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Identification of novel CK2 kinase substrates using a versatile biochemical approach --Manuscript Draft--

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TITLE:

Identification of Novel CK2 Kinase Substrates Using a Versatile Biochemical Approach

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KEYWORDS:

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SUMMARY:

The objective of this protocol is to label, enrich, and identify substrates of protein kinase CK2 from a complex biological sample such as a cell lysate or tissue homogenate. This method leverages unique aspects of CK2 biology for this purpose.

ABSTRACT:

The study of kinase-substrate relationships is essential to gain a complete understanding of the functions of these enzymes and their downstream targets in both physiological and pathological states. CK2 is an evolutionarily conserved serine/threonine kinase with a growing list of hundreds of substrates involved in multiple cellular processes. Due to its pleiotropic properties, identifying and characterizing a comprehensive set of CK2 substrates has been particularly challenging and remains a hurdle in the study of this important enzyme. To address this challenge, we have devised a versatile experimental strategy that enables the targeted enrichment and identification of putative CK2 substrates. This protocol takes advantage of the unique dual co-substrate specificity of CK2 allowing for specific thiophosphorylation of its substrates in a cell or tissue lysate. These substrate proteins are subsequently alkylated, immunoprecipitated, and identified by liquid chromatography/tandem mass spectrometry (LC-MS/MS). We have previously used this approach to successfully identify CK2 substrates from *Drosophila* ovaries and here we extend the application of this protocol to human glioblastoma cells, illustrating the adaptability of this method to investigate the biological roles of this kinase in various model organisms and experimental systems.

INTRODUCTION:

Protein kinases are key components of signal transduction cascades. Phosphorylation of

substrate proteins by these enzymes elicits biological responses that regulate critical events controlling cell division, metabolism, and differentiation, among others. CK2 is a ubiquitously expressed, acidophilic serine/threonine kinase that is conserved from yeast to humans and that plays important roles in many cellular processes ranging from transcriptional regulation to cell cycle progression to apoptosis¹⁻³. The enzyme is a heterotetramer composed of two catalytic α (or α') subunits and two regulatory β subunits⁴. In addition to being highly pleiotropic, CK2 exhibits two other unusual characteristics that complicate its analysis, namely constitutive activity⁵ and dual co-substrate specificity⁶. This latter property endows CK2 with the ability to use GTP as well as ATP for phosphorylation of substrate proteins.

Genetic deletion of the catalytic or regulatory subunits of CK2 in mice results in embryonic lethality indicating that it plays crucial roles during development and organogenesis^{7,8}. CK2 is also overexpressed in several types of cancer and thus represents a promising therapeutic target⁹⁻¹¹. Indeed, specific inhibitors that target CK2 kinase activity are currently under investigation for this purpose¹²⁻¹⁴. While inhibition of CK2 is a viable option, given its pleiotropic nature, an alternative and perhaps more rational approach would be to target critical CK2 substrates that underlie the progression of certain cancers. Therefore, the comprehensive identification and characterization of CK2 substrate proteins would be of significant benefit for elucidating the specific function(s) of this kinase within a particular tissue or tumor type.

Here, we describe a versatile biochemical method for identifying CK2 substrates from a complex biological sample such as a cell or tissue lysate. This protocol takes advantage of the dual co-substrate specificity of CK2 by use of the GTP analogue GTP γ S (guanosine 5'-[γ -thio]triphosphate) that other endogenous kinases cannot use. This effectively allows the kinase to "label" its substrates within this sample for subsequent isolation and identification.

PROTOCOL:

NOTE: Ensure that the required materials are available and properly prepared (see **Table of Materials**).

1. Preparation

1.1. Mechanically lyse tissue sample (1-2 mg of tissue in 100 μ L of lysis buffer, **Table 1**) or cultured cells (10 cm plate that is 80-90% confluent in 350 μ L of lysis buffer), with the goal being to collect a total of 900 μ L of sample for the experiment. Note that this volume is in slight excess of what is required for the experiment described below.

1.2. Spin down the samples by centrifugation at 17,500 $\times g$ for 3 minutes at 4 °C. Upon completion, transfer 270 μ L of the supernatant to each of three new 1.7 mL microcentrifuge tubes. There will be approximately 90 μ L remaining. Remove 40 μ L to be used as an "input control" and place in a new tube. Place all samples on ice.

2. Kinase assay: thiophosphorylation and alkylation

2.1. Label the three tubes containing 270 μL each as follows: "kinase rxn", "GTP γ S only", and "PNBM (p-nitrobenzyl mesylate) only". Prepare the kinase reactions.

2.1.1. To the "kinase rxn" tube, add 2.7 μL (equivalent to 1,350 U) of CK2, then add 2.7 μL of 2.5 mM GTP γ S.

2.1.2. To the "GTP γ S only" tube, add 2.7 μL of 2.5 mM GTP γ S, and then add 2.7 μL of lysis buffer.

2.1.3. To the "PNBM only" tube, add 5.4 μL of lysis buffer. Flick all tubes to mix and then immediately place on ice.

2.2. Incubate all three tubes for 1 min in a 30 $^{\circ}\text{C}$ water bath. Following incubation, add 13.5 μL of 12 mg/mL PNBM to all three tubes. Invert to mix samples. Incubate these samples at room temperature for 1 h.

2.3. After starting the incubation in step 2.2, prepare the desalting columns as soon as possible as the process takes approximately 45 min.

3. Preparation of desalting columns

3.1. Initially prepare columns (3 for this example experiment) by inverting each column several times to re-suspend the Sephadex G-25 resin in the storage buffer. Allow the resin to settle by attaching each column to a clamp stand and letting it sit undisturbed for approximately 5 min.

3.2. Following settling of the Sephadex G-25 resin, remove the caps from both the top and bottom of the column to allow the storage buffer to drain by gravity and have a tube placed below the bottom opening to collect the flow-through for discard.

3.3. Once storage buffer is depleted, add approximately 2.7 mL of lysis buffer in order to equilibrate the columns. Collect the flow-through and discard. Repeat 3 times. Following the final equilibration, the columns are ready for step 4.1.

4. Removal of PNBM

4.1. After the 1 h incubation (step 2.2) and column preparation (step 3) are complete, apply the samples to the columns. Label each column as follows: "kinase rxn", "GTP γ S only", and "PNBM only". Load all of each sample onto its respective column. Collect and discard the flow-through.

131 4.2. Wash samples by adding 420 μ L of lysis buffer to each column. Allow lysis buffer to filter
132 through the column and collect the flow-through for discard. Following this wash step, place
133 tubes in position for collection of samples.

135 4.3. Elute samples by adding 500 μ L of lysis buffer to each column. Collect the flow-through
136 that now contains thiophosphorylated and alkylated CK2 substrates.

138 5. Immunoprecipitation: Part I

140 5.1. Prepare samples for immunoprecipitation by first removing 80 μ L for an "elution input
141 control" sample from each of the respective elutions ("kinase rxn", "GTP γ S only", and "PNBM
142 only") collected in step 4.3. Following removal of 80 μ L from each sample, there will be
143 approximately 420 μ L remaining per sample.

145 5.1.1. Split each sample into 2 tubes containing 200 μ L each. Label each respective tube:
146 "kinase rxn anti-thiophosphate ester", "kinase rxn IgG", "GTP γ S anti-thiophosphate ester",
147 "GTP γ S IgG", "PNBM anti-thiophosphate ester", and "PNBM IgG".

149 5.2. Add 2.8 μ g of anti-thiophosphate ester antibody to each of the anti-thiophosphate
150 ester-labeled tubes and 2.8 μ g of isotype control antibody to each of the IgG-labeled tubes.
151 Place tubes on a rotator at 4 $^{\circ}$ C for 2 h.

153 5.3. Begin preparation of protein A/G bead during the last 15 min of the 2 h incubation in
154 step 5.2.

156 6. Protein A/G agarose bead preparation

158 6.1. Briefly vortex the storage tube to ensure beads are completely re-suspended. Cut off
159 the end of a P200 pipette tip using a clean razor blade in order to increase gauge size. Pipet 100
160 μ L of the bead slurry per immunoprecipitation into a new 1.7 mL microcentrifuge tube. For this
161 example, a total of 6 tubes are needed.

163 6.2. Centrifuge the tubes at 17,500 $\times g$ for 1 min at 4 $^{\circ}$ C. Remove the supernatant and
164 discard. Re-suspend the beads in 200 μ L of lysis buffer and briefly vortex. Repeat the spin and
165 wash steps 3 times.

167 6.3. Following the final wash, place the beads on ice until the incubation in step 5.2 is
168 complete.

170 7. Immunoprecipitation: Part II

172 7.1. After the 2 h incubation from step 5.2, spin down the samples at 17,500 $\times g$ for 3 min at
173 4 $^{\circ}$ C. Following centrifugation add 200 μ L from each sample to the tubes with the washed
174 beads. Place the tubes on a rotator at 4 $^{\circ}$ C for 1 h.

175
176 7.2. Following the 1 h incubation, centrifuge the tubes at 17,500 x g for 1 min at 4 °C. Next
177 remove 40 µL of supernatant from each sample and save as a “depletion control” (total 6
178 tubes). Remove the remainder of the supernatant and discard. Take care not to disturb the
179 beads.

180
181 7.3. Wash the samples by adding 200 µL of lysis buffer and vortexing briefly. Then centrifuge
182 at 17,500 x g for 1 min at 4 °C. Remove the supernatant and discard. Repeat the wash and spin
183 steps 3 times. Take care not to disturb the beads during these steps.

184
185 7.4. After completing the wash steps, add 50 µL of 2x sample buffer to each sample
186 containing beads. For all other samples, add 8 µL of 6x sample buffer: “input control”, “elution
187 input controls”, and “depletion controls”.

188
189 7.5. Once buffer is added to the tubes, pipet up and down to mix, and heat all samples at 95
190 °C for 5 min before proceeding with SDS-PAGE.

191 192 8. Analysis/Validation of results

193
194 8.1. Validate successful CK2-dependent thiophosphorylation and alkylation.

195
196 8.1.1. To determine the efficacy of CK2-mediated thiophosphorylation in step 2.2, perform SDS-
197 PAGE and Western blotting by running 15-20 µL of the “elution input controls” collected in step
198 5.1 on a 12.5% polyacrylamide gel.

199
200 8.1.2. Probe membranes with the following antibodies: anti-thiophosphate ester, anti-CK2α,
201 and anti-GAPDH (or other appropriate loading control). If this step was successful, an enhanced
202 anti-thiophosphate ester signal should be apparent in the “kinase rxn” lane compared to the
203 other two lanes (**Figure 2**).

204
205 8.2. Visualize enriched putative substrates of CK2 and determine protein identity.

206
207 8.2.1. To assess if the immunoprecipitation steps were successful, run 25-30 µL of the samples
208 eluted from the beads in step 7.4 on a separate 12.5% polyacrylamide gel. Ensure that all
209 equipment is clean and wear gloves at all times during this step to minimize contamination.

210
211 8.2.2. Stain the gel with Coomassie blue to visualize enriched proteins from various stages of
212 the experimental protocol (**Figure 3**). Using new razorblades, carefully excise unique bands
213 present in the “kinase rxn anti-thiophosphate ester IP” lane, noting their approximate
214 molecular weights.

215
216 8.2.3. Submit these bands for protein identification by liquid chromatography/tandem mass
217 spectrometry (LC-MS/MS) (**Figure 4**). If antibodies directed against the identified proteins are

available, confirm the results of mass spectrometry by SDS-PAGE and immunoblotting of input, depleted, and IP fractions collected during the course of the protocol (**Figure 4**).

REPRESENTATIVE RESULTS:

A schematic diagram of the experimental procedure is provided in **Figure 1**. The underlying basis of the technique is the unusual ability of CK2 to use GTP for phosphoryl group transfer. Addition of exogenous CK2 holoenzyme along with the GTP analogue, GTP γ S, to a cell lysate results in thiophosphorylation of endogenous CK2 substrates. Subsequent treatment of the lysate with the alkylating reagent *p*-nitrobenzyl mesylate (PNBM) generates a thiophosphate ester moiety on these specific substrate proteins that can then be immunoprecipitated using an anti-thiophosphate ester antibody and ultimately identified by mass spectrometry. **Figure 2** depicts a positive result following the addition of CK2 and GTP γ S and then PNBM to T98G (glioblastoma) cell lysate. These results demonstrate that CK2-dependent thiophosphorylation and subsequent alkylation were successful. As expected, an enhanced anti-thiophosphate ester signal by Western blotting is observed only in the lane containing the complete kinase reaction and not in the GTP γ S only- and PNBM only-treated samples. Shown in **Figure 3** is a Coomassie blue-stained gel of the immunoprecipitated and eluted proteins using isotype control IgG or anti-thiophosphate ester antibodies in the presence or absence of excess CK2 and/or GTP γ S. These data also demonstrate a positive result as multiple unique bands are evident only in the anti-thiophosphate ester IP lane in which the lysate was incubated with exogenous CK2 and GTP γ S. The band indicated with an asterisk was excised from the gel and submitted for protein identification by mass spectrometry. **Figure 4** illustrates representative data obtained by mass spectrometric analysis including protein identification, percent coverage, and number of unique peptides identified per protein within the band. Shown are the top ten hits from the submitted band (**Figure 3**) and information regarding whether or not the protein has been previously identified as a substrate of CK2¹⁵⁻¹⁷. The identity of one of the known immunoprecipitated CK2 substrates, nucleolin¹⁵, was confirmed by SDS-PAGE and immunoblotting of the indicated fractions using an anti-nucleolin antibody.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the experimental strategy. The GTP analogue, GTP γ S, along with excess recombinant CK2 holoenzyme is added to a cell or tissue lysate and allows for thiophosphorylation of substrates by CK2 but not by other endogenous kinases. Thiophosphorylated substrates are next alkylated with PNBM, generating a thiophosphate ester moiety on these proteins, which are then captured via immunoprecipitation (IP) for subsequent identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Figure 2: Validation of CK2-dependent thiophosphorylation in whole cell lysate. Whole cell lysates prepared from T98G cells were incubated with GTP γ S in the presence (kinase reaction) or absence (GTP γ S only) of exogenous recombinant CK2 holoenzyme. PNBM was subsequently added to the indicated reactions, and samples were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Protein molecular weight markers are indicated in kDa.

Figure 3: Immunoprecipitation of putative CK2 substrate proteins. Enrichment and visualization

of putative CK2 substrates (third lane) is evident as multiple unique bands following immunoprecipitation with anti-thiophosphate ester antibodies. Immunoprecipitates were resolved by SDS-PAGE and the gel was stained with Coomassie blue. The band marked with an asterisk was excised from the gel and submitted for protein identification by mass spectrometry. Protein molecular weight markers are indicated in kDa. IP=immunoprecipitation.

Figure 4: Identification and confirmation of proteins as substrates of CK2 in vitro. Data obtained by mass spectrometry demonstrates that both previously known¹⁵⁻¹⁷ as well as putative novel CK2 substrates were identified using this experimental approach. Shown are the top ten proteins identified from the excised band (top). The identity of nucleolin, a known CK2 substrate, was confirmed by immunoblotting of the indicated fractions using an anti-nucleolin antibody (bottom). Protein molecular weight markers are indicated in kDa. IP=immunoprecipitation.

Table 1. Lysis buffer recipe.

DISCUSSION:

Here, we describe a relatively simple biochemical method for identifying substrates of protein kinase CK2 from a complex biological sample. The critical steps of this protocol are based on the unusual enzymatic properties of CK2 and include CK2-dependent thiophosphorylation of specific substrate proteins using GTP γ S and their subsequent immunoprecipitation and identification. With these results, we have demonstrated the utility and versatility of this approach as we have now applied this strategy in both human glioblastoma cells and *Drosophila* ovaries¹⁸.

A number of previously published studies using quantitative phosphoproteomics approaches have indeed proven successful in identifying novel CK2 substrates¹⁹⁻²³. However, some of these strategies make use of immobilized substrate arrays, and it is possible that the conformation of an immobilized protein may render a potential phosphorylation site inaccessible to the kinase. The technique described here permits phosphorylation within a more physiological or native environment (i.e., a cell lysate), thereby reducing the probability of site inaccessibility. Another benefit of this strategy is that once protein identification is determined by mass spectrometry, validation of putative CK2 substrates can easily be performed on samples collected during the procedure if antibodies directed against the substrate protein(s) of interest are available. For example, using standard western blotting, one should observe a reduction in the level of the relevant protein in the depleted (post-IP) samples and its presence in the anti-thiophosphate ester immunoprecipitates as we have demonstrated for the known CK2 substrate nucleolin (**Figure 4**).

A notable limitation of this method is that the final step of the procedure prior to analysis by mass spectrometry relies on the ability to discern discrete differences in banding pattern on a gel. Thus, it is certainly possible that specific CK2 substrates may be missed if they are of low abundance and therefore below the limit of visual detection. If one is concerned about this possibility, a more sensitive method such as silver staining can be used to visualize these proteins instead of using Coomassie blue. An additional consideration that should be acknowledged is that a number of CK2 substrates may not be identified using this strategy since the physiologically

relevant sites will already be phosphorylated in vivo. This is almost certainly to be the case given the constitutive activity of CK2. Finally, it should also be noted that this methodology only identifies proteins as putative substrates of CK2 in vitro. Subsequent assays to validate that these are physiologically relevant substrates of CK2 in vivo are required and should entail identification of CK2-dependent phosphorylation sites and assessing if phosphorylation of these particular residues is altered in response to manipulation of CK2 kinase activity.

In summary, this strategy coupled with downstream experimental approaches (phosphorylation site mapping by truncation/deletion analysis, site-directed mutagenesis, functional in-vitro and in-vivo assays, etc.) will facilitate the study of this unusual kinase and will increase our understanding of the various roles that CK2 plays in multiple biological systems.

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DISCLOSURES:

The authors have nothing to disclose.

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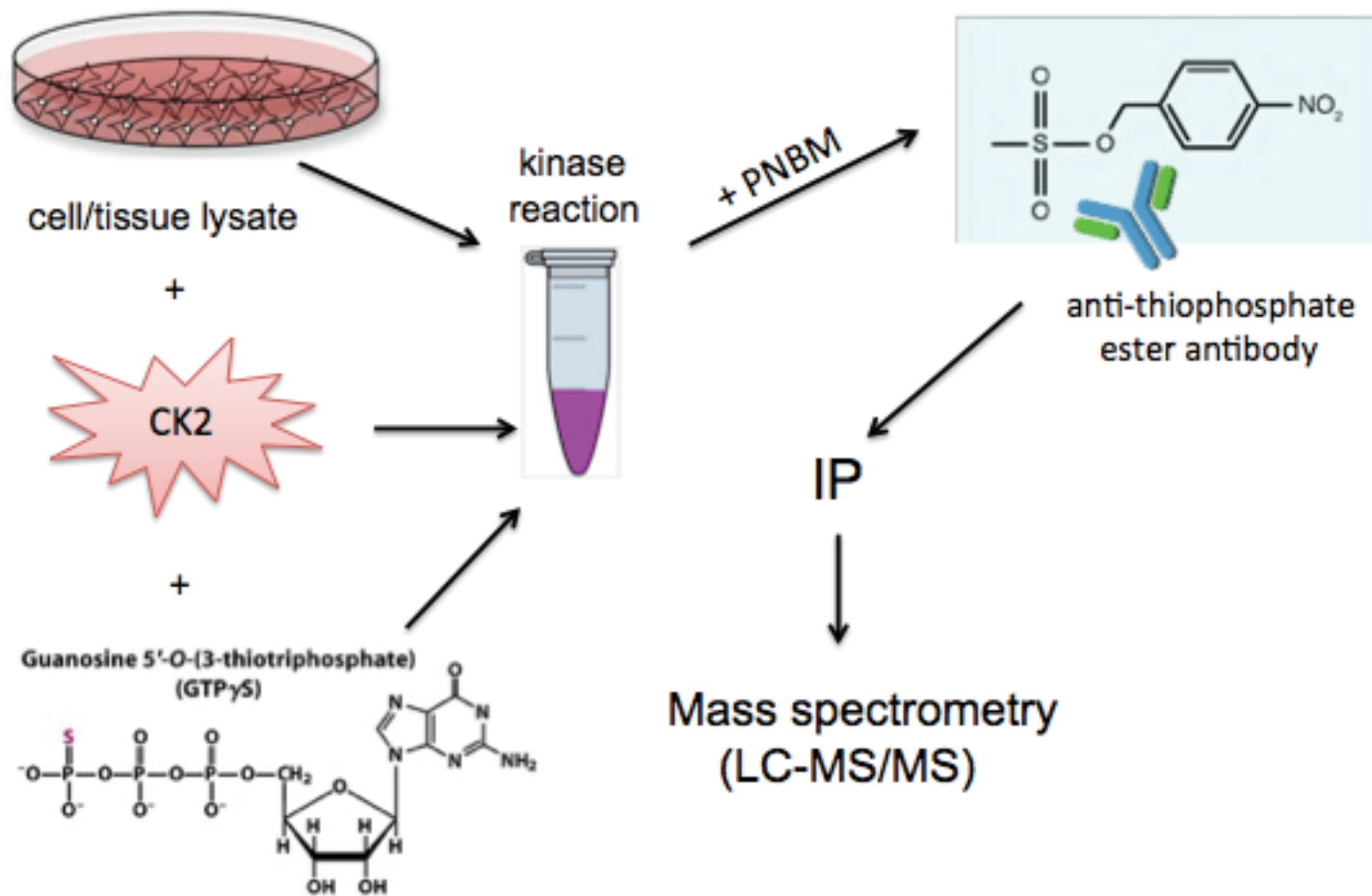
Figure 1

Figure 2

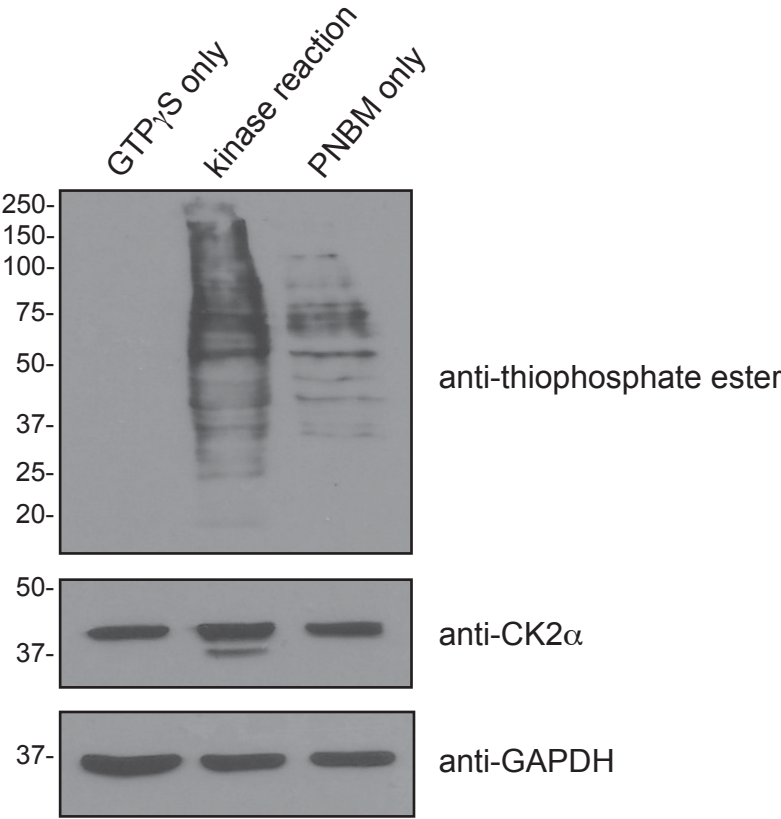


Figure 3

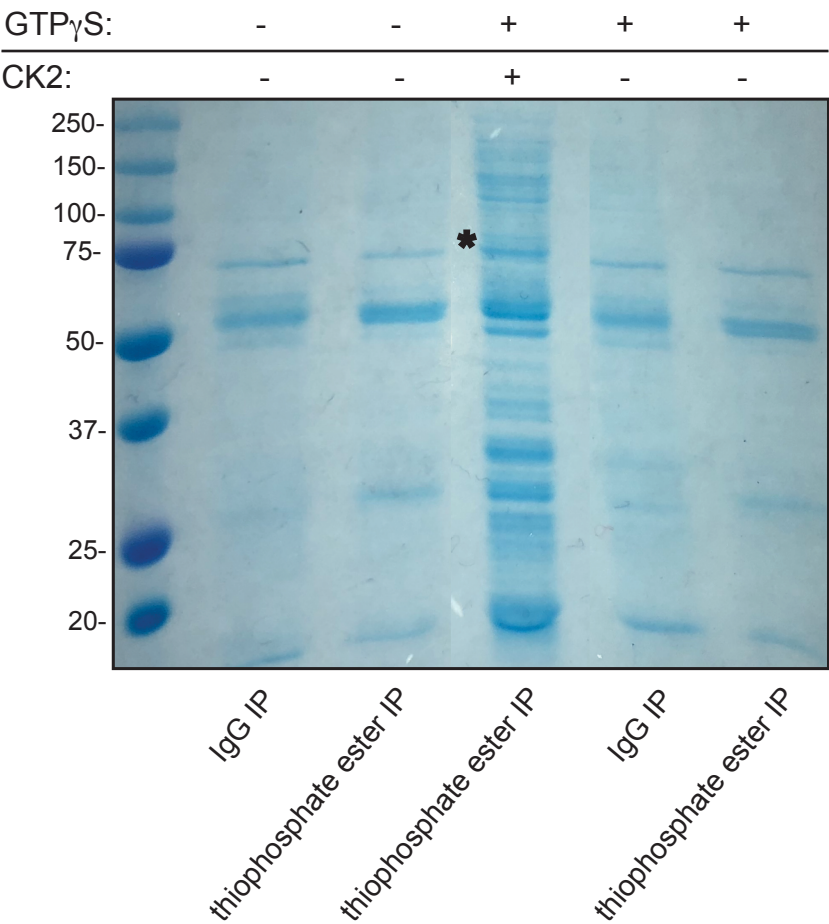
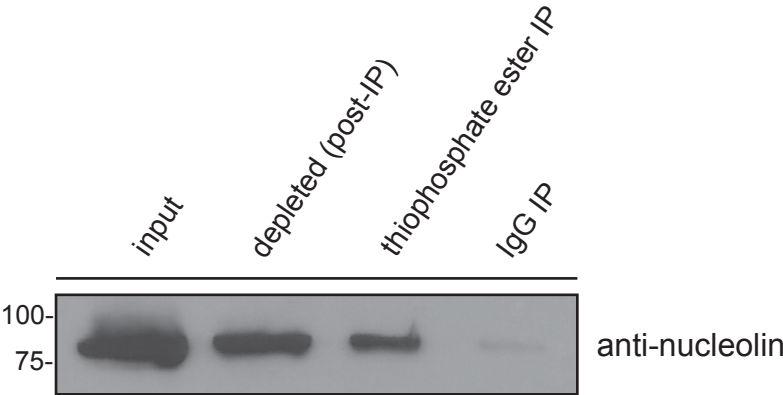


Figure 4

Identified Proteins	Percent Coverage	# Of Unique Peptides Identified	Known CK2 Substrate?
Elongation factor 2	60%	154	
N-alpha-acetyltransferase 15, NatA auxiliary subunit	50%	94	
Nucleolin	50%	88	✓
Interleukin enhancer-binding factor 3	41%	74	
Heat shock protein HSP 90-alpha	44%	71	✓
Heat shock protein HSP 90-beta	49%	71	✓
Eukaryotic translation initiation factor 4 gamma 2	45%	71	
Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	44%	69	
Nucleolar RNA helicase 2	51%	64	
NF-kappa-B-repressing factor	53%	63	



Lysis Buffer Recipe (10 mL, 1X)

<i>Reagent</i>	<i>Stock Concentration</i>	<i>Reagent</i>	<i>Final Concentration</i>
	1 M Tris pH 7.4		20.0 mM
	4 M NaCl		20.0 mM
	20% Triton X-100		0.50%
	1 M MgCl ₂		10.0 mM
	1 M DTT		0.5 mM
	200 mM Na ₃ VO ₄		1.0 mM
	500 mM NaF		10.0 mM
	500 mM β-glycerol phosphate		10.0 mM
	ddH ₂ O		
	+1 miniComplete tab/10 mL		

Example volumes added based on stock concentrations

200 mL

50 mL

250 mL

100 mL

5 mL

50 mL

200 mL

200 mL

8.945 mL

1 tablet

Materials Needed

12 mg/mL PNBM

2.5 mM GTP γ S

Anti-CK2 α (E-7) mouse monoclonal antibody

Anti-GAPDH (6C5) mouse monoclonal antibody

Anti-nucleolin rabbit polyclonal antibody

Anti-thiophosphate ester [51-8] rabbit monoclonal antibody

Centrifuge pre-set to 4 °C

cOmplete Mini EDTA-Free Protease Inhibitor

Lysis Buffer

Normal rabbit IgG antibody (isotype control)

PD MiniTrap Column

Protein A/G Plus Agarose Beads

Recombinant human CK2 holoenzyme

Rotator

T98G human glioblastoma cells

Water bath pre-set to 30 °C

Company/Catalog Number

Abcam: ab138910

Sigma-Aldrich: G8634-1MG

Santa Cruz Biotechnology: sc-373894

Santa Cruz Biotechnology: sc-32233

Abcam: ab22758

Abcam: ab92570

ThermoScientific: Sorvall Legend Micro 21R Cat# 75-772-436

Roche: #11836170001

See recipe below

Cell Signaling Technology: #2729S

GE Healthcare: #28-9180-10

Santa Cruz Biotechnology: sc-2003

New England Biolabs: #P6010S

Labnet: Mini Labroller SKU# H5500

ATCC: CRL-1690

Shel Lab: H20 Bath Series Model# SWB15

Approximate Volume/Concentration

40.5 mL

5.4 mL

1:1000 for Western blotting

1:1000 for Western blotting

1:1000 for Western blotting

Varies (final concentration 2.8 mg for each sample)

30 mL

Varies (final concentration 2.8 mg for each sample)

3 columns

600 mL

2.7 mL



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Article Title: **Identification of novel CK2 kinase substrates using a versatile biochemical approach**
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We sincerely thank the reviewers for their constructive comments on our manuscript. We have incorporated their suggestions into a revised version of the manuscript that we believe is much improved.

Comments from Reviewer #1

Major Concerns

- 1) The last step of the new methodology is missing. Identification by LC-MS/MS of putative CK2 substrates in at least one unique band should be illustrated.**

This is a critical point (also raised by Reviewer #3) and we have now included data in a new figure (Figure 4) demonstrating that several previously identified CK2 substrates as well as some potential novel substrates were indeed identified using the described method. In addition, we have included confirmation by Western blot analysis for one known CK2 substrate protein (nucleolin), demonstrating that it was specifically thiophosphorylated and subsequently immunoprecipitated.

- 2) The reviewer questions to what extent endogenous CK2 contributes to the overall phosphorylation of endogenous substrates and suggests that this contribution may vary depending on the tissue/cell type used. Likewise, it is expected that free -SH groups will be alkylated and contribute to the cross-reactivity disclosed in Figure 2.**

The reviewer is correct on both accounts. It is almost certain that endogenous CK2 contributes to the phosphorylation of endogenous substrates and that this likely varies between cell and tissue types. Furthermore, as the reviewer surmises, the background signal observed in Figure 2 is likely due to alkylation of free thiol groups on various proteins.

- 3) The reviewer is concerned whether previous/variable occupancy of CK2 phospho-sites interferes with the methodology and asks whether dephosphorylation of proteins prior to the addition of exogenous CK2 was attempted in order to determine if the results of the experiment would be altered.**

While we have not directly tested if dephosphorylation of proteins prior to the addition of CK2 would change the outcome of the experiment, we predict (as probably the reviewer does as well) that it would. This is therefore a drawback to the methodology and may result in some CK2 substrates not being identified via this strategy. We have added a sentence acknowledging this limitation in the revised Discussion section. This same comment was also made by Reviewer #3.

- 4) In the Discussion section, an important limitation of the new methodology should be highlighted: the identified thiophosphorylated proteins are thus far only in vitro substrates of CK2. The actual occurrence of their phosphorylation in vivo remains to be demonstrated.**

We agree with the reviewer, and we suggest that this approach simply provides an initial platform for identifying potential novel CK2 substrates. Subsequent validation of these proteins as *bona fide* CK2 substrates in cell-based or in-vivo assays is absolutely essential and we have highlighted this point in the Discussion section.

Minor Concerns

It would be advisable to include a list of abbreviations used.

We have made sure to fully define all abbreviations the first time they are used in the manuscript.

While mentioning previous studies using quantitative phosphoproteomics approaches, a reference describing the strategy of knocking out both CK2 catalytic subunits (Franchin *et al.*, 2018, *Cell. Mol. Life Sci.*) should also be cited.

We thank the reviewer for catching this omission. The suggested additional reference is now cited appropriately within the manuscript and in the References section.

Comments from Reviewer #3

Major Concerns

- 1) **The reviewer states that the results, as shown, are promising but preliminary. The overall impact of this manuscript is diminished because it is unclear whether the method, as presented, has been used successfully to identify CK2 substrates (either known or novel) and whether sufficient protein has been thiophosphorylated and isolated to enable identification by mass spectrometry.**

This is definitely an important point, shared by Reviewer #1 (see comment 1 above), and we believe this concern is now adequately addressed with the inclusion of the data in Figure 4.

- 2) **The reviewer comments that certain aspects of the experimental procedure are described qualitatively instead of quantitatively. For instance, the amount of CK2 enzymatic activity should be indicated rather than listing the volume of CK2 enzyme added to the reaction.**

We have now listed the activity of the enzyme (in U) that corresponds to the volume of CK2 used in the kinase reactions.

- 3) **To ensure that the substrates identified are physiologically relevant substrates of CK2, the manuscript should consider validation strategies that include the demonstration that sites of thiophosphorylation are actual phosphorylation sites in living cells (and are not simply sites that are phosphorylated when there is an excess of CK2 in a cell lysate).**

This is also a concern expressed by Reviewer #1 (see comment 4 above). These types of validation studies are now highlighted in the Discussion section as being essential follow-up experiments to demonstrate that an identified substrate of CK2 is physiologically relevant.

- 4) **Similar to point #3, the utility of this method would be increased if the authors could demonstrate that specific sites of thiophosphorylation/phosphorylation could be identified.**

We likewise agree and in the Discussion section propose identification of specific CK2 phosphorylation sites as logical next steps in the workflow.

- 5) **The reviewer comments that, given the constitutive activity of CK2, it is possible that a number of CK2 substrates will not be identified using this strategy since the physiologically relevant sites will already be fully occupied in cell lysates.**

The reviewer is absolutely correct, and this issue was also raised by Reviewer #1 as a potential drawback of the approach (see comment 3 above). We have therefore added a sentence to the Discussion section that describes this particular scenario as a limitation of the methodology.

Minor Concerns

Column Preparation: It would be helpful to the reader to identify the specific resin that is used here.

The type of resin (Sephadex G-25 desalting resin) is now indicated.

Figure 2: It is unclear why all lanes show relatively similar levels of CK2 by immunoblotting since excess CK2 was not added to all lanes.

This discrepancy is most likely due to a dilutional effect. The kinase was diluted 100-fold in the reaction volume, and then only a small fraction of this was loaded onto the gel. The anti-CK2 α antibody may not be sensitive enough to detect this minor difference in protein levels.

Table of Materials: What is the concentration of the stock lysis buffer?

The recipe listed in the table is to generate 10 ml of 1X lysis buffer.