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RESEARCH ARTICLE

Analyzing the interactome of human CK2β in prostate carcinoma cells reveals HSP70-1 and Rho guanin nucleotide exchange factor 12 as novel interaction partners

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Abstract

CK2β is the non-catalytic modulating part of the S/T-protein kinase CK2. However, the overall function of CK2β is poorly understood. Here, we report on the identification of 38 new interaction partners of the human CK2β from lysates of DU145 prostate cancer cells using photo-crosslinking and mass spectrometry, whereby HSP70-1 was identified with high abundance. The K_D value of its interaction with CK2β was determined as 0.57 µM by microscale thermophoresis, this being the first time, to our knowledge, that a K_D value of CK2β with another protein than CK2α or CK2α′ was quantified. Phosphorylation studies excluded HSP70-1 as a substrate or activity modulator of CK2, suggesting a CK2 activity independent interaction of HSP70-1 with CK2β. Co-immunoprecipitation experiments in three different cancer cell lines confirmed the interaction of HSP70-1 with CK2β in vivo. A second identified CK2β interaction partner was Rho guanin nucleotide exchange factor 12, indicating an involvement of CK2β in the Rho-GTPase signal pathway, described here for the first time to our knowledge. This points to a role of CK2β in the interaction network affecting the organization of the cytoskeleton.

KEYWORDS

HSP70-1, photo-crosslinking mass spectrometry, prostate cancer, protein kinase CK2, Rho guanin nucleotide exchange factor 12

1 | INTRODUCTION

Human CK2β is an 24.9 kDa protein with 215 amino acids encoded by the gene *CSNK2B*. It is usually considered as an integral part of human protein kinase CK2, with a $CK2\alpha_2\beta_2$ composition. The CK2β amino acid sequence is highly conserved among species,[1–3] however, no other homologous protein has been described so far for CK2β, except the stellate protein from *Drosophila melanogaster*.[4] It is known to interact with CK2α as well, but of unknown function.[5] In human, single point mutations within *CSNK2B* leads to developmental disorders and reduced brain function, such as in the Poirier-Bienvenu syndrome.[6,7] In mice, a complete knockout of *CSNK2B* results

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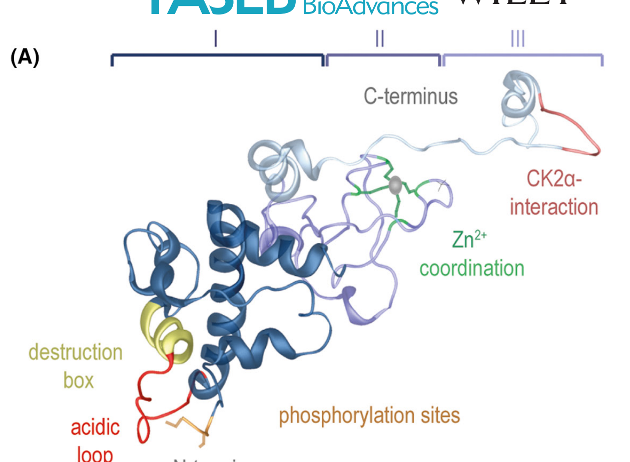
in embryonic stage mortality.[8] This indicates an essential and non-substitutable function of CK2β.

The CK2β protein structure can be divided into three main domains (Figure 1A). The N-terminal domain contains the phosphorylation sites S2 and S3,[9,10] the destruction box and the acidic loop. Phosphorylation of CK2β enhances its stability,[11] while the destruction box is a motif that has been described also in other proteins, in which it is responsible for recognition by the cellular degradation machinery.[12] The acidic loop is mediating the interaction with positively charged surface patches of proteins, other polycations and the plasma membrane.[13,14] The second domain harbors a zinc finger motif for dimerization of two CK2β subunits.[15] The third domain, also called CK2β tail, supports this dimerization and provides the interaction site with the catalytic subunit CK2α or its isoform CK2α′.[16]

Eponymous and non-arguably most important is the role of CK2β as the non-catalytic subunit of the S/T-protein kinase CK2, in which it is indispensable for the assembly of the tetrameric holoenzyme. This consists of two catalytic CK2α or α′ subunits binding to a CK2β dimer[17] (Figure 1B,1). One CK2β subunit of the tetrameric CK2 holoenzyme can interact via its acidic loop with the basic cluster of a further catalytic subunit of another CK2 holoenzyme.[18] Thereby, oligomeric CK2 super-structures, such as highly active linear oligomers and trimeric rings or less active polymeric filaments, were found to be formed[19] (Figure 1B,2).

CK2 is a highly pleiotropic, ubiquitous, and constitutively active protein kinase and due to these properties known to be involved in a variety of physiological and pathophysiological processes. For example, dysregulated CK2 activity is related to cancer, neurodegenerative diseases, and viral infections like HIV or COVID-19.[20] CK2β stabilizes the catalytic CK2 subunits, enhances their activity[21] and modulates their substrate specificity.[22] Thereby CK2β acts as a kind of binding platform for substrates and regulators of CK2, suggesting a key role in the CK2 interactome.

Beside its CK2 dependent functions, CK2β was also found in absence of or in excess to CK2α and CK2α′.[23] Moreover, it was shown that CK2β interacts independently with non-kinase proteins such as the lysosomal-trafficking regulator LYST[24] or the NADPH oxidase cytosolic protein p47phox,[25] as well as with kinase activity exhibiting proteins beyond CK2α or CK2α′ (Figure 1B,3 and 4). It shows inhibiting effects as reported for protein kinase c-Mos[26] and p21-activated kinase PAK,[27] or it is activating as reported for A-Raf[28] and Chk1.[29] Altogether, this suggests an additional CK2α and CK2α′ independent role of CK2β.



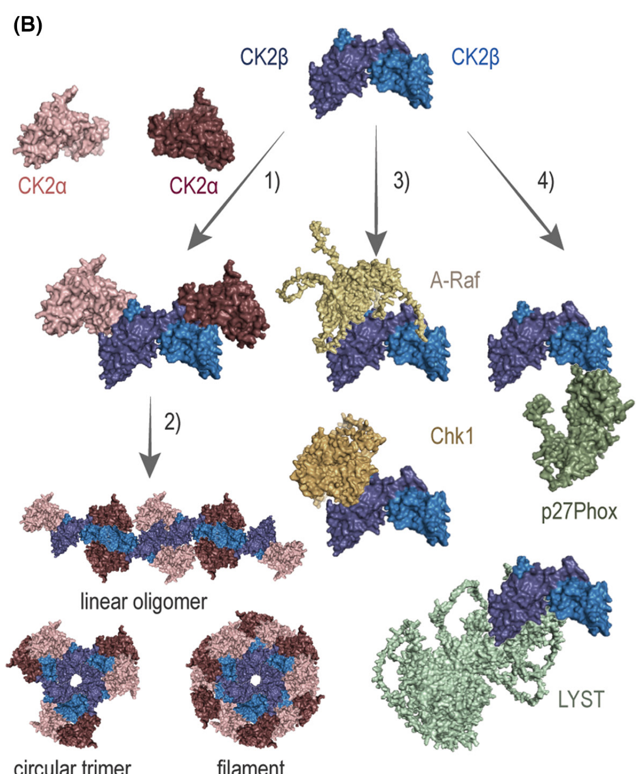


FIGURE 1 Schematic structure of CK2β as well as interaction sites and interaction partners. (A) Structure of human CK2β monomer (PDB: 1jwh) sectioned into (I) N-terminal region (E5-G104), (II) juxta dimer interface (D105-T161) and (III) C-terminal region (G162-R215). Functional areas were highlighted: phosphorylation sites (S2, S3; orange), destruction box (R47-D54; yellow), acidic loop (D55-P66; red), zinc coordinating amino acids (C109, C114, C137 and C140; green), main interaction surface with CK2α (N181-A203; light red). (B) Known interactions of the CK2β dimer. (1) Binding of CK2 catalytic subunit CK2α assembling the tetrameric CK2 holoenzyme. (2) Aggregation of several CK2 holoenzymes. (3) interaction with other kinases shown as a model, A-Raf (AF-P10398-F1-mod), Chk1 (AF-014757-F1-mod), (4) interaction with regulatory proteins shown as a model, p27Phox/NCF1 (AF-P14598-F1-mod), LYST (AF-E9QFZ1-F1-mod).

A deeper background on such interaction partners of CK2β appears to be the first step toward the understanding of its essential and obviously non-substitutable function.

Interaction partners of CK2β have already been identified by yeast-two-hybrid,[30] affinity pulldown coupled to mass spectrometry (MS)[31] or immunoprecipitation.[32] In the present study, photo-crosslinking was used for this purpose. Crosslinking in general is a widely used method for a covalent fixation of protein–protein interactions, with the advantage that also transient, rare and weak interactions can be detected.[33] Photo-crosslinking via an unnatural amino acid such as *para*-azido-phenylalanine (*p*AzF) in a biorthogonal manner enables a covalent and site-directed capturing of interaction partners, thus additionally offering the possibility to unveil the binding site.[34,35] Applying genetic code expansion, *p*AzF can be incorporated into bait proteins during protein biosynthesis.[35] UV light induced activation of the azido-group results in a nitrene, which reacts non-specifically with C-H, N-H, or C-C double bonds, resulting in a covalent bond.[36,37] For this purpose, the interaction partner must be in a proximity of at least 10 Å to the azido-group. The site-directed coupling with a small radius for reaction allowing approximate localization of the binding site in CK2β may provide information about the nature and purpose of the interaction. This is a distinct advantage over methods already used to identify binding partners of CK2β such as affinity pull down or yeast-two-hybrid. Due to covalent coupling, binding strength as well as the binding duration are not critical for the identification process such as separation from unbound proteins. Furthermore, only directly interacting proteins and not proteins bound indirectly via other proteins to CK2β should be identified using this strategy.

In this work, photo-crosslinking using *p*AzF was used for a site-specific identification of CK2β interaction partners from human cancer cell lysates to gain a closer view on possible functions of CK2β in malignant cells.

2 | METHODS

2.1 | Chemicals

The unnatural amino acid *para*-azidophenylalanine was purchased from Bachem GmbH (Bubendorf, Germany). Isopropyl β-d-1-thiogalactopyranoside (IPTG), KCl, leupeptin, pepstatin A and KH_2PO_4 were purchased from AppliChem (Darmstadt, Germany), $MgCl_2$ was purchased from VWR (Leuven, Belgium), NaCl, thiamin and imidazole from Sigma-Aldrich (Schnelldorf, Germany), NaOH and Na2HPO4 from VWR Chemicals (Darmstadt, Germany) and $FeCl_3$ was purchased from Merck (Darmstadt, Germany). Standard media ingredients, EDTA and SDS were purchased from Roth (Karlsruhe, Germany).

2.2 | Plasmids

The DNA sequence encoding for a truncated human CK2β (1–193) was cloned into a pET vector with an N-terminal hexahistidine tag by overlap PCR and restriction free Infusion cloning using the In-Fusion HD EcoDry Cloning Kit from Clontech (Saint-Germain-en-Laye, France) and was called $pCK2\beta^{His}$. The CK2β encoding sequence was under control of a T7 promotor. The plasmid contained a ColE1 origin of replication and a β-lactamase gene for carbenicillin resistance. The amber stop codon TAG was cloned into $pCK2\beta^{His}$ at three different positions resulting in the plasmids $pCK2\beta^{His}{}_{34stop}$, $pCK2\beta^{His}{}_{62stop}$ and $pCK2\beta^{His}{}_{80stop}$. The plasmid pEVOL-*p*AzF[35] encodes for the *para*-azidophenylalanine aminoacyl-tRNA synthetase and tRNA.

2.3 | Protein expression and purification

For expression of CK2β variants $CK2\beta_{34pAzF}$, $CK2\beta_{62pAzF}$ and $CK2\beta_{80pAzF}$ *Escherichia coli* BL21(DE3) was used for the biosynthesis of proteins. Cells were transformed with plasmids pEVOL-*p*AzF and $pCK2\beta^{His}{}_{34stop}$ or $pCK2\beta^{His}{}_{62stop}$ or $pCK2\beta^{His}{}_{80stop}$ by electroporation. An overnight culture (lysogeny broth, LB) was inoculated with a single colony. For main cultures 2×500 ml minimal medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCL, 14.7 mg/L $CaCl_2$, 120 mg/L $MgSO_4$, 5.9 μg/L Fe(III) Cl_3, 30 mg/L Thiamin, 1 g/L NH_4Cl, 2.2 g/L D-Glucose (pH 7.0)) containing 50 μg/μl carbenicillin and 30 μg/ml chloramphenicol was supplemented with 10 ml overnight culture each. Cells were cultivated at 37°C until an optical density of 0.5 measured at 578 nm was reached. Cell suspension was concentrated to a volume of 200 ml and supplemented with 1 mM *p*AzF. Expression was induced by addition of 1 mM IPTG and 0.2% (w/v) L-arabinose. Cells were shaken for 18 h at 18°C and 200 rpm and afterwards sedimented by 10 min centrifugation at 3000×g. For the expression of unmodified CK2β, *E. coli* BL21(DE3) cells were transformed with plasmid $pCK2\beta^{His}$. An overnight culture in LB medium was inoculated with a single colony. For main cultures 2×500 ml LB medium containing 50 μg/μl carbenicillin was supplemented with 5 ml overnight culture each. Cells were cultivated at 37°C until an optical density of 0.5 measured at 578 nm was reached. Expression was induced by addition of 1 mM IPTG. Cells were shaken for 18 h at 18°C and 200 rpm and afterwards

sedimented by 10 min centrifugation at 3000×g. The cell sediments were stored at −80°C until purification.

For purification, the cell sediment was suspended in lysis buffer containing 25 mM Tris/HCl (pH 8.5), 300 mM NaCl, 1.3 mg/ml lysozyme, 13 µg/ml DNase I, 2 mM PMSF, 0.5 µg/ml pepstatin A and 0.7 µg/ml leupeptin. Bacterial cells were disrupted by sonication (six 20 s cycles, with 20 s intervals and 20 s brake) on ice. Cell debris were removed by centrifugation (10 min, 4000×g, 4°C and 1 h, 100,000×g, 4°C). The obtained supernatant was loaded on a 2 ml NiNTA-agarose column (Mackerey-Nagel, Düren, Germany) that was equilibrated with buffer P500 (25 mM Tris/HCl (pH 8.5), 500 mM NaCl). Unbound protein was removed by washing with 10 column volumes (cv) buffer P500 and 10 cv buffer P500 containing 50 mM imidazole. Proteins were eluted with 5 ml buffer P500 containing 500 mM imidazole and concentrated using an Amicon Ultra-0.5 spin filter (Merck, Darmstadt, Germany). Furthermore, the buffer was exchanged to P500 in this step. Protein solution was frozen in liquid nitrogen and stored at −80°C.

Human CK2α was expressed and purified as described earlier.[38]

Human HSP70-1 was purchased from Sigma-Aldrich (Saint Louis, MO, USA) as His-tagged protein.

2.4 | SDS-PAGE and western blot

SDS-PAGE analysis was performed with 12.5% acrylamide gels. Protein solution was supplemented with 5× preparation buffer and gels were stained with Coomassie brilliant blue. For western blot analysis proteins were transferred from SDS-PAGE gel to a polyvinylidenfluoride membrane (Machery-Nagel, Düren, Germany). The membrane was blocked with 5% milk in TBS (25 mM Tris/HCl (pH 7.4)), 137 mM NaCl, 2.7 mM KCl and stained with primary antibody against CK2α (1 AD9[39]) or primary antibody against the 6xHis-tag in CK2β (anti-6xHis, Thermo Scientific, Braunschweig, Germany) for 18 h at 4°C. A horseradish peroxidase coupled second antibody (Antibodies Online, Atlanta, GA, USA) was used and the proteins were detected using the Immuno Cruz™ luminol reagent from Santa Cruz Biotechnology (Santa Cruz, USA) following the manufactures instructions. The PageRuler prestained protein ladder from Fisher Scientific (Braunschweig, Germany) was used for sizing.

2.5 | Cell culture

For identification of interaction partners, the human prostate carcinoma cell line DU145 was used (ATCC-number: HTB-81). Cells were cultivated in 12 ml Dulbecco's modified eagle medium (DMEM, Gibco, Darmstadt, Germany) supplemented with 10% (v/v) fetal calf serum (FCS, Life Technologies, Darmstadt, Germany) at 37°C and 5% CO_2. Medium was changed every second or third day. Cells reaching a confluence of 90% were harvested by washing cells with 5 ml phosphate buffered saline (PBS) and incubation with 1 ml 1x trypsin/EDTA solution (Gibco by Life Technologies, Darmstadt, Germany) for 3 min at 37°C. Digestion was stopped by the addition of 5 ml medium. Cells were washed by sedimentation (5 min at 300×g) and suspension in PBS. Afterwards cells were sedimented again and were suspended in lyses buffer for eukaryotic cells containing 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM Na-orthovanadate, 50 mM NaF, 0.1 mM PMSF, 1 mM benzamidin and were lysed by sonication (five cycles, with 5 pulse intervals and 5 s brake). For protein quantification Bradford assay was performed with ROTI®Quant (Roth, Karlsruhe, Germany) according to manufacturer's instruction. LNCaP (ATCC: CRL-1740) were maintained at 37°C in RPMI 1640, supplemented with 10% fetal calf serum (FCS) in an atmosphere enriched with 5% CO_2. HeLa (ATCC: CCL-2) were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS.

2.6 | Photo-crosslinking

Photo-crosslinking reaction was performed using a 96 well plate (Greiner Bio-One, Kremsmünster, Austria) with 40 µl for reaction with purified proteins or 200 µl for reaction in cell lysate. For proof-of-concept analysis 10 µM CK2β_{34pAzF} was mixed with 3 µM CK2α with or without the lysate of DU145 cells having a protein concentration of 5 mg/ml. For identification of CK2β interaction partners 20 µM of each CK2β variant was mixed with the lysate of DU145 cells having a protein concentration of 2.5 mg/ml. Mixtures were incubated for 15 min at 37°C and afterwards irradiated with UV light ($\lambda = 365$ nm, 14,000 mA) for 2 × 30 s on ice using a UV/LED-lamp (M365LP1–365 nm, Thorlabs Inc, Newton, USA).

2.7 | Separation of cross-linked products by affinity chromatography

For separation of cross-linked proteins affinity chromatography using 0.7 ml NiNTA agarose (Mackerey-Nagel, Düren, Germany) was performed. The column was equilibrated with buffer P500 (25 mM Tris/HCl (pH 8.5), 500 mM NaCl). Unbound protein was removed by washing with 10 cv buffer P500 and 10 cv buffer P500 containing 50 mM imidazole. For identification of CK2β interaction partners

from DU145 cell lysate an additional washing step with buffer P500 containing 50 mM imidazole and 8 M urea was performed. Proteins were eluted with 5 ml buffer P500 containing 500 mM imidazole. For SDS-Page and western blot analysis elution fractions were concentrated using an Amicon Ultra-0.5 spin filter (Merck, Darmstadt, Germany).

2.8 | Mass spectrometry

Gel bands were destained, reduced and alkylated. They were in-gel digested with trypsin and the peptides were extracted and dried. Peptides were dissolved in 10 µl 0.1% formic acid containing 5% acetonitrile, and 0.5 µl was analyzed by reversed-phase liquid chromatography (LC) coupled to high-resolution MS with Synapt G2 Si/M-Class nanoUPLC (Waters Corp., Manchester, UK) using C18 µPAC columns (trapping and 50 cm analytical; PharmaFluidics, Ghent, Belgium) with a 90 min gradient (solvent system 100% water versus 100% acetonitrile, both containing 0.1% formic acid). For identification of CK2β interaction partners from DU145, cell lysate elution fractions of affinity chromatography for separation of cross-linked products were processed as described using filter-aided tryptic digestion.[40] Comparative analyzes of the protein samples were performed using data independent MS with above instrumentation and the human Uniprot database as detailed elsewhere.[41] Data were analyzed with PLGS and Progenesis software.

2.9 | Microscale thermophoresis

For analysis of protein–protein interaction by microscale thermophoresis (MST) HSP70-1 was labeled with the Monolith NT™ Protein Labeling Kit RED-NHS (NanoTemper Technologies GmbH, München, Germany) according to the manufacturer's instructions. CK2β was serially diluted in P500 to 16 concentrations ranging from 4 nM to 100,000 nM. Each concentration was mixed with fluorescently labeled HSP70-1 in the ratio 1:1 to a final volume of 20 µl. The resulting preparations contained 10 nM HSP70-1 in a final buffer of 10.6 mM Tris/HCl, 3.75 mM NaH_2PO_4 400 mM NaCl, 0.4% Tween 20, 11.3 mM imidazole, 7.5 µM DTT, and 1.9% glycerol. Samples were analyzed by the Monolith NT.115 (NanoTemper Technologies GmbH, München, Germany). The measurement was performed at an excitation strength of 95% and an MST-strength of 60% at 23°C for 30 s. Data were evaluated using the software MO Affinity Analysis v2.1.3 (NanoTemper Technologies GmbH, München, Germany).

2.10 | Phosphorylation assay

Capillary electrophoresis assay was performed as described before.[42] Deviating from this, a buffer containing 40 nM CK2α and 80 nM CK2β or CK2β variant as well as 300 µM substrate peptide RRRDDDSDDD (GenicBio, Shanghai, China) and 250 µM ATP in 150 mM NaCl, 25 mM $MgCl_2$ and 25 mM Tris/HCl, pH 7.5 was used. Kinase reaction was performed at 37°C and 300 rpm shaking and was stopped at four different timepoints within the linear range of reaction by addition of 17 µl 0.5 M EDTA and storage on ice. Phosphorylated and unphosphorylated substrate peptide were separated by capillary electrophoresis and detected via UV/Vis detector at 195 nm. Acetic acid (2 M, pH 1.8) served as the background electrolyte.

ADP-Glo™ assay (Promega, Madison, WI, USA) was performed according to manufacturer's instruction using a 384 well plate. Kinase reaction was performed in 5 µl combining 1 mM ATP (ADP-GloTM Kit, Promega, Madison, WI, USA), 40 nM CK2α, 80 nM CK2β, and optional 1 µM HSP70-1 and/or 300 µM CK2 substrate peptide RRRDDDSDDD in buffer containing 12 mM Tris/HCl, 18 mM NaH_2PO_4, 240 mM NaCl, 5 mM $MgCl_2$, 53 mM imidazole, 35 µM DTT, 9% glycerol. Samples were incubated for 10 min at 37°C and 300 rpm shaking and were cooled down to room temperature for 5 min. Afterwards, 5 µl ADP-Glo reagent were added and samples were incubated for 40 min at room temperature. Finally, 10 µl kinase detection reagent was supplemented, and luminescence was determined after 60 min with an Infinite M200Pro (Tecan, Männedorf, Switzerland).

2.11 | Co-immunoprecipitation of HSP70 with CK2β

Co-immunoprecipitation experiments were performed as described earlier.[43] In short, DU145 (ATCC-number: HTB-81), LNCaP (ATCC-number: CRL-1740), and HeLa (ATCC-number: CCL-2) cells were subjected to a heat shock for 30 min at 42°C and then, they were allowed to recover for 3 h at 37°C. Cells treated accordingly and cells without heat shock used as control cells were harvested and washed with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Cell extracts were prepared using RIPA buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% sodium desoxycholate, 1% Triton X-100, 0.1% sodium dodecylsulfate) supplemented with the protease inhibitor cocktail completeTM (Roche Diagnostics, Mannheim, Germany) as previously described.[44] Total protein content was quantified by Bradford assay and 1 mg of total protein was subjected to immunoprecipitation. For this purpose, cell extracts were

at first incubated three times with fresh, pure protein G sepharose beads (Immobilized Protein G Plus, Pierce) for 1 h at 4°C. Protein G sepharose beads were removed subsequently by centrifugation, to get rid of all proteins binding non-specifically to the beads. The remaining supernatant was then incubated with the same beads carrying serum #32,[45] a specific CK2β antiserum, overnight at 4°C. Beads were washed three times with PBS/0.1% tween20 and once with PBS. Bound proteins were eluted with SDS sample buffer (130 mM Tris–HCl, pH 6.8, 0.02% bromophenol blue (w/v), 10% β-mercaptoethanol, 20% glycerol (v/v), and 4% SDS) and separated by SDS-PAGE (12.5% acrylamide). Samples were subsequently analyzed by western blot sequentially using the mouse monoclonal HSP70 specific antibody (MAB1663 from R&D systems, Minneapolis, USA), the mouse monoclonal antibody 1A5 against CK2α[39] and the mouse monoclonal antibody 6D5 against CK2β.[46] Proteins were visualized by enhanced chemiluminescence as described.[43]

2.12 | Statistical analysis

Photo-crosslinking coupled with affinity chromatography and mass spectrometry experiments were performed in two independent replicates. Data were given as mean ± range. Binding affinity was determined in four independent replicates. Data were given as mean ± SD. Activity measurements were performed at least in three independent replicates. Data were given as mean ± SD. To proof significance of results, an unpaired, two-tailed Student's *t*-test with 95% confidence intervals was performed using the software GraphPad Prism v5.02 (La Jolla, CA, USA).

3 | RESULTS

3.1 | Construction of CK2β variants for photo-crosslinking

To identify new interaction partners of CK2β, three variants of CK2β were created. In the first variant, F34, was replaced by the unnatural amino acid *p*AzF. F34 contributes to the interaction of CKβ with CK2α and may interact with other kinases. In the second variant, L62 was chosen for replacement, because it is located in the acidic loop regulating oligomerization and other interactions. For the third variant, Y80 was chosen, as it is already known for being involved in interactions with proteins such as p53.[47] All CK2β variants contained an N-terminal 6xHis epitope to facilitate a subsequent isolation from cell lysates by affinity chromatography. Furthermore, a C-terminally truncated variant $CK2\beta^{1-193}$ was used as it has been previously shown that absence of the $CK2\beta^{194-215}$ amino acid sequence favors higher CK2β stability but has almost no effect on dimerization as shown in the crystal structure, as well as on binding affinity to CK2α[48,49] or CK2α′.[50] To verify an unaltered CK2α stabilizing effect, the three variants $CK2\beta_{34pAzF}$, $CK2\beta_{62pAzF}$ and $CK2\beta_{80pAzF}$ were compared to unmodified CK2β in a capillary electrophoresis-based activity assay with CK2α. At 37°C CK2α exhibits low activity, which can be increased by the formation of the tetrameric complex with CK2β, indicated by a substantially increased enzymatic activity (Figure 2). CK2 holoenzymes based on $CK2\beta_{62pAzF}$ and $CK2\beta_{80pAzF}$ showed no difference in activity compared to the holoenzyme with unmodified CK2β. This indicates that the incorporation of *p*AzF at positions 62 or 80 did not influence the function of CK2β. In presence of the CK2β variant with *p*AzF at position 34, a slightly decreased activity was observed, in comparison to the holoenzyme with unmodified CK2β. F34 is directly involved in CK2β-CK2α interaction, which may be impaired by the azido-group, leading to a reduced stability and, hence, a reduced CK2 activity. This shows that, in principle, *p*AzF might reduce the affinity to interaction partners binding close to the *p*AzF position. However, this does not play a major role here, since the identification of binding partners is not supposed to depend on binding affinity, but on the formation of a covalent bond by photo-crosslinking. Nevertheless, incubation of CK2α with all variants led to a remarkable increase in enzymatic activity, clearly showing CK2α/β interaction. In consequence, the incorporation of the unnatural amino acid at the different positions did not break CK2β structure.

As a proof-of-concept, $CK2\beta_{34pAzF}$ was tested for its suitability for being photo-crosslinked with CK2α. For this purpose, both purified proteins were mixed and irradiated with UV light (λ = 365 nm). Photo-crosslinked products were identified by Coomassie staining after SDS-PAGE and western blot analysis, in comparison to control samples (Figure 3A). Beside CK2α, appearing at 40 kDa and $CK2\beta_{34pAzF}$ appearing at 23 kDa, an additional protein band at 46 kDa was observed after UV-light irradiation. This band appeared in the Coomassie stained SDS-PAGE gel and in the western blot with a primary antibody against the 6xHis-epitope, but not in the western blot with a primary antibody against CK2α. With 46 kDa, the band corresponded to the calculated molecular mass of a β-β-dimer, indicating photo-crosslinking of $CK2\beta_{34pAzF}$ with itself. A protein band at 63 kDa, corresponding to the calculated molecular mass of an β-α-dimer, was observed in the Coomassie staining as well as in all western blots. It clearly indicated a photo-crosslinking of $CK2\beta_{34pAzF}$ with CK2α. To purify photo-crosslinked $CK2\beta_{34pAzF}$, affinity chromatography

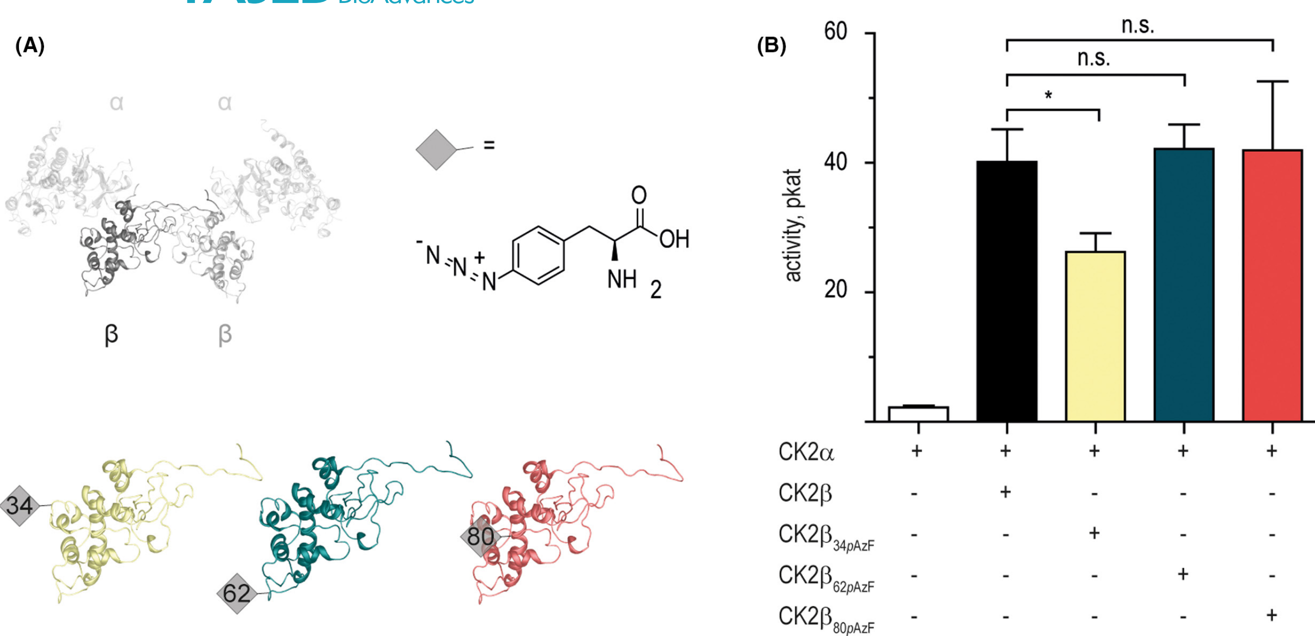


FIGURE 2 Incorporation sites of *p*AzF in CK2β and enzyme activity of CK2 holoenzymes containing the different variants in comparison to unmodified CK2β. (A) CK2 holoenzyme consisting of two CK2α and two CK2β subunits (PDB: 1jwh) and the CK2β variants with *p*AzF at position 34 (yellow), 62 (petrol) and 80 (red). (B) Phosphorylation of CK2 substrate peptide RRRDDDSDDD (300 μM) by CK2α or CK2α based holoenzymes was analyzed using the capillary electrophoresis-based activity assay. CK2α (40 nM) was incubated with 80 nM CK2β or modified variants $CK2\beta_{34pAzF}$, $CK2\beta_{62pAzF}$ or $CK2\beta_{80pAzF}$ at 37°C. Mean ± SD, $n = 3$, *: $p \leq 0.05$, n.s.: $p > 0.05$.

using NiNTA was performed after UV-light irradiation. Both crosslinked products (β-β, β-α) were recovered as their protein bands appeared also after NiNTA affinity chromatography. The photo-crosslinking of $CK2\beta_{34pAzF}$ with CK2α was also possible in the presence of cell lysate from DU145 prostate cancer cells (Figure 3B). Moreover, the same photo-crosslinked proteins as described above could be isolated from cell lysates by NiNTA affinity chromatography. The presence of CK2β in the protein band assigned to the β-β-crosslink, as well as the presence of CK2β and CK2α in the protein band assigned to the α-β-crosslink was verified after in-gel digestion by mass spectrometry, as additional control (Tables S1 and S2). Representative peptides were identified for CK2β in the band corresponding to the β-β crosslink or for both CK2β and CK2α in the β-α crosslink, which clearly supported the corresponding crosslinking. Interestingly, the intensity of the β-β-crosslink was reduced in presence of CK2α. Consequently, binding to CK2α reduced the interaction of two $CK2\beta_{34pAzF}$ via position 34. Interaction of two CK2β subunits within a dimer via position 34 is rather unlikely, as this position is distant from the contact surface. This suggests that two CK2β subunits from different dimers were covalently linked by UV light-induced coupling through a different contact surface involving position 34 and could be explained by the presence of CK2β aggregates. Beside the crosslinked proteins and the free CK2β variants, also non-photo-crosslinked CK2α was separated in the purification step as well, obviously due to its high affinity to CK2β.

3.2 | Identification of CK2β interacting proteins from human DU145 prostate carcinoma cell lysate

To identify CK2β interaction partners from the lysate of human prostate carcinoma cell line DU145 cells a workflow according to the scheme shown in Figure 4A was performed. Prostate carcinoma is the most common cancer among American men[51] and one of the four most common cancers in Europe,[52] accompanied by CK2 hyperactivity.[53] Lysates of DU145 cells were mixed with one of the three CK2β variants $CK2\beta_{34pAzF}$, $CK2\beta_{62pAzF}$, $CK2\beta_{80pAzF}$ or without any added protein as control and irradiated with UV light ($\lambda = 365$ nm). Subsequently, samples were loaded on a NiNTA agarose column and were washed under denaturing conditions (8 M urea) to get rid of proteins not covalently bound to CK2β. After elution, proteins were digested with trypsin and analyzed by liquid chromatography (LC) coupled to high-resolution MS.

In samples containing $CK2\beta_{34pAzF}$, 71 proteins were detected with a higher intensity than in the sample without a single CK2β variant (control). In samples containing $CK2\beta_{62pAzF}$, 72 proteins, and in $CK2\beta_{80pAzF}$ containing samples, 74 proteins with an intensity higher than in the control

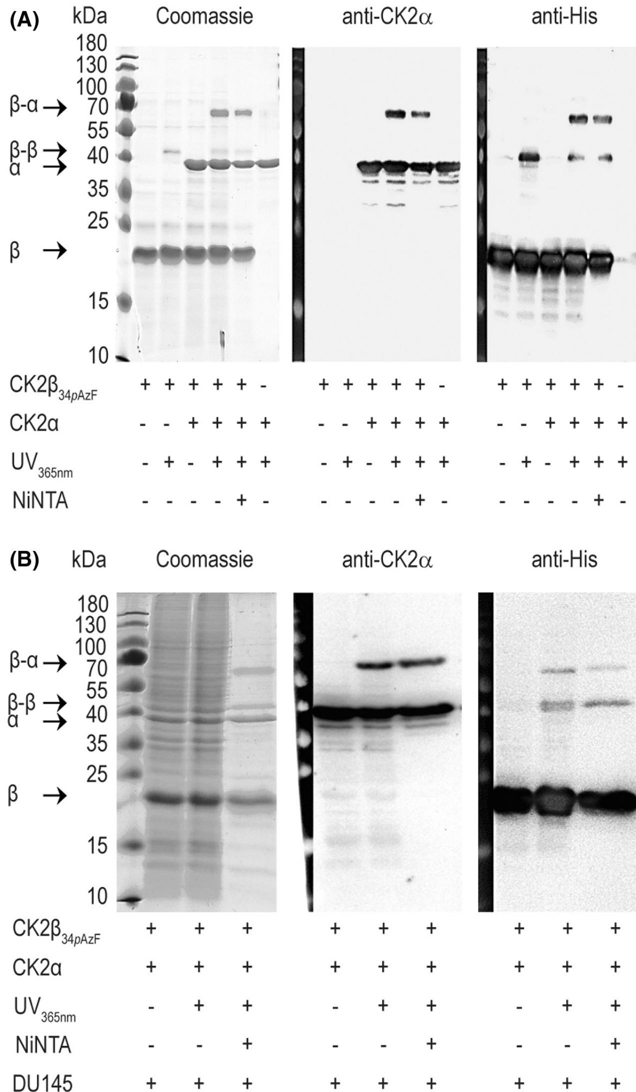
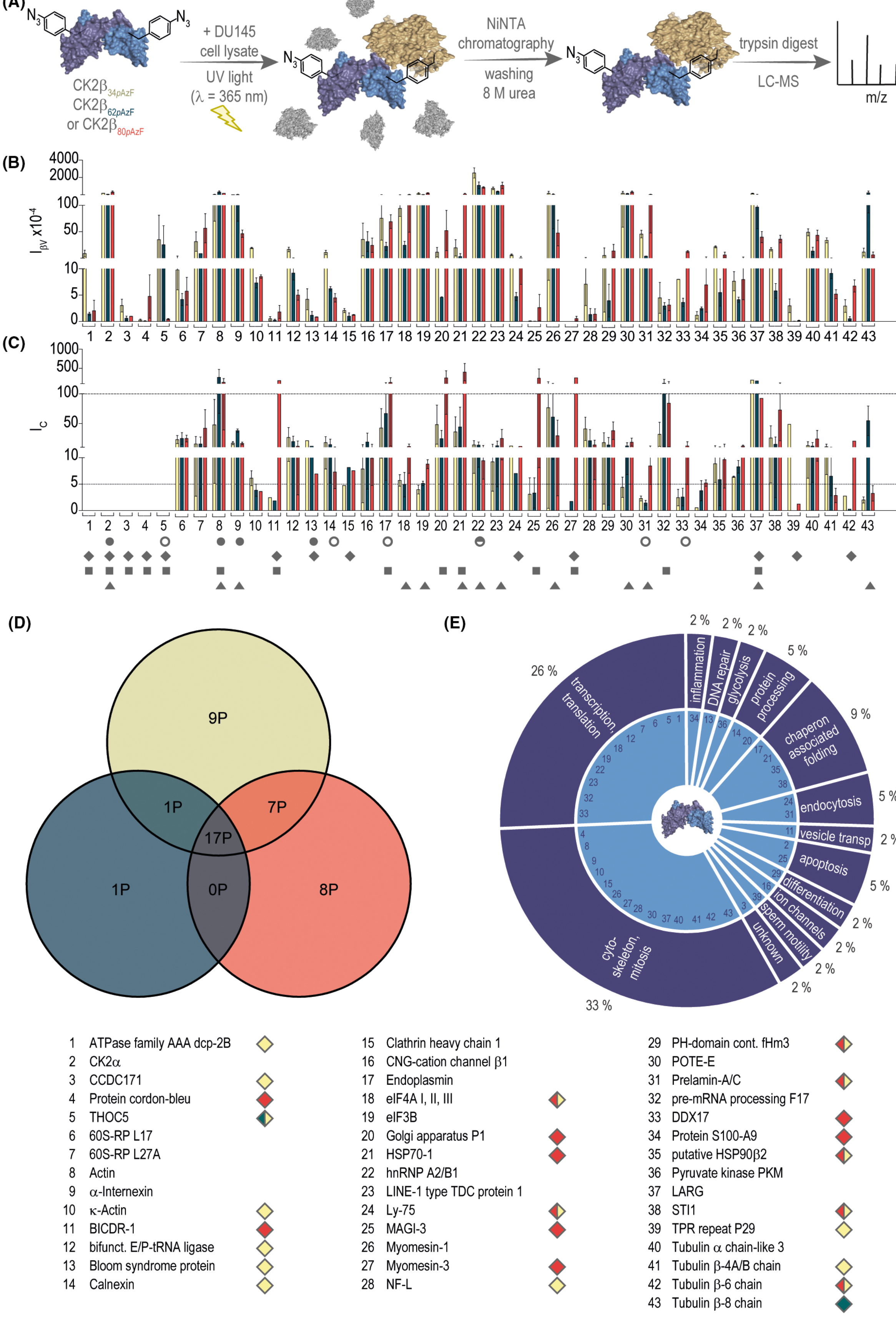


FIGURE 3 UV light induced crosslinking of CK2β_{34pAzF} with CK2α. (A) CK2β_{34pAzF} (10 μM) and CK2α (3 μM) were incubated for 15 min at 37°C, UV light irradiated (λ = 365 nm) and purified by NiNTA affinity chromatography. (B) CK2β_{34pAzF} (10 μM) and CK2α (3 μM) were incubated in DU145 cell lysate (5 mg/ml protein), UV light irradiated and purified by NiNTA affinity chromatography. Proteins were separated by SDS-PAGE and analyzed by Coomassie staining or western blot with primary antibody against CK2α or against 6xHis-tag.

(Table S3) were found. In summary, a total of 95 different proteins were assigned with all three variants. For further analysis, a fold-value of five was selected for the abundance of a protein detected in presence of one of the three CK2β variants compared to control. Of the 95 proteins, 27 proteins exceeded the threshold in samples with CK2β_{34pAzF} (yellow), 24 proteins with CK2β_{62pAzF} (petrol) and 36 proteins with CK2β_{80pAzF} (red); a total of 43 different proteins because of double or triple appearance (Figure 4B,C, Table S3). For further investigation, the ion intensity of individual proteins was set into relation to that of the CK2β variant in the same sample ($I_{\beta V}$, Figure 4B). This allowed a better comparison between the results obtained with the different CK2β variants. Furthermore, the ion intensity was set into relation to that of the same protein in the control sample without CK2β variant (I_C, Figure 4C). The greater the value of I_C, the clearer the identified protein appears as an interaction partner of CK2β, since it is not present in the control sample, or only to a small extent. Among the 43 proteins, 18 proteins were identified with an $I_{\beta V}$ value at least 2.5-fold higher for one variant than for the other two variants (Figure 4, colored diamonds). A distribution among the respective variants is shown in Figure 4D. It is noticeable that most of the proteins interact with CK2β particularly via position 34 or 80, but less frequently via position 62. Furthermore, an interaction of some proteins at more than only one position in CK2β simultaneously was observed, that can be explained by multiple interaction sites of large proteins with CK2β. Besides, it cannot be excluded that they bind to oligomeric CK2 superstructures and thus interact with two CK2β subunits of different holoenzymes at different sites.

Four of the 43 proteins have already been reported as interaction partners of CK2β (Figure 4, filled circles; CK2α, Actin,[32] α-Internexin, Bloom syndrome protein[54]), while further five proteins were known as interaction partners of CK2α (Figure 4, unfilled circles; THO complex subunit 5 homolog,[55] Calnexin,[56] Endoplasmin,[57] Prelamin-A/C[58] and probable ATP-dependent RNA-helicase DDX17[55]). These were therefore considered also to be potential CK2β interaction partners. The heterogeneous nuclear

FIGURE 4 Identification and analysis of CK2β interaction partners. (A) Experimental workflow. One of the CK2β variants (20 μM) or no external protein as control were mixed each with lysate of DU145 cell lysate, photo-crosslinking was performed by UV light irradiation. Cross-linked products were enriched by NiNTA affinity chromatography followed by harsh washing conditions. After tryptic digestion, proteins coupled to CK2β were analyzed using LC–MS. (B) Ion intensities of the proteins identified in samples containing CK2β_{34pAzF} (yellow), CK2β_{62pAzF} (petrol) or CK2β_{80pAzF} (red) were related to the intensity of the corresponding CK2β variant ($I_{\beta V}$) or (C) to the intensity of the respective protein in the control (I_C). Proteins already described as interacting partner of CK2β (filled circles) or CK2α (unfilled circles) as well as proteins showing at least for one variant a mean $I_{\beta V} > 0.01$ (triangles) or a mean $I_C > 100$ (squares) were marked. Experiment was performed in two independent replicates and data are shown as mean including range. Proteins that were not detected in control in at least one replicate were marked with a gray diamond. (D) Distribution of proteins (P) on the respective variant in which presence they were found with an $I_{\beta V}$ value at least 2.5-fold higher than in the presence of the other variants. These proteins were also marked in the list with a diamond in the respective color. (E) Assignment of identified proteins to their physiological function.



(A)
N3
+ DU145
cell lysate
UV light
(λ = 365 nm)
NiNTA
chromatography
washing
8 M urea
trypsin digest
LC-MS
m/z
CK2β34pAzF
CK2β62pAzF
or CK2β80pAzF
(B)
Iβv x10-4
(C)
Ic
(D)
9P
1P
7P
17P
1P
0P
8P
(E)
transcription, translation
inflammation
DNA repair
glycolysis
protein processing
chaperon associated folding
endocytosis
vesicle transp
apoptosis
differentiation
ion channels
sperm motility
unknown
cyto-skeleton, mitosis
26 %
2 %
2 %
2 %
5 %
9 %
5 %
2 %
5 %
2 %
2 %
2 %
2 %
33 %
1 ATPase family AAA dcp-2B
2 CK2α
3 CCDC171
4 Protein cordon-bleu
5 THOC5
6 60S-RP L17
7 60S-RP L27A
8 Actin
9 α-Internexin
10 κ-Actin
11 BICDR-1
12 bifunct. E/P-tRNA ligase
13 Bloom syndrome protein
14 Calnexin
15 Clathrin heavy chain 1
16 CNG-cation channel β1
17 Endoplasmin
18 eIF4A I, II, III
19 eIF3B
20 Golgi apparatus P1
21 HSP70-1
22 hnRNP A2/B1
23 LINE-1 type TDC protein 1
24 Ly-75
25 MAGI-3
26 Myomesin-1
27 Myomesin-3
28 NF-L
29 PH-domain cont. fHm3
30 POTE-E
31 Prelamin-A/C
32 pre-mRNA processing F17
33 DDX17
34 Protein S100-A9
35 putative HSP90β2
36 Pyruvate kinase PKM
37 LARG
38 STI1
39 TPR repeat P29
40 Tubulin α chain-like 3
41 Tubulin β-4A/B chain
42 Tubulin β-6 chain
43 Tubulin β-8 chain

ribonucleoproteins (hnRNP) A2/B1 are described as interaction partners of CK2β as well as CK2α.[59]

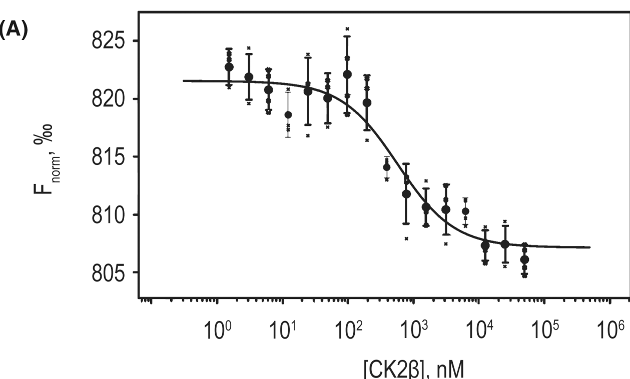
For a further classification of the physiological and pathophysiological significance of the observed interactions, the proteins were grouped according to general functions (Figure 4E). It should be noted that some proteins, such as CK2α, have multiple functions, but have been listed only once according to a main function. One third of the identified proteins are involved in the organization of the cytoskeleton, and one-fourth are involved in transcription and translation.

To verify and characterize an interaction with CK2β, the proteins with the highest intensities were further investigated. Four, namely CK2α,[2] Actin,[8] heat shock 70 kDa protein 1 (HSP70-1, 21) and Rho guanine nucleotide exchange factor 12 (leukemia-associated RhoGEF, LARG, 37) showed a mean $I_{\beta V}$ value higher than 0.01 (Figure 4B,C, triangles), as well as a mean I_C value higher than 100 (Figure 4B,C, squares). Since both, CK2α and Actin, were already known as interaction partners of CK2 and LARG was not available commercially, only HSP70-1 was further studied.

3.3 | Quantification and characterization of HSP70-1 binding to CK2β

Microscale thermophoresis (MST) with fluorescently labeled HSP70-1 was used to analyze the binding to CK2β. For this purpose, HSP70-1, labeled with NT-647-NHS (NanoTemper Technologies GmbH, Munich, Germany), was applied in a concentration of 10 nM and mixed with 2–50,000 nM CK2β (Figure 5A). By this strategy, a dissociation constant of 0.57 μM was determined for HSP70-1 with CK2β. To our knowledge, this is the first experimentally determined affinity for a binding partner of human CK2β beyond CK2α and CK2α′.

To exclude, that HSP70-1 could be a substrate of CK2, phosphorylation studies using an ADP-Glo assay were performed and indicated that HSP70-1 is neither a substrate of CK2 and nor does it influence the phosphorylation of the CK2 substrate peptide (Figure 5B). In assay as performed, the ADP produced during the phosphorylation reaction is converted back to ATP, which is then detected by a luciferase reaction. CK2α supplemented with CK2β showed an increased ADP generation compared to the buffer control sample. This appears be due to either a phosphorylation of CK2β within the CK2 oligomeric superstructures, a so called 'CK2 autophosphorylation'[60] or to an ATPase activity of CK2 in absence of substrate. In the presence of HSP70-1, ADP generation was not increased, which excluded a phosphorylation of HSP70-1 by CK2 as substrate. As positive control, in the presence of the standard CK2 substrate peptide (RRRDDDSDDD) the luminescence signal largely



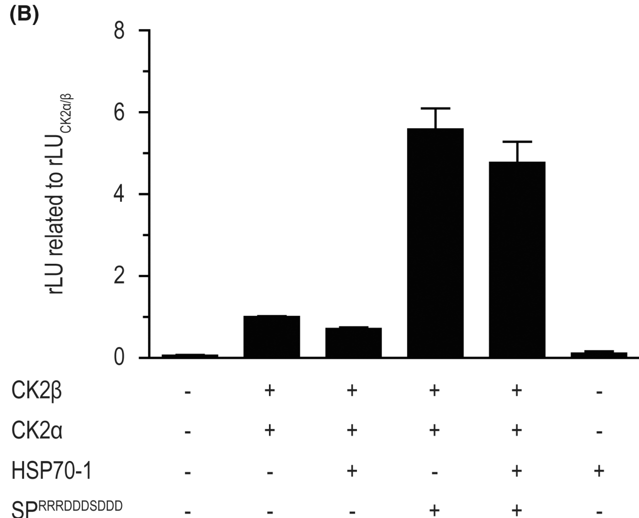


FIGURE 5 Characterization of the interaction of CK2β and HSP70-1. (A) The binding affinity of fluorescently labeled HSP70-1 to CK2β was measured by microscale thermophoresis. The K_D-value was determined to 0.57 μM. Data is shown as mean ± SD, $n = 4$. (B) Phosphorylation through CK2α supplemented with CK2β in the ratio 1:2 was analyzed in presence or absence of 1 μM HSP70-1 and 300 μM of the CK2 substrate peptide (SP) RRRDDDSDDD by ADP-Glo™ assay. The rLU were related to the mean rLU value of the samples containing only CK2α and CK2β. Data are shown as mean ± SD, $n = 3$.

increased, indicating phosphorylation as expected. Adding HSP70-1 to this sample did not lead to a significantly higher or lower phosphorylation signal. This indicated that HSP70-1 is not modulating the enzyme activity of CK2. The slight reduction of the luminescence signal in presence of HSP70-1 could have been due to nucleotide binding of HSP70-1 at its known nucleotide binding site, reducing the amount of ATP available for luciferase reaction.

3.4 | Interaction of CK2β and HSP70 in tumor cells

To confirm the in vitro observed interaction of CK2β and HSP70 in living cancer cells, co-immunoprecipitation

experiments were performed. For this purpose, extracts of DU145 cells, LNCaP cells and HeLa cells treated with and without heat shock were prepared and precleared with pure protein G sepharose beads. Afterwards, the supernatant from this preclearing step was incubated with protein G sepharose beads loaded with a specific serum against CK2β.[45] Bound proteins from the preclearing step as well as immunoprecipitated proteins were analyzed by immunoblot, subsequently using an antibody against CK2β first (Figure S1), an antibody against HSP70 (Figure S2) and finally an antibody directed against CK2α on the same blot (Figure 6). Co-immunoprecipitation was performed with DU145 cells as used for photo-crosslinking experiments. In addition, another prostate carcinoma cell line, LNCaP, and the cervix carcinoma cell line HeLa were applied. In all three cell lines, HSP70 was found to be co-precipitating with CK2β. Treating the cells with a heat shock before preparing the extracts subjected to co-immunoprecipitation resulted in a further increase of the amount of HSP70. This was to expect for a protein related to cellular stress response. For comparison, levels of CK2α, also co-precipitating with CK2β as expected, remained constant in the precipitates before and after heat shock.

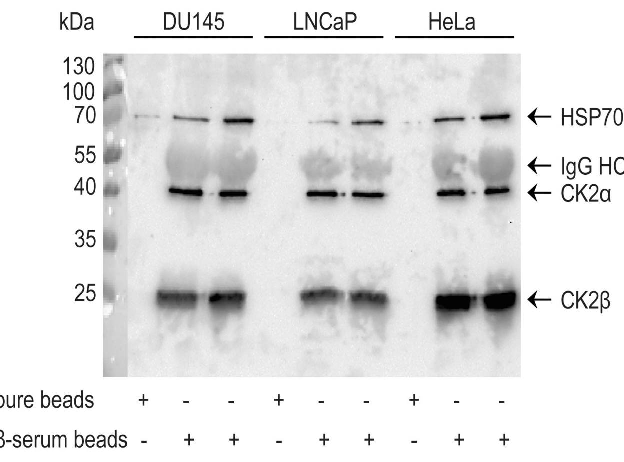


FIGURE 6 Co-immunoprecipitation of CK2β and HSP70 in extracts of DU145, LNCaP and HeLa cells. Cells were harvested after or before heat shock at 42°C for 30 min. Cell extracts were prepared, and an amount corresponding to 1 mg protein was incubated with pure protein G sepharose beads (pure beads). After removing pure beads, cell extracts were incubated with beads carrying an CK2β serum for co-immunoprecipitation (β-serum beads). Precipitates were separated by SDS-PAGE and analyzed by western blot subsequently using a primary antibody against CK2β first, a primary antibody against HSP70 and finally a primary antibody against CK2α. Extracts of all cell lines were incubated with mere protein G sepharose beads prior to co-immunoprecipitation to remove proteins binding non-specifically. IgG HC: IgG heavy chains.

4 | DISCUSSION

The human non-catalytic CK2 subunit CK2β plays a central role in the activity regulation and substrate specificity modulation of the multifunctional protein kinase.[21,22] It is becoming more prominent that CK2β has also CK2α-independent functions.[61] However, the general regulation as well as the function of CK2β in the human cell remains elusive, not least in malignant cells.

In this study, 38 new interaction partners of CK2β were found in the lysate of DU145 prostate carcinoma cells. Among these, five known interaction partners of CK2α were detected (Figure 7, red bordered ellipses), being potential CK2β dependent CK2 substrates. Further 12 proteins, such as 60 S ribosomal proteins or eukaryotic initiation factors,[62,63] are related to known interaction partners of CK2β as they belong to the same protein family (Figure 7, blue filled ellipses). However, half of the 38 proteins have not yet been linked to CK2β in humans before (Figure 7, orange filled ellipses). Nevertheless, it appear worth emphasizing that some of these interactions with homologous proteins have been observed in other species, such as an interaction between the clathrin light chain and CK2β in mouse,[62] while an interaction of human clathrin heavy chain 1 with human CK2β was observed in this work. As an internal positive control, the strategy as applied here, led to five already known interaction partners of CK2β (Figure 7, blue bordered ellipses).

The total of 43 interaction partners of CK2β contribute to cellular mechanisms ranging from DNA-replication over transcription and translation to protein processing and folding, reflecting a widespread interactome. This versatility was proposed before for CK2 as well as for CK2β.[63,64] The high appearance of cytoskeletal proteins as interaction partners of CK2β may indicate an involvement in the reorganization of the cytoskeleton, which in turn is important for the migration and invasion of cancer cells. Furthermore, binding to cytoskeletal proteins might be an underestimated role of CK2β.

On a side note, an interaction between two proteins could occur also within a complex of several proteins. In addition, several proteins are related in certain physiological functions. Therefore, a consideration of the interactions between the binding partners of CK2β identified in this study could help to understand the relevant interactome. As shown in Figure 7, some of the proteins, in particular hnRNP A2/B1, Calnexin and Actin appear to be interconnected in several pathways. This could be an indication of a presence in common functional complexes or an interaction of CK2β with multiple steps of the same physiological process.

In this study, a photo-crosslinking and MS-based approach was applied for the identification of CK2β

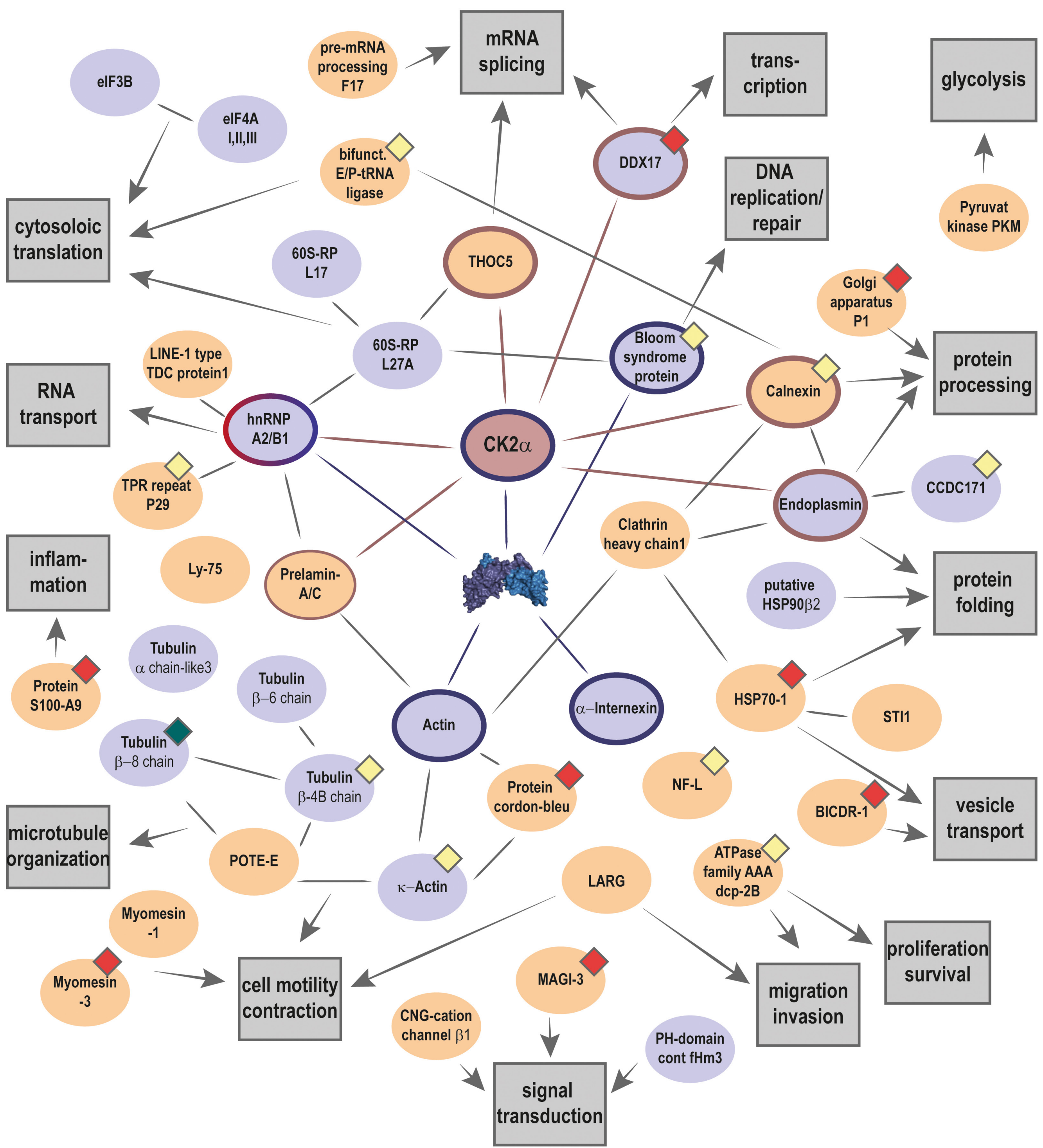
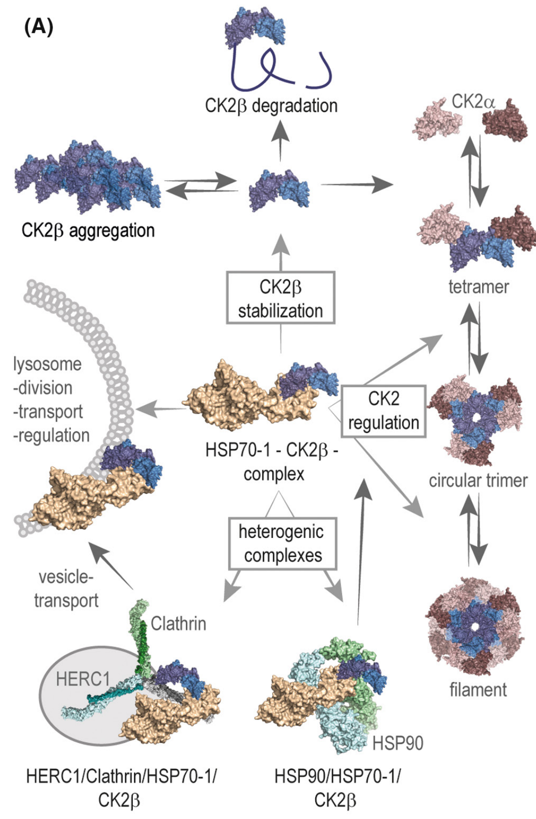


FIGURE 7 Interactome of the 43 CK2β binding proteins identified in this study with regard to their physiological function. Protein interactions listed on BioGrid[65] are marked by lines. Proteins already known to interact with CK2β are framed in blue, proteins that have a homolog known to interact with CK2β are highlighted in blue. Known interaction partners of CK2α are framed in red. Proteins that have not been listed as interaction partners of CK2β or CK2α before or that belong to protein families that are known to interact with CK2β are colored in orange. A rough classification according to the physiological function of the proteins as presented is indicated by arrows. Proteins found with a 2.5-fold excess in the presence of one of the three variants compared to the others are marked with a colored diamond: CK2β_{34pAzF} (yellow), CK2β_{62pAzF} (petrol) and CK2β_{80pAzF} (red).

interaction partners. Three different positions in CK2β were chosen for *p*AzF incorporation. Some proteins, such as CK2α, cytoplasmic Actin or POTE ankyrin domain family member E, were identified equally in the presence of all three variants. Nine proteins were found mainly in samples containing CK2β_{34pAzF} (Figure 7, yellow



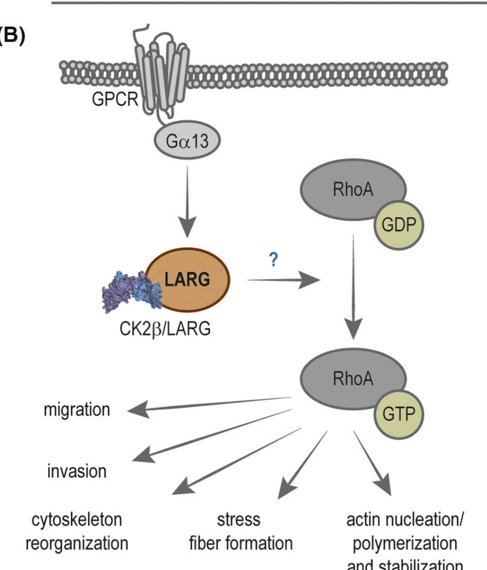


FIGURE 8 Hypothetical model of possible interaction pathways of CK2β with HSP70-1 and the influence of CK2β-LARG interaction on RhoA signaling. (A) The interaction of CK2β (PDB 1jwh) with HSP70-1 (PDB 3jxu—Nucleotide binding domain, PDB 4wv5—Substrate binding domain) may affect CK2β or CK2 regulation, various ongoing processes through interaction in heterogenic multicomplexes with clathrin (PDB 3lvh) and HERC1 or with HSP90 (PDB 2iop) as well as it may affect the regulation of lysosomes. (B) LARG is activated by G-protein coupled receptor activated G-protein Gα13, and normally activates RhoA responsible for migration and invasion of prostate cancer cells as well as for cytoskeleton reorganization, stress fiber formation and actin polymerization. The function of CK2β binding to LARG thereon is still unclear.

diamond). Since position 34 in CK2β is partly responsible for direct interaction with CK2α, these proteins probably interact with CK2β instead of CK2α. This could apply to the already described interaction of CK2β with the Bloom syndrome protein,[54] which is not described as a substrate of CK2, as well as to newly discovered interaction partners such as ATPase family AAA domain containing protein 2B. This is a histone binding protein functioning as an oncogenic transcription co-factor[66] and may be regulated by CK2β or interact with CK2β in larger protein complexes supporting migration and invasion in cancer cells. In contrast, calnexin was also found mainly in presence of $CK2\beta_{34pAzF}$. It is already known as a CK2 substrate,[67] so its recruitment by CK2β may occur first, whereupon CK2α binds to the CK2β-calnexin complex and finally phosphorylates calnexin. Proteins such as Tubulin β-8 chain that bind mainly to position 62 (Figure 7, petrol diamond) in the acidic loop of CK2β will most likely not interact with CK2 highly active ring structures, since the acidic loop of CK2β is occupied by a CK2α molecule of a neighboring holoenzyme in these CK2 superstructures.[18] At this point it appears worth mentoring that this CK2 superstructures were detectable in vitro. Whether this happens also inside the cell is subjected to further investigations. Interestingly, Tubulin β-8 was the only protein, that was mainly identified with $CK2\beta_{62pAzF}$. This could suggest that the acidic loop serves less as an interaction site for binding partners of CK2β. Eight proteins, for example HSP70-1, were mainly identified with $CK2\beta_{80pAzF}$ (Figure 7, red diamond). To accurately classify the interaction partners as CK2 substrate, CK2 regulator or pure CK2β interactor, further investigations are indispensable, such as described here for HSP70-1.

This is the first time, to our knowledge, that an interaction of human HSP70-1 and human CK2β has been reported. The existence of CK2β in complex with single members of the heat shock 70 kDa protein family, however, has already been described in other species like *Saccharomyces cerevisiae*[63] or mice.[62] As mentioned above, this does not necessarily imply a direct interaction of CK2β with HSP70 proteins but can also be attributed to a common occurrence in a multiprotein complex. The interaction of HSP70-1 and CK2β was additionally proven by MST and thereby a K_D value of 0.57 μM was determined. In comparison to the interaction of porcine CK2α and mouse Hsp90 with a dissociation constant of 1 μM,[68] HSP70-1 shows nearly twice the affinity to CK2β. Co-immunoprecipitation experiments supported an in vivo interaction of HSP70-1 and CK2β. However, it is worth mentioning, that from the results of

this experiment it gets not clear, whether HSP70-1 interacts with free CK2β or the CK2 holoenzyme. Whereby it was clearly shown by photo-crosslinking coupled mass spectrometry-based experiments that HSP70-1 directly interacts with CK2β. Furthermore, MST experiments confirmed this interaction also in absence of CK2α or CK2α′ in vitro. HSP70-1 is a chaperon and involved in protein stress-protection, folding and transport of newly synthesized polypeptides, activation of proteolysis of misfolded proteins as well as in formation and dissolution of protein complexes.[69]

In this work, no phosphorylation of HSP70-1 by CK2 was detectable. This agrees with phospho-proteome studies where HSP70-1 was not significantly phosphorylated by CK2.[70] Furthermore, HSP70-1 showed no effect on CK2-activity toward the CK2 substrate peptide, thus suggesting HSP70-1 not as a modulator of CK2-activity. Hence, HSP70-1 seems to be an interaction partner of free CK2β or of CK2β as part of the CK2 holoenzyme but not as a CK2 substrate or activity modulator, rather interacting with holoenzyme integrated CK2β due to its function as a binding platform in multiprotein complexes.[22] Possible functions of the interaction are summarized in Figure 8A, including the formation of superordinate complexes. For example, a complex of CK2β or CK2 holoenzyme, HSP70-1 and HSP90 would be conceivable since HSP70 is a co-chaperon of HSP90.[71] Furthermore, HSP90 has been described as a modulator of CK2 superstructures,[72] as well as a substrate of CK2.[73] Another possible complex could result from the interaction of the already described p532-clathrin-HSP70 complex[74] with CK2β, since clathrin heavy chain 1 was also one of the proteins identified here. Further possible effects of the HSP70-1 – CK2β interaction could be the regulation of lysosomes, since HSP70-1 stabilizes the lysosomal membrane[75] and CK2β is known to interact with the lysosomal-trafficking regulator (LYST), regulating lysosomal exocytosis.[24] Not least, the interaction could have a stabilizing effect on free CK2β, which is rapidly ubiquinated and degraded in the cell in a proteasome dependent manner.[11,76] Phosphorylation of the N-terminus of CK2β within oligomeric CK2 structures protects it from degradation.[11] Since CK2β exists also in absence of CK2α, there must be some stabilization event apart of its CK2 dependent phosphorylation. HSP70-1 could stabilize CK2β as a chaperon and thus regulate the presence of CK2β unbound in the CK2 holoenzyme. The enhancement of the interaction after heat shock of cells as observed in the co-immunoprecipitation experiments support the assumption, that HSP70-1 prevents CK2β from heat associated unfolding followed by aggregation or degradation. However, an increased interaction of HSP70-1 and CK2β can also be due to an enhanced formation of complexes with CK2α and HSP90 in frame of stress induced chaperon machinery as mentioned already above. To our knowledge, this is the first time that binding of a protein other than CK2α or CK2α′ to CK2β has been quantified by a biochemically based setup. Furthermore, the verification of this interaction by MST validated the method as developed to be a valid tool suitable for the identification of CK2β interacting proteins.

As both, HSP70 and CK2β, are on one hand present in various tissues and on the other hand related to cancer,[77,78] the interaction was supposed to appear not only in the prostate carcinoma cell line DU145, but also in other carcinoma cell lines. Co-immunoprecipitation experiments with two additional cancer cell lines, the prostate carcinoma cell line LNCaP and the cervix carcinoma cell line HeLa, confirmed an in vivo interaction of CK2β and HSP70 across different cell lines and cancer types.

Another highly interesting interaction partner of CK2β present to the same extent with all three variants was the leukemia-associated RhoGEF (LARG). The 173 kDa protein is a guanine nucleotide exchange factor for the GTPase RhoA.[79] Previously reported interaction between CK2β and CDC42BPA and CDC42BPB were the first hints for a connection of CK2β to the Rho signaling pathways. In this work, however, a direct interaction between CK2β and the leukemia-associated RhoGEF was shown. Thus, this is the initial connection of CK2β and maybe CK2 with RhoA associated signal transduction. RhoA is predominantly involved in the organization of the cytoskeleton and thus in migration and cell cycle.[80,81] In tumor cells, the RhoA/ROCK signal transduction supports invasion and metastasis. In prostate cancer LARG was shown to link bombesin receptors to RhoA signaling pathway stimulating migration and actin stress fiber formation.[82] Since LARG activates RhoA, it could be assumed that the interaction with CK2β has a regulating effect of its activating properties on RhoA, hypothetically illustrated in Figure 8B. This would be an interesting function of CK2β to investigate in the future.

In summary, using the newly established photo-crosslinking based method, 38 human proteins were elucidated as new interaction partners of CK2β in human prostate carcinoma cells. A third of them is involved in cytoskeleton regulation, which on the one hand indicates a role of CK2β in its organization, but on the other hand can also mean a regulation mechanism of CK2β itself. The interaction of CK2β with HSP70-1 shown here may also represent a new factor in CK2β regulation and stabilization. But this needs to be further investigated, as well as the interaction between CK2β and LARG, providing a

promising new insight into the physiological role of CK2β intervening in the Rho signaling pathway.

AUTHOR CONTRIBUTIONS

Anna Nickelsen and Joachim Jose conceived and designed the research; Anna Nickelsen, Claudia Götz, and Simone König performed the research and acquired the data; Anna Nickelsen, Claudia Götz, Simone König, Karsten Niefind and Joachim Jose analyzed and interpreted the data. All authors were involved in drafting and revising the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and/or supplementary material of this article. The data for the protein interaction network were derived from the following resources at https://thebiogrid.org.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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