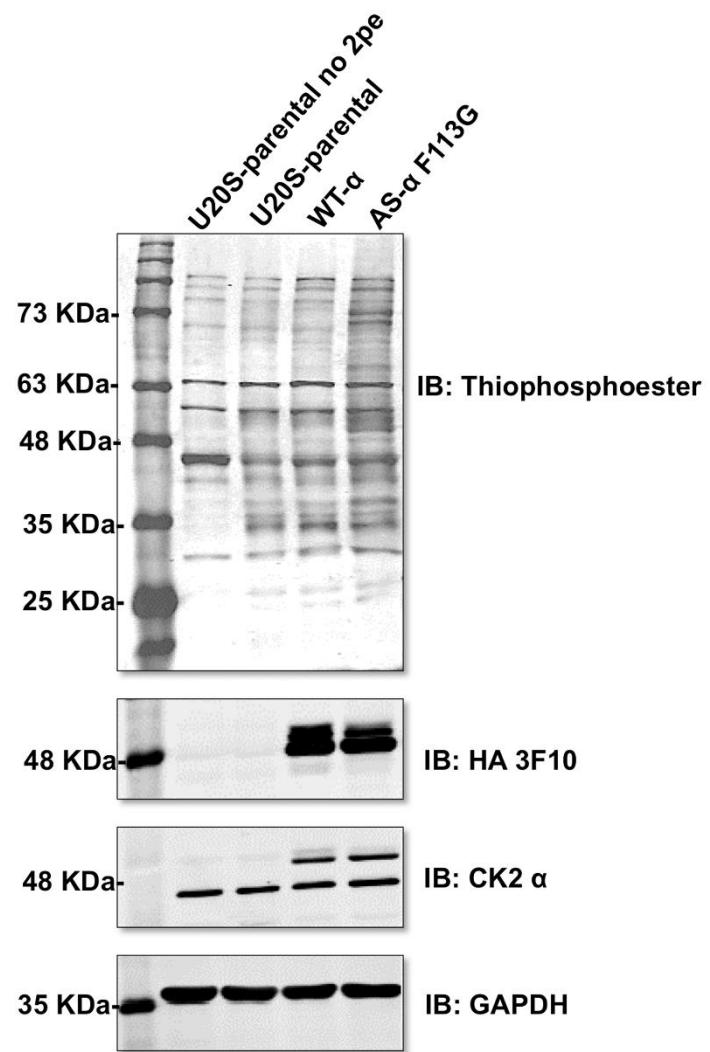


Figure 12: Proteomic workflow for the identification of CK2 substrates: The SILAC labelled cells wt-CK2 α and α' (Medium), as-CK2 α and α' (Heavy) and U2OS-parental cell line (Light) were supplemented with 2peATP γ S for thiophosphorylation reaction to take place. The lysates of all three labels were combined in 1:1:1 ratio and digested with trypsin. The peptides were added with iodoacetyl beads which capture the thiophosphorylated peptides only. The thio-peptides were run through LC-MS/MS mass spectrometer for the identification of these peptides. The raw data was analyzed through Peaks8 data analysis software. The peptides unique in as-CK2 were considered as CK2 substrates.



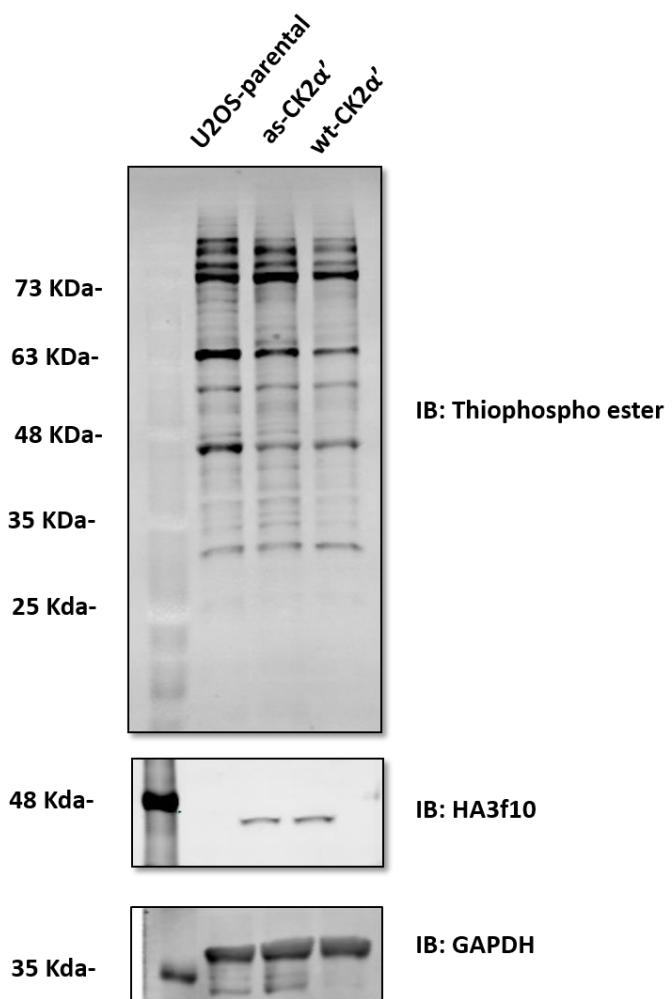


Figure 13: Thiophosphorylation reaction and utilization of 2peATP γ S by stable cell lines. Kinase assays were performed by supplementing 2peATP γ S to stable cell lines wt-CK2 α (Medium), as-CK2 α (Heavy) and U20S-parental cell (Light) (A). wt-CK2 α' (Medium), as-CK2 α' (Heavy) and U20S-parental cell (Light) (B). After the kinase reaction, samples were alkylated by treatment with PNBM, and thiophosphorylation was detected by immunoblotting with an anti-thiophosphate ester antibody. An additional control was added in which reaction was not supplemented with 2peATP γ S, but was alkylated to check the background. The expression of exogenous kinase was detected by HA3F10 antibody. The expression of the total Ck2 kinase was detected by CK2 α antibody.

2.2.9 Data analysis and identification of CK2 substrates

Peptides identification from tandem mass spectrometry (MS/MS) data is a central task in proteomics (Ma et al, 2003). Peaks studio is data analysis and statistics package for analyzing LC-MSMS data. MS/MS spectra are analyzed for identification of peptides, and the sequences of amino acids generated are used to identify the protein from the data bases. The PTM's are also determined by calculating the confidence of the modification site (Ascore values). This Ascore is the calculation of ambiguity score as $-10 \times \log_{10} P < 0.005$ and higher the Ascore there is more confidence in the modification (Ma et al, 2003).

The proteins only found in as-CK2 α or α' were unique to considered as CK2 substrates and the other were considered as background. A total of 80 unique phosphopeptides/thiophosphopeptides were identified based on FDR 1% ($-10\log P \geq 18.59$) in both as-CK2 α and α' . Unique peptides only present in heavy label are indicated in black font (Table 1). Unique peptides present in light and/or medium label in addition to heavy label are indicated in red font. Due to a high degree of irreproducibility across nine analyzed replicates, additional restrictions were applied using phosphorylation sites' Ascore values. Those phosphorylation sites that contain an Ascore < 19 (less than 99% site confidence) are considered ambiguous; however, phosphorylation sites with an Ascore from 10 - 19 (90% - 99% site confidence) are reported in the table along with the phosphorylation sites having an Ascore ≥ 19 ($\geq 99\%$ site confidence). Phosphorylation sites with an Ascore ≥ 19 ($\geq 99\%$ site confidence) are indicated in bold, while phosphorylation sites with an Ascore between 15 - 19 (97% - 99% site confidence) and 10 - 15 (90% - 97% site confidence) are indicated as non-bold and highlighted in grey respectively. The unique thiophosphorylated peptides found in as-CK2 α' were not according to the Ascore ≥ 19 (so were not considered), this could be due to the low level of expression of the exogenous as-CK2 α' , which was not able to utilize the 2peATP γ S. In summary, 27 non-redundant phosphorylation sites were identified at $\geq 97\%$ site confidence, of which 24 phosphorylation sites were identified at $\geq 99\%$ site confidence (Table 2). These 24 proteins are considered as CK2 α substrates after having a strict scoring system. The biological functions of these proteins are given in detail in table 3.

Table 1: Unique phosphopeptide in as-CK2 and Scoring system

Protein Accession	Phosphopeptide	Mass	-10 ³ gP	A-Score	Replicate 1	-10 ³ gP	A-Score	Replicate 2	-10 ³ gP	A-Score	Replicate 3	-10 ³ gP	A-Score	Replicate 4	-10 ³ gP	A-Score	Replicate 5	-10 ³ gP	A-Score	Replicate 6	-10 ³ gP	A-Score	Replicate 7	-10 ³ gP	A-Score	Replicate 9	-10 ³ gP	A-Score
D36818UASP2	G14+2.0TK(+8.0IPK(+7.9715GACAT	1537.73				20.7	55:1000.00																					
D95218L2HAB2	EVEIK(+8.0IE5(+7.971GEFEEDEC	2434.92				21	Y11:40.00																					
P153801R5Z	LLMMAGIDDCY(+7.971TSAR(+10.01	1748.72	21	Y11:40.00																								
P235281C0F1	AK(+2.0ISL(+7.971G5WAVASDGVK(+8.0	1231.59																										
P235431HMRH	DLWCFS(+7.971G5WHDHR(+10.01	1633.59																										
P386461GHP75	AK(+8.0IAVAVIT(+7.971PAVYNDSQF	1980.96																										
P43243IMATR3	L(+42.0MESSSGVGDETOALNLGIVAS	3157.42	19	521:29.98																								
P46861INEST	PESEGHPEST(+7.971PEPK(+8.01	1460.64	36	S9:1000.00																								
P46861INEST	IPSEGHPEST(+7.971PEPK(+8.01EGRK	1883.85																										
P50914IRL14	APGTC(+7.971KGTC(+7.971AAAAGAA	1636.75																										
P53639LMU11	ARGAAMAEESGGPAAAGPSGCR(+10.01T	1650.98																										
P03970PLC93	KRT(+10.01HPPS(+7.971AGGPDSKF	1637.78																										
Q07020IRL18	ALDLS(+7.971PCKL(+8.01GCGTVLLSGP	1763.88																										
Q44351FLNC	URASL(+7.971GCPGK(+10.01	1130.55																										
Q4516761MUC1	LLAEDSI(+7.971EEEVDFLSEK(+10.01	2082.93																										
Q150791SER12	MLPDAESE(+7.971IDEDEDFK(+8.01E	2534.06																										
Q2N205IK124	T(+42.01H(+7.971K(+8.01CNAMEYKQ	1924.91																										
Q882891THOC4	Q882891THOC4	1237.52																										
Q81W91MGAP	ESI(+42.01AGGKGGSGLG511(+7.971BRK(+	1554.83																										
Q8VUW1ERK1	IADLNSFMPSL(+7.971ICR(+10.01	1607.64																										
Q8VUW1ERK1	K14.8.01IAUDFINSFMSL(+7.971ICR(+1	1743.75																										
Q8BBD02KSC3	AFRBD(+7.971S5A1WHBK(+10.01)	1487.75																										
Q8BY771PDP3	NENWVTC(+7.971SDOKC(+8.01PILRL(+10.01	1622.8	19	T6:40.00																								
Q3NQ55SSF1	WGGS(+7.971DEEASGIPKSR(+10.01	1443.6	32	S4:51.00																								
P235281C0F1	AL(+42.01S(+7.971GVAASDGVK(+8.01	1231.59	47	S2:102.43																								
P235281C0F1	AT(+42.01S(+7.971GVAASDGVK(+8.01	1231.59	47	S2:102.43																								
P437361MCM2	GLL-YDLS(+7.971DEEDEK(+10.01P	1932.82	36	S6:27.83																								
Q049171433F	LAEGAEK(+10.01DMD(+15.591A(+7.971	1941.18																										
Q132831G3BP1	YDDEEVFVTFPES(+7.971PEEV	3305.33	19	S17:36.87																								
Q150841PDM6	DEH(+10.01DSELVPM(+10.01	3183.4																										
Q9H093INAC2	ALK(+8.01NNNNNNNNNN(+15.591E(+7.971	2712.31	36.1	S24:18.04																								
Q005551C1A	NNKK(LAT)ESAAHPIGSLGAGL(FQ(+395(+7.971	1765.683	26.3	S15:18.33	T6:11.52																							
Q052310H16	LEEDSL(+7.971EES(+7.971PEVSK(+10.01	1654.54	413	S3:16.90																								
P047391HSB1	QLL(+7.971S5(+10.01)	164.545																										
Q94593K1C17	S(+42.01NSKKL(+8.01G5(+7.971	2073.8538																										
Q136281TCOF	GA(+7.971PAPGKGKA(+7.971GKAWAS(+7.971	1775.699																										
Q860K7M5L1	K14.42.01H(+8.01NSK(+7.971PLGUGGGSASS(+8.01	1661.8484																										
Q8641HIF2P	NK(+8.01GPMK(+7.971NEODCA(+7.971	1768.892	33.3	S9:78.75																								
Q86841HIF2P	K(+8.01NSPFDQNSL(+7.971NEODCA(+8.01	1693.7488																										
P047391HSB1	QLL(+7.971S5(+10.01)	164.545																										
Q9N1301DX21	VTR(+8.01MEFP(+7.971TEEDK(+8.01P	2143.016	40.6	S8:19.69																								
P276351PBL10	VEELPLGCHWMSL(+7.971DEYDLSSEALEA(+7.971	3114.3225																										
P276351PBL10	VIDELPLGCHWMSL(+7.971DEYDLSSEALEA(+7.971	1653.6394																										
Q8TERABD01	SAS(+7.971S5AEGSVVWASER(+10.01P	1653.6394																										
Q13231G3BP1	S(+7.971S5AEGSVVWASER(+10.01P	1913.897																										

A total of 80 unique phosphopeptides were identified based on FDR 1% (-10³gP > 18.59). Unique peptides present in heavy label are indicated in bold font. Unique peptides present in light and/or medium label in addition to heavy label are indicated in red font. Due to a high degree of irreproducibility across nine analysis replicates, additional restrictions were applied using phosphorylation sites' A-Score values. Those phosphorylation sites that contain an A-Score < 19 (less than 99% site confidence) are considered ambiguous. However, phosphorylation sites with an A-Score from 10 - 19 (90% - 99% site confidence) are indicated in grey. Those phosphorylation sites with an A-Score ≥ 19 (≥ 99% site confidence) and 10 - 15 (90% - 99% site confidence) are indicated in bold, while phosphorylation sites with an A-Score between 10 - 19 (≥ 99% site confidence) and 10 - 15 (90% - 99% site confidence) are indicated in grey respectively. In summary, 27 non-redundant phosphorylation sites were identified at ≥ 97% site confidence, of which 24 phosphorylation sites were identified at ≥ 99% site confidence, and 99% site confidence sites that contain an A-Score < 19 (less than 99% site confidence) are considered ambiguous. However, phosphorylation sites with an A-Score from 10 - 19 (90% - 99% site confidence) are indicated in grey. Those phosphorylation sites with an A-Score ≥ 19 (≥ 99% site confidence) and 10 - 15 (90% - 99% site confidence) are indicated in bold, while phosphorylation sites with an A-Score between 10 - 19 (≥ 99% site confidence) and 10 - 15 (90% - 99% site confidence) are indicated in grey respectively.

Table 2: Proteins with Ascore ≥ 19 ($\geq 99\%$ site confidence)

Protein Accession	Phosphopeptide	Mass	-10lgP	AScore
O43818 U3IP2_HUMAN	G(+42.01)K(+8.01)PAS(+79.97)GAGAGAGAGKRR	1597.7928	20.7	S5:1000.00
O95218 ZRAB2_HUMAN	EVEDK(+8.01)ES(+79.97)EGEEEDEDEDLSK(+8.01)	2434.9243	21.8	S7:54.40
P15880 RS2_HUMAN	LLMMAGIDDCY(+79.97)TSAR(+10.01)	1748.7212	21.47	Y11:40.00
P23528 COF1_HUMAN	A(+42.01)S(+79.97)GVAVSDGVIK(+8.01)	1231.594	35.56	S2:86.57
P31943 HNRH1_HUMAN	DLNYCFS(+79.97)GMSDHR(+10.01)	1633.593	21.51	S7:22.45
P38646 GRP75_HUMAN	AK(+8.01)NAVIT(+79.97)VPAYFNDSQR	1980.955	29.14	T7:86.34
P43243 MATR3_HUMAN	DETDLANLGDVAS(+79.97)DGKK(+8.01)EPSDK(+8.01)	3157.4158	18.87	S21:29.98
P48681 NEST_HUMAN	IPGEGHPE(+79.97)PEPK(+8.01)	1460.6428	36.3	S9:1000.00
P48681 NEST_HUMAN	IPGEGHPE(+79.97)PEPK(+8.01)EQR(+10.01)	1883.8533	30.56	S9:1000.00
P50914 RL14_HUMAN	APGT(+79.97)KGT(+79.97)AAAAAAAAAAK(+8.01)	1636.7466	21.42	T4:1000.00;T7:1000.00
P83369 LSM11_HUMAN	ARGAAAGSGVPAAPGPSGR(+10.01)T(+79.97)R(+6.02)	1958.9769	20.84	T20:20.70
Q01970 PLCB3_HUMAN	KR(+10.01)HRPS(+79.97)AGGPDSAGR	1637.7775	26.81	S6:90.89
Q07020 RL18_HUMAN	ALDS(+79.97)PK(+8.01)GCGTVLLSGPR(+6.02)	1763.8827	18.9	S4:23.15
Q14315 FLNC_HUMAN	VRAS(+79.97)GPGLER(+10.01)	1130.5472	19.61	S4:1000.00
Q14676 MDC1_HUMAN	LLLAEDS(+79.97)EEEVDLFSER(+10.01)	2082.9272	36.89	S7:113.22
Q15019 SEPT2_HUMAN	IYHLPAES(+79.97)DEDEDFK(+8.01)EQTR(+10.01)	2534.0605	28.47	S9:37.29
Q2M2I5 K1C24_HUMAN	T(+42.01)(+79.97)K(+8.01)CQNAEYKQ(+.98)LLDIK	1924.9097	23.01	T1:27.25
Q86V81 THOC4_HUMAN	G(+42.01)AGGFGGGGT(+79.97)RR(+10.01)	1237.5228	19.87	T11:1000.00
Q8IW19 MGAP_HUMAN	ES(+79.97)K(+8.01)VMPTLAPVVAK(+8.01)	1564.827	20.92	S2:23.70
Q8WUW1 BRK1_HUMAN	IADFLNSFDMS(+79.97)CR(+10.01)	1607.6389	33.71	S11:44.28
Q8WUW1 BRK1_HUMAN	K(+8.01)IADFLNSFDMS(+79.97)CR(+10.01)	1743.748	29.09	S12:52.86
Q9BRR0 ZKSC3_HUMAN	AFIGS(+79.97)SALVIHQR(+10.01)	1487.7524	24.48	S5:20.92
Q9BY77 PDIP3_HUMAN	NGNVIT(+79.97)SDQ(+.98)PILLR(+10.01)	1629.8003	18.59	T6:40.00
Q9NQ55 SSF1_HUMAN	VGGS(+79.97)DEEASGIPS(+10.01)	1449.6012	32.46	S4:51.00
O00555 CAC1A_HUMAN	NNKLATAESAAPHGSLGHAGLPQ(+.98)S(+79.97)PAK(+8.01)	2712.311	36.09	S24:18.04
P04792 HSPB1_HUMAN	QLS(+79.97)SGVSEIR(+10.01)	1164.5415	41.29	S3:16.90
Q68DK7 MSL1_HUMAN	K(+42.01)(+8.01)S(+79.97)PLGGGGSGASSQAACLK	1861.8484	21.85	S2:17.65

Table 3: Biological functions of Potential substrates of CK2

Proteomics data were analyzed using PEAKS, version 8.1, by applying the following parameters: 4.5 ppm Parent Mass Error Tolerance (Orbitrap mass analyzer) and 0.5 Da Fragment Mass Error Tolerance (Linear Ion Trap mass analyzer). Based on FDR 1% (peptide -10logP ≥ 1.859), a total of 27 nonredundant unique heavy labeled phosphopeptides were identified, which includes 24 phosphorylation sites identified at ≥ 99% site confidence (indicated in bold) and 3 phosphorylation sites identified at ≥ 97% site confidence.

Protein Accession	Biological function
043818 U3BP2	Nucleolus; RNA-binding protein, thought to participate in the processing and modification of pre-ribosomal RNA.
055218 ZRA82	RNA Binding protein; Splice factor required for alternative splicing of TRA2B/SRS510 transcripts. May interfere with constitutive 5'-splice site selection
P15880 RS2	mRNA catabolic process; nonsense-mediated decay; positive regulation of transferase activity; protein amino acid methylation; rRNA processing; SRP-dependent cotranslational protein targeting to membrane; translation; translational initiation; viral transcription
P23281 C0F1	actin cytoskeleton organization and biogenesis; actin filament depolymerization; cytoskeleton organization and biogenesis; negative regulation of apoptosis; regulation of cell morphogenesis; response to virus; Rho protein signal transduction
P31043 HNRH1	fibroblast growth factor receptor signaling pathway; nuclear mRNA splicing, via spliceosome; regulation of RNA splicing; RNA metabolic process; RNA processing
P38646 GPBP75	erythrocyte differentiation; negative regulation of apoptosis; negative regulation of erythrocyte differentiation
P43243 MTR3	RNA processing; RNA-binding
P48881 NEST	brain development; embryonic camera-type eye development; G2/M transition of mitotic cell cycle; negative regulation of catalytic activity; negative regulation of protein binding; positive regulation of intermediate filament depolymerization
P48881 NEST	brain development; embryonic camera-type eye development; G2/M transition of mitotic cell cycle; negative regulation of catalytic activity; negative regulation of protein binding; positive regulation of intermediate filament depolymerization
P50914 RIL14	mRNA catabolic process; nonsense-mediated decay; ribosomal large subunit biogenesis and assembly; rRNA processing; SRP-dependent cotranslational protein targeting to membrane; translation; translational initiation; viral transcription
P83369 LSM11	histone mRNA 3'-end processing; histone mRNA metabolic process; termination of RNA polymerase II transcription
Q01970 PLCB3	inositol phosphate metabolic process; regulation of systemic arterial blood pressure; Wnt receptor signaling pathway; calcium modulating pathway
Q07202 RIL18	mRNA catabolic process; nonsense-mediated decay; rRNA processing; SRP-dependent cotranslational protein targeting to membrane; translation; translational initiation; viral transcription
Q14315 F1NC	Muscle-specific filamin, which plays a central role in muscle cells, probably by functioning as a large actin-cross-linking protein.
Q14676 MDC1	double-strand break repair; via nonhomologous end joining; intra-S DNA damage checkpoint
Q15059 SEBP2	smoothened signaling pathway
Q2MA25 K1C24	member of the type I (acidic) keratin family, which belongs to the superfamily of intermediate filament (IF) proteins
Q86831 THOC4	intronless viral mRNA export from host nucleus; mRNA 3'-end processing; mRNA export from nucleus; nuclear mRNA splicing, via spliceosome; osteoblast differentiation; positive regulation of RNA elongation; regulation of DNA recombination; replication fork processing; RNA export from nucleus; termination of RNA polymerase II transcription
Q8W19 MGAP	Functions as a dual-specificity transcription factor, regulating the expression of both MAX-network and T-box family target genes. Functions as a repressor or an activator.
Q8WUW1 BRK1	cell motility involved in cell locomotion; Rac protein signal transduction; vascular endothelial growth factor receptor signaling pathway
Q8WUW1 BRK1	cell motility involved in cell locomotion; Rac protein signal transduction; vascular endothelial growth factor receptor signaling pathway
Q8R80 ZESC3	lysosome organization and biogenesis; negative regulation of autophagy; negative regulation of transcription from RNA polymerase II promoter; negative regulation of transcription, DNA-dependent
Q8BY77 PDB3	mRNA 3'-end processing; mRNA export from nucleus; poly(A)+ mRNA export from nucleus; positive regulation of translation; RNA export from nucleus; termination of RNA polymerase II transcription
Q9HQ55 SSF1	ribosomal large subunit assembly and maintenance
000555 CAC1A	cell death; elevation of cytosolic calcium ion concentration; membrane depolarization; regulation of insulin secretion
P04792 HS981	positive regulation of blood vessel endothelial cell migration; positive regulation of interleukin-1 beta production; positive regulation of tumor necrosis factor biosynthetic process; regulation of I-kappaB kinase activity; positive regulation of angiogenesis; cell motility; negative regulation of apoptosis; negative regulation of protein kinase activity; positive regulation of angiogenesis;
Q8BDK7 MSL1	Component of histone acetyltransferase complex responsible for the majority of histone H4 acetylation at 'Lys-16' which is implicated in the formation of higher-order chromatin structure

2.3 Conclusion and future prospect

In conclusion, we have generated an analog-sensitive CK2 α and α' kinase by mutating the gatekeeper/bulky residue to glycine to enlarge the nucleotide-binding pocket. The mutants, as-CK2 α and α' efficiently utilize a bulky ATP analog, 2peATP γ S to phosphorylate substrates. Phosphorylation of proteins by as-CK2 α and α' using 2peATP γ S in cell lysate illustrates the specificity and sensitivity of the as-CK2 strategy as a novel molecular tool to identify direct substrates and, finally, to study the cellular roles of CK2. A common limitation in other approaches is their inability to confidently identify direct substrates of CK2 on a large-scale. The strength of the as-CK2 strategy is the enlarged nucleotide-binding pocket that uniquely and efficiently utilizes 2peATP to phosphorylate cellular substrates of CK2. The advantage in the use of 2peATP lies with an additional tag on the γ -phosphate to specifically label direct substrates of CK2. Thus, the labeled substrates could be captured with affinity enrichment, and can be identified by mass spectrometry. We validated our strategy by thiophosphorylating proteins in cell lysate, and used thiopeptide capture strategy by using iodoacetyl beads to capture the digested thiopeptides and identified them using mass spectrometry. The data was analyzed using Peaks package to identify thiophospho peptides. Unique thiophosphorylated peptides generated by the as-CK2 catalytic subunits were considered as potential CK2 substrates. A stringent scoring system was used to have more confidence in identifying the substrates. The peptides which had an Ascore ≥ 19 ($\geq 99\%$ site confidence) were considered as substrates of CK2.

This approach further outlines a framework that can be used in future to further improve and develop this approach to globally identify direct substrates of CK2 and further discover the cellular roles of CK2 and strengthen the foundation for future drug development. To further explore the cellular roles of CK2, mouse models and mammalian cell lines could be established which have the endogenous CK2 gene replaced with the as-CK2 allele. This would prove to be extremely useful in identifying more bona fide substrates of CK2, as this would eliminate the role of wt-CK2 endogenous interference of CK2. One approach to mutate the endogenous CK2 catalytic subunits, could be to use

CRISPR/Cas genome editing technique. As seen in figure 3A and 3B that as-CK2 α and α' can slightly utilize ATP, this could be applied when gatekeeper mutation is introduced endogenously. There would be a decrease in fold change of phosphopeptides in as-CK2 samples compared to wt-CK2, as as-CK2 has less affinity for ATP. The SILAC approach could then be used for quantifying the phosphoproteome of as-CK2 endogenous mutant and wt-CK2. This approach would give global prospective of bona fide substrates of CK2 kinase. Moreover, these quantitative phosphoproteomic results may be compared with those obtained by using different known competitive inhibitors of CK2, like TBBz or CX4946. Since the structural studies have shown that F113 residue in CK2 active site interacts with the TBBz inhibitor (Battistutta et al, 2001; Battistutta et al, 2007) the mutation of this hydrophobic residue, as seen in figure 1, would open the active site and the interaction of inhibitor would be less strong, in this way it makes less inhibition. This would allow the quantification of treated samples compared to control.

The other approach to complement this chemical genetic approach is to use of PP1 (4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine) analogue inhibitors (Bishop et al, 1999). Originally PP1 inhibitors had been developed to inhibit Src tyrosine kinases (Hanke et al, 1996). The analogues of PP1 have bulky chains which would only allow these inhibitors to attach to as-CK2 protein as they have larger ATP binding site, this would allow to use quantitative phosphoproteomic approach to identify substrates of CK2. In summary, using the above-mentioned approaches, will increase identification the number of *bona fide* CK2 substrates and further help in studying the kinase-substrate relationships of CK2.

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