Journal of proteome research

ARTICLE

pubs.acs.org/jpr

Unbiased Functional Proteomics Strategy for Protein Kinase Inhibitor Validation and Identification of *bona fide* Protein Kinase Substrates: Application to Identification of EEF1D as a Substrate for CK2

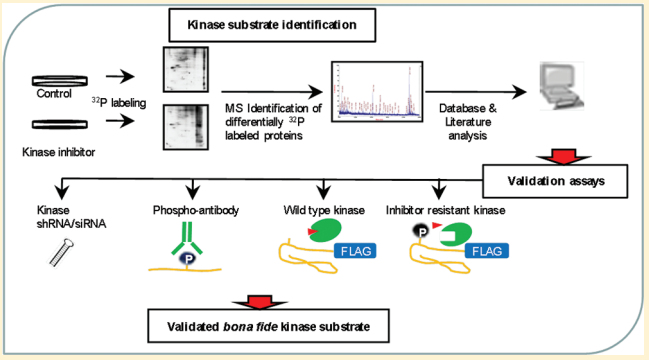
Laszlo Gyenis,[†] James S. Duncan,[†] Jacob P. Turowec,[†] Maria Bretner,[‡,§] and David W. Litchfield*[,†]

[†]Department of Biochemistry, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, N6A 5C1, Canada

[‡]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, and [§]Chemistry Department, Warsaw University of Technology, Warsaw, Poland

Supporting Information

ABSTRACT: Protein kinases have emerged as attractive targets for treatment of several diseases prompting large-scale phosphoproteomics studies to elucidate their cellular actions and the design of novel inhibitory compounds. Current limitations include extensive reliance on consensus predictions to derive kinase−substrate relationships from phosphoproteomics data and incomplete experimental validation of inhibitors. To overcome these limitations in the case of protein kinase CK2, we employed functional proteomics and chemical genetics to enable identification of physiological CK2 substrates and validation of CK2 inhibitors including TBB and derivatives. By 2D electrophoresis and mass spectrometry, we identified the translational elongation factor EEF1D as a protein exhibiting CK2 inhibitor-dependent decreases in phosphorylation in ^{32}P-labeled HeLa cells. Direct phosphorylation of EEF1D by CK2 was shown by performing CK2 assays with EEF1D-FLAG from HeLa cells. Dramatic increases in EEF1D phosphorylation following λ−phosphatase treatment and phospho-EEF1D antibody recognizing EEF1D pS162 indicated phosphorylation at the CK2 site in cells. Furthermore, phosphorylation of EEF1D in the presence of TBB or TBBz is restored using CK2 inhibitor-resistant mutants. Collectively, our results demonstrate that EEF1D is a *bona fide* physiological CK2 substrate for CK2 phosphorylation. Furthermore, this validation strategy could be adaptable to other protein kinases and readily combined with other phosphoproteomic methods.



KEYWORDS: 2D electrophoresis, CK2 inhibitor, functional proteomics, chemical genetics, protein kinase, inhibitor-resistant kinase, unbiased validation strategy

INTRODUCTION

The human genome encodes more than 500 protein kinases that catalyze the reversible phosphorylation of proteins, a regulatory mechanism controlling diverse cellular processes.[1,2] Notably, perturbations in protein kinase-mediated signaling pathways have been reported in many human diseases including cancer.[3,4] Protein kinases have thus emerged as promising candidates for molecular-targeted therapy.[5] A prime example of this selective therapy is the development of imatinib (Gleevec), dasatinib and nilotinib as ATP-competitive inhibitors targeting the BCR-Abl fusion protein in chronic mylogenous leukemia (CML).[6−9] These successes have demonstrated that protein kinases can be exploited to treat diseases and have encouraged efforts to develop inhibitors directed against other protein kinases implicated in a number of human diseases. In addition to their therapeutic potential, the development of many novel protein kinase inhibitors has yielded new opportunities to evaluate enzyme−substrate relationships for protein kinases and to further elucidate their regulatory participation in biological events.[10−14] However, to capitalize on the promise of protein kinase inhibitors for therapy and as tools to elucidate the role(s) of protein kinases in the regulation of biological processes, it is imperative that unbiased approaches be developed to evaluate inhibitor specificity[15] and to validate that inhibitors are effective when used in cells or *in vivo*.

Protein kinase CK2 (collectively referring to two isoforms designated CK2α and CK2α′ in mammals) is one family of protein kinases that has attracted attention as potential therapeutic targets because of its elevated expression in cancer cells, and participation in a broad spectrum of biological processes.[16] CK2 is classified as a protein serine/threonine kinase[17,18] that has also been shown to phosphorylate tyrosine residues.[19] CK2 regulates multiple cellular processes such as cell cycle progression,[20−22]

Received: August 4, 2010
Published: September 21, 2011

apoptosis,[23−27] transcription,[28] circadian rhythms,[29] and has been demonstrated to promote tumorigenesis in mouse models.[30] While the understanding of its precise roles in tumorigenesis remains incomplete, its ever-expanding lists of potential interaction partners[31,32] and substrates are consistent with complex roles in regulatory signaling networks.[33] Furthermore, large scale phosphoproteomic studies that have identified phosphorylated proteins and phosphorylation sites at unprecedented rates offer the promise of revealing a thorough description of the role of CK2 and other protein kinases in cellular regulation.[34−37] Information about the substrate specificity and consensus motifs for individual protein kinases such as CK2[38,39] has aided the prediction of possible protein kinase:substrate relationships from these studies. However, to maximize the benefits that emerge from these large-scale studies, it is important that complementary unbiased experimental strategies be devised to verify that predicted substrates are in fact direct substrates of the protein kinase of interest.

The development of cell permeable inhibitors of CK2[10,40] has represented a major advance for the discovery of its substrates and elucidation of its roles in cell regulation. Furthermore, since CK2 appears to be constitutively active,[41,42] it is expected that CK2 inhibitors could be valuable agents to neutralize the dramatic increases in CK2 activity that accompany its elevated expression that is frequently observed in human cancers.[43,44] Since many protein kinase inhibitors, including the majority of CK2 inhibitors, are ATP-competitive inhibitors, there is a clear need for methods that evaluate their specificity and the potential for off-target effects involving other members of the protein kinase family or other ATP-binding proteins that are abundant in human cells.[15,45] For example the CK2 catalytic site has unique features that can be exploited in the design of specific inhibitors and in devising strategies to evaluate the specificity of these inhibitors. Notably, CK2 is one of the few protein kinases with the ability to utilize GTP as a substrate[46] and the catalytic pocket that binds ATP/GTP is somewhat more compact than in other kinases because of the presence of bulky hydrophobic residues.[13,47,48] Mutation of these residues in the catalytic site of CK2α (V66A and I174A) or CK2α' (V67A and I175A) renders the kinase less sensitive to the CK2 inhibitor TBB and its derivates.[13,40,49] With the objective of devising a systematic strategy to identify *bona fide* substrates of CK2 and with the expectation that substrates could be used as indicators to validate inhibition of CK2 in cells, we have coupled a functional proteomics strategy with chemical genetics. We employed two-dimensional electrophoresis to identify proteins exhibiting diminished phosphorylation in cells treated with CK2 inhibitors based on its capacity to fractionate thousands of individual protein variants, including separation of different phosphorylated forms of individual proteins, and its demonstrated ability to identify substrates for protein kinases such as MAP kinase.[50] To extend these studies, we generated inhibitor-resistant mutants of CK2[15] to evaluate whether the identified proteins are indeed direct substrates for CK2. Utilizing these strategies, we identified EEF1D, a translational elongation factor implicated as a potential prognostic indicator in cancer (including medulloblastoma[51] and esophageal carcinoma[52]) as a *bona fide* cellular target of CK2. Given its potential prognostic value, its ubiquitous expression and abundant nature, our results suggest that EEF1D may be a viable marker for CK2 inhibition. Furthermore, the unbiased validation strategies utilizing functional proteomics and chemical genetic methods that we have employed can be readily adapted to identify and validate *bona fide* substrates of other kinases.

EXPERIMENTAL SECTION

Cell Culture and CK2 Inhibitors

The HeLa (Tet-Off, Clontech) cells used in all experiments were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 units/mL penicillin (Invitrogen) at 37 °C with 5% CO_2 in 10 or 15 cm dishes (Falcon). The CK2 inhibitors were obtained from commercial suppliers as follows: 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) was purchased from Calbiochem, 4,5,6,7-tetrabromobenzotriazole (TBB) and 4,5,6,7-tetrabromobenzimidazole (TBBz) were from Sigma. Dimethyl sulfoxide (DMSO, Caledon) was used as solvent for the inhibitors in all experiments.

^{32}P Labeling and 2D Gel Analysis

HeLa cells (plated at 10^6 cells per 10 cm dish) were grown for 48 h to approximately 80% confluency in regular DMEM media. In preparation for biosynthetic labeling, the culture media was replaced with phosphate-free DMEM (Chemicon) supplemented with dialyzed 10% FBS, 100 μg/mL streptomycin and 100 units/ml penicillin (Invitrogen) just prior to ^{32}P labeling. Biosynthetic labeling was achieved by adding 800 μCi ^{32}P-orthophosphate in the presence or absence of 25 μM DMAT or TBBz. For untreated controls, DMSO was used in equal volumes as in the inhibitor treatments. After 12 h of ^{32}P orthophosphate labeling, the media was removed and the cells were washed twice with cold PBS on ice. The cells were lifted from the dish with PBS containing 5 mM EDTA and the cellular proteins were extracted with Trizol and separated with two-dimensional (2D) electrophoresis using pI 4−7 NL strips (GE Healthcare) for the first dimension (equal cpm of ^{32}P was loaded for each sample). Following SDS-PAGE for the second dimension, gels were dried and ^{32}P incorporation was detected with autoradiography. The autoradiograph images were scanned on an Epson 4990 flatbed scanner at 16-bit Grayscale and quantified with ImageQuant Version 5.2 software (Molecular Dynamics). ^{32}P incorporation differences were quantified by calculating volume ratios of the corresponding areas from 2D images of 25 μM TBBz, 25 μM DMAT or DMSO-treated samples. Proteins from nonradioactive experiments, processed with identical conditions as the ^{32}P-labeled samples, were stained with Pro-Q Diamond phosphoprotein gel stain (Invitrogen) and then with SYPRO Ruby stain (Invitrogen). Spots in the 2D gels showing significant inhibitor-dependent decreases in ^{32}P incorporation and Pro-Q Diamond staining were isolated from nonradioactive gels using an Ettan Spot Picker (GE Healthcare) and processed further for analysis by mass spectrometry as described below.

Sample Preparation and Identification with Mass Spectrometry

Excised gel pieces were processed using a MASSPrep Automated Digestor (Waters/Micromass) in our Functional Proteomics Facility (http://www.biochem.uwo.ca/wits/fpf/index.html). Briefly, the gel pieces were destained using 50 mM ammonium bicarbonate and 50% Acetonitrile (ACN), the proteins were reduced in 10 mM DL-Dithiothreitol (DTT), then alkylated using 55 mM iodoacetamide (IAA) and subjected to in-gel digestion with Trypsin. Following digestion, peptides were extracted using 1% formic acid and 2% ACN and lyophilized. The lyophilized peptides were resuspended in 10% ACN and 0.1% Trifluoroacetic acid (TFA) and mixed at a 1:1 ratio (v/v) with α-cyano-4-hydroxycinnamic acid (CHCA) matrix that was prepared using 5 mg/mL in 6 mM ammonium phosphate monobasic, 50% ACN,

0.1% TFA. Samples (0.7 μL) were spotted on MALDI plates and analyzed on a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) by MALDI-TOF or MALDI-TOF/TOF. The data were acquired and processed using 4000 Series Explorer and Data Explorer (both from Applied Biosystems). The mass spectrometer is equipped with a 355 nm Nd:YAG laser with a laser rate of 200 Hz and was run in a reflectron positive ion mode collecting a sum of 1000 (MS) or 2000 (MS/MS) laser shots for each mass spectrum. Calibration was performed with a mass tolerance of 50 ppm. Masses obtained for each sample were submitted to the MASCOT search engine for peptide mass fingerprint identification (http://www.matrixscience.com/cgi) using the SwissProt database of *Homo sapiens* with carbamidomethyl (C) fixed and oxidation (M) plus phosphorylation (ST) variable modification and a peptide tolerance of 15−35 ppm not allowing any missed cleavages. Identified proteins were subsequently examined using existing phosphorylation databases PhosphoSitePlus (www.phosphosite.org), Phosida (www.phosida.de),[53] PhosphoELM (phospho.elm.eu.org) and by literature search to determine if they were previously identified as phosphorylated proteins in human cells.

Plasmid Construction and Transfection

The elongation factor 1-delta (designated as EEF1D) cDNA clone was purchased from American Type Culture Collection. To incorporate a FLAG epitope tag at either its N- or C- terminus, the 846 bp EEF1D cDNA encoding the 281 amino acid 31 kDa EEF1D transcript variant 2 (gi|25453472) was PCR amplified with the following primers: N-terminal (FLAG-EEF1D) (Forward: 5′-gctaagcttatggactacaaagacgatgacgacaagatggctacaaacttccta-3′, Reverse: 5′-tgcgaattctcagatcttgttgaaagc-3′) or C-terminal (EEF1D-FLAG) (Forward primer: 5′-gctggtaccatggctacaaacttccta-3′, Reverse primer: 5′-tgcgaattctcacttgtcgtcatcgtctttgtagtccatgatct-tgttgaaagc-3′). Amplification products were cloned into pcDNA3.1+ expression vector (Invitrogen) with *Hin*dIII/*Eco*RI or *Kpn*I/*Eco*RI cloning sites for FLAG-EEF1D or EEF1D-FLAG, respectively. The nonphosphorylatable S162A (Forward primer: 5′-tgacattgacctgtttggcgctgacaatgaggaggaggac-3′, Reverse: 5′-gtcctc-ctcctcattgtcagcgccaaacaggtcaatgtca-3′) or phopshomimic S162D (Forward primer: 5′-tgacattgacctgtttggcgatgacaatgaggaggaggac-3′, Reverse: 5′-gtcctcctcctcattgtcatcgccaaacaggtcaatgtca-3′) mutants of FLAG tagged EEF1D were generated following Quik-Change Site-Directed Mutagenesis (Stratagene, San Diego, CA). The plasmids were propagated in DH5α bacterial cells, isolated with standard methods[54] and verified by sequencing. In the CK2 siRNA experiments, the individual subunits of CK2 or scambled control (Dharmacon SMART pools) were transfected at 200nM final concentration for 96 h following the manufacturer's recommendations. In cell rescue experiments HA tagged form at C-terminus of wild type (pRc/CMV-CK2α-HA) or inhibitor resistant form of CK2α (pRc/CMV-CK2α(V66A/I174A)-HA)[15] with N-terminal MYC tagged wild type CK2β (pRc/CMV-Myc-CK2β)[55] were used.

HeLa cells were transfected at approximately 60% confluency in 10 cm tissue culture dishes using PEI reagent (Linear, MW 25 000, Polysciences, Inc.) in most experiments. Lipofectamine2000 (Invitorgen) was used for CK2 siRNA and experiments with inhibitor-resistant CK2 constructs. For PEI transfections,[56] a construct encoding Enhanced Green Fluorescent Protein (EGFP) to monitor transfection efficiency (1 μg) and either FLAG-EEF1D or EEF1D-FLAG pDNA (5 μg) in 440 μL of 150 mM filter sterilized NaCl were mixed with 60 μL of PEI (1 mg/mL, in filter sterilized 10 mM pH5.5 MES). The pDNA-PEI mixtures were vortexed for 10 s, incubated for 10 min at room temperature and then added to the culture media with cells in the plates. In transfections with Lipofectamine2000 manufacturer recommendations were followed using 5 μL of Lipofectamine2000 per 10 cm plate with 6 mL media. Following transfection, cells were harvested at the times indicated.

Antibodies, Immunoblotting and Other Reagents

Western blotting[57] was performed using EEF1D antibody (Novus), FLAG M2Monoclonal Antibody (Sigma) and Anti-β-lactoglobulin (CedarLane) primary antibodies at 1:1000 dilution in LiCor Blocking Buffer:PBS containing 0.1% Tween 20 (1:1). The phospho-EEF1D antibody was raised against CDIDLFGpSD-NEEEDK phospho-peptide recognizing CK2 phosphorylation at the EEF1D pS162 site. Antibody production from peptide synthesis to affinity purification was completed at YenZym Antibodies, LLC, San Francisco, CA following their proprietary company protocols. Western blotting with phospho-EEF1D polyclonal antibody was done at 1:20 000 dilutions in 1% BSA/TBST. Endogenous CK2α, CK2α′ or CK2β were immunoblotted with polyclonal antibodies using previously established protocols.[15,58,59] For the HA- or MYC-tagged form of CK2 detection monoclonal anti-HA 3F10 (BabCO, Berkley, CA) or anti-MYC 9E10 antibodies were used as described.[60] For loading controls, anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), clone 6C5 (Millipore) in 3%BSA/PBST was used at a 1:1000 dilution. Secondary Infrared IRDye-labeled antibodies (LiCor) (1:10 000 dilution in PBS containing 1% BSA and 0.1% Tween 20) were visualized on the LiCor Odyssey Infrared Imaging System and evaluated using Odyssey V3.0 software. All 2D electrophoresis reagents and Protein G Sepharose used for immuno-precipitation were from GE Amersham.

Purification of Recombinant CK2

An inhibitor resistant mutant of GST-CK2α (ie. V66A/I174A) designated CK2αR[15] was generated with the Quikchange Mutagenesis Kit (Stratagene). Wild type GST-CK2α or GST-CK2αR were expressed in bacteria and purified according to previously established protocols.[61] To obtain the wild type (holo-CK2WT) or the inhibitor resistant form of holo-CK2 (holo-CK2R), the bacterial pellets from GST-CK2α-expressing or GST-CK2αR-expressing bacteria were mixed with twice the volume of pellet from bacteria expressing His-tagged CK2β bacterial pellet and the enzymes were purified by Glutathione affinity chromatography as for GST-CK2α or GST-CK2αR. The protein concentrations of the purified enzymes were determined by BioRad Protein Assay. Purified enzymes were also separated by 10% SDS-PAGE and stained with GelcodeBlue (Thermo) protein stain to confirm the monomeric or holo-enzyme form of CK2.[62]

In vitro Phosphorylation Assays

The CK2 substrate peptide RRRDDDSDDD (100 μM) was used to measure the activity of the purified enzymes. Each kinase reaction (40 μL) contained 50 mM Tris pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, 0.01 mM ATP supplemented with 1.6 μCi γ-^{32}P-ATP per reaction. Prior to the phosphorylation reactions, the inhibitors at 25 μM concentration were preincubated on ice with CK2 for 18 min and the enzyme:inhibitor was diluted into the kinase reaction to achieve a 5 μM final concentration of the inhibitor. Kinase reactions (10 min at 37 °C) were performed according to established protocols.[10,63,64] ^{32}P incorporation, residual kinase activity was calculated for each experiment and

the representative result of the two experiments was displayed on the final figures.

For immuno-kinase assays, EEF1D-FLAG or the mutant forms of S162A and S162D were immuno-precipitated from 500 μg of transfected HeLa lysate with 2.5 μg of FLAG M2 antibody following the protocol outlined previously[19] and then used for substrate in kinase reactions. Where indicated, immuno-precipitated EEF1D-FLAG and mutants were preincubated with λ-phosphatase (New England Biolabs). Kinase reactions were terminated by the addition of 50 μL 2× Laemmli sample buffer and 25% of the reaction was separated on 10% SDS-PAGE gels. Each reaction was separated twice on SDS-PAGE gels (technical replicate) and the experiment was repeated twice. All phosphorylation reactions were exposed to PhosphorImager screens, scanned on Storm 820 scanner (GE Healthcare) and quantified with ImageQuant Version 5.2 software. One PhosphorImager scan of the two experiments was displayed on the figures.

Alternatively, the CK2 phosphorylation dependent change of EEF1D-FLAG was visualized with a nonradioactive method. The immunoprecipitated EEF1D-FLAG were treated with λ-phosphatase and/or subjected to kinase reactions as indicated and the reactions were separated with 2D electrophoresis followed by immuno-blotting with FLAG M2 antibody. The immuno-blots were scanned on the LiCor Odyssey System and the signal intensity change of the 2D profile was analyzed by Odyssey V3.0 software (LiCor).

Analysis of Phosphorylation in Cells Expressing Inhibitor-Resistant CK2 Constructs

For rescue experiments with inhibitor-resistant CK2, HeLa cells were transfected in 15 cm size dishes using DMEM media without FBS or antibiotic at 80% confluency. Equal amount of wild type pRc/CMV-CK2α-HA and pRc/CMV-Myc-CK2β (10 μg each) plus 5 μg of EGFP to monitor transfection efficiency, or inhibitor resistant pRc/CMV-CK2α(V66A/I174A)-HA and pRc/CMV-Myc-CK2β (10 μg each) plus 5 μg of EGFP plasmid DNA were transfected with 12.5 μL of Lipofectamine2000 following manufacturer's recommendations. After 12 h incubation, the media was replaced with DMEM/0.1% FBS/100 μg/mL streptomycin and 100 units/mL penicillin and cells incubated for an additional 6 h. The cells were then transfected again with EEF1D-FLAG (20 μg) and EGFP (5 μg). Seven hours after the second transfection all cells were lifted with PBS containing 5 mM EDTA, pooled together into a single 50 mL tube, and then distributed equally into six of 10 cm plates containing 10 mL of DMEM/10% FBS/100 μg/mL streptomycin and 100 units/mL penicillin. One fraction of the cells were pelleted (100 xg, 4 °C), and were frozen at −80 °C to be used as 0 h control for EEF1D-FLAG protein expression level at the time of kinase inhibitor treatment. The cells in 10 cm plates were treated with CK2 inhibitors TBB (25 or 15 μM) or TBBz (15 or 8 μM). Dimethyl sulfoxide (DMSO) was added to control cells (DMSO cont.) or cells were kept without any treatment (12 h Untrd.). Following 12 h of incubation, all cells were harvested and washed four times with ice cold PBS on ice prior to lysis in Tris Lysis Buffer (TLB, 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% Deoxycholic Acid, 29.4 μg/mL Aprotinin, 1 mM PMSF, 20 μg/mL Leupeptin, 7 μg/mL Pepstatin A, 1 μM Okadaic acid, 1 μM Microcystin, 1 mM Sodium orthovanadate, 5 mM NaF). Cells from the 0 h treatment were lysed in the same buffer without phosphatase inhibitors. Cells were sonicated for 3 × 5 s on ice and then kept on ice for an additional 30 min to achieve complete lysis. Lysates were cleared by centrifugation (2330× g, 4 °C) and protein concentrations were measured with BCA Protein Assay Kit (Pierce) using BSA as standard.

EEF1D-FLAG was immuno-precipitated according to the protocol described above from 400 μg of lysate per treatment with 2.5 μg of FLAG M2 antibody. For phospho-EEF1D antibody immuno-blotting, one reaction of the immuno-precipitated EEF1D-FLAG protein of 0 h control was treated with λ-phosphatase and then 20% of each reaction was separated on 10% SDS-PAGE. For detection of endogenous or transfected CK2, 12.5 μg of lysate was separated on 10% SDS-PAGE gels. Proteins were transferred to Immobilon-FL PVDF membrane (Millipore) and immuno-blotted with antibodies indicated in the figures and following the protocol described above.

2D Electrophoresis to Monitor Phosphorylation Profiles of EEF1D

Proteins were immuno-precipitated prior to 2D electrophoresis and 2D profiles of EEF1D-FLAG examined with or without subsequent λ-phosphatase treatments. Briefly, transfected HeLa cells were harvested with Tris Lysis Buffer (TLB, 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% Deoxycholic Acid, 29.4 μg/mL Aprotinin, 1 mM PMSF, 20 μg/mL Leupeptin, 7 μg/mL Pepstatin A) and subjected to immuno-precipitation with FLAG M2 antibody (2.5 μg) using Protein G-Sepharose at 4 °C. Immune-complexes were collected by centrifugation and washed four times with NP-40 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 29.4 μg/mL Aprotinin, 1 mM PMSF, 20 μg/mL Leupeptin, 7 μg/mL Pepstatin A) followed by two washes with the same buffer lacking NP-40. After treatment with or without λ-phosphatase, the reactions were washed once (50 mM Tris pH 7.5, 150 mM NaCl, 29.4 μg/mL Aprotinin, 1 mM PMSF, 20 μg/mL Leupeptin, 7 μg/mL Pepstatin A, 1 μM Okadaic acid, 1 μM Microcystin, 1 mM Sodium orthovanadate) and then the buffer was removed completely. The proteins were eluted using 80 μL of 2D lysis buffer (8 M Urea, 2% CHAPS, 1 mM Benzamidine, 25 μg/mL Leupeptin, 20 μg/mL Pepstatin A, 20 μg/mL Aprotinin, 1 μM Okadaic acid, 1 μM Microcystin, 1 mM Sodium orthovanadate, 0.5% Ampholyte and 40 mM DTT) with constant rotation at 4 °C for 15 min. β-lactoglobulin (100 ng, Sigma) was added to 50 μL of each sample as an isoelectric focusing marker prior to sample application to 4−7 NL 2D strips for the first dimension separation. Following second dimensional separation on 10% SDS-PAGE, proteins were transferred to Immobilon-FL PVDF membrane (Millipore) and immuno-blotted with FLAG-M2 and β-lactoglobulin antibodies.

In CK2 inhibitor experiments, when GFP expression was first detectable by fluorescence microscopy after transfection, the cells were treated with CK2 inhibitors (25 μM) or an equivalent volume of DMSO for 12 h. After treatment, cells were washed with ice cold PBS (4×) on ice and harvested with 2D lysis buffer. Cell lysates were cleared by centrifugation (2330× g, 4 °C) to remove insoluble materials and the protein concentration was measured with the BioRad Protein Assay (BioRad) using BSA as standard. The proteins were separated by 2D electrophoresis and then immono-blotted and visualized as described above.

RESULTS

Development of a Strategy to Identify and Validate Protein Kinase Substrates

The systematic unbiased strategy that we developed to identify and validate *bona fide* substrates for protein kinases is

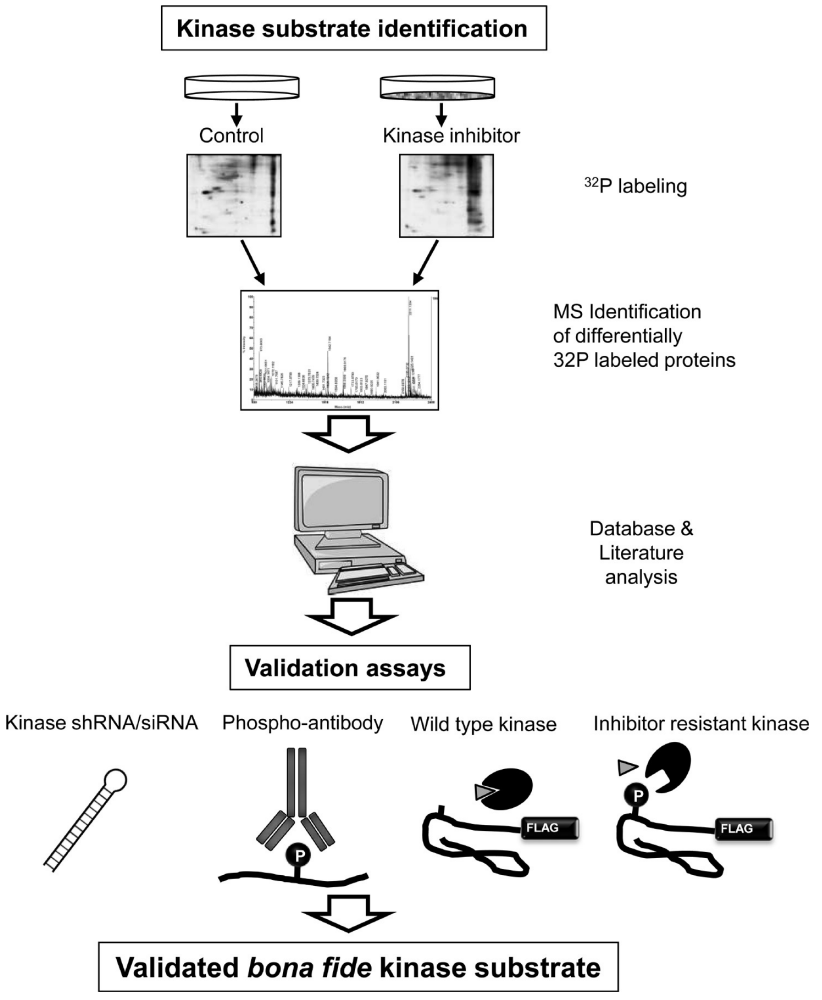


Figure 1. Schematic representation of the unbiased experimental strategy to identify and validate *bona fide* kinase substrates using functional proteomics methods in combination with chemical genetics. The main components of the validation strategy include: (1) identification of proteins exhibiting inhibitor-dependent changes in phosphorylation, (2) validating inhibition of phosphorylation with selective knockdown of the kinase, (3) development of a phospho-specific antibody to specifically detect phosphorylation, and (4) performing rescue experiments where inhibitor-resistant kinase is employed to overcome inhibitor effects on phosphorylation.

outlined in Figure 1. The starting point is the identification of proteins that exhibit inhibitor-dependent decreases in phosphorylation. Since inhibitor-dependent decreases in phosphorylation could arise from direct effects on the activity of the protein kinase targeted by the inhibitor or from indirect effects on downstream protein kinases within a signaling pathway, an important component of the strategy is to demonstrate direct phosphorylation of the target by the protein kinase under investigation. To perform these studies, the substrate can be isolated from mammalian cells and evaluated as a direct protein kinase substrate. The isolated protein can also be treated with phosphatase prior to the protein kinase assay to assess whether the relevant phosphorylation sites are occupied in cells (i.e., prior treatment with phosphatase would enhance the level of phosphorylation observed in the protein kinase reaction). Additionally, the phosphorylation of the substrate in cell by the selected kinase can be demonstrated by developing phospho-site specific antibodies recognizing the predicted phosphorylated residue on the protein that is modified by the kinase. The phospho-antibody is a valuable tool to monitor kinase activity dependent change following targeted knockdown of the kinase by siRNA or shRNA and evaluate the efficacy of kinase inhibitors. As a companion to these studies, inhibitor-resistant mutants of the protein kinase can be used to overcome (i.e., rescue) the effects of the inhibitors.

While there are now a number of proteomics strategies that enable the comparative analysis of protein abundance or extent of phosphorylation, many of these methods require access to dedicated mass spectrometers and extensive analysis of large data sets. By comparison, despite suggested limitations such as limited dynamic range and relatively low sample throughput, 2D gels that are readily available and routinely performed in many laboratories, have the capacity to fractionate and distinguish individual protein variants on the basis of covalent modifications such as phosphorylation. Accordingly, since our goals were primarily to devise a validation strategy for the identification of direct substrates for protein kinases, the limitations of 2D gels would not be of consequence especially when using ^{32}P incorporation to directly identify proteins that are phosphorylated in cells. To apply this strategy to the identification of substrates for CK2, ^{32}P labeled HeLa cells were treated with CK2 inhibitors and proteins were separated by 2D gel electrophoresis (Figure 2). Proteins exhibiting inhibitor-dependent decreases in ^{32}P incorporation were identified

dx.doi.org/10.1021/pr2008994 |*J. Proteome Res.* 2011, 10, 4887–4901

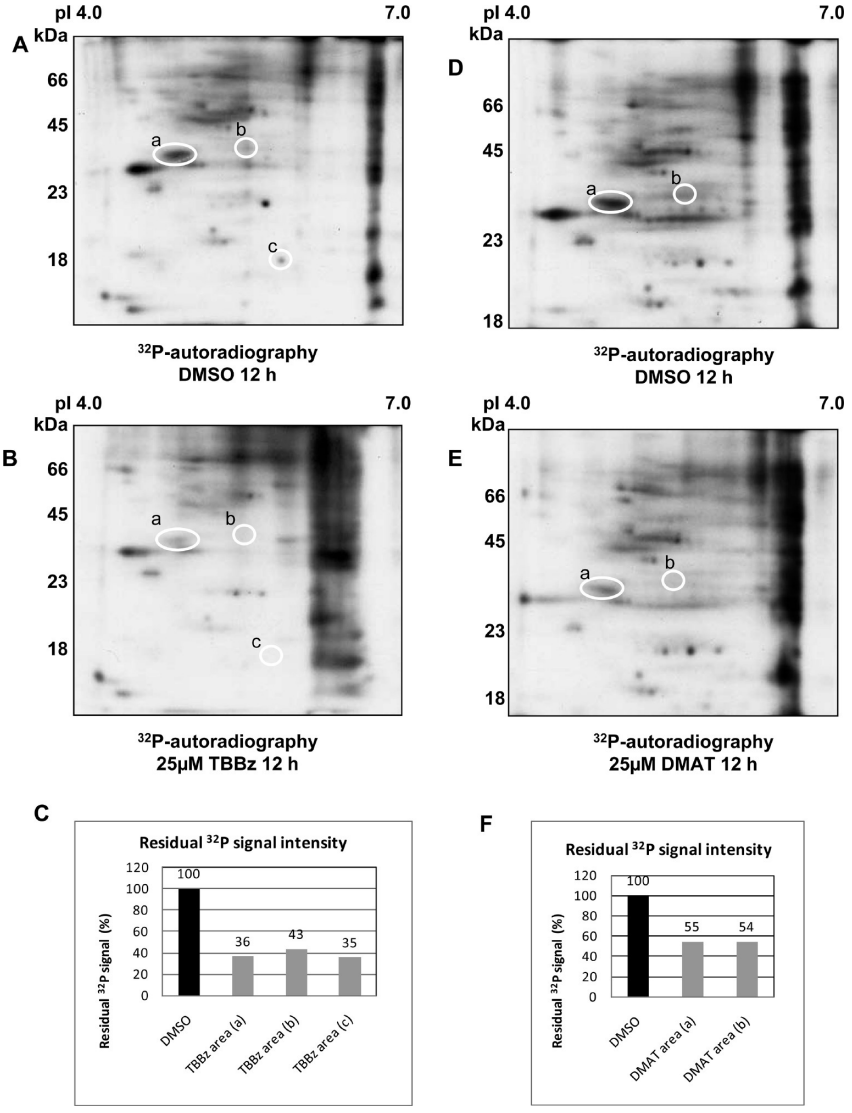


Figure 2. Identification of proteins with inhibitor-dependent decreases in ^{32}P labeling. The ^{32}P labeled HeLa cells were treated with DMSO (A and D), 25 μM TBBz (B) or 25 μM DMAT (E) for 12 h. Proteins were separated by 2D electrophoresis and ^{32}P incorporation was detected with autoradiography. ImageQuant quantification (C, F) of the areas circled in white (a,b,c) showed significant reduction in ^{32}P incorporation. The differences in ^{32}P incorporation were quantified as described in the Experimental Section. Circled spots were excised from unlabeled gels and analyzed by mass spectrometry to identify candidate substrates for CK2. Images are representative of duplicate experiments.

by mass spectrometry. Identified proteins were searched in existing phosphorylation databases (including Phosphosite, Phosida, PhosphoELM) and in the literature to determine if there was any prior evidence of phosphorylation. Three proteins, exhibiting a CK2 inhibitor-dependent decrease in phosphorylation, were identified by mass spectrometry from areas circled in white on Figure 2. These proteins identified by MALDI-TOF, had also been listed as phosphoproteins by previous phosphoproteomics studies, and were identified as elongation factor 1-delta (UniProt name: EEF1D_HUMAN, area "a" Figure 2), F-actin-capping protein subunit alpha-1 (UniProt name: CAZA1_HUMAN, area "b" Figure 2) and nucleoside diphosphate kinase A (UniProt name: NDKA_HUMAN, area "c" Figure 2). Mass spectra and peptide sequences are listed in Figure S3 (Supporting Information). Out of the three candidate substrates, we selected EEF1D for testing our validation strategy on the bases that EEF1D was listed in multiple phosphorylation databases (Table S2, Supporting Information), confirming that it was phosphorylated in cells at sites matching the CK2 consensus phosphorylation sequence (S/TXXD/E/pS/pY). Notably, recombinant EEF1D had also been shown to be a substrate for purified CK2 *in vitro*. However, like the vast majority of predicted protein kinase substrates, there were no studies performed to manipulate its phosphorylation in cells nor to determine whether it is a *bona fide* physiological substrate for CK2.

Validation of EEF1D as a Direct CK2 Substrate

The EEF1D protein sequence alignment clearly demonstrated that the CK2 site was highly conserved among different species (Table S1, Supporting Information). These findings provided additional assurance that EEF1D was a suitable candidate to carry out our validation strategy. Accordingly, EEF1D was FLAG tagged on the N- and C-termini and expressed in HeLa cells. The expressed protein was immuno-precipitated using the FLAG

dx.doi.org/10.1021/pr2008994 |*J. Proteome Res.* 2011, 10, 4887–4901

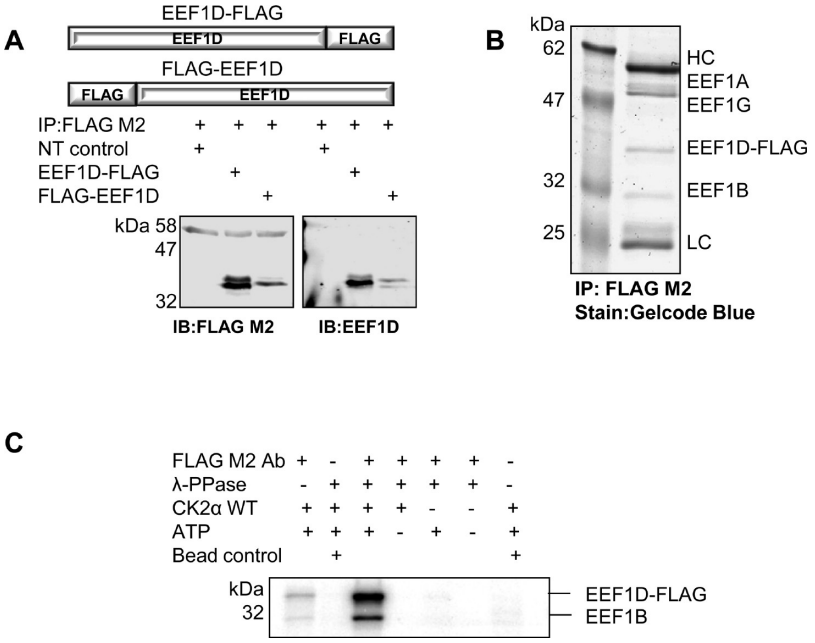


Figure 3. Investigation of EEF1D as a candidate CK2 substrate. (A) Schematic representation of EEF1D with N- (FLAG-EEF1D) or C- (EEF1D-FLAG) terminal FLAG tag. Overlapping signals of FLAG M2 monoclonal and EEF1D polyclonal antibodies identified the FLAG tagged form of EEF1D after the proteins were immuno-precipitated with FLAG M2 antibody. Nontransfected HeLa cells lysate was used as control (NT control) (B) EEF1D-FLAG expressed in HeLa cells coimmuno-precipitated with other three members of the EEF1 protein complex that were identified by MALDI TOF. (C) Phosphorylation of EEF1D-FLAG and elongation factor 1-beta (EEF1B) by CK2αWT. ^{32}P-ATP incorporation in the *in vitro* kinase assays on both proteins greatly increased after λ-phosphatase treatment indicating that both proteins were phosphorylated in human cells. Results are representative of two experiments.

M2 antibody and immuno-blotted with both FLAG M2 and EEF1D antibodies (Figure 3A). The C-terminally tagged form of EEF1D (EEF1D-FLAG) showed higher expression in HeLa cells (Figure 3A) and was selected for further experiments. Importantly, EEF1D-FLAG expressed in HeLa cells was functional in mammalian cells as indicated by its complex formation with endogenous proteins. In this respect, EEF1D-FLAG formed complexes with three endogenous members of the EEF1 complex that could be isolated by coimmuno-precipitation (Figure 3B) and identified by MALDI-TOF analysis as elongation factor 1-alpha (UniProt name: EEF1A_HUMAN), elongation factor 1-beta (UniProt name: EEF1B_HUMAN) and elongation factor 1-gamma (UniProt name: EEF1G_HUMAN). Mass spectra and other MALDI-TOF identification parameters are listed in Figure S4 (Supporting Information). Immuno-kinase assays with the wild type catalytic CK2α subunit (designated CK2αWT) *in vitro* demonstrated that both EEF1D-FLAG and the endogenous EEF1B were substrates of CK2 (Figure 3C). Furthermore, prior treatment of immunoprecipitates with λ-phosphatase led to a dramatic increase in phosphorylation of both proteins by CK2 (Figure 3C). The enhanced phosphorylation following phosphatase treatment clearly indicates substantial occupancy of the CK2 phosphorylation sites of the candidate substrates in human cells.

To further examine the phosphorylation profiles of EEF1D, we performed 2D electrophoresis (Figure S1 and S2, Supporting Information). Initially, the 2D profile of EEF1D-FLAG was evaluated $\pm$ λ-phosphatase treatment revealing a profile shift toward the less acidic region following phosphatase treatment (Figure S1, Supporting Information). Visualization of the profile shift could be further enhanced with the use of beta-lactoglobulin as an isoelectric-focusing marker. The shift toward the less acidic region in the 2D profile could also be observed expressing EEF1D-FLAG in HeLa cells treated with CK2 inhibitor at different times or concentrations (Figure S2A, Supporting Information). The CK2-dependent 2D profile change was also confirmed by separating the immune-complex kinase assays (Figure S2B, Supporting Information). In order to quantify changes in the 2D profile, we compared intensity changes after CK2 phosphorylation and CK2 inhibitor treatment. We determined that changes in spot numbers 5 and 6 were the most prominent and reflected the activity of CK2 in the assays (Figure S2B, Supporting Information). Collectively, these results suggested that phosphorylation of EEF1D was responsive to CK2 inhibitors in HeLa cells.

While 2D electrophoresis suggested that the phosphorylation profile of EEF1D was affected by CK2 inhibition, these analyses did not provide a direct assessment of specific site(s) of phosphorylation. Therefore, in order to monitor CK2 activity dependent phosphorylation on EEF1D we developed a phospho-EEF1D antibody against a EEF1D phospho-peptide shown in Figure 4A (top sequence) that corresponds to a predicted CK2 site that is present in both Phosida and PhosphoELM (Table S2, Supporting Information). Using this phospho-EEF1D antibody, the immuno-blots in Figure 4B showed two distinct groups of bands, corresponding to EEF1D and EEF1B on Figure 3B. Moreover, our results indicated that our phospho-EEF1D antibody could recognize EEF1D and EEF1B from HeLa cell lysates only when lysates were not previously treated by λ-phosphatase. The alignment of EEF1D and EEF1B protein sequences demonstrated that only one amino acid difference was found between the two proteins near the recognition site of the phospho-EEF1D antibody. The pSer in the immunizing peptide corresponds to pS162 on EEF1D and pS106 on EEF1B (Figure 4A). In peptide

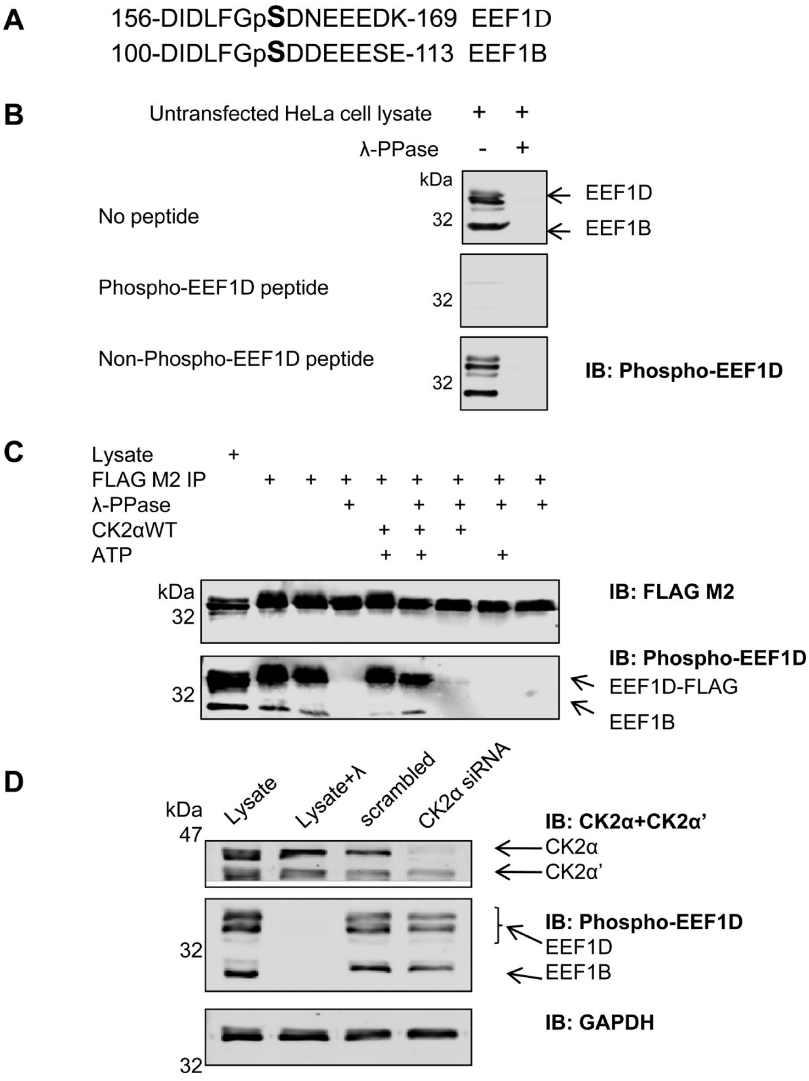


Figure 4. Characterization of a phosphospecific antibody recognizing S162 of EEF1D. (A) EEF1D peptide sequence (top) used for phospho-antibody development. High level of similarity between EEF1D and EEF1B near the CK2 phosphorylation site explains why phospho-EEF1D antibody can recognize phosphorylation on both proteins. (B) Peptide competition using molar excess (200×) of phospho- or nonphospho-peptide on immunoblots demonstrates specificity of phospho-EEF1D antibody. (C) Phosphorylation on EEF1D-FLAG and EEF1B by CK2 is detected by the phospho-EEF1D antibody. (D) Targeted knock down of CK2α by siRNA (200 nM of Dharmacon SMART pools) for 96 h in HeLa cells results in reduced EEF1D phosphorylation by CK2. The level of phosphorylated EEF1D, endogenous CK2α and CK2α' were measured with immunoblotting. GAPDH was used to demonstrate equal gel loading. Results are representative of two independent experiments.

competition assays, the phospho-EEF1D signal could be competed out with an excess of phospho-EEF1D peptide and there was no signal loss with the same molar excess of nonphospho-EEF1D peptide (Figure 4B). We also tested the CK2 dependent phosphorylation specificity of the phospho-EEF1D antibody. For these studies, we used EEF1D-FLAG as CK2 substrate in *in vitro* immuno-kinase CK2 assays. In these assays the antibody recognized phosphorylation of EEF1D-FLAG by CK2 and on the coimmuno-precipitated endogenous EEF1B (Figure 4C).

To further examine the dependence of the phosphorylation of S162 of EEF1D on CK2, we performed CK2 knockdown studies (Figure 4D). Treatment with siRNA targeting CK2α resulted in decreased phosphorylation of EEF1D and EEF1B, as compared to the level of phosphorylation observed in cells treated with a control (scrambled) siRNA. These studies further suggest that EEF1D is a direct substrate of CK2. Furthermore, detection of endogenous EEF1D with the phospho-specific S162 antibody definitively demonstrates that this residue is phosphorylated in human cells.

Validation of CK2 Substrates with Inhibitor-resistant CK2 Mutants Using Recombinant Enzymes

Since protein kinase inhibitors are often prone to off-target effects, our substrate validation strategy also incorporated the use of inhibitor resistant mutants of CK2 to evaluate the ability of these mutants to restore substrate phosphorylation in the presence of the inhibitor. Inhibitor-resistant variants of CK2α were prepared both as free catalytic subunits (CK2αR) and as holoenzyme complexes comprised of both catalytic and regulatory CK2 subunits (holo-CK2R). As expected, wild-type CK2 holoenzyme (holo-CK2WT) displayed more activity toward the synthetic CK2 substrate peptide RRRDDDSDDD than did the

dx.doi.org/10.1021/pr2008994 |*J. Proteome Res.* 2011, 10, 4887–4901

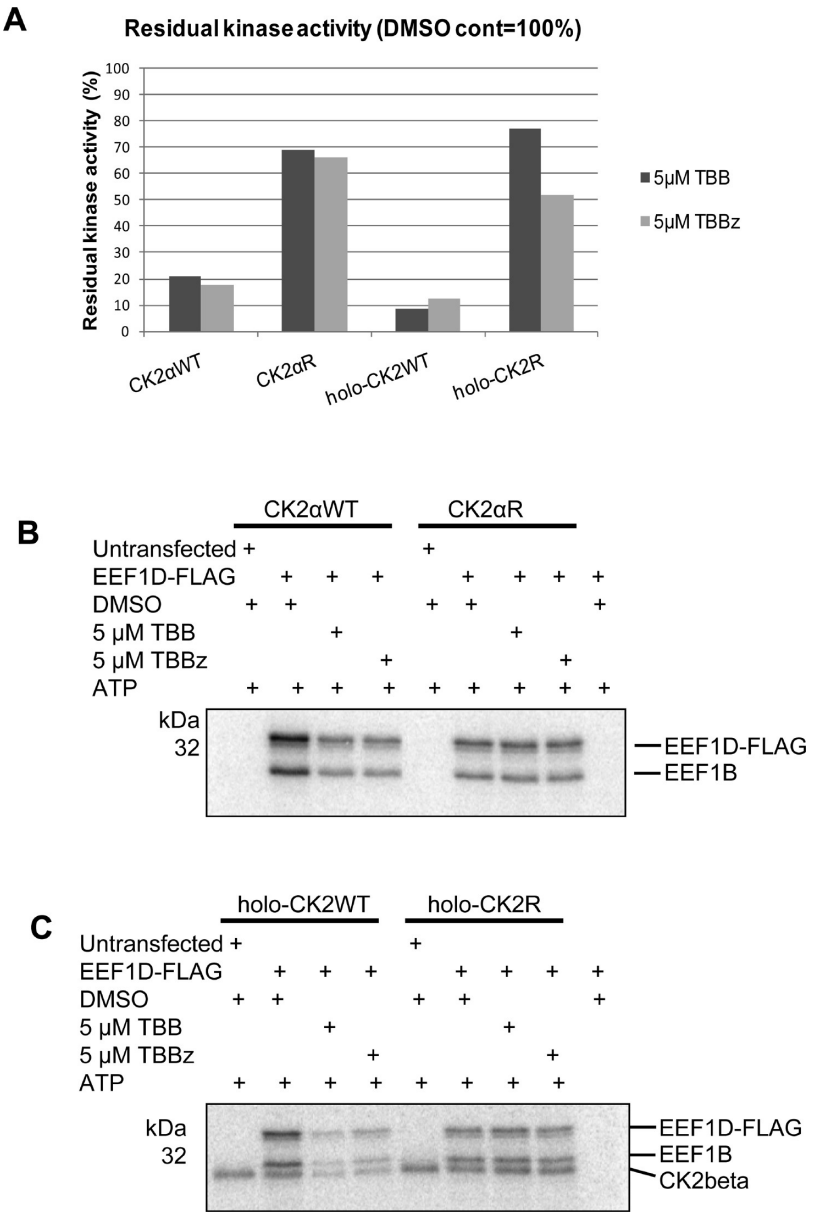


Figure 5. In vitro phosphorylation of EEF1D using inhibitor resistant forms of CK2. (A) Residual kinase activity, as compared to DMSO control = 100% activity, with wild type (WT) or inhibitor-resistant (R) CK2α or holo-enzyme on RRRDDDSDDD peptide using 5 μM TBB or TBBz. Kinase activity was restored using the inhibitor-resistant form of CK2. The bars in panel A represent the range of two samples of the reaction in one of two independent experiments. (B and C) Rescue of kinase activity with the inhibitor resistant CK2 on EEF1D-FLAG, EEF1B and CK2beta (C only) in *in vitro* immune-kinase assays. ^{32}P-ATP incorporation was measured on λ-phosphatase treated EEF1D-FLAG that was phosphorylated in immune-kinase assay by wild type (WT) or inhibitor resistant (R) CK2α or holo-CK2 enzyme. Representative results from two independent experiments are shown.

isolated CK2αWT subunit. A similar observation was made when comparing holo-CK2R to CK2αR indicating that the ability of CK2β to enhance CK2 activity is not compromised by the V66A/I174A substitutions that are introduced to generate the inhibitor-resistant forms of CK2. Notably, in these kinase assays, inhibitor-resistant forms (both catalytic CK2αR and holo-CK2R) restored the substrate phosphorylation in the presence of the inhibitor. Restoration of kinase activity was more evident after the residual kinase activities of the reactions were calculated and compared (kinase activity with DMSO alone=100%, Figure 5A). These results clearly indicated that TBB and TBBz could both effectively inhibit CK2, and that the inhibitor-resistant forms of CK2 could rescue activity toward a peptide substrate in the presence of the CK2 inhibitors.

As a next step, we proceeded to utilize the inhibitor resistant CK2 mutants to evaluate their ability to rescue the phosphorylation of EEF1D-FLAG in the presence of the CK2 inhibitors. CK2 kinase assays using EEF1D-FLAG (immuno-precipitated from HeLa cells and the treated with λ-phosphatase) as substrate showed multiple phosphorylated bands, most notably the EEF1D and EEF1B that were previously identified by mass spectrometry (Figure S4, Supporting Information). In the assays performed with the holo-enzyme forms of CK2, CK2β autophosphorylation by CK2α was also evident (Figure 5C). With wild-type forms of CK2, a significant decrease in phosphorylation of both EEF1D and EEF1B was visible in the presence of TBB or TBBz. By comparison, while inhibitor resistant forms do show a modest decrease in

dx.doi.org/10.1021/pr2008994 |*J. Proteome Res.* 2011, 10, 4887–4901

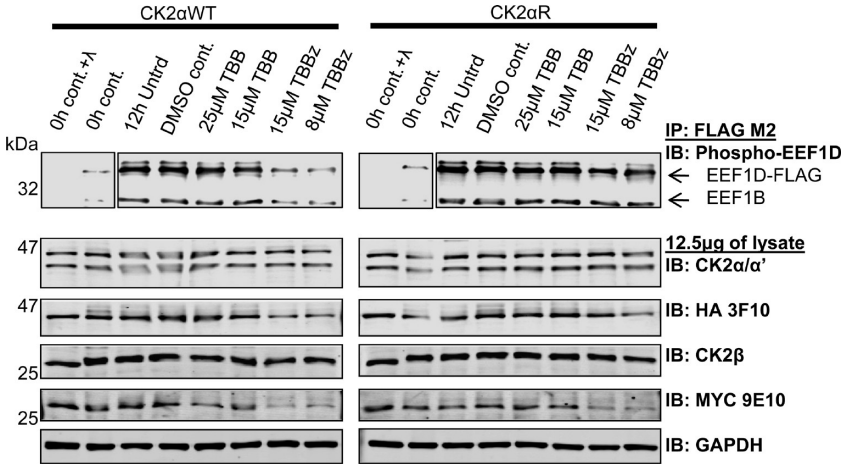


Figure 6. Expression of inhibitor-resistant CK2 restores phosphorylation of EEF1D in the presence of CK2 inhibitors. HeLa cells were transfected with wild type CK2 (pRc/CMV-CK2α-HA and pRc/CMV-Myc-CK2β) or an inhibitor resistant form of CK2 (pRc/CMV-CK2α(V66A/I174A)-HA and pRc/CMV-Myc-CK2β) prior to transfection again with EEF1D-FLAG. After an additional 7 h, cells were treated with CK2 inhibitors as indicated. Cells harvested at time 0 (incubated with or without λ phosphatase as indicated), cells treated for 12 h with DMSO only (DMSO cont.) or cells incubated for 12 h without treatment (12 h Untrd) were experimental controls. Anti-Flag immunoprecipitates were analyzed by immunoblotting with phospho-specific anti-EEF1D antibodies. For better visualization of 0 h control bands (run on the same gel), the image intensities were enhanced with the LiCor Odyssey software and the images were displayed separated from the rest of the immunoblot (top panel). Alternatively, cell lysates were immunoblotted with a mixture of CK2α and CK2α' antibodies, with anti-HA antibodies, anti-CK2β antibodies or anti-Myc antibodies as indicated to detect both endogenous and transfected CK2. Anti-GAPDH was used to confirm equal loading. Results are representative of two independent experiments.

kinase activity, neither TBB nor TBBz have an appreciable effect on the phosphorylation of either EEF1D or EEF1B (Figure 5B and C). Similar effects are observed with the phosphorylation of CK2β when using holo-CK2 R. Notably, CK2αWT and holo-CK2WT activity were higher on EEF1D and EEF1B than the activity measured with the inhibitor resistant form in the DMSO control reactions. However, there was no difference in kinase activity between the wild type and inhibitor resistant form of holo-CK2 in the autophosphorylation reaction on CK2β (Figure 5B and C). Collectively, these results indicated that inhibitor-resistant mutants of CK2 can be employed to ensure that TBB- or TBBz-dependent decreases in phosphorylation do indeed arise through CK2 inhibition.

Validation of CK2 Substrates by Expressing Inhibitor-resistant CK2 Mutants in Cells

To further our validation strategy, we expressed wild type or inhibitor resistant forms of the CK2 in cells. By monitoring CK2 activity with the phospho-EEF1D antibody we observed that EEF1D-FLAG was immediately phosphorylated by CK2 in cells after expression as even a weak band was observed at time 0 in all experiments (Figure 6, top panel). Time 0 was defined as the earliest time point when green fluorescent protein could be detected in living cells by fluorescence microscopy (typically ~6 h following transfection). At this time point, we did not observe notable differences in kinase activity between wild type and inhibitor resistant forms of CK2 expressed in HeLa cells (Figure 6, IB:phospho-EEF1D). Furthermore, we did not detect differences in expression of endogenous (IB:CK2$\alpha/\alpha'/\beta$) or transfected (IB:HA 3F10 and MYC 9E10) CK2 comparing immunoblots with the wild type or inhibitor resistant CK2 in Figure 6. However, CK2 inhibition for 12 h resulted in a significant decrease in phosphorylation of EEF1D-FLAG and endogenous EEF1B with TBBz (last 2 lanes of Figure 6; top left panel) and to a lesser extent with TBB. This difference in potency of TBBz over TBB is consistent with our previous findings.[15] Notably, the signal intensity of Phospho-EEF1D and EEF1B was partially restored by the expression of inhibitor-resistant CK2 (compare last 2 lanes of right panel and left panel of Figure 6). Again, the effect was more evident in cells treated with TBBz presumably because the inhibitory effect of TBBz was more dramatic, which made the rescue more readily detectable.

Evaluation of EEF1D WT and S162A or S162D Mutants

To further reinforce the conclusion that S162 is phosphorylated in cells, we engineered mutants harboring nonphosphorylatable and phospho-mimetic substitutions of this residue (Figure 7). In HeLa cells, S162A and S162D mutants of EEF1D-FLAG were expressed to similar levels as wild-type EEF1D in multiple experiments (Figure 7). Immuno-blotting with FLAG M2 did not show differences in EEF1D-FLAG wild type or mutant expression levels in HeLa cells, however our phospho-EEF1D antibody did only recognize the wild type form of EEF1D-FLAG (Figure 7A). The mutants formed complexes with the endogenous EEF1A, EEF1B, EEF1G and also with EEF1D as shown on the Gelcode Blue stained gel in Figure 7A. As was expected only the EEF1D-FLAG WT could be phosphorylated by the catalytic or holo-form of CK2 as demonstrated by both autoradiography or by immuno-blotting with our phospho-EEF1D antibody (Figure 7B). Coupled with the observations that CK2 can directly phosphorylate EEF1D, phosphatase treatment enhances phosphate incorporation, and that targeted siRNA knock down of CK2 resulted in decreased CK2 site specific phosphorylation, these results support the conclusion that EEF1D is a *bona fide* physiological substrate of CK2 in human cells.

DISCUSSION

The perturbation of cell signaling networks in human diseases (e.g., cancer) have generated a high level of attention in protein kinases and fuelled interest in protein kinase inhibitors as tools to manipulate specific signaling pathways and as potential therapeutic

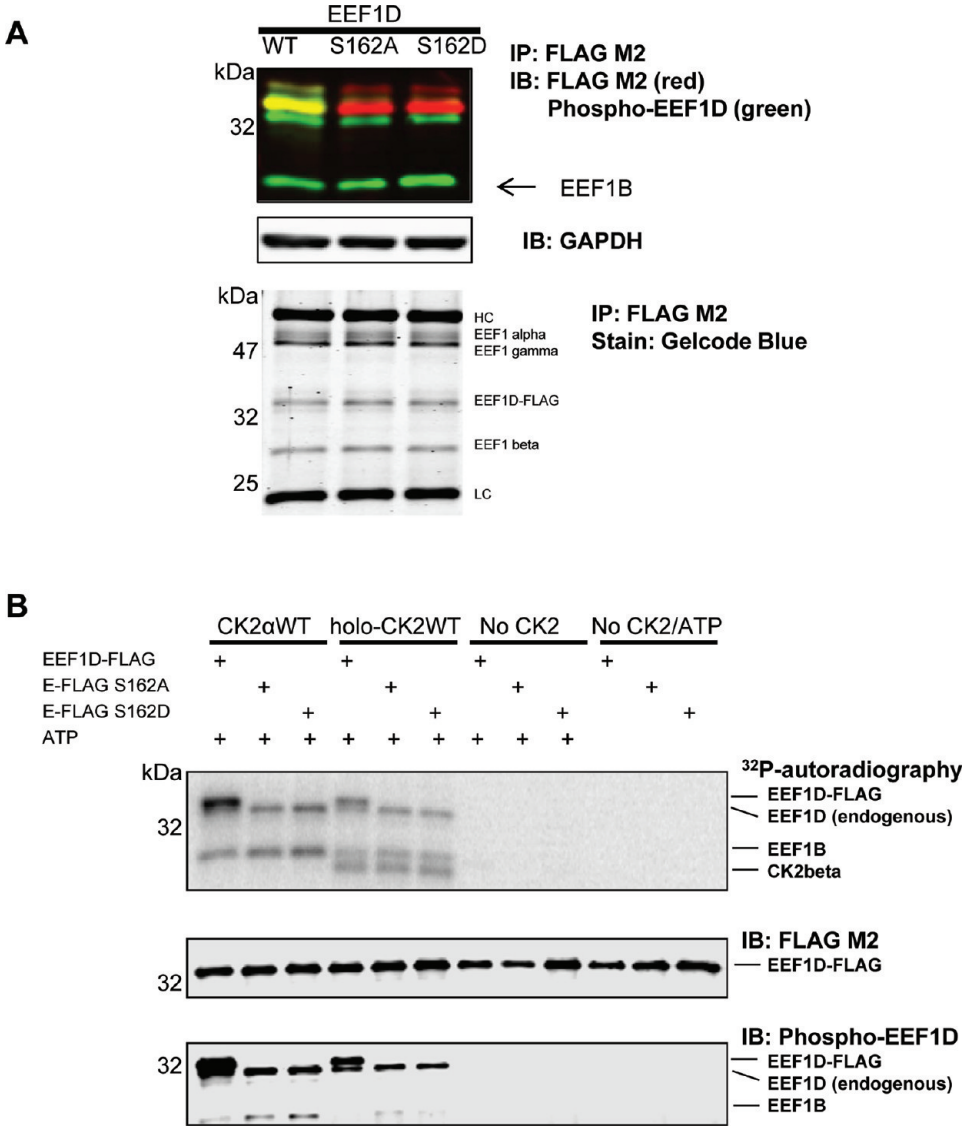


Figure 7. Evaluation of wild type and mutant forms of EEF1D-FLAG. (A) Wild type and S162A or S162D forms of EEF1D were transiently expressed in HeLa cells. Proteins were immuno-precipitated with FLAG M2 antibody from equal amount of cell lysates according to the experimental protocols and then immuno-blotted or stained by GelcodeBlue as indicated on the figure. Anti-GAPDH was used to demonstrate equal loading. (B) Immuno-kinase assays indicated that CK2αWT or holo-CK2WT could phosphorylate only the wild type EEF1D-FLAG in *in vitro* kinase assays. Results are representative of two independent experiments.

agents.[3−5] With the objective of comprehensively describing protein phosphorylation events in cells, a number of global phosphoproteomic studies have been performed employing mass spectrometry and other strategies.[34−37,65] However, to fully capitalize on the potential of protein kinase inhibitors and the wealth of information that is emerging from phosphoproteomic studies, it is necessary to develop systematic and unbiased approaches for validation of protein kinase inhibitors and protein kinase−substrate relationships. In this study, we combined the use of 2D electrophoresis coupled to mass spectrometry[50] together with the use of protein kinase inhibitors and inhibitor-resistant protein kinase mutants to identify elongation factor 1-delta (EEF1D) as a *bona fide* physiological substrate for CK2.

EEF1D may be an attractive candidate as a biomarker since elevated levels of EEF1D are associated with transformation,[52] invasiveness[66,67] and poor survival in cancer.[51] Furthermore, EEF1D has been implicated in the pathogenesis of amyotrophic lateral sclerosis.[68] EEF1D is one of the members of the EEF1 complex[69] that catalyze GDP/GTP exchange on EEF1A in complex with EEF1G and EEF1B subunits (Figure 3C) with the ability to regulate translation.[70] EEF1D derived from rabbits was reported to be phosphorylated *in vitro* by CK2 at Ser162[71−73] a residue that is highly conserved between species (Table S1, Supporting Information). Furthermore, phosphoproteomics databases (Table S2, Supporting Information) and global proteomics studies indicated that EEF1D is indeed phosphorylated in cells with two residues, namely Ser162 and Thr147 identified as sites of phosphorylation upstream to the N-terminus.[34−37,74,75] While these findings suggest that EEF1D is an attractive candidate as a physiological substrate for CK2, there was a lack of experimental evidence to confirm that EEF1D is phosphorylated directly by CK2 in human cells. Consequently, to build on these

dx.doi.org/10.1021/pr2008994 |*J. Proteome Res.* 2011, 10, 4887–4901

earlier findings, we performed the first experimental studies that conclusively demonstrate that EEF1D is phosphorylated by CK2 in cells. This conclusion is supported by the observations that ^{32}P incorporation into endogenous EEF1D is diminished in cells treated with CK2 inhibitors (Figure 2) and that EEF1D-FLAG is directly phosphorylated by CK2 (Figure 3−7). The dramatic increase in the *in vitro* phosphorylation of immuno-precipitated EEF1D-FLAG by CK2 that is observed following λ-phosphatase treatment indicates that the CK2 site is phosphorylated in cells, a conclusion that is reinforced through the use of a phospho-specific antibody recognizing pS162 of EEF1D. The use of this phospho-specific antibody to detect a decreased phosphorylation of EEF1D-FLAG in cells treated with CK2 inhibitors or cells with siRNA-mediated knockdown of CK2 further supports the conclusion that EEF1D is a direct physiological substrate for CK2. Furthermore, the demonstration that phosphorylation of EEF1D in the presence of CK2 inhibitors is restored by inhibitor-resistant forms of CK2 (Figures 5B−C and 6) further suggests that EEF1D is a *bona fide* CK2 substrate. Given the emergence of CK2 as a candidate for molecular-targeted therapy,[76,77] it could thus be envisaged that the phosphorylation of EEF1D could be used to validate the effectiveness of CK2 inhibitor treatments in cells or tissues.

While our studies clearly indicate that EEF1D is a *bona fide* physiological substrate for CK2, it is important to note that treatment with CK2 inhibitors or siRNA-mediated knockdown of CK2 had a relatively modest effect on the levels of EEF1D phosphorylation as detected by the phospho-specific S162 antibody. By comparison, there was a striking decrease in ^{32}P incorporation into EEF1D seen upon treatment of ^{32}P-labeled cells with CK2 inhibitors. This discrepancy may arise because the phosphospecific antibodies will detect total steady-state levels of EEF1D whereas ^{32}P labeling will only detect the population of the EEF1D that is phosphorylated during the labeling period. Furthermore, 2D profiles of transfected EEF1D-FLAG suggest that the phosphorylation of EEF1D may occur shortly after, and possibly coincident with, its synthesis and that once it is phosphorylated, the phosphorylation of S162 may be relatively stable. Our findings are also consistent with the prospect that CK2 may indeed be constitutively active in cells, a conclusion that would be in keeping with structural insights and with its high specific activity when expressed as a recombinant enzyme in bacteria. Collectively, these results suggest that the phosphorylation of S162 would be most useful as a marker for CK2 inhibition under circumstances of long-term CK2 inhibition as would be the case when CK2 inhibitors are used therapeutically.[77]

Overall, many protein kinases have emerged as attractive targets for cancer therapy, an observation that has prompted interest in the development of many novel protein kinase inhibitors with a wide range of specificities.[5,15,45,78] Given the close similarity between many members of the protein kinase family, the prospect for off-target effects is a major concern particularly for ATP competitive inhibitors that includes TBB and its derivates used in our study.[15,45,78,79] Accordingly, it is imperative that systematic and unbiased methods be available to evaluate specificity and identify any possible side effects of these compounds.[15] Additionally, identification of good biomarker candidates for kinase inhibition is important for confirmation that the inhibitors effectively neutralize the appropriate target in cells or tissues.[80] Toward these goals, the strategy used here for CK2 is not only a powerful and reliable tool for protein kinase substrate or biomarker candidate identification and validation, but may also be easily adapted to other protein kinases. Furthermore, this method can be combined with other known phosphoproteomic strategies[81,82] to validate candidate substrates for protein kinases. The adaptability of this strategy for inhibitor validation relies on the availability of kinase inhibitors and information regarding the structural basis of inhibition to enable the generation of the inhibitor-resistant form of the kinase. It can also be readily envisaged that this strategy could be modified to employ shRNA/siRNA or RNAi in combination with knockdown-resistant constructs. Overall, it is evident that protein kinase inhibitors are promising agents for therapy. Furthermore, the emergence of new strategies to identify phosphorylation sites and phosphopeptides at unprecedented rates offers the promise of yielding a comprehensive understanding of the role of protein kinases and protein phosphorylation in cell regulation. By combining functional proteomics with chemical genetics, as illustrated by the strategy employed in this study, it is possible to capitalize on advances in both of these areas to reveal *bona fide* protein kinase substrates and identify biomarkers for protein kinase inhibition.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1, S2, and Figures S1, S2, S3, S4. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Biochemistry, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, N6A 5C1, Canada. Tel: 1-519 661-4186. Fax: 1-519 661-3175. E-mail: litchfi@uwo.ca.

■ ACKNOWLEDGMENT

We thank Dr. Ken Yeung, Cunjie Zhang and Kristina Jurcic for their help with the MS identification and Ashley French for her assistance with plasmid construction. We also thank Dr. Silvia Penuela and the members of the Litchfield lab for their help with manuscript editing. Protein separation and identification were performed in the MALDI/Mass Spectrometry Facility and the Functional Proteomics Facility within the Schulich School of Medicine and Dentistry at the University of Western Ontario. This work was supported by the Canadian Cancer Society Research Institute with funds from the Canadian Cancer Society.

■ REFERENCES

(1) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912–34.

(2) Johnson, S. A.; Hunter, T. Kinomics: methods for deciphering the kinome. *Nat. Methods* **2005**, *2* (1), 17–25.

(3) Channavajhala, P.; Seldin, D. C. Functional interaction of protein kinase CK2 and c-Myc in lymphomagenesis. *Oncogene* **2002**, *21* (34), 5280–8.

(4) Kelliher, M. A.; Seldin, D. C.; Leder, P. Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase II alpha. *EMBO J.* **1996**, *15*, 5160–6.

(5) Bettayeb, K.; Tirado, O. M.; Marionneau-Lambot, S.; Ferandin, Y.; Lozach, O.; Morris, J. C.; Mateo-Lozano, S.; Drueckes, P.; Schächtele, C.; Kubbutat, M. H.; Liger, F.; Marquet, B.; Joseph, B.;

Echalier, A.; Endicott, J. A.; Notario, V.; Meijer, L. Meriolins, a new class of cell death inducing kinase inhibitors with enhanced selectivity for cyclin-dependent kinases. *Cancer Res.* **2007**, *67* (17), 8325–34.

(6) Bantscheff, M.; Eberhard, D.; Abraham, Y.; Bastuck, S.; Boesche, M.; Hobson, S.; Mathieson, T.; Perrin, J.; Raida, M.; Rau, C.; Reader, V.; Sweetman, G.; Bauer, A.; Bouwmeester, T.; Hopf, C.; Kruse, U.; Neubauer, G.; Ramsden, N.; Rick, J.; Kuster, B.; Drewes, G. Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat. Biotechnol.* **2007**, *25* (9), 1035–44.

(7) Jabbour, E.; Cortes, J. E.; Ghanem, H.; O'Brien, S.; Kantarjian, H. M. Targeted therapy in chronic myeloid leukemia. *Expert Rev. Anticancer Ther.* **2008**, *8* (1), 99–110.

(8) Quintás-Cardama, A.; Kantarjian, H.; Cortes, J. Flying under the radar: the new wave of BCR-ABL inhibitors. *Nat. Rev. Drug Discovery* **2007**, *6* (10), 834–48.

(9) Shah, N. P.; Tran, C.; Lee, F. Y.; Chen, P.; Norris, D.; Sawyers, C. L. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* **2004**, *305* (5682), 399–401.

(10) Cozza, G.; Mazzorana, M.; Papinutto, E.; Bain, J.; Elliott, M.; di Maira, G.; Gianoncelli, A.; Pagano, M. A.; Sarno, S.; Ruzzene, M.; Battistutta, R.; Meggio, F.; Moro, S.; Zagotto, G.; Pinna, L. A. Quinalizarin as a potent, selective and cell-permeable inhibitor of protein kinase CK2. *Biochem. J.* **2009**, *421* (3), 387–95.

(11) Gianoncelli, A.; Cozza, G.; Orzeszko, A.; Meggio, F.; Kazimierczuk, Z.; Pinna, L. A. Tetraiodobenzimidazoles are potent inhibitors of protein kinase CK2. *Bioorg. Med. Chem.* **2009**, *17* (20), 7281–9.

(12) Najda-Bernatowicz, A.; Lebska, M.; Orzeszko, A.; Kopanska, K.; Krzywinska, E.; Muszynska, G.; Bretner, M. Synthesis of new analogs of benzotriazole, benzimidazole and phthalimide-potential inhibitors of human protein kinase CK2. *Bioorg. Med. Chem.* **2009**, *17* (4), 1573–8.

(13) Sarno, S.; Ruzzene, M.; Frascella, P.; Pagano, M. A.; Meggio, F.; Zambon, A.; Mazzorana, M.; Di Maira, G.; Lucchini, V.; Pinna, L. A. Development and exploitation of CK2 inhibitors. *Mol. Cell. Biochem.* **2005**, *274* (1−2), 69–76.

(14) Zhu, D.; Hensel, J.; Hilgraf, R.; Abbasian, M.; Pornillos, O.; Deyanat-Yazdi, G.; Hua, X. H.; Cox, S. Inhibition of protein kinase CK2 expression and activity blocks tumor cell growth. *Mol. Cell. Biochem.* **2010**, *333* (1−2), 159–67.

(15) Duncan, J. S.; Gyenis, L.; Lenehan, J.; Bretner, M.; Graves, L. M.; Haystead, T. A.; Litchfield, D. W. An unbiased evaluation of CK2 inhibitors by chemo-proteomics: characterization of inhibitor effects on CK2 and identification of novel inhibitor targets. *Mol. Cell. Proteomics* **2008**, *7* (6), 1077–88.

(16) Litchfield, D. W. Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem. J.* **2003**, *369*, 1–15.

(17) Hanks, S. K.; Hunter, T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* **1995**, *9* (8), 576–96.

(18) Hunter, T.; Plowman, G. D. The protein kinases of budding yeast: six score and more. *Trends Biochem. Sci.* **1997**, *22* (1), 18–22.

(19) Vilk, G.; Weber, J. E.; Turowec, J. P.; Duncan, J. S.; Wu, C.; Derksen, D. R.; Zien, P.; Sarno, S.; Donella-Deana, A.; Lajoie, G.; Pinna, L. A.; Li, S. S.; Litchfield, D. W. Protein kinase CK2 catalyzes tyrosine phosphorylation in mammalian cells. *Cell Signal.* **2008**, *11*, 1942–51.

(20) Litchfield, D. W.; Lüscher, B. Casein kinase II in signal transduction and cell cycle regulation. *Mol. Cell. Biochem.* **1993**, *127−128*, 187–99.

(21) St-Denis, N. A.; Derksen, D. R.; Litchfield, D. W. Evidence for regulation of mitotic progression through temporal phosphorylation and dephosphorylation of CK2alpha. *Mol. Cell. Biol.* **2009**, *29* (8), 2068–81.

(22) St-Denis, N. A.; Bailey, M. L.; Parker, E. L.; Vilk, G.; Litchfield, D. W. Localization of phosphorylated CK2{alpha} to the mitotic spindle requires the peptidyl-prolyl isomerase Pin1. *J. Cell Sci.* **2011**, *124* (Pt 14), 2341–8.

(23) Ahmad, K. A.; Wang, G.; Unger, G.; Slaton, J.; Ahmed, K. Protein kinase CK2 - A key suppressor of apoptosis. *Adv Enzyme Regul* **2008**, *48*, 179–87.

(24) Wang, G.; Ahmad, K. A.; Harris, N. H.; Ahmed, K. Impact of protein kinase CK2 on inhibitor of apoptosis proteins in prostate cancer cells. *Mol. Cell. Biochem.* **2008**, *316* (1−2), 91–7.

(25) Duncan, J. S.; Turowec, J. P.; Vilk, G.; Li, S. S.; Gloor, G. B.; Litchfield, D. W. Regulation of cell proliferation and survival: Convergence of protein kinases and caspases. *Biochim. Biophys. Acta* **2010**, *1804* (3), 505–10.

(26) Duncan, J. S.; Turowec, J. P.; Duncan, K. E.; Vilk, G.; Wu, C.; Lüscher, B.; Li, S. S.; Gloor, G. B.; Litchfield, D. W. A Peptide-based target screen implicates the protein kinase CK2 in the global regulation of caspase signaling. *Sci. Signal.* **2011**, *4* (172), ra30.

(27) Turowec, J. P.; Duncan, J. S.; Gloor, G. B.; Litchfield, D. W. Regulation of caspase pathways by protein kinase CK2: identification of proteins with overlapping CK2 and caspase consensus motifs. *Mol. Cell. Biochem.* **2011**, DOI: 10.1007/s11010-011-0972-5.

(28) Cabrejos, M. E.; Allende, C. C.; Maldonado, E. Effects of phosphorylation by protein kinase CK2 on the human basal components of the RNA polymerase II transcription machinery. *J. Cell. Biochem.* **2004**, *93* (1), 2–10.

(29) Tsuchiya, Y.; Akashi, M.; Matsuda, M.; Goto, K.; Miyata, Y.; Node, K.; Nishida, E. Involvement of the protein kinase CK2 in the regulation of mammalian circadian rhythms. *Sci. Signal.* **2009**, *2* (73), ra26.

(30) Landesman-Bollag, E.; Romieu-Mourez, R.; Song, D. H.; Sonenshein, G. E. Cardiff RD; Seldin DC, Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* **2001**, *20* (25), 3247–57.

(31) Gyenis, L.; Litchfield, D. W. The emerging CK2 interactome: insights into the regulation and functions of CK2. *Mol. Cell. Biochem.* **2008**, *316*, 5–14.

(32) Olsten, M. E.; Weber, J. E.; Litchfield, D. W. CK2 interacting proteins: emerging paradigms for CK2 regulation? *Mol. Cell. Biochem.* **2005**, *274* (1−2), 115–24.

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

(34) Dai, J.; Jin, W. H.; Sheng, Q. H.; Shieh, C. H.; Wu, J. R.; Zeng, R. Protein phosphorylation and expression profiling by Yin-yang multidimensional liquid chromatography (Yin-yang MDLC) mass spectrometry. *J. Proteome Res.* **2007**, *6* (1), 250–62.

(35) Gevaert, K.; Staes, A.; Van Damme, J.; De Groot, S.; Hugelier, K.; Demol, H.; Martens, L.; Goethals, M.; Vandekerckhove, J. Global phosphoproteome analysis on human HepG2 hepatocytes using reversed-phase diagonal LC. *Proteomics* **2005**, *5* (14), 3589–99.

(36) Olsen, J. V.; Blagoev, B.; Gnad, F.; Macek, B.; Kumar, C.; Mortensen, P.; Mann, M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **2006**, *127* (3), 635–48.

(37) Olsen, J. V.; Vermeulen, M.; Santamaria, A.; Kumar, C.; Miller, M. L.; Jensen, L. J.; Gnad, F.; Cox, J.; Jensen, T. S.; Nigg, E. A.; Brunak, S.; Mann, M. Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci. Signal.* **2010**, *3* (104), ra3.

(38) Salvi, M.; Sarno, S.; Cesaro, L.; Nakamura, H.; Pinna, L. A. Extraordinary pleiotropy of protein kinase CK2 revealed by weblogo phosphoproteome analysis. *Biochim. Biophys. Acta* **2009**, *1793* (5), 847–59.

(39) Yoo, P. D.; Ho, Y. S.; Zhou, B. B.; Zomaya, A. Y. SiteSeek: Post-translational modification analysis using adaptive locality-effective kernel methods and new profiles. *BMC Bioinform.* **2008**, *9* (1), 272.

(40) Zien, P.; Duncan, J. S.; Skierski, J.; Bretner, M.; Litchfield, D. W.; Shugar, D. Tetrabromobenzotriazole (TBBt) and tetrabromobenzimidazole (TBBz) as selective inhibitors of protein kinase CK2: evaluation of their effects on cells and different molecular forms of human CK2. *Biochim. Biophys. Acta* **2005**, *1754* (1−2), 271–80.

(41) Duncan, J. S.; Litchfield, D. W. Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. *Biochim. Biophys. Acta* **2008**, *1784* (1), 33–47.

(42) Sarno, S.; Ghisellini, P.; Pinna, L. A. Unique activation mechanism of protein kinase CK2. The N-terminal segment is essential for constitutive activity of the catalytic subunit but not of the holoenzyme. *J. Biol. Chem.* **2002**, *277* (25), 22509–14.

(43) French, A. C.; Luscher, B.; Litchfield, D. W. Development of a stabilized form of the regulatory CK2beta subunit that inhibits cell proliferation. *J. Biol. Chem.* **2007**, *282* (40), 29667–77.

(44) Zhang, C.; Vilk, G.; Canton, D. A.; Litchfield, D. W. Phosphorylation regulates the stability of the regulatory CK2beta subunit. *Oncogene* **2002**, *21* (23), 3754–64.

(45) Pagano, M. A.; Bain, J.; Kazimierczuk, Z.; Sarno, S.; Ruzzene, M.; Di Maira, G.; Elliott, M.; Orzeszko, A.; Cozza, G.; Meggio, F.; Pinna, L. A. The selectivity of inhibitors of protein kinase CK2: an update. *Biochem. J.* **2008**, *415* (3), 353–65.

(46) Mazzorana, M.; Pinna, L. A.; Battistutta, R. A structural insight into CK2 inhibition. *Mol. Cell. Biochem.* **2008**, *316* (1−2), 57–62.

(47) Niefind, K.; Guerra, B.; Pinna, L. A.; Issinger, O. G.; Schomburg, D. Crystal structure of the catalytic subunit of protein kinase CK2 from Zea mays at 2.1 A resolution. *EMBO J.* **1998**, *17* (9), 2451–62.

(48) Niefind, K.; Pütter, M.; Guerra, B.; Issinger, O. G.; Schomburg, D. GTP plus water mimic ATP in the active site of protein kinase CK2. *Nat. Struct. Biol.* **1999**, *6* (12), 1100–3.

(49) Zhang, N.; Zhong, R. Structural basis for decreased affinity of Emodin binding to Val66-mutated human CK2alpha as determined by molecular dynamics. *J. Mol. Model.* **2010**, *16* (4), 771–80.

(50) Roberts, E. C.; Hammond, K.; Traish, A. M.; Resing, K. A.; Ahn, N. G. Identification of G2/M targets for the MAP kinase pathway by functional proteomics. *Proteomics* **2006**, *6* (16), 4541–53.

(51) De Bortoli, M.; Castellino, R. C.; Lu, X. Y.; Deyo, J.; Sturla, L. M.; Adesina, A. M.; Perlaky, L.; Pomeroy, S. L.; Lau, C. C.; Man, T. K.; Rao, P. H.; Kim, J. Y. Medulloblastoma outcome is adversely associated with overexpression of EEF1D, RPL30, and RPS20 on the long arm of chromosome 8. *BMC Cancer* **2006**, *6*, 223.

(52) Ogawa, K.; Utsunomiya, T.; Mimori, K.; Tanaka, Y.; Tanaka, F.; Inoue, H.; Murayama, S.; Mori, M. Clinical significance of elongation factor-1 delta mRNA expression in oesophageal carcinoma. *Br. J. Cancer* **2004**, *91* (2), 282–6.

(53) Gnad, F.; Ren, S.; Cox, J.; Olsen, J. V.; Macek, B.; Oroshi, M.; Mann, M. PHOSIDA (phosphorylation site database): management, structural and evolutionary investigation, and prediction of phosphosites. *Genome Biol.* **2007**, *8* (11), R250.

(54) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboroatory Manual*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

(55) Penner, C. G.; Wang, Z.; Litchfield, D. W. Expression and localization of epitope-tagged protein kinase CK2. *J. Cell. Biochem.* **1997**, *64* (4), 525–37.

(56) Reed, S. E.; Staley, E. M.; Mayginnes, J. P.; Pintel, D. J.; Tullis, G. E. Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. *J Virol. Methods* **2006**, *138* (1−2), 85–98.

(57) Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **1979**, *76* (9), 4350–4.

(58) Litchfield, D. W.; Lozeman, F. J.; Cicirelli, M. F.; Harrylock, M.; Ericsson, L. H.; Piening, C. J.; Krebs, E. G. Phosphorylation of the beta subunit of casein kinase II in human A431 cells. Identification of the autophosphorylation site and a site phosphorylated by p34cdc2. *J. Biol. Chem.* **1991**, *266* (30), 20380–9.

(59) Litchfield, D. W.; Lüscher, B.; Lozeman, F. J.; Eisenman, R. N.; Krebs, E. G. Phosphorylation of casein kinase II by p34cdc2 in vitro and at mitosis. *J. Biol. Chem.* **1992**, *267* (20), 13943–51.

(60) Vilk, G.; Saulnier, R. B.; Pierre, R., St; Litchfield, D. W. Inducible expression of protein kinase CK2 in mammalian cells. Evidence for functional specialization of CK2 isoforms. *J. Biol. Chem.* **1999**, *274* (20), 14406–14.

(61) Bosc, D. G.; Slominski, E.; Sichler, C.; Litchfield, D. W. Phosphorylation of casein kinase II by p34cdc2. Identification of phosphorylation sites using phosphorylation site mutants in vitro. *J. Biol. Chem.* **1995**, *270* (43), 25872–8.

(62) Turowec, J. P.; Duncan, J. S.; French, A. C.; Gyenis, L.; Denis, N. A., St; Vilk, G.; Litchfield, D. W. Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in mammalian cells. *Methods Enzymol.* **2010**, *484*, 471–93.

(63) Bosc, D. G.; Graham, K. C.; Saulnier, R. B.; Zhang, C.; Prober, D.; Gietz, R. D.; Litchfield, D. W. Identification and characterization of CKIP-1, a novel pleckstrin homology domain-containing protein that interacts with protein kinase CK2. *J. Biol. Chem.* **2000**, *275* (19), 14295–306.

(64) Litchfield, D. W.; Lozeman, F. J.; Piening, C.; Sommercorn, J.; Takio, K.; Walsh, K. A.; Krebs, E. G. Subunit structure of casein kinase II from bovine testis. Demonstration that the alpha and alpha' subunits are distinct polypeptides. *J. Biol. Chem.* **1990**, *265* (13), 7638–44.

(65) Rigbolt, K. T.; Prokhorova, T. A.; Akimov, V.; Henningsen, J.; Johansen, P. T.; Kratchmarova, I.; Kassem, M.; Mann, M.; Olsen, J. V.; Blagoev, B. System-wide temporal characterization of the proteome and phosphoproteome of human embryonic stem cell differentiation. *Sci. Signal.* **2011**, *4* (164), rs3.

(66) Keenan, J.; Murphy, L.; Henry, M.; Meleady, P.; Clynes, M. Proteomic analysis of multidrug-resistance mechanisms in adriamycin-resistant variants of DLKP, a squamous lung cancer cell line. *Proteomics* **2009**, *9* (6), 1556–66.

(67) Yang, S.; Du, J.; Wang, Z.; Yuan, W.; Qiao, Y.; Zhang, M.; Zhang, J.; Gao, S.; Yin, J.; Sun, B.; Zhu, T. BMP-6 promotes E-cadherin expression through repressing deltaEF1 in breast cancer cells. *BMC Cancer* **2007**, *7*, 211.

(68) Wain, L. V.; Pedroso, I.; Landers, J. E.; Breen, G.; Shaw, C. E.; Leigh, P. N.; Brown, R. H.; Tobin, M. D.; Al-Chalabi, A The role of copy number variation in susceptibility to amyotrophic lateral sclerosis: genome-wide association study and comparison with published loci. *PLoS One* **2009**, *4* (12), e8175.

(69) Riis, B.; Rattan, S. I.; Clark, B. F.; Merrick, W. C. Eukaryotic protein elongation factors. *Trends Biochem. Sci.* **1990**, *15* (11), 420–4.

(70) Sivan, G.; Aviner, R.; Elroy-Stein, O. Mitotic modulation of translation elongation factor 1 leads to hindered tRNA delivery to ribosomes. *J. Biol. Chem.* **2011**, *286* (32), 27927–35.

(71) Palen, E.; Venema, R. C.; Chang, Y. W.; Traugh, J. A. GDP as a regulator of phosphorylation of elongation factor 1 by casein kinase II. *Biochemistry* **1994**, *33* (28), 8515–20.

(72) Sheu, G. T.; Traugh, J. A. Recombinant subunits of mammalian elongation factor 1 expressed in Escherichia coli. Subunit interactions, elongation activity, and phosphorylation by protein kinase CKII. *J. Biol. Chem.* **1997**, *272* (52), 33290–7.

(73) Sheu, G. T.; Traugh, J. A. A structural model for elongation factor 1 (EF-1) and phosphorylation by protein kinase CKII. *Mol. Cell. Biochem.* **1999**, *191* (1), 181–6.

(74) Linding, R.; Jensen, L. J.; Ostheimer, G. J.; van Vugt, M. A.; Jørgensen, C.; Miron, I. M.; Diella, F.; Colwill, K.; Taylor, L.; Elder, K.; Metalnikov, P.; Nguyen, V.; Pasculescu, A.; Jin, J.; Park, J. G.; Samson, L. D.; Woodgett, J. R.; Russell, R. B.; Bork, P.; Yaffe, M. B.; Pawson, T. Systematic discovery of in vivo phosphorylation networks. *Cell* **2007**, *129* (7), 1415–26.

(75) Linding, R.; Jensen, L. J.; Pasculescu, A.; Olhovsky, M.; Colwill, K.; Bork, P.; Yaffe, M. B.; Pawson, T. NetworKIN: a resource for exploring cellular phosphorylation networks. *Nucleic Acids Res.* **2008**, *36* (Database issue), D695–9.

(76) Perez, D. I.; Gil, C.; Martinez, A. Protein kinases CK1 and CK2 as new targets for neurodegenerative diseases. *Med Res Rev* **2010**in press.

(77) Pierre, F.; Chua, P. C.; O'Brien, S. E.; Siddiqui-Jain, A.; Bourbon, P.; Haddach, M.; Michaux, J.; Nagasawa, J.; Schwaebe, M. K.; Stefan, E.; Vialettes, A.; Whitten, J. P.; Chen, T. K.; Darjania, L.; Stansfield, R.; Bliesath, J.; Drygin, D.; Ho, C.; Omori, M.; Proffitt, C.; Streiner, N.; Rice, W. G.; Ryckman, D. M.; Anderes, K. Pre-clinical characterization of CX-4945, a potent and selective small molecule inhibitor of CK2 for the treatment of cancer. *Mol. Cell. Biochem.* **2011**, DOI: 10.1007/s11010-011-0956-5.

(78) Schneider, C. C.; Hessenauer, A.; Montenarh, M.; Götz, C. p53 is dispensable for the induction of apoptosis after inhibition of protein kinase CK2. *Prostate* **2010**, *70* (2), 126–34.

(79) Zien, P.; Bretner, M.; Zastapilo, K.; Szyszka, R.; Shugar, D. Selectivity of 4,5,6,7-tetrabromobenzimidazole as an ATP-competitive potent inhibitor of protein kinase CK2 from various sources. *Biochem. Biophys. Res. Commun.* **2003**, *306* (1), 129–33.

(80) Mizuarai, S.; Yamanaka, K.; Itadani, H.; Arai, T.; Nishibata, T.; Hirai, H.; Kotani, H. Discovery of gene expression-based pharmacodynamic biomarker for a p53 context-specific anti-tumor drug Wee1 inhibitor. *Mol. Cancer* **2009**, *8*, 34.

(81) Morandell, S.; Stasyk, T.; Grosstessner-Hain, K.; Roitinger, E.; Mechtler, K.; Bonn, G. K.; Huber, L. A. Phosphoproteomics strategies for the functional analysis of signal transduction. *Proteomics* **2006**, *6* (14), 4047–56.

(82) Thingholm, T. E.; Jensen, O. N.; Larsen, M. R. Analytical strategies for phosphoproteomics. *Proteomics* **2009**, *9* (6), 1451–68.