Regulation of CK2 by Phosphorylation and O-GlcNAcylation Revealed by Semisynthesis

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Supplementary Materials
Supplementary Results: Tables, Figures,
Legend for Supplementary Dataset 1 (separate excel file)
Supplementary Methods
**Supplementary Results**

**Supplementary Table 1: Specific activities for CK2α kinases.** To evaluate the effects of the truncation and G342C mutation in the prepared semisynthetic proteins, kinase assays were performed with the recombinant full-length WT, recombinant full-length G342C mutant, and semisynthetic unmodified CK2α proteins. The assay used is a continuous enzyme-coupled spectrophotometric assay that uses pyruvate kinase and lactic dehydrogenase, and monitors the disappearance of NADH (absorbance at 340nm) as an indicator of CK2α kinase activity.

| Enzyme                              | V/[E] (min\(^{-1}\))\(^{†}\) |
|-------------------------------------|--------------------------------|
| Full-length WT CK2α (aa 1-391)      | 170 ± 5                        |
| Full-length G342C CK2α\(^{‡}\) (aa 1-391) | 170 ± 9                        |
| unmodified CK2α\(^{‡}\) (semisynthetic aa 1-365) | 150 ± 4                        |

\(^{†}\)Specific activities were measured with 2.2 mM ATP and 400μM peptide substrate.

\(^{‡}\)These enzymes contain a Gly→Cys mutation at ligation site.
Supplementary Table 2. Statistical analysis of validity of protein microarrays identifying known CK2 substrates based on relative signal intensities. We compiled a list of validated CK2 substrates from the literature\(^1\,^2\). For those validated substrates that are also on our protein microarray chip, signal intensities are significantly higher than all the proteins printed on the chips in all the experiments, except for the negative control experiments. For these comparisons, a t test was used to compute p values and false discovery rate (FDR) was used to correct multiple testing. P value being less than 0.05 was defined as “significant”.

|                        | Adjusted p value |
|------------------------|------------------|
| S-GlcNAc CK2α + β (1)  | 0.005            |
| S-GlcNAc CK2α + β (2)  | 0.02             |
| Pfa CK2α + β (1)       | 0.01             |
| Pfa CK2α + β (2)       | 0.01             |
| Unmodified CK2α + β (1)| 0.01             |
| Unmodified CK2α + β (2)| 0.01             |
| S-GlcNAc CK2α alone (1)| 0.02             |
| S-GlcNAc CK2α alone (2)| 0.02             |
| Pfa CK2α alone (1)     | 0.02             |
| Pfa CK2α alone (2)     | 0.02             |
| Unmodified CK2α alone (1)| 0.01         |
| Unmodified CK2α alone (2)| 0.02         |
| No kinase              | 0.08             |
Supplementary Table 3. Statistical analysis of validity of protein microarrays identifying known CK2 substrates based on 3 SD cutoff. The chip assay recovered significant number of previous known substrates. Statistical significance was calculated using hypergeometric test and FDR was used to correct multiple testing. P value being less than 0.05 was defined as “significant”.

|                   | Adjusted p value |
|-------------------|------------------|
| S-GlcNAc CK2α + β (1) | 1.2x10^{-5}      |
| S-GlcNAc CK2α + β (2) | 0.008            |
| Pfa CK2α + β (1)    | 6.4x10^{-6}      |
| Pfa CK2α + β (2)    | 0.0005           |
| Unmodified CK2α + β (1) | 0.002         |
| Unmodified CK2α + β (2) | 1.5x10^{-5}    |
| S-GlcNAc CK2α alone (1) | 0.0005       |
| S-GlcNAc CK2α alone (2) | 0.007          |
| Pfa CK2α alone (1)  | 0.0002           |
| Pfa CK2α alone (2)  | 0.001            |
| Unmodified CK2α alone (1) | 9.0x10^{-5}    |
| Unmodified CK2α alone (2) | 0.0006        |
| No kinase          | 1                |
**Supplementary Table 4.** Protein microarray results for Pin1 effects on substrate selectivity. Gene names listed with common protein name in parentheses. See Supplementary Methods for criteria for positive hits.

| Substrate is blocked by phospho-dependent interaction with Pin1 | Substrate is enhanced by phospho-dependent interaction with Pin1 |
|---------------------------------------------------------------|---------------------------------------------------------------|
| DUSP7 (Dual specificity phosphatase 7),                      | EIF2AK1 (eukaryotic translation initiation factor 2-alpha kinase 1), |
| MTF2 (Metal-response element-binding transcription factor 2), | RASSF5 (Ras association RalGDS/AF-6 domain family 5 or Rap1-binding protein), |
| NIPBL (Nipped-B-homolog (delangin)),                         | FLVCR2 (feline leukemia virus subgroup C receptor-related protein 2) |
| EPN1 (Epsin 1),                                               |                                                               |
| MTL5 (Tesmin),                                                |                                                               |
| FAM89B (Mammary tumor virus receptor homolog 1),              |                                                               |
| LRFN1 (Synaptic adhesion-like molecule 2),                    |                                                               |
| THUMPD3 (THUMP domain-containing protein 3)                   |                                                               |
Supplementary Figure 1: Identification of O-GlcNAc modification site Ser347. (a) The majority of active CK2 from bovine brain specifically binds WGA-sepharose and is eluted by 0.5 M GlcNAc. CK2 activity was assayed using the CK2 peptide substrate RRREETEEE. (b) A bovine milk galactosyltransferase probe detects terminal GlcNAc residues on CK2α and α’ subunits but not the β subunit. Purified bovine brain CK2 was incubated with UDP-[³H]galactose and
galactosyltransferase for 24 h at 4 °C. Proteins were resolved by 12% SDS-PAGE and visualized by Coomassie staining and autoradiography. (c) Reversed-phase HPLC separation of CK2α CNBr fragments bearing O-GlcNAc. CK2 (0.1 mg) was digested with 200 mg of cyanogen bromide, O-GlcNAc-bearing peptides were radiolabeled with galactosyltransferase and UDP-[3H]galactose, and the peptides were fractionated by reversed-phase HPLC. (d) The major site of O-GlcNAcylation is Ser347. Peaks 1, 2, and 3 from panel c were further purified by additional rounds of RP-HPLC. Peak 1 contained multiple peptides which were not further resolved. Peaks 2 and 3 each yielded a single component and were subjected to gas-phase sequencing. Both peaks 2 and 3 yielded the identical sequence, PXXSTPVSSANh (where ‘X’ could not be determined from the gas phase sequencing and ‘h’ is homoserine lactone, the product produced from methionine upon CNBr digestion). This sequence corresponds to residues 340-351 of human CK2α. Manual Edman degradation of the purified, radiolabeled peptide showed that the saccharide is released at cycle 8, which corresponds to Ser347 in the CK2 sequence.
Supplementary Figure 2: MALDI-MS spectra for CK2α C-terminal tail peptides used in ligation reaction for semisynthesis. Peptides were purified by reversed-phase HPLC. (a) Unmodified CK2 peptide (expected mass, 2306 Da), positive mode. (b) S-GlcNAc-Ser347 CK2 peptide (expected mass, 2525 Da), positive mode. (c) Pfa344 CK2 peptide (expected mass, 2405 Da), negative mode. (d) pThr344 CK2 peptide (expected mass, 2384 Da), negative mode. (e) T344E CK2 peptide (expected mass, 2334 Da), positive mode.
Supplementary Figure 3: Coomassie stained SDS-PAGE of purified proteins. (a) Semisynthetic and recombinant CK2α proteins run on 12% gel. (b) Recombinant CK2β proteins run on 12% gel.
Supplementary Figure 4: Confirmation of CK2α semisynthetic proteins. Representative MALDI spectra of prepared semisynthetic proteins. Calculated MW (m/z) for N-terminal methionine-clipped CK2α proteins are: 43060 Da for unmodified; 43280 Da for SGlcNAc; and 43140 Da for phospho. Std error for these measurements +/- 100 Da.
Supplementary Figure 5: Localization of CK2α two hours following microinjection in the cytoplasm of REF52 cells. Cells were injected with CK2α or CK2α + β in the cytoplasm and returned to incubator for 2h. Following incubation, the cells were fixed and immunostained for HA-tagged CK2α.
Supplementary Figure 6: Cellular Stability of CK2α. Representative immunofluorescence staining of cells following microinjection of CK2α proteins.
Supplementary Figure 7: Cell cycle analysis by FACS for cells treated with Cdk inhibitors. HeLa cells were treated with 2 μM flavopiridol, 3 μM 9-cyanopaullone, or vehicle (DMSO) for 18 h, fixed with ethanol and stained with propidium iodide. (a) Representative chromatograms. (b) Bar graph indicating percent cells in different cell cycle stages. Error bars indicate the range for duplicate samples.
Supplementary Figure 8: Western blot for total CK2α protein in HeLa cells following pharmacological treatment. (a) Treatment with Cdk inhibitors. Full gel for blot shown in Figure 2d. (b) Treatment with nocodazole to arrest cells in mitosis. Full gel for blot shown in Figure 2e.
Supplementary Figure 9: Cell cycle analysis by FACS for nocodazole treated cells. HeLa cells were treated with 100 ng/mL nocodazole or vehicle (DMSO) for 24 h, fixed with ethanol and stained with propidium iodide. (a) Representative chromatograms. (b) Bar graph indicating percent cells in different cell cycle stages. Error bars indicate the range for duplicate samples.
Supplementary Figure 10: Pull-down with semisynthetic HA-CK2α and recombinant Pin1.

(a) CK2α was immobilized using anti-HA antibodies bound to protein G beads. Pin1 was incubated with immobilized CK2α for 20 min at 4°C. Full gel for blot shown in Figure 3a. (b) Pull-down experiments with Pfa344 HA-CK2α protein and recombinant Pin1 in the presence of anti-Pin1 or non-specific IgY. Full gel for blot shown in Figure 3c.
Supplementary Figure 11: Co-immunoprecipitation of endogenous Pin1 with CK2α. Pfa containing and unmodified HA-CK2α semisynthetic proteins were spiked into REF52 cell lysates and immobilized using anti-HA antibodies. Input samples are shown for loading control. Full gel for blot shown in Figure 3b.
Supplementary Figure 12: Relative CK2α protein levels over time following microinjection of Pfa344 CK2α with anti-Pin1 IgY. Representative immunofluorescence staining of cells following microinjection of CK2α proteins.
Supplementary Figure 13: Role of O-GlcNAc modification in CK2α. Phosphorylation of C-terminal tail CK2α peptides (residues 337 to 352) by Cdk1/cyclin B. In vitro Cdk1/cyclin B kinase assays were performed using the following peptide substrates: SSMPGGSTPVXSANMMK(εBiotin) where X = serine or S-GlcNAc-serine. After reactions were quenched, peptides were separated from reaction mixture on Tris-tricine gels. Representative phosphorimage of peptide substrates is shown.

Supplementary Figure 14: IP-Western blot for O-GlcNAc CK2α in HeLa cells following treatment with O-GlcNAcase inhibitor, TMG, for 12 h. Full gel for blot shown in Figure 4b.
Supplementary Figure 15: Western blot for total CK2α protein in HeLa cells following treatment with TMG for 12 or 16 h. Full gel for blot shown in Figure 4c (top left) and biological replicated for results shown in Figure 4c.
Supplementary Figure 16: CK2α phosphorylation of calmodulin. CK2α alone phosphorylates calmodulin and the presence of CK2β inhibits calmodulin phosphorylation. Reaction conditions: 200 μM ATP, 0.25 mg/mL calmodulin, 100 nM CK2α and various CK2β (0, 50, 100, 200 nM).
Supplementary Figure 17: Data analysis for human proteome microarray kinase assays. Histogram of signal intensities of all the proteins follows a normal distribution. Each duplicate experiment shown for unmodified CK2α alone, unmodified CK2α+β, GlcNAc CK2α alone, GlcNAc CK2α+β, Pfa CK2α alone, Pfa CK2α+β.
Supplementary Figure 18: Coomassie stained SDS-PAGE of purified protein substrates. (a) GST-tagged substrate proteins purified from yeast. (b) His6-tagged NIPBL purified from *E. coli*. 
Supplementary Figure 19: Phosphorimage analysis of in vitro CK2 kinase assays with AHCYL2 and NAP1L3 substrates. (a) 25 ng/μL AHCYL2 protein substrate with 100 μM ATP and 10 nM CK2α ± 11 nM CK2β for 6 min at 30 °C. Full gel for phosphorimage shown in Figure 5a. (b) 15 ng/μL NAP1L3 protein substrate with 100 μM ATP and 10 nM CK2α ± 11 nM CK2β for 6 min at 30 °C. Full gel for phosphorimage shown in Figure 5b.
Supplementary Figure 20: Comparison of pThr344 and T344E for CK2 substrate selectivity. Phosphorimage analysis of in vitro CK2 kinase assays with protein substrates. All reactions performed at 30 °C. Numbers indicate nM product for each kinase reaction. (a) NAP1L3 substrate (15 ng/μL) with 100 μM ATP and 5 nM CK2α ± 6 nM CK2β for 8 min. (b) ACHYL2 (25 ng/μL) with 100 μM ATP and 5 nM CK2α ± 6 nM CK2β for 8 min.
Supplementary Figure 21: Substrate selectivity for CK2. Phosphorimage analysis of in vitro CK2 kinase assays with protein substrates. All reactions performed at 30 °C with 100 μM ATP and 10 nM CK2α ± 11 nM CK2β. Numbers indicate nM product for each kinase reaction. (a) EEF2K (10 ng/μL) for 6 min. (b) WASPL substrate (10 ng/μL) for 18 min.
**Supplementary Figure 22: Substrate selectivity for CK2.** Phosphorimage analysis of in vitro CK2 kinase assays with protein substrates. All reactions performed at 30 °C with 100 μM ATP and 10 nM CK2α ± 11 nM CK2β. Numbers indicate nM product for each kinase reaction. (a) UBE2O substrate (22 ng/μL) for 6 min. (b) PCDL2 substrate (22 ng/μL) for 6 min.
**Supplementary Figure 23: Effect of Pin1 on CK2 activity with peptide substrate.** 100 μM ATP and various concentrations of optimized peptide substrate. (a) CK2α at 10 nM ± 25 nM Pin1. (b) 10 nM CK2α + 20nM CK2β ± 25nM Pin1.
Supplementary Figure 24: Data analysis for human proteome microarray kinase assays with Pin1. Histogram of signal intensities of all the proteins follows a normal distribution. Each duplicate experiment shown for unmodified CK2α alone, unmodified CK2α + Pin1, pThr CK2α alone, pThr CK2α + Pin1.
Supplementary Figure 25: Substrate selectivity for CK2α in the presence of Pin1.
Phosphorimage analysis of in vitro CK2 kinase assays with NIPBL protein substrate (50 ng/μL). All reactions performed at 30 °C for 5 min with 100 μM ATP and 5 nM CK2α ± 11 nM Pin1. Full gel for phosphorimage shown in Figure 5c.
Supplementary Figure 26: MALDI-MS spectra for substrate peptides used in kinase assays. Peptides were purified by reversed-phase HPLC. (a) Optimized peptide (expected mass, 1803 Da), positive mode. (b) Casein peptide (expected mass, 2192 Da), positive mode. (c) XRCC1 peptide (expected mass, 2261 Da), positive mode. (d) Cdk1 substrate peptide unmodified (expected mass, 1935 Da), positive mode. (e) Cdk1 substrate peptide with S-GlcNAc-Serine (expected mass, 2154 Da), positive mode.
Supplementary Figure 27: Cell permeability as a marker for viability following microinjection. Following microinjection with HA-CK2α recombinant protein, REF52 cells were allowed to incubate for 0-8 h before being treated with diacetyl fluorescein and propidium iodide. Viable cells take up diacetyl fluorescein and hydrolyze it to fluorescein, to which the cell membrane of viable cells is impermeable (green). Nonviable cells take up propidium iodide (red). Representative images for each time point are shown.
Supplemental Figure 28: Nuclear staining as a marker for cell viability following microinjection. Intact nuclei are used as a marker for cell viability following microinjection. HA-CK2α recombinant protein is injected into REF52 cells which are returned to incubator for 2 h at 37°C prior to being fixed and stained with DAPI and immunostained for HA-CK2α protein and lamin A. (a) Representative images obtained with ApoTome feature (100X). (b) Representative epifluorescence images (40X).
Description of Supplementary Dataset 1: Excel file with the relative signal intensities for individual protein substrates for each of the CK2 enzyme conditions are shown. Gene symbol and IOH ID (Invitrogen open-reading-frame ID numbers) are listed for each protein substrate.

Results of microarray array experiments are also deposited in the GEO database.
Supplementary Methods

Identification of O-GlcNAc site:
To follow CK2 during the purification from bovine brain, kinase activity was measured by incorporation of label from \([\gamma^{-32P}]ATP\) into the CK2 peptide substrate, RRREEETEEE, in a reaction mixture containing 24 mM MOPS, pH 7.6, 80 mM NaCl, 9 mM MgCl\(_2\) 200 µM \([\gamma^{-32P}]ATP\) (100- 250 µCi/ µmol). Reactions were started by the addition of \([\gamma^{-32P}]ATP\), incubated for 10 min at 30°C, and terminated by spotting sample onto P-81 phosphocellulose paper (Whatman). P-81 papers were washed in a flask shaking with seven changes of 75 mM H\(_3\)PO\(_4\), rinsed with acetone, and incorporation of \(^{32}\)P into substrate was determined using liquid scintillation spectrometry.

CK2 was purified from bovine brain (RJO Biologicals) by modification of a previously described procedure\(^3\). Briefly, 1 kg of bovine brain was homogenized in 1,500 mL of ice-cold buffer (40 mM Hepes, pH 7.6, 200 mM (NH\(_4\))\(_2\)SO\(_4\), 10% glycerol, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 10 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride) in a Waring blender. Homogenization was performed in the presence of 50 µM of PUGNAC (O-(2-acetamido-2-deoxy-D-glycopyranosylidene) amino-N-phenylcarbamate, Calbiochem) in order to inhibit endogenous hexosaminidases. The homogenate was centrifuged at 4°C for 20 min at 12,000 g, the supernatant was filtered through cheese cloth, and the pH was adjusted to 7.0 ± 0.1. All chromatographic steps were performed at 4°C. After successive chromatography over Heparin-Toyopearl and Polyethyleneimine Toyopearl, CK2 active fractions were pooled and dialyzed against 50 mM sodium phosphate buffer (pH 6.8). The sample was loaded onto a 2.5 cm-diameter column packed with 40 mL of P-11 phosphocellulose (Whatman) at flow rate of 120 mL/h. The column was washed with 100 mL of dialysis buffer, and a linear gradient of 0 - 2.0M NaCl was developed over 150 min at a flow rate of 2 mL/min. CK2 containing fractions were pooled and dialyzed to remove NaCl, then diluted with glycerol to 50% (v/v), and stored by aliquot at -20°C for one year without loss of activity.

CK2 purified from bovine brain was labeled with UDP-[6-\(^{3}\)H] galactose (Amersham) and galactosyltransferase (bovine milk galactosyltransferase from Sigma, pre-galactosylated in buffer containing 5mM MnCl\(_2\) as previously described\(^4\)). The CK2 sample was incubated with 2 µM of UDP-[6-\(^{3}\)H] galactose and 0.1 unit of galactosyltransferase for 2 d at 4°C in galactosyltransferase buffer. Galactosylated CK2 was resuspended in 1.0 mL of 0.1 M NaOH,
1.0 M NaBH₄ and incubated at 37°C for 16 h. Sample tubes were placed on ice and neutralized with cold 4 M glacial acetic acid. After neutralization, the tube was vortexed extensively and left on ice to allow trapped gas to escape. The sample was then centrifuged in a tabletop centrifuge to remove any debris. The supernatant was applied to a Sephadex G-50 column equilibrated in 0.1% SDS and 50 mM ammonium formate. The fractions were pooled and the SDS was removed by KCl precipitation and centrifugation. The supernatant was analyzed on a TSK-Fractogel HW-40 (1.5 x 200 cm) column in 200 mM ammonium acetate and 10% ethanol at 55°C. Dextran and galactose were used as standards (Dionex). A single tritium peak was collected and lyophilized. Next, the sample was resuspended in 50 µL of water and analyzed on CARBO-PAC-MA-1 column (Dionex) by high pH anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) in 0.3 M NaOH, at 0.4 mL/min according to the previously described method⁵. Each fraction was neutralized with 50 µL of HCl and 1 M Tris HCl, pH 6.8 prior to scintillation counting. In parallel, 1 nmol of authentic Galβ1-3GlcNAcitol, Galβ1-4GlcNAcitol, Galβ1-3GalNAcitol, Galβ1-4GalNAcitol were run on the same column as standards.

Purified CK2 was desalted over a 1 mL Sephadex G-50 column into 50 mM sodium phosphate buffer (pH 7.5) to eliminate glycerol. WGA-Sepharose chromatography was performed by the previously described method⁶. Samples were applied to a 300 WGA-Sepharose column and were washed with 5 ml of 50 mM sodium phosphate buffer (pH 7.5). The column was washed with 6 mL of 50mM sodium phosphate buffer (pH 7.5) containing 0.5 M galactose to elute nonspecifically bound proteins. Finally, CK2 activity was specifically eluted with 0.5 M N-acetylglucosamine in the same buffer. Aliquots (50 µL) of each 1 mL fraction were desalted over Sephadex G-50 equilibrated in CK2 assay buffer prior to assaying kinase activity.

Purified CK2 (100 µg) was dialyzed against deionized water to remove glycerol and lyophilized. The lyophilized sample was dissolved in 20 µL of 70% formic acid and 200 µg of cyanogen bromide was added. The sample was kept in the dark at room temperature for 24 h. Peptide fractions were purified by binding to a Sep-Pak C18 cartridge (Waters, Millipore Division), washing in water, eluting with 60% CH₃CN, and then lyophilized. The lyophilized sample was labeled with galactosyltransferase, as previously described. The resulting labeled peptide was purified over a Sep-Pak C-18 cartridge and analyzed by RP-HPLC on a Dynamax C-18 column with a 90 minute 0-60% CH₃CN in 0.05% trifluoroacetic acid gradient. The flow rate was 1 mL/min, the absorbance was monitored at 214 nm. Fractions were collected every
minute, and 1% of each fraction was analyzed by liquid scintillation spectrometry. Each tritium-labeled peak was pooled and separated by a second dimension RP-HPLC over a Nanobore C18 column (Rainin) with a 90 min, 0-30% CH₃CN gradient in 0.1 M NaClO₄, 5 mM sodium phosphate, pH 7.4. The flow rate was 0.2 mL/min, the absorbance was monitored at 214 nm and fractions were collected every min. The third dimension RP-HPLC was performed under the same conditions as the second dimension, but with the addition of 0.05% trifluoroacetic acid in the buffers instead of sodium phosphate. The tritium labeled peaks were pooled and analyzed by gas phase sequencing and manual Edman degradation.

Pure glycopeptides were sequenced by automated Edman degradation in a Model 470A Applied Biosystems gas-phase sequencer. Peptides were also subjected to the manual Edman modification protocol as previously described⁷. Prior to manual Edman degradation, peptide samples were incubated with 0.1% NH₄OH (pH 10.7) for one day at ambient temperature and lyophilized. Incubation with dilute alkali was necessary to open the lactone ring of the C-terminal homoserine residues and allow covalent coupling to the arylamine solid support used in the Edman procedure.

**Molecular cloning:**
DNA encoding full-length human CK2α (aa 1-391) and truncated CK2α (aa 1-341) were each subcloned into the pTYB2 vector (New England Biolabs) using NdeI and SmaI restriction sites. The pTYB2 vector is a C-terminal fusion vector in which the C-terminus of the target protein is fused to the Vent intein followed by CBD (chitin-binding domain). DNA encoding human CK2β was subcloned into pTYB2 using NdeI and XmaI restriction sites. Full-length G342C CK2α was prepared using the Quikchange mutagenesis protocol and the following primers: 5’-CATCTAGCATGCCAGGCTAGTACGCCCCGTAGCAGCAG-3’ and 5’-CTGCTGACGGGCGTACTACACCTGGCATGCTAGATG-3’. N-terminal HA-tags (YPYDVPDY) were added to full-length CK2α, full-length G342C CK2α, and truncated CK2α (aa 1-341) using the Quikchange mutagenesis protocol and the following primers: 5’-GGAGATACATATATGACCATTACCGCTTCCAGATTACGCTTCCGGACCCGTGCAAGC -3’ and 5’-GCTTGGCACGGGTCCCGAAGCGTAATGGAACATCGTATGGGTACATATGCTATAGTATATTCC -3’. N-terminal FLAG-tag (DYKDDDDK) was added to CK2β using the Quikchange mutagenesis protocol and the following primers: 5’-
GGAGATATACATGGACTACAAGGATGACGACGATAAGAGCAGCTCAGAGGAGG-3’ and 5’-CCTCCTCTGAGCTGCTCTTATCGTCGTCATCCTTGTAGTCCATATGTATATCTCC-3’.

Synthesis of non-natural amino acids and peptide synthesis:
Phosphonodifluoromethylene alanine (Pfa) was prepared according to the procedures previously described and characterization matched literature values. N-Fmoc-S-GlcNAc-Serine was prepared according to the procedures previously described and characterization matched literature values. Fmoc-Thr(PO(OBzl)OH)-OH, Fmoc-Lys(Dde)-OH, and Biotin-NHS were all purchased from Novabiochem. All peptides were prepared using the standard Fmoc solid phase peptide synthesis strategy with Rink Amide resin (Novabiochem) which leaves a carboxamide at the C-terminus. For Fmoc-Pfa and Fmoc-S-GlcNAc-Ser, which were used in substoichiometric amounts, acetic anhydride capping was performed after coupling. For preparation of biotinylated peptides, Fmoc-Lys(Dde) was incorporated at the C-terminal end of the peptide, the Dde protecting group was removed with 2% hydrazine, and biotin was coupled to ε amino group using NHS-activated biotin. All synthetic peptides were purified by reverse phase HPLC on a preparative C-18 column. The molecular weights of the peptides were confirmed by MALDI-MS using α-Cyano-4-hydroxycinnamic acid (CHCA) matrix (Supplementary Results, Fig. 2 and 26). The concentrations of the substrate peptides were determined by amino acid analysis (Harvard Microanalysis facility or the Yale Keck facility). Some of the peptides corresponding to the C-terminal tail of CK2α prepared for expressed protein ligation showed evidence on the HPLC chromatograms of multiple conformations attributed to the several prolines found in these peptides. In these cases, multiple peaks corresponding to pure material with the same mass were collected during HPLC purification and combined. The ultimate purity of the peptides used was based on the MALDI-MS spectra.

CK2 substrate peptides (from N to C direction)
- Optimized peptide substrate: RRRADDSDDDDK(εBiotin)
- Casein peptide substrate: SKDIGAESTEDQAMEDIK(εBiotin)
- XRCC1 peptide substrate: PYAGSTDENTDSEEHQEK(εBiotin)

Cdk1 substrate peptides (from N to C direction)
- Unmodified CK2α 337-352: SSMPGGSTPVSSANMMK(εBiotin)
- S-GlcNAc CK2α 337-352: SSMPGGSTPVSS-GlcNAcSANMMK(εBiotin)
Peptides for expressed protein ligation (CK2α aa 342-365)
Unmodified: CSTPVSSANMMSGISSVPTPSLG
S-GlcNAc: CSTPVS(S-GlcNAc)SANMMSGISSVPTPSLG
Phospho: CSpTPVSSANMMSGISSVPTPSLG
Pfa: CSZPVSSANMMSGISSVPTPSLG (where Z=Pfa)
T344E: CSEPVSSANMMSGISSVPTPSLG

Preparation of semisynthetic CK2α proteins:
The plasmid corresponding to truncated CK2α (aa 1-341) was transformed into E. coli BL21 (DE3) codon plus cells. Transformed cells were grown at 37°C in LB media for 2 h until cell density reached OD600=0.6, then induced at 16°C with 350 μM IPTG for overexpression for 20 h. Cell pellets were harvested by centrifugation and lysed via double-pass on French press in 25 mM Hepes pH 8.0, 150 mM NaCl, 1 mM MgSO4, 5% glycerol, 5% ethylene glycol, 1 mM EDTA, and 100 μM PMSF. Cell lysates were clarified by centrifugation at 15,000 rpm (25,000g), supernatant was loaded onto chitin beads (New England BioLabs) for affinity purification and washed with 25 mM Hepes pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 followed by a wash with cleavage buffer (50 mM Hepes pH 7.5, 250 mM NaCl, 1 mM EDTA). After the CK2α-intein-CBD fusion protein is bound to the chitin beads and washed thoroughly, the column is quickly equilibrated with 200 mM 2-Mercaptoethane sulfonate (MESNA) in cleavage buffer, the ligation reaction mixture is added (2 mM peptide, 200 mM MESNA, in cleavage buffer, pH 7.5), and the column is sealed and purged with nitrogen gas. The ligation reaction is allowed to proceed for approximately 60 h at rt. Upon completion of ligation reaction, the semisynthetic CK2α protein is washed and collected from the column with cleavage buffer plus 1 mM phenylmethysulfonylfluoride (PMSF), dialyzed extensively against 50 mM Hepes, 500 mM NaCl, 1 mM EDTA, 10 mM DTT, 10% glycerol to remove the MESNA and unligated peptide, concentrated to >1 mg/mL, and stored at -80°C. Protein purity and concentrations were evaluated by Coomassie stained 12% SDS-PAGE and Bradford assay (BioRad) using bovine serum albumin (BSA) as a standard.

The other recombinant CK2 proteins (full-length CK2α, G342C CK2α, and CK2β) were expressed and purified in a manner similar to that described above with the noted exceptions. Following the wash steps, instead of adding ligation reaction mixture, the full-length
recombinant proteins were eluted from the chitin beads with either 50 mM DTT or 4 mM L-cysteine in 50 mM Hepes pH 7.5, 250 mM NaCl, 1 mM EDTA at rt for 16 h.

**Steady-state kinetic kinase assays using peptide substrates:**

To determine the kinetic parameters, radiometric kinase assays were carried out in 50 mM Hepes (pH 7.5), 75 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 5% glycerol, 250 μg/mL BSA using the biotinylated peptide substrates described above in a 25 μL reaction volume with various ATP and peptide concentrations. Reactions were initiated by the addition of kinase (10 nM final concentration), carried out at 30°C for 2-16 min, and stopped by the addition of 10 μL of 100 mM EDTA. To each sample, 10 μL of 10 mg/mL avidin (Thermo Scientific) was added, and all samples were transferred to centrifugal filtration units with 30,000 nominal molecular weight limit (NMWL) membranes (Millipore) and washed three times with 100 μL wash solution (0.5 M Phosphate, 0.5 M NaCl, pH 8.5). The K_	ext{m}^{\text{app}} values for the optimized peptide substrates were determined with varying peptide concentrations (800, 360, 80, 36, 8, 4 μM), fixed ATP concentration (100 μM), and 10 nM CK2α enzyme ± 20 nM CK2β. The K_	ext{m}^{\text{app}} for the casein peptide substrate was determined with varying peptide concentrations (1600, 800, 360, 80, 36, 8 μM), fixed ATP concentration (100 μM), and 10 nM CK2α enzyme ± 20 nM CK2β. The K_	ext{m}^{\text{app}} for the XRCC1 peptide substrate was determined with varying peptide concentrations (800, 360, 80, 36, 8, 4 μM), fixed ATP concentration (100 μM), and 10 nM CK2α enzyme ± 20 nM CK2β. The K_	ext{m}^{\text{app}} for ATP was determined using varying ATP concentrations (128, 64, 32, 16, 8, 4, 2 μM), fixed optimized peptide substrate concentration (800 or 360 μM), and 10 nM CK2α enzyme ± 20 nM CK2β. The α and β subunits were allowed to incubate on ice for at least 30 min prior to initiating the assays. The K_	ext{m}^{\text{app}} values for peptide substrates in the presence of Pin1 were determined with varying optimized peptide concentrations (800, 360, 80, 36, 8, 4 μM), fixed ATP concentration (100 μM), and 10 nM CK2α enzyme ± 20 nM CK2β and ± 25 nM Pin1. The Pin1 was allowed to incubate on ice with CK2 for at least 30 min prior to initiating the assays. Activities proved to be linear with time and kinase concentration in the ranges used and the limiting substrate turnover was less than 10% for all rate measurements. Duplicate measurements were generally within 15%. Apparent K_	ext{m} and k_	ext{cat} values were obtained from non-linear curve fits to the Michaelis-Menten equation.
Cdk1/cyclin B kinase assays:
To determine the kinase activity for recombinant Cdk1/cyclin B (Upstate Millipore) toward non-
modified and S-GlcNAc-modified CK2α tail peptides, radiometric kinase assays were carried
out in 20 mM MOPS (pH 7.2), 10 mM MgCl₂, 80 mM NaCl, 1 mM DTT, 1 μCi [³²P]-γ-ATP, 250
μg/mL BSA, 0.005% Triton, 1% glycerol, 0.05% βME, 100 μM ATP, and 100 μM peptide
substrate in a 25 μL reaction volume with 125 ng enzyme. Reactions were initiated by the
addition of kinase (46 nM final concentration), carried out at 30°C for 20 min, and stopped by
the addition of 10 μL of 100 mM EDTA and 7 μL 6x-SDS loading buffer. Samples were loaded
onto 20% Tris-Tricine gels (100V), fixed in 40% MeOH, 10% acetic acid for 30 min, washed
20 min in ddH₂O, dried on filter paper, and exposed to phosphoimager screen for 2 h. Exposed
screen was developed on Typhoon 9410 Variable Mode Imager (Amersham Biosciences).

Evaluation of cell viability following microinjection:
Even though about 75% of the cells that were injected had sample efficiently loaded, survived
injection and wash steps, and could be visualized by immunostaining, there were two separate
additional methods used to evaluate the viability of the REF52 cells following microinjection
with HA-CK2α protein. The first method involved examining the permeability of the cells to two
dyes, diacetyl fluorescein¹⁰ (Sigma) and propidium iodide¹¹ (Sigma). Cells were microinjected
with recombinant HA-CK2α protein in the same way as described above and were incubated at
37 °C in 5% CO₂ for various time points (0, 1, 4, 8 h). Cells were seeded on grided glass
coverslips (Bellco) and all of the cells that were in various grided squares (at least 4 per
sample time point) were injected. Following the incubation period for each sample time point,
the dishes were washed with HBSS buffer, then treated with 10 μg/mL diacetyl fluorescein and
50 μg/mL propidium iodide in HBSS for 15-20 min at 37 °C, and the grided squares where the
cells were injected were imaged (without fixation) using the Leica SDC fluorescence
microscope (Supplementary Results, Fig. 27). Viable cells fluoresce green and non-viable
cells will take-up the propidium iodide and fluoresce red.

In addition to determining cell viability using diacetyl fluorescein and propidium iodide¹¹,
the viability of the microinjected cells was also evaluated by examining the nuclei and
demonstrating that they are still intact following injections. REF52 cells were microinjected
with recombinant HA-CK2α protein in the same way as described above and were incubated at
37 °C in 5% CO₂ for 2 h before being fixed and immunostained (see description above). In
addition to anti-HA (1:4000 Covance) for detecting CK2α injected protein, cells were also
stained with anti-lamin A (1:200 Santa Cruz) and DAPI (33 ng/mL). Images of samples were obtained with both an Olympus BX61 microscope (as described before) and also with a Zeiss Ax10 equipped with the ApoTome feature (Supplementary Results, Fig. 28). If the nuclei are intact, then lamin A and DAPI staining is found only in the nucleus with lamin A primarily around the nuclear periphery.

**Cell cycle analysis using flow cytometry:**
HeLa cells were treated with either 2 μM flavopiridol (18 h), 3 μM 9-cyanopaullone (18 h), DMSO (18 h), nocodazole (24 h) or DMSO (24 h). Following treatment, cells were collected, washed with PBS, and fixed in 70% ethanol at 4 °C. Cells were stained with a solution of 0.02 mg/mL propidium iodide (Sigma) in 0.1% (v/v) Triton X-100 (Sigma) in PBS containing RNAse cocktail (Ambion) for 30 min at 37 °C. Cell cycle analysis was performed by FACS using FACSCalibur flow cytometer (BD). Data were analyzed with the CellQuest software (BD). Each treatment condition was performed with at least 2 biological duplicate samples.

**Data analysis of protein microarray data:**
The scanned images of the exposed film for the microarray chips were imported into GenePix Pro Software. Signal intensity of each spot on the microarray chips was quantified by dividing the median foreground intensity by its median background intensity. To correct for the variance that are non-uniformly distributed on the chips, signal intensities of a set of spots within a block on a chip were normalized by setting the median intensity of that block equal to one, based on the assumption that proteins are randomly printed and only a limited number of proteins on a microarray are substrates of a kinase. As the distribution of signal intensities of all the proteins follows a normal distribution (see Supplementary Results Fig. 17 and 24), we used the standard deviation (SD) value to determine the cutoff value as previously described method. A stringent cutoff of three SDs was used to increase the chances of a low false positive rate. Moreover, proteins identified as “positive hits” in both duplicate experiments were qualified as a “substrate” for a given experiment. To be included in Table 2, the “positive hit” could not have shown to be positive in either of the duplicate experiments for the other CK2 enzyme forms. See Supplementary Dataset 1 for signal intensities.
Purification of substrate proteins from yeast:
The following proteins were selected for the validation studies and were expressed and purified in yeast: AHCYL2, NAP1L3, EEF2K, WASPL, PCDL2, and UBE2O. DNA encoding these proteins was subcloned into the pEGH-A vector using Gateway LR Clonase reactions (Invitrogen) and the plasmids were transformed into WT Y258A yeast strain using standard LiOAc protocol. Transformed cells were grown in URA-/raffinose liquid media (50-mL culture) at 30°C until cell density reached OD600=0.6-0.8, then induced with 2% galactose for overexpression for 8 h. Cells were collected by centrifugation at 4500 RPM, washed with ddH2O, and flash frozen in liquid N2. Frozen cell pellets were lysed in lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100, 0.1% βME, 1 mM PMSF, plus Roche Protease inhibitor tablet) with 2 mL zirconia beads (Biospec) and 16 x 100 s intervals of shaking on paint shaker at 4°C. Lysed samples were centrifuged at 4000 RPM for 22 min and the clarified supernatant was loaded onto 225 μL of glutathione agarose beads. Protein was bound to glutathione beads at 4°C on nutator for 1 h and beads were washed 3x with 50 mM Tris pH 7.5, 500 mM NaCl, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100, 0.1% βMe, 1 mM PMSF and washed 3x with 50 mM Hepes pH 7.5, 100 mM NaCl, 1 mM EGTA, 10% glycerol, 0.1% βMe, 1 mM PMSF. Proteins were eluted with 50 mM Hepes pH 8.0, 100 mM NaCl, 10% glycerol, 40 mM glutathione (reduced form), 0.03% Triton X-100, 1 mM PMSF. Protein purity and concentrations were evaluated by Coomassie stained 10% SDS-PAGE using BSA as a standard.

Purification of NIPBL from E. coli:
NIPBL was selected for validation studies and was expressed and purified in E. coli. NIPBL DNA was subcloned into pDEST17 (N-terminal 6xHis tag) using a Gateway LR Clonase reaction and the plasmid was transformed into E. coli BL21 DE3 cells. Transformed cells were grown at 37°C in LB media for 2 h until cell density reached OD600=0.6, then induced at 16°C with 350 μM IPTG for overexpression for 20 h. Cell pellet was harvested by centrifugation and lysed via double-pass on French press in 25 mM Tris pH 8, 500 mM NaCl, 10 mM imidazole, 10 mM βME, 0.1% Tween, and cell lysates were clarified by centrifugation at 15,000 RPM. The supernatant was loaded onto Ni-NTA column, washed with 25 mM Tris pH 8, 500 mM NaCl, 10 mM imidazole, 10 mM βME, and protein was eluted with wash buffer containing 50, 100, 250, and 500 mM imidazole. Purified NIPBL was dialyzed into 50 mM Hepes pH 7.5, 100
mM NaCl, 10% glycerol, 0.1% βME. Protein purity and concentration was evaluated by 12% SDS-PAGE stained with Coomassie using BSA as a standard.

**Solution phase kinase assays with selected protein substrates from protein microarrays:**
To determine the kinase activity for the differently modified CK2α enzymes with candidate protein substrates from protein chips, solution phase radiometric kinase assays were carried out in 50 mM Hepes (pH 7.5), 10 mM MgCl₂, 80 mM NaCl, 1 mM DTT, 0.08 μg/μL BSA, 0.015% Triton, 6.6% glycerol, 100 μM ATP, and 20 mM glutathione with various protein substrates in a 24 μL reaction volume with 10 nM CK2α ± 11 nM CK2β. The glutathione was present as a result of the preparation and purification of the substrate proteins as GST fusions. Reactions were initiated by the addition of kinase carried out at 30°C for 6-18 min, and stopped by the addition of 5 μL of 200 mM EDTA and 6 μL 6x-SDS loading buffer. The CK2 α and β subunits were allowed to incubate on ice for at least 30 min prior to initiating the assays. Each sample was loaded into two 10% Tris-Glycine gels (170V). One set of gels was washed in ddH₂O, dried on filter paper, and exposed to phosphoimager screen for 2-8 h. The other set of gels were stained with Coomassie, and the bands corresponding to protein substrates were cut and counted using liquid scintillation counter to determine the amount of ³²P incorporated into the band. Kinase assays for NIPBL were carried out by the same procedure with the following conditions: 50 mM Hepes (pH 7.5), 10 mM MgCl₂, 80 mM NaCl, 1 mM DTT, 250 μg/mL ovalbumin, 0.1% NP-40, 5% glycerol, 100 μM ATP, and with 50 ng/μL NIPBL protein substrate in a 24 μL reaction volume with 10 nM CK2α ± 11 nM Pin1. CK2α and Pin1 were allowed to incubate on ice for at least 30 min prior to initiating the assays. Reactions were carried out at 30°C for 5 min and samples were loaded onto 12% Tri-Glycine gel.

**CK2 kinase assays for calmodulin substrate:**
To determine the extent that the semisynthetic CK2α enzymes are able to phosphorylate calmodulin protein substrate, radiometric kinase assays were carried out in 50 mM Tris (pH 7.5), 75 mM NaCl, 12 mM MgCl₂, 1 mM DTT, 5% glycerol, 100 μg/mL BSA using 0.25 mg/mL calmodulin (Sigma) and 200 μM ATP. Reactions were initiated by the addition of kinase (100 nM final concentration CK2α), carried out at 30°C for 10 min in a 28 μL reaction volume, and stopped by the addition of 10 μL of 100 mM EDTA and 10 μL 5x-SDS loading buffer. Samples were loaded into 15% Tris-Glycine gel and gels were run at 130V, stained with Coomassie,
and the bands corresponding to calmodulin were cut from the gel. The amount of $^{32}$P incorporated into the band was determined using liquid scintillation counting. Various amounts of CK2β (0, 50, 100, 200 nM final concentration) were added to CK2α sample prior to the initiation of the reaction.

**Spectrophotometric kinase assays for CK2 specific activities:**
To evaluate the effects of the truncation and G342C mutation in the prepared semisynthetic proteins, continuous enzyme-coupled spectrophotometric assays were performed using pyruvate kinase and lactic dehydrogenase, and monitoring the disappearance of NADH (absorbance at 340nm) as an indicator of CK2α kinase activity. Final enzyme assay conditions were 50 mM Hepes pH 7.5, 30 mM NaCl, 10 mM MgCl$_2$, 2.2 mM ATP, 400 μM optimized peptide substrate, 2 mM phosphoenol pyruvate, 200 μM NADH, 400 μM optimized peptide substrate, 5% glycerol, 7.4 units pyruvate kinase (Sigma), 9.3 units lactic dehydrogenase (Sigma), and 200 nM CK2α enzyme in 100 μL reaction volume at 30 °C. The decrease in absorbance over time was converted to reaction rates using 6200 M$^{-1}$cm$^{-1}$ as the extinction coefficient. Specific activities were determined using the linear range during the 6 min reaction time for each of the following enzymes: HA-CK2α full-length wild-type, HA-CK2α full-length G342C, and semisynthetic HA-CK2α unmodified

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