Biochimica et Biophysica Acta 1833 (2013) 439–451

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Functional interaction of protein kinase CK2 and activating transcription factor 4 (ATF4), a key player in the cellular stress response

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ARTICLE INFO

Article history:
Received 9 August 2012
Received in revised form 5 October 2012
Accepted 23 October 2012
Available online 31 October 2012

Keywords:
Phosphorylation
Protein kinase
Transcription
Transcription factor
Protein stability

ABSTRACT

Protein kinase CK2 is a pleiotropic enzyme, which is implicated in the regulation of numerous biological processes. It seems to regulate the various functions by binding to other proteins and by phosphorylation of many different substrates. Here, we identified the activating transcription factor 4 (ATF4), an essential component of the ER stress signaling, as a new binding partner and a new substrate of CK2 in vitro and in vivo. Bifluorescence complementation analysis (BiFC) revealed that CK2α and ATF4 associate in the nucleus. By using mutants of ATF4 we identified serine 215 as the main CK2 phosphorylation site. The ATF4 S215A mutant turned out to be more stable than the wild-type form. We further noticed that an inhibition of CK2 caused an increased transcription of the ATF4 gene. Analyses of the transcription factor activity revealed an impaired activity of the CK2 phosphorylation mutant of ATF4. Thus, we show that (i) ATF4 is a binding partner of CK2α (ii) ATF4 is a substrate of CK2, (iii) the phosphorylation of ATF4 by CK2 influences the stability of ATF4, (iv) the transcription of ATF4 is regulated by CK2 and (v) the transcription factor activity of ATF4 is regulated by the CK2 phosphorylation of ATF4. Thus, CK2 plays an essential role in the regulation of the ER-stress induced signaling pathway.

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1. Introduction

Protein kinase CK2, formerly known as casein kinase 2 is a ubiquitously expressed pleiotropic serine and threonine kinase, which is absolutely essential for life of eukaryotes. In many organisms protein kinase CK2 is a heterotetramer composed of two catalytic α- or α′-subunits and two non-catalytic β-subunits [1]. CK2 is known to phosphorylate more than 300 different substrates including many proteins involved in transcription. In addition to the holoenzyme there is increasing evidence for CK2 subunits either in free form or associated with other proteins [2–5]. Substrates can be phosphorylated by the catalytic subunits alone or by the holoenzyme. By using a yeast two-hybrid screen with CK2α as a bait, we recently identified the splicing factor hPrp3p, the motor neuron protein KIF5C, the transcription factor Pdx-1 and the transition nuclear protein TNP-1 as new binding partners of CK2 [6–11]. With the exception of TNP-1, all other new binding partners also served as substrates for CK2. Binding partners of CK2 were found to be associated within the plasma membrane, and are implicated in the regulation of gene expression by targeting intracellular signaling cascades, DNA replication, transcription or the translation machinery, cell cycle regulatory proteins or guardian angels maintaining cellular integrity or apoptosis [12]. Here, we identified the transcription factor ATF4, a central molecule in the ER stress signaling pathway, as a new binding partner and as a new substrate for CK2.

The activating transcription factor 4 (ATF4) also known as CREB-2 was first identified as a transcription factor that can negatively regulate transcription from the cAMP responsive element [13]. ATF4 is a member of the ATF family of basic leucine zipper transcription factors, which are implicated in stress responses [14,15], development [16] and learning memory [17]. ATF4 can be transcriptionally [18] and translationally [15,19] regulated. In response to unfolded protein response in the endoplasmic reticulum (ER) the PERK kinase phosphorylates the eukaryotic initiation factor eIF2α thereby down-regulating global translation. However, a group of stress-responsive mRNAs including ATF4 is translated more efficiently when eIF2α is phosphorylated. ATF4 is expressed only at low concentrations, but becomes rapidly induced under stress conditions. ATF4 binds to promoter regions of genes whose products are implicated in adaptation, redox control or depending on the extent of ER stress can trigger apoptosis. Forced expression of ATF4 was sufficient to promote cell death [20]. In the ER-stress pathway there are two proteins, CHOP/gadd153 and Nrf2 [21,22] whose activities are regulated by CK2 phosphorylation and whose transcription is regulated by ATF4. There is increasing evidence that ATF4 is also regulated by phosphorylation [23,24]. Therefore in the present study we analyzed whether ATF4 might be a substrate for CK2 and how CK2 phosphorylation might regulate ATF4 properties and functions. By subjecting ATF4 to an in silico phosphorylation, we identified 12 putative CK2 phosphorylation sites. By in vitro and in vivo assays, we identified ATF4 as a new substrate for CK2. Using deletion as well as point mutants of ATF4, we identified serine 215 as the main CK2 phosphorylation site. Finally, it turned out that CK2 phosphorylation at serine

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http://dx.doi.org/10.1016/j.bbamcr.2012.10.025

215 influences the stability as well as the transcription factor activity of ATF4.

2. Material and methods

2.1. Chemicals and biological reagents

DNA modifying enzymes were purchased from Fermentas (Thermo scientific, St. Leon-Rot, Germany). Tissue culture media were purchased from GIBCO. The fetal calf serum (FCS) was from PAA (Pasching, Austria) and $\gamma[^{32}P]$ATP from Hartmann Analytic (Braunschweig, Germany). Cycloheximide (CHX) was purchased from Roth (Karlsruhe, Germany), MG132 and thapsigargin were purchased from ENZO life sciences (Lörrach, Germany). 4,5,6,7-Tetrabromobenzotriazole (TBB, VWR, Darmstadt) [25] and quinalizarin (Labotest OHG, Niederschöna) [26], both specific CK2 inhibitors, were dissolved in dimethyl sulfoxide (DMSO) to 10 mM stock solutions. We used TBB and quinalizarin in a concentration of 50 μM over a period of 24 h. 3,3′,5,5′ Tetramethylbenzidine (TMB) was from Sigma-Aldrich (München, Germany), 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was from Roche (Mannheim, Germany).

2.2. Cell lines

HeLa cells (ATCC; CCL-2) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and 1% glutamine. Cells were cultured at 37 °C and 5% CO_2 in a humidified atmosphere in an incubator.

2.3. Plasmid DNA constructs

Deletion mutants of ATF4 were created by PCR and inserted into the plasmid pGEX-4T-1 in frame with the GST-coding sequence. Point mutations were generated by overlapping extension PCR and the mutated DNA was cloned into the plasmid pGEX-4T-1 and p3XFLAG-CMV7.1 (Sigma-Aldrich, München, Germany). The integrity of the DNA constructs was verified by sequencing. The ATF4 5′UTR and AUG-Luc (No. 21850) were purchased from Addgene (Cambridge, USA). The 2xAARE-Luc was kindly provided by Dr. A. Bruhat [27]. The 5′UTR ATF3-Luc was a kind gift from G. Thiel (Homburg, Germany) [28,29].

For the BiFC analysis (bimolecular fluorescent complementation), pEYFPc1 vectors containing the full-length CK2α and the full-length CK2β were a kind gift of O. Filhol (Grenoble, France) [30]. To generate an YN- and YC-ATF4 we digested ATF4 wild-type, which was cloned into p3XFLAG-CMV7.1 with EcoRI and BamHI. Subsequently the fragment was cloned into pEYFPc1, which contains either the N-terminal fragment of EYFP (AS 1–154) or the C-terminal fragment (AS 155–238). The dominant-negative mutants CK2α K68M and CK2α′ K69M (generous gift of D. Litchfield, London, Ontario, Canada) were generated by cloning the cDNA from the corresponding pRC/CMV vectors into Hind III/BamHI sites of p3xFlag/CMV-7.1 [31]. Constructs generated by cloning were checked by DNA sequencing.

2.4. Antibodies

The antibodies eIF2α (# 9722) and phospho-eIF2α (# 9721) were purchased from New England BioLabs (Frankfurt am Main, Germany). The anti-ATF4 antibodies (sc200) and (sc22800) and also the anti-GAPDH antibody were purchased from Santa Cruz Inc. (Heidelberg, Germany); anti-GST antibody (27–4577) was from Pharmacia Biotech (Freiburg, Germany); anti-FLAG M2 (F1804) antibody and the anti-α-tubulin (T9026) antibody were purchased from Sigma-Aldrich (München, Germany); anti-GFP antibody, which recognizes only full-length YFP, was from Abcam (Cambridge, UK). The antibodies anti-CK2α antibody #26, anti-CK2β antibody #32, the monoclonal antibodies 1A5 (anti-CK2α) and 6D5 (anti-CK2β) were previously described [32]. Goat anti-mouse IgG (No. 115-035-146) and goat anti-rabbit IgG (No. 111-035-144) were from Dianova (Hamburg, Germany), Alexa Fluor546 goat anti-mouse IgG and Alexa Fluor488 goat anti-rabbit IgG were from Molecular Probes (Invitrogen, Darmstadt, Germany).

2.5. Transient transfection and luciferase assay

24 h before transfection with Turbofect® (Fermentas, Thermo scientific, St. Leon-Rot, Germany), HeLa cells were seeded on a 10 cm cell culture plate (for co-immunoprecipitation) in a total volume of 5 ml/plate of DMEM plus 10% FCS and 1% glutamine. 24 h later cells were transfected with 5 μg DNA by using Turbofect®. For a 6-well plate (CHX and MG132 experiments), HeLa cells were seeded in a total volume of 3 ml/well of DMEM supplemented with 10% FCS and 1% glutamine and 24 h later transfected using Turbofect® with 4 μg DNA. For a 24-well plate (reporter assay), HeLa cells were seeded in a total volume of 1 ml/well of DMEM plus 10% FCS and 1% glutamine and 24 h later transfected using Turbofect® with 1 μg DNA. To normalize the experimental variations such as differences in transfection efficiencies we calculated the relative intensities of FLAG tagged protein amounts first in relation to GAPDH and then in relation to the luciferase activity.

2.6. Extraction of proteins

For Western blot analysis, HeLa cells were lysed with RIPA buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% sodium desoxycholate, 1% Triton X-100, 0.1% sodium dodecylsulfate (SDS)), supplemented with complete® EDTA free (Roche Diagnostics, Mannheim, Germany). After lysis cell debris was removed by centrifugation at 13,000×g. The protein content was determined with the BioRad reagent dye (BioRad, München, Germany). Protein extracts were immediately used for Western blot analysis.

2.7. Western blot analyses

Proteins were separated through a 12.5% SDS polyacrylamide gel, and transferred onto a PVDF membrane. The membrane was blocked with 5% dry milk in PBS-Tween20 for 1 h and then incubated with the specific antibodies which were diluted in PBS-Tween20 containing 1% dry milk. The membrane was incubated with a peroxidase-coupled secondary antibody (anti-rabbit 1:30,000 or anti-mouse 1:10,000) for 1 h. Signals were visualized by the Lumilight system of Roche Diagnostics (Mannheim, Germany).

2.8. Co-immunoprecipitation

HeLa cells were harvested and lysed 24 h post transfection with RIPA buffer. After lysis cell debris was removed by centrifugation at 13,000×g. The protein content was determined with the BioRad reagent dye (BioRad, München, Germany). For co-immunoprecipitation cell lysates were pre-cleared twice with a mixture of protein A agarose beads and CL 4-B agarose beads over a period of 45 min. The supernatant was incubated with a combination of the ATF4 specific rabbit polyclonal antibodies sc200 and sc-22800 (Santa Cruz Inc. Heidelberg, Germany) (15 μl of each) for 90 min. Beads were washed twice with PBS, pH 7.4. Bound proteins were eluted with SDS sample buffer (195 mM Tris/HCl, pH 6.8, 6% SDS, 15% β-mercaptoethanol, 30% glycerol, and 0.03% bromophenol blue) and separated on a 12.5% SDS polyacrylamide gel, transferred to a PVDF membrane and analyzed by Western blot with specific antibodies.

2.9. Purification of recombinant proteins

Overnight cultures of *Escherichia coli* BL21 (DE3) transformed with GST-fused constructs were diluted 1:10 in LB medium containing ampicillin. The cultures were grown up to an $OD_{600\ nm}$ of 0.6. Protein expression was induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM and incubated for 3 h at 37 °C. After centrifugation (5500×g; 10 min; 4 °C), cells were resuspended in buffer R1 (100 mM Tris/HCl, pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$, and 0.1% (w/v) Tween-20) supplemented with complete® (EDTA free) and 1 mg/ml lysozyme. Cells were lysed by sonification (3 times for 1 min) and centrifuged at 13,000×g for 15 min at 4 °C. The supernatants were incubated overnight at 4 °C with glutathione-agarose 4B (Macherey Nagel, Düren, Germany). Beads were washed 3 times with buffer R1. The GST-fused constructs were eluted with buffer R1 containing 20 mM glutathione.

2.10. In vitro phosphorylation with protein kinase CK2

To determine the incorporation of phosphate into the polypeptide chain of ATF4, GST-ATF4 (0.1 μg) was mixed with 0.6 μg CK2 holoenzyme in a volume of 25 μl of kinase buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 50 μM ATP, and 1 mM DTT). The reaction was started by adding 2 μCi γ[^{32}P]ATP and incubated for 5, 15, 30 and 45 min at 37 °C. The reaction was stopped by adding 10 μl of sample buffer. Finally samples were separated through a 12.5% SDS polyacrylamide gel. Phosphorylated proteins were detected by autoradiography and the protein bands were densitometrically scanned. The incorporation rate for the phosphorylation of ATF4 was performed as described earlier [7]. Briefly ATF4 protein was mixed with protein kinase CK2 in 20 μl of kinase buffer. The phosphorylation reaction was started by the addition of γ[^{32}P]ATP. After 30 min at 37 °C the reaction was stopped by the addition of 10 μl sample buffer. Proteins were separated in an SDS polyacrylamide gel and visualized by autoradiography. The incorporated radioactivity was measured from the excised bands by scintillation counting.

For the analysis of the phosphorylation of truncated versions of ATF4 recombinant GST-tagged ATF4 fragments (0.2 μg of GST-C1, 0.2 μg of GST-C2, 0.8 μg of GST-C3, 0.1 μg of GST-N1 and 0.6 μg of GST-N2) were mixed with equal amounts of CK2α, CK2α′ and CK2 holoenzyme (0.6 μg) in a volume of 25 μl of kinase buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 50 μM ATP, and 1 mM DTT). To start the reaction we added 2 μCi γ[^{32}P]ATP and incubated the samples for 30 min at 37 °C. The reaction was stopped by adding 10 μl of sample buffer. Finally samples were separated through a 12.5% SDS polyacrylamide gel. Phosphorylated proteins were detected by autoradiography.

2.11. Determination of the CK2 kinase activity

Cells were lysed and the extracts were used for kinase filter assays. The incorporation rate of [^{32}P] phosphate into the CK2-specific substrate peptide with the sequence RRRDDDSDDD was measured. 20 μl of kinase buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT) containing 30 μg of proteins was mixed with 30 μl CK2 mix (25 mM Tris/HCl, pH 8.5, 150 mM NaCl, 5 mM $MgCl_2$, 50 μM ATP, 1 mM DTT, and 0.19 mM substrate peptide) containing 10 μCi/500 μl γ[^{32}P]ATP. The mixture was spotted onto a P81 ion exchange paper. The paper was washed with 85 mM H_3PO_4 for 3 times. After treatment with ethanol the paper was dried and the Čerenkov-radiation was determined in a scintillation counter.

2.12. Pulse-labeling with [^{35}S]methionine

HeLa cells were transfected with FLAG-ATF4 or FLAG-ATF4 S215A for 24 h. Cells were washed three times with methionine-free DMEM plus 10% FCS and 1% glutamine and for 1 h labeled with 50 μCi of [^{35}S] methionine. The [^{35}S]methionine was removed by washing and the cells were incubated with DMEM plus 10% FCS and 1% glutamine. The cells were harvested at indicated times, lysed and FLAG-ATF4 and FLAG-ATF4 S215A were precipitated as described above. The [^{35}S]methionine labeled proteins were visualized by the Typhoon-Trio imaging system (GE Healthcare, Munich, Germany).

2.13. GST-pull-down-assay

15 μg of the GST-tagged ATF4 proteins was added to 30 μl of the GSH sepharose and incubated in 800 μl PBS with 0.1% Tween20 for 1 h. The beads were washed for 3 times in PBS/0.1% Tween-20. The pull-down reaction was carried out with 15 μg of recombinant CK2 holoenzyme or the individual CK2 subunits. Beads were washed with PBS/0.1% Tween20 for 4 times and the complex was separated on a 12.5% SDS polyacrylamide gel and visualized by Coomassie Blue staining.

2.14. ELISA based binding assay

The ELISA based binding assay was carried out as described by Hübner et al. [33]. The GST-ATF4 proteins (1 μg/well) or the CK2 holoenzyme in 50 mM $NaHCO_3$, pH 9.8, was coated on 96 well polystyrene microtiter plates (Nunc, Thermo scientific, Langenselbold) for 16 h at 4 °C. After blocking in 50 mM $NaHCO_3$, pH 9.8, with 5% bovine serum albumin (BSA) for 90 min at 22 °C, appropriate dilutions of the binding protein in 50 mM $NaHCO_3$, pH 9.8 with 1% bovine serum albumin (BSA) were added to the microtiter plates and incubated for 16 h at 4 °C. After washing with 50 mM $NaHCO_3$, pH 9.8 containing 1% BSA, bound complexes were detected as described for the ligand-Western blot assay and visualized with 3,3′,5,5′ tetramethylbenzidine (TMB).

2.15. BiFC (bimolecular fluorescent complementation) analysis

HeLa cells were seeded on coverslips and transfected with the BiFC plasmids for 24 h. After 24 h, cells were shifted to 28 °C for 24 h to promote the fluorophore maturation. Immunostaining of YFP was performed by indirect immunofluorescence using a GFP antibody that recognizes only the complete YFP. Cells were fixed in PBS, pH 7.4 with 4% paraformaldehyde for 10 min, permeabilized in PBS with 0.5% Triton X-100 for 10 min and pre-incubated in PBS with 5% BSA for 30 min at room temperature. Cells were incubated with the GFP antibody for 30 min at 37 °C, washed with PBS and incubated with Alexa Fluor 546 goat anti-mouse antibody for 30 min at 37 °C. The coverslips were washed and incubated with DAPI for 30 min at 37 °C before being sealed with mounting media.

2.16. Co-localization experiment

HeLa cells were seeded on coverslips. Immunostaining of ATF4 and CK2 was performed using sc200 and 1A5 antibody, respectively. Cells were fixed in PBS, pH 7.4 with 2% paraformaldehyde for 10 min, permeabilized in PBS with 0.5% Triton X-100 for 10 min and pre-incubated in PBS with 5% BSA for 30 min at room temperature. Cells were incubated with the ATF4 and CK2 antibodies for 30 min at 37 °C, washed with PBS and incubated with Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse antibody for 30 min at 37 °C. The coverslips were washed and incubated with DAPI for 30 min at 37 °C. The coverslips were sealed with mounting media.

3. Results

CK2 is known to be implicated in the regulation of many different basic cellular processes including transcription. An increasing number of transcription factors were identified as binding partners of protein

kinase CK2 [34–36]. ATF4 is a key player in the ER stress induced signaling cascade leading to growth arrest and eventually apoptosis. Since also CK2 was shown to be involved in the regulation of members of the ER stress pathway, we now wanted to know whether there might be a direct interaction between ATF4 and CK2. For this type of experiment we first used purified GST-tagged ATF4 and purified CK2$\alpha_2\beta_2$ holoenzyme. GST-tagged ATF4 was precipitated with GSH-sepharose and the associated proteins were analyzed on a 12.5% SDS polyacrylamide gel. As shown in Fig. 1 a GST-ATF4 bound to the CK2 holoenzyme. Control experiments showed that the CK2 subunits bound neither to GSH-sepharose nor to the GST-tag alone. In a separate lane we loaded the CK2 holoenzyme as a control. Thus, these pull-down experiments showed that ATF4 bound to the CK2 holoenzyme in vitro.

Next, we wanted to know whether the interaction between CK2 and ATF4 also exists in vivo. Therefore, we performed co-immunoprecipitation experiments in mammalian cells. HeLa cells were transfected with FLAG-tagged ATF4. Twenty-four hours after transfection, the cell extract was incubated with protein A sepharose as a control (C) or with an anti-ATF4 antibody linked to protein A sepharose (IP). The immunoprecipitate as well as the supernatant of the immunoprecipitate were analyzed on a 12.5% SDS polyacrylamide gel followed by a transfer to a PVDF membrane. The membrane was either incubated with an anti-FLAG-tag antibody to detect ATF4 or with a CK2α- or a CK2β-specific antibody. As shown in Fig. 1 b CK2α was co-immunoprecipitated with ATF4 (IP). The supernatant of the immunoprecipitate (S) contained a small amount of ATF4 and considerable amounts of CK2α and CK2β indicating that the antibody efficiently precipitated ATF4 and that only CK2α and no visible amount of CK2β was bound. To further support the observation above, we performed a co-immunoprecipitation experiment with endogenous proteins. To get detectable amounts of endogenous ATF4, HeLa cells were treated with thapsigargin for 4 h, which induces ER stress [37]. Four hours after treatment with thapsigargin, cells were extracted and CK2 was immunoprecipitated with the CK2 specific monoclonal antibody 1A5. Immunoprecipitates were analyzed by Western blot. As shown in Fig. 1 c CK2α bound to ATF4. As expected CK2α was also co-precipitated with CK2β in the CK2 holoenzyme (IP). ATF4 was also found in the supernatant (S) indicating that not all of ATF4 is complexed to CK2. Thus, we conclude from these experiments that ATF4 binds to CK2α not to CK2β.

Having shown that ATF4 bound to CK2, in the next step we wanted to confirm this interaction by immunofluorescence analysis and to analyze where in the cell this interaction takes place. HeLa cells were treated with MG132 for 24 h, a proteasome inhibitor, which is known to activate ATF4 [38]. Cells were stained with an antibody directed against ATF4 followed by an Alexa Fluor 488 coupled secondary antibody (green). CK2α was detected with antibody 1A5 followed by Alexa Fluor 546 coupled secondary antibody (red). The overlay shows a yellow staining for co-localized CK2α and ATF4 (Fig. 2).

To demonstrate a direct interaction between ATF4 and CK2 we performed a bimolecular fluorescent complementation (BiFC) experiment. The YN and the YC fragments of YFP were fused to CK2α, CK2β and ATF4. First of all, we analyzed the expression of the YN-ATF4, YC-ATF4, YC-CK2α, YN-CK2β, YN-CK2α and YC-CK2β hybrid proteins by Western blot. As shown in Fig. 3 a, fusion proteins were expressed and found to migrate at the expected size on a SDS-polyacrylamide gel. In the next step we transfected HeLa cells with different combinations of the YN-/YC-constructs for testing the interaction between CK2 and ATF4. As positive control, there was a clear signal for the interaction of YN-CK2α and YC-CK2β (Fig. 3 b). We found positive staining after transfection of YC-CK2α and YN-ATF4 fragments. BiFC signals were obtained exclusively in the nucleus of the transfected HeLa cells indicating that CK2α bound to ATF4 in the nucleus. There was no signal for YC-CK2α or YN-ATF4 alone. Although ATF4 was also expressed in combination with CK2β, we never detected positive signals (data not shown). These results confirmed the co-immunoprecipitation experiments, demonstrating that ATF4 and CK2α interact in vivo and in vitro, and this interaction takes place in the nucleus.

ATF4 is known to be a phosphoprotein [24]. Since most of the binding partners of CK2 are also substrates of CK2, we asked whether ATF4 might be also phosphorylated by CK2. Before we started to analyze the phosphorylation of different truncated forms of ATF4 we first analyzed kinetic parameters of the phosphorylation reaction. ATF4 was incubated with γ[^{32}P]ATP for various times. Phosphorylated

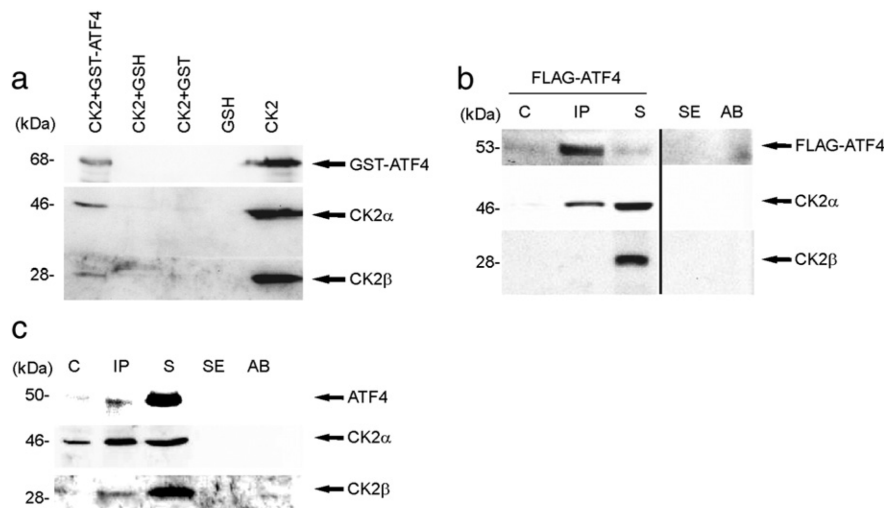


Fig. 1. CK2 interacts with ATF4 in vitro and in vivo. a) GST pull down assay. Bacterially expressed and purified CK2 holoenzyme and GST-tagged ATF4 were incubated together for 3 h. GST-ATF4 was bound by GSH-sepharose and then bound proteins were separated on a 12.5% SDS-polyacrylamide gel followed by Western blot. ATF4, CK2α and CK2β were detected using specific antibodies. b) Co-immunoprecipitation of endogenous CK2 with FLAG-ATF4. HeLa cells were transfected with p3XFLAG-CMV ATF4 for 24 h. Lysates were incubated with ATF4 specific antibody sc200. Eluted proteins from immunoprecipitates were detected with an anti-FLAG antibody, CK2α with antibody 1A5 and CK2β with antibody 6D5. c) Co-immunoprecipitation of endogenous ATF4 with endogenous CK2. HeLa cells were treated with 1 μM thapsigargin for 24 h to induce expression of endogenous ATF4. Lysates were incubated with CK2α specific antibody 1A5. Eluted proteins from immunoprecipitates were detected with antibody #26 against CK2α, CK2β with antibody 6D5 and ATF4 with antibody sc200. C, pre-precipitate; IP, immunoprecipitate; S, supernatant; SE, sepharose; AB, antibody; molecular markers are indicated on the left part of the gels.

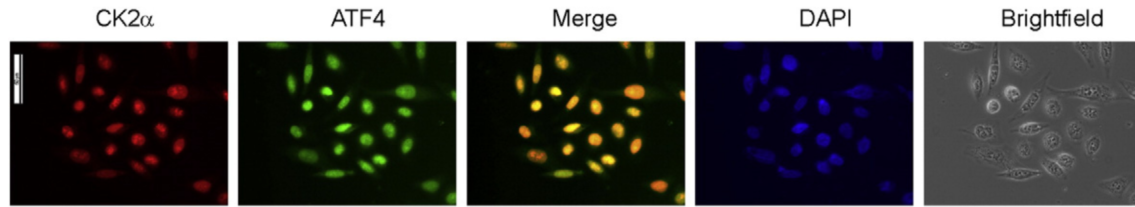


Fig. 2. Co-localization of ATF4 and CK2. Immunofluorescence images from HeLa cells treated with MG132 for 24 h. ATF4 was detected with the specific antibody sc200 and labeled by Alexa Fluor 488-coupled secondary antibody (green). CK2α was detected by specific antibody 1A5 and labeled by Alexa Fluor 546-coupled secondary antibody (red). Overlay of the images shows co-localization of ATF4 and CK2α (yellow). Scale bars: 50 μm.

proteins were analyzed on a SDS polyacrylamide gel followed by autoradiography (Fig. 4 a). The densitometry of the protein bands showed that the ATF4 protein was saturated with phosphate after 30 min. We choose this time point to determine the phosphate incorporation into ATF4. We performed 3 independent experiments and determined the incorporation rate to be 0.6 ± 0.2 mol phosphate into 1 mol of ATF4 (Fig. 4 b), which suggests that not every ATF4 molecule is phosphorylated.

By a computer based search for CK2 phosphorylation sites with the consensus sequence S/TXXD/E [39] on the polypeptide chain of ATF4 (in silico), we identified 12 putative CK2 sites, which are almost all localized within the first 220 amino acids (Fig. 4 c). In order to narrow down the putative CK2 sites we cloned various fragments of the ATF4 cDNA into a bacterial expression vector. A scheme which represents the fragments including the respective CK2 sites is shown in Fig. 4 c. The ATF4 fragments were expressed as fusion proteins with a GST-tag in *E. coli*, purified and then used for an in vitro kinase reaction with the CK2 holoenzyme and γ[^{32}P]ATP. Phosphorylated proteins were analyzed on a 12.5% SDS polyacrylamide gel followed by autoradiography. As a control we also stained the gel with Coomassie Blue in order to show that we used the same amount of protein (lower part of Fig. 4 d). Fragment N1, which contains the putative CK2 sites serine 18, serine 74 and serine 80 was not phosphorylated by CK2. Thus, these three N-terminal sites can be excluded as CK2 sites. Fragment N2 which represents the first 200 amino-terminal amino acids of ATF4 was also not phosphorylated by CK2, which excluded also threonine 107, threonine 114, threonine 115, threonine 149, serine 172, serine 184 and threonine 189 as CK2 phosphorylation sites. On the other hand, three C-terminal fragments of ATF4, namely C1, ranging from amino acids 94 to 351, C2 ranging from amino acids 173 to 351 and C3 ranging from amino acids 190 to 351 were considerably phosphorylated by CK2. Together with the results obtained

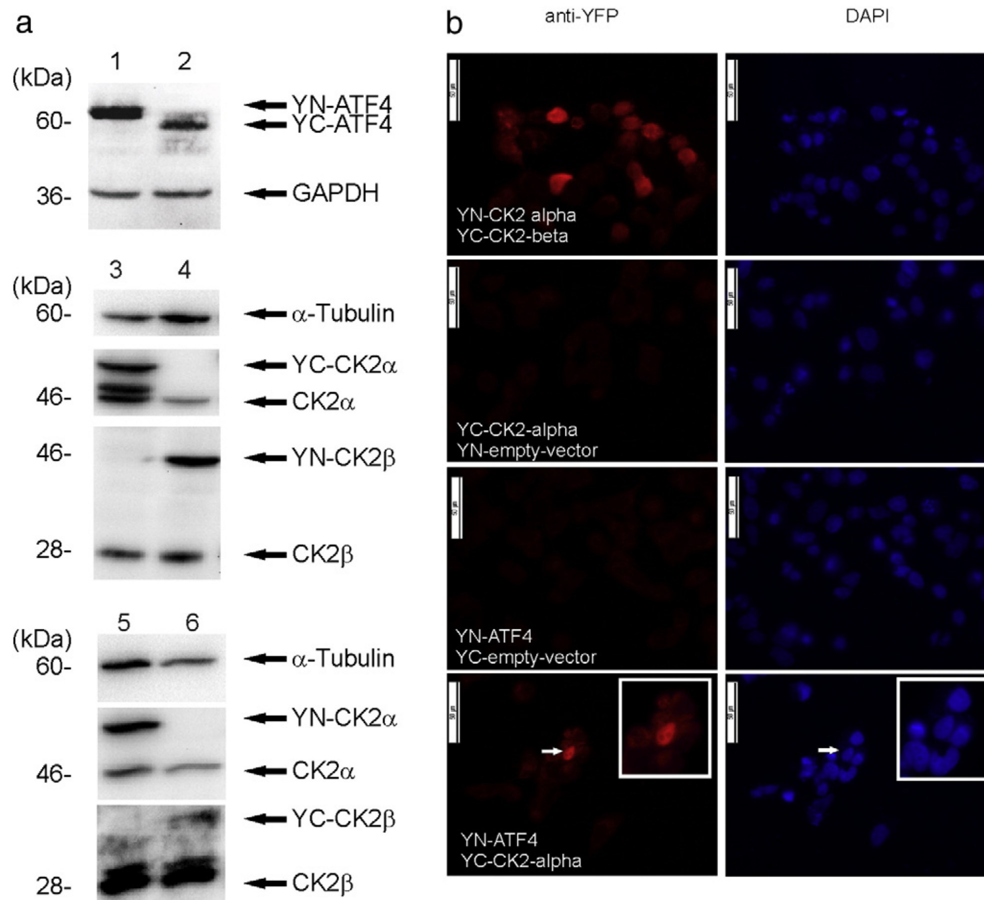


Fig. 3. In situ interaction of CK2 with ATF4. a) Hela cells were transfected with the plasmids expressing the N- or C-terminal fragments of YFP fused to ATF4 or CK2α and CK2β for 24 h. Proteins were separated on a 12.5% SDS-polyacrylamide gel followed by Western blot with anti-ATF4 antibody sc200, CK2α and CK2β-specific antibodies and with anti-GAPDH antibody as a loading control. Molecular weight markers are indicated at the left part of the gel. b) Immunofluorescence analysis of HeLa cells transfected with plasmids expressing the N- or C-terminal fragments of YFP fused to CK2α, CK2β or ATF4. The complemented YFP was detected with anti-GFP antibody. Scale bars: 50 μm.

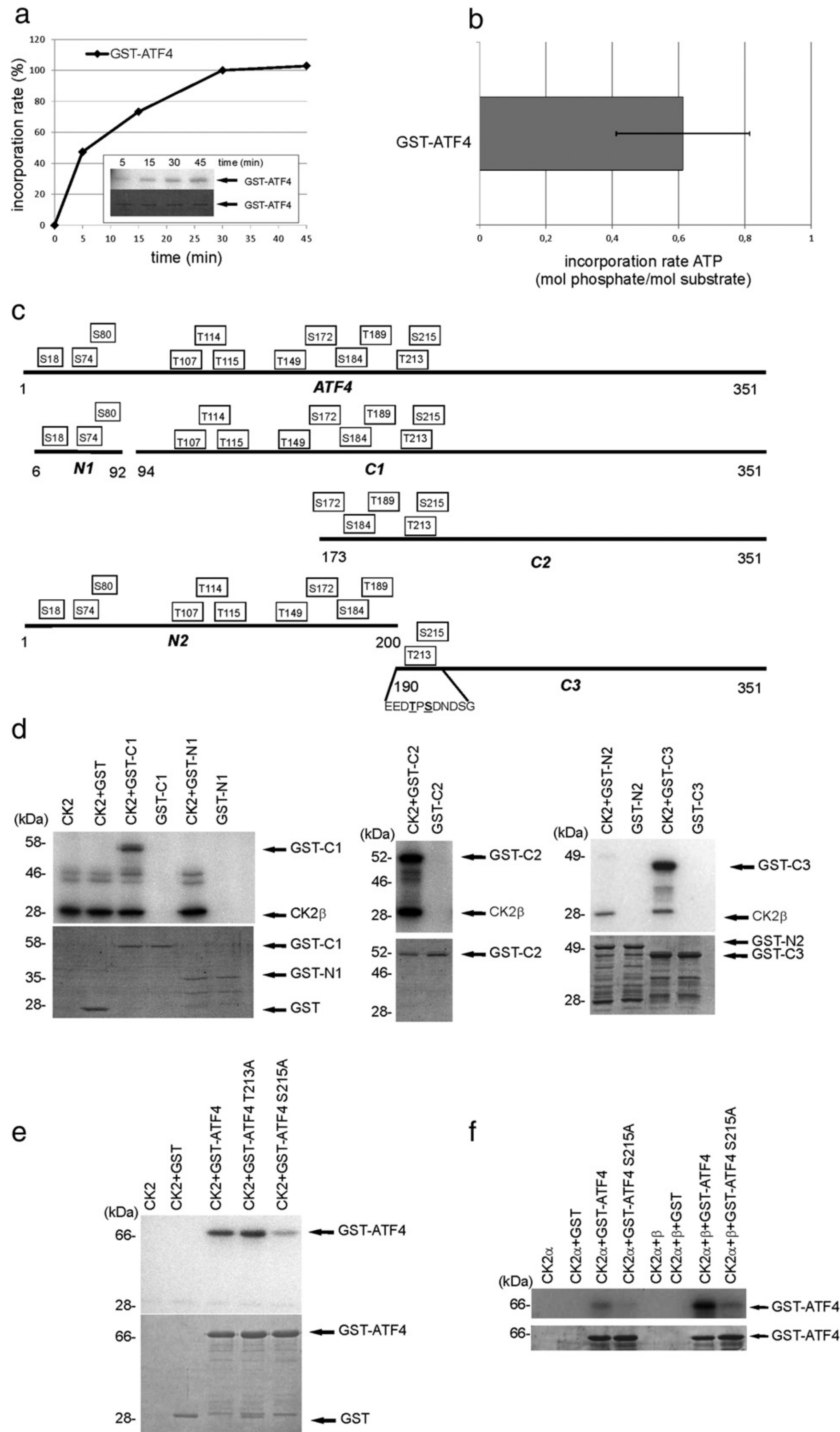


Fig. 4. Identification of the CK2 phosphorylation sites of ATF4. a) Time course study of the phosphorylation of ATF4. ATF4 was phosphorylated using the CK2 holoenzyme. After 5, 10, 15, 30 and 45 min the reaction was stopped adding sample buffer. After SDS polyacrylamide gel electrophoresis and autoradiography the bands were subjected to a densitometric analysis. The relative intensity of the bands at the different time points is shown in the graph. b) Phosphate incorporation rate into the polypeptide chain of ATF4. ATF4 was phosphorylated with CK2 in the presence of γ[^{32}P]ATP. Phosphorylated ATF4 was analyzed on a SDS polyacrylamide gel. The band was excised from the gel and incorporated phosphate was counted in a scintillation counter. The graph shows their incorporation of phosphate [mol] into protein [mol] as a result of three independent experiments. c) Schematic representation of N-terminal and C-terminal deletion fragments of ATF4 with the putative CK2 phosphorylation sites. d) Phosphorylation of N-terminal and C-terminal deletion mutants of GST-ATF4 by CK2 in vitro kinase assay. The recombinant GST-ATF4 fragments were incubated with or without recombinant CK2 holoenzyme in the presence of kinase buffer and 2 µCi of γ[^{32}P]ATP for 30 min at 37 °C. The samples were separated in a 12.5% SDS-polyacrylamide gel and the radioactivity was detected by X-ray films (upper part). The lower part shows Coomassie Blue staining of the gel. e) The phosphorylation of the recombinant GST-ATF4, GST-ATF4 T213A and GST-ATF4 S215A proteins were made as described in d). Molecular weight markers are indicated on the left. f) The phosphorylation of recombinant GST-ATF4 and GST-ATF4 S215A proteins by recombinant CK2α or by recombinant CK2 holoenzyme was made as described in. d) Molecular weight markers are indicated on the left.

with the N-terminal fragments, we have to conclude that serine 215 and threonine 213 are potential phosphorylation sites for CK2.

In the next step we mutated threonine 213 and serine 215 to alanine residues. The alanine mutants were expressed in bacteria and the purified proteins were used as substrates for CK2 together with $\gamma[^{32}P]$ATP. As a control, gels were also stained with Coomassie Blue to demonstrate the protein amount which was used for the phosphorylation reaction (lower part of Fig. 4 e). As shown in Fig. 4 e (upper part), the ATF4 T213A mutant was phosphorylated by CK2 as strong as wild-type ATF4, whereas ATF4 S215A was markedly less phosphorylated than the two other ATF4 forms. These data strongly suggest that serine 215 is the main CK2 phosphorylation site on the polypeptide chain of ATF4.

Most of the CK2 substrates are phosphorylated by the CK2 holoenzyme and by CK2α alone. A few substrates are not phosphorylated by CK2α or CK2α′ but they are efficiently phosphorylated by the holoenzyme. Finally, a limited number of substrates are not phosphorylated by the holoenzyme but by the CK2α or CK2α′-subunits (for review see [40]). Therefore, in the next step we asked to which class of substrates ATF4 might belong. Phosphorylation was performed as above but with the variation that we used CK2α alone or together with CK2β. As shown in Fig. 4 f, ATF4 was weakly phosphorylated by CK2α alone, whereas the holoenzyme consisting of CK2α/CK2β efficiently phosphorylated ATF4. The lower part of Fig. 4 f shows the gels, stained with Coomassie Blue to demonstrate the protein amount which was used for the phosphorylation reaction. We made the same observation when phosphorylating ATF4 with CK2α′-subunit alone or with a holoenzyme consisting of CK2α′/CK2β (data not shown). Thus, we have demonstrated that ATF4 is efficiently phosphorylated only by the CK2 holoenzyme.

Having shown that the CK2 holoenzyme bound to ATF4 and that serine 215 seems to be the major CK2 phosphorylation site, we asked whether the CK2 phosphorylation of ATF4 might influence its binding to CK2. We performed the same experiments as described above, but instead of wild-type ATF4 we used the S215A mutant. In a first set of experiments we performed a pull-down assay with GST-tagged ATF4 S215A and CK2 holoenzyme. As shown in Fig. 5 a, both CK2α and CK2β were co-precipitated with GST-ATF4 S215A. In another experiment FLAG-tagged ATF4 S215A was transfected into HeLa cells. FLAG-ATF4 S215A was precipitated with the antibody sc200 against ATF4. As shown in Fig. 5 b, CK2α was clearly co-precipitated (IP). Thus, mutant S215A bound to CK2 in vitro and in vivo. To verify the pull down assay and to compare the binding capacity of wild-type and mutant ATF4 we used an ELISA based binding assay as shown in Fig. 5 c. Recombinant CK2 holoenzyme bound to a microtiter plate was incubated with recombinant GST tagged ATF4 or ATF4 S215A. We found that both ATF4 and ATF4 S215A bound to CK2 holoenzyme with no significant difference. Thus, binding of CK2 to ATF4 is independent of the phosphorylation of ATF4 by CK2.

In the course of our experiments with wild-type ATF4 and the S215A mutant of ATF4, we always noticed a stronger expression of mutant ATF4. Therefore, it was an obvious question whether the stability of ATF4 wild-type and the CK2 phosphorylation mutant ATF4 S215A might be different. After transfection of cells with ATF4 and ATF4 S215A, protein synthesis was inhibited with cycloheximide. Cells were harvested immediately after incubation with cycloheximide (0), after 45 or 90 min incubation and the cell extracts were analyzed by Western blot with an anti-FLAG antibody. Fig. 6 a shows that the level of FLAG-ATF4 decreased rapidly whereas the level of FLAG-ATF4 S215A remained nearly constant over the

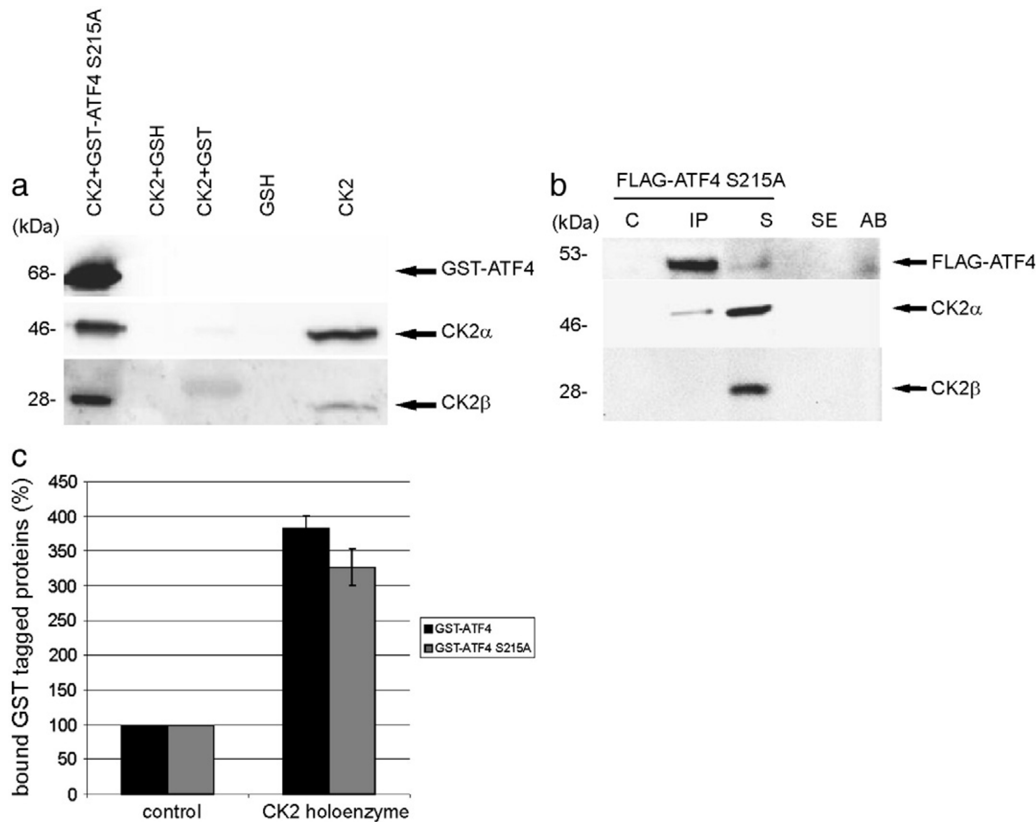
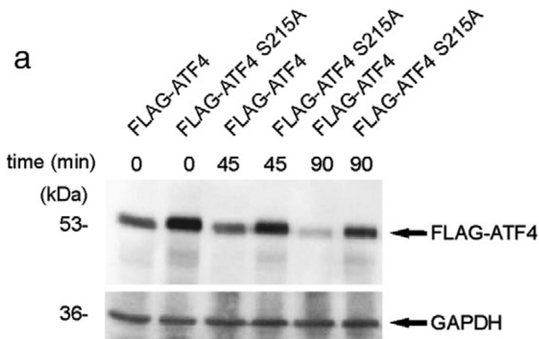
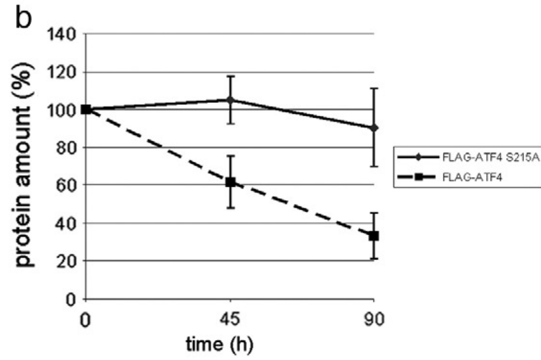
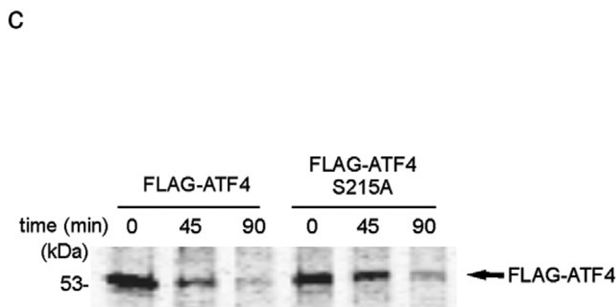


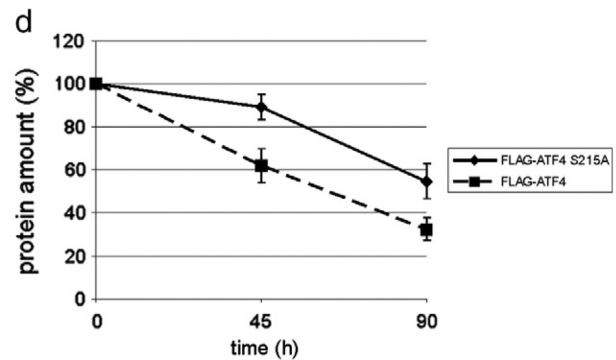
Fig. 5. CK2 interacts with ATF4 S215A in vitro and in vivo. a) Interaction between CK2 and ATF4 S215A in vitro as demonstrated by GST pull down assay. Proteins were separated on a 12.5% SDS-polyacrylamide gel followed by Western blot where ATF4, CK2α and CK2β were detected using the corresponding antibodies. b) Co-immunoprecipitation of endogenous CK2 with FLAG-ATF4 S215A. HeLa cells were transfected with p3XFLAG-CMV ATF4 S215A for 24 h. Lysates were incubated with anti-ATF4 antibody sc200. Eluted proteins from immunoprecipitates were detected with anti-FLAG antibody, CK2α with antibody 1A5 and CK2β with antibody 6D5. C, pre-precipitate; IP, immunoprecipitate; S, supernatant; SE, sepharose; AB, antibody; molecular weight markers are indicated at the left side of the gels. c) GST-ATF4 and GST-ATF4 S215A were coated on 96 wells microtiter plates for 16 h at 4 °C and then incubated with recombinant CK2 holoenzyme. The bound complexes were detected as described for the ligand-Western blot assay and visualized with TMB.

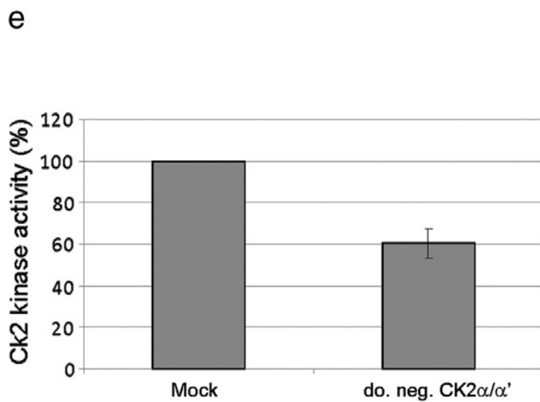
time. In order to determine the half-life, the protein bands were densitometrically scanned and plotted against time (Fig. 6 b). The half-life of FLAG-ATF4 was around 60 min, which is in the same range as previously described [24]. In contrast the ATF4 phosphorylation mutant was significantly more stable. To exclude secondary effects of cycloheximide on ATF4 and ATF4 S215A, we performed a pulse-chase labeling experiment with [^{35}S]methionine followed by immunoprecipitation of ATF4. The labeled and precipitated proteins were analyzed on a 12.5% SDS-polyacrylamide gel followed by autoradiography (Fig. 6 c). Radioactive labeled protein bands were

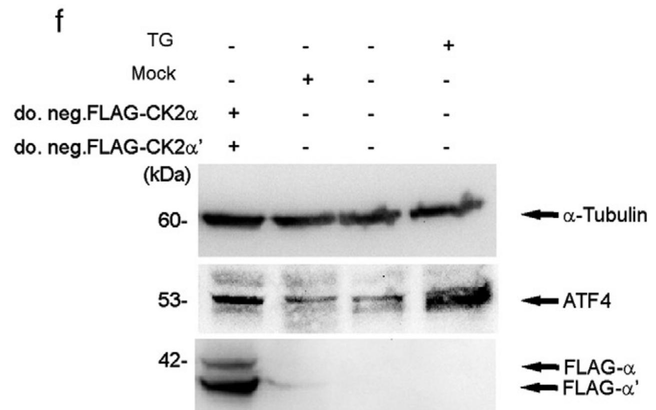


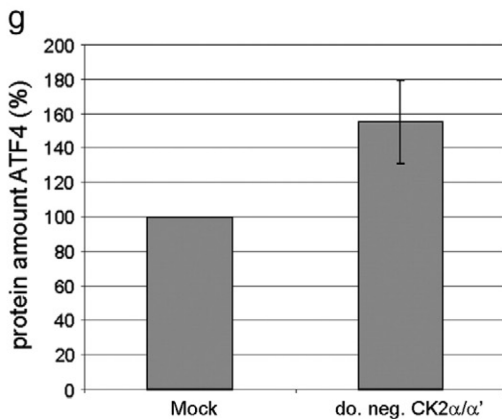












quantified by densitometry (Fig. 6 c). Also with this type of experiment we found that FLAG-ATF4 S215A is more stable than FLAG-ATF4.

In a next step we analyzed the influence of CK2 phosphorylation on the expression of endogenous ATF4. For inhibition of CK2 we used also CK2 dominant negative CK2α and CK2α′ mutants. We transfected HeLa cells with the dominant negative mutants of CK2α and CK2α′ to inhibit the CK2 holoenzyme as well as both catalytic CK2 subunits. First, we determined the CK2 kinase activity after inhibition with the dominant negative mutants. The kinase activity was reduced by about 40% (Fig. 6 e). Next, we analyzed the protein level of endogenous ATF4 after transfection of the dominant negative mutants of CK2α and CK2α′. We found an elevated protein level of endogenous ATF4 (Fig. 6 f and g). In another approach we used two specific and efficient CK2 inhibitors, TBB or quinalizarin (Q) [25,26] and analyzed accumulation of ATF4 over a time period of 24 h. As shown in Fig. 7 a, b the level of ATF4 increased transiently between 8 and 12 h after treatment with either of the two CK2 inhibitors. It was previously shown that inhibition of CK2 kinase activity or silencing of CK2α induced the ER-stress signaling pathway [41–43]. Within this pathway the eIF2α kinase, which is phosphorylated and activated by PERK kinase, is an upstream element of ATF4. This activation of eIF2α leads to a down-regulation of general protein synthesis whereas a few proteins including ATF4 are upregulated. Therefore, we analyzed whether we could detect an upregulation of phosphorylated (p-eIF2α). Cells were treated as described above with TBB or quinalizarin. Proteins were analyzed on an SDS polyacrylamide followed by Western blot with antibodies that detect p-eIF2α or total eIF2α. As shown in Fig. 7 a and b there is an increase in the phosphorylation of eIF2α which goes along with the increase in ATF4. Thus, the data about the activation of eIF2 are in agreement with recently published data by Manni et al. Here, we identified ATF4 as a down-stream factor of p-eIF2α.

In order to analyze the effect of CK2 on the transcription of ATF4, we transfected HeLa cells with a luciferase reporter containing the promoter region of ATF4 in front of the luciferase reporter gene (Fig. 8 a). Cells were treated with TBB or quinalizarin (Q) and as a control with DMSO for 24 h to inhibit CK2 activity (Fig. 8 b and c). After this treatment we detected a strong increase in the luciferase activity. Thus, we conclude that TBB or quinalizarin treatment of HeLa cells led to an enhanced transcription of the ATF4 gene which might also be responsible for a higher ATF4 level. Since we have already shown an elevated stability of the CK2 phosphorylation mutant of ATF4, there are obviously two mechanisms contributing to the elevated ATF4 level; first, enhanced transcription of the ATF4 gene and second, elevated stability of ATF4 protein.

It is well known that ATF4 binds to different promoter sequences and thereby affects the transcription of different genes [44]. We would like to know whether the CK2 phosphorylation of ATF4 might influence its transcription factor activity. We analyzed two different promoter elements, a sequence from −1850 to +34 in the 5′UTR from the ATF3 promoter [29] called 5′UTR ATF3-Luc (Fig. 9 a) and a 19 bp long sequence from the CHOP promoter called 2xAARE-Luc [27] (Fig. 9 c). We detected a reduced transcription activity of FLAG-ATF4 S215A compared to wild-type FLAG-ATF4 by using both elements. The relative luciferase activity was normalized to the protein amount of

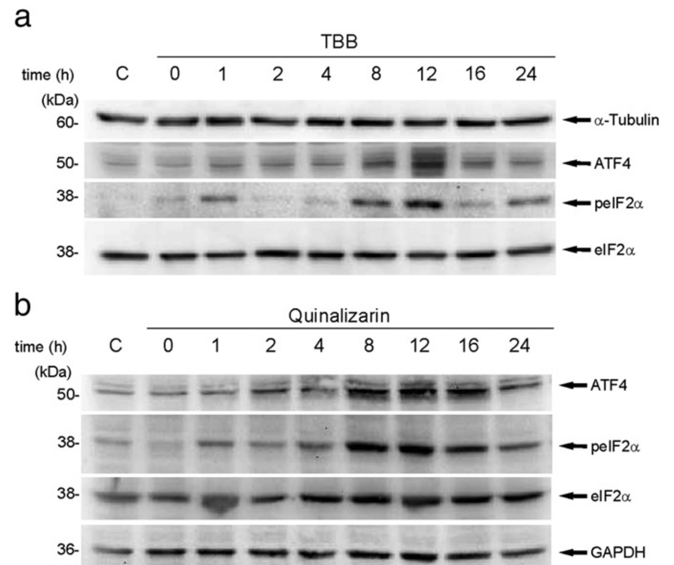


Fig. 7. Influence of the CK2 inhibition on endogenous ATF4. HeLa cells were treated with a) 50 μM TBB or b) 50 μM quinalizarin (Q) for the indicated time period. Control cells were treated with equal amounts of DMSO (4 h). Cell lysates were prepared and analyzed on a 12.5% SDS polyacrylamide gel followed by Western blotting using anti-ATF4 antibody sc-200, anti-GAPDH antibody, anti-α-tubulin antibody and the antibodies anti-eIF2α and anti-p-eIF2α. Molecular weight markers are indicated at left side of the gels.

FLAG-ATF4 and FLAG-ATF4 S215A as detected by Western blot analysis (Fig. 9 b and d). Thus, we conclude that the CK2 phosphorylation at serine 215 activates the transcription factor activity of ATF4 in HeLa cells by using the AARE element of the CHOP promoter or a sequence from −1850 to +34 in the 5′UTR from the ATF3 promoter.

4. Discussion

Protein kinase CK2 is a ubiquitous, highly pleiotropic protein kinase which is implicated in the regulation of multiple cellular processes [45]. Although CK2 is known for more than 55 years [46] it is still an enigma how this enzyme regulates so many different processes. One key to the understanding of the multiple functions seems to be its localization in the cell [47]. Targeting CK2 as a holoenzyme or as individual subunits to a specific cellular compartment as a response to various signals and stimuli might be a mechanism for regulating the phosphorylation of particular substrates. A second mechanism might be phosphorylation of specific substrates by the holoenzyme or by the catalytic α- or α′-subunits. The β-subunit seems to be responsible for the regulation of the substrate specificity of the holoenzyme [40]. Here, we showed that ATF4 is only efficiently phosphorylated by the CK2-holoenzyme and not by the catalytic subunits alone. A third mechanism for the regulation of the enzymatic activity could be the interaction of the holoenzyme or individual subunits with proteins or other factors in the cell (for review see: [48]). Thus, the identification of new interacting proteins is an attractive approach to understand the complex functions of CK2. Using a yeast-two-hybrid system over the last ten years, a number of different

Fig. 6. Stability of ATF4. a) HeLa cells were transfected with p3XFLAG-CMV ATF4 and p3XFLAG-CMV ATF4 S215A for 24 h. Cells were treated with cycloheximide for the indicated times, cell lysates were prepared and analyzed by Western blotting using anti-FLAG antibody and anti-GAPDH antibody. b) The ratios of the amounts of FLAG-ATF4 and FLAG-ATF4 S215A at each time point relative to that of the starting point (untreated) were plotted as a function of time. The diagram shows five independent experiments c) HeLa cells were transfected with p3XFLAG-CMV ATF4 and p3XFLAG-CMV ATF4 S215A for 24 h after the cells were incubated in methionine-free DMEM containing 50 μCi/ml of [^{35}S]methionine for 1 h and then lysed. Cell lysates were immunoprecipitated with anti-ATF4 antibody sc200. The precipitates were subjected to SDS polyacrylamide gel electrophoresis and methionine imaging was performed with the Typhoon-Trio imaging system. d) The ratios of the amounts of methionine labeled FLAG-ATF4 and FLAG-ATF4 S215A at each time point relative to that of the starting point were plotted as a function of time. e) HeLa cells were transfected with dominant negative mutants of CK2α and CK2α′ or empty vector (mock) for 24 h. Proteins were extracted and the CK2 activity was determined by the incorporation of [^{32}P]phosphate into the synthetic substrate RRRDDDSDDD. f) Cell lysates from a) and cell lysates treated with and without thapsigargin as control were analyzed by Western blotting using anti-FLAG antibody, anti-ATF4 antibody (sc200) and anti-α-tubulin antibody. g) The amount of endogenous ATF4 after transfection with dominant negative mutants of CK2α and CK2α′ was compared to the amount of empty vector transfected cells. The diagram shows four independent experiments.

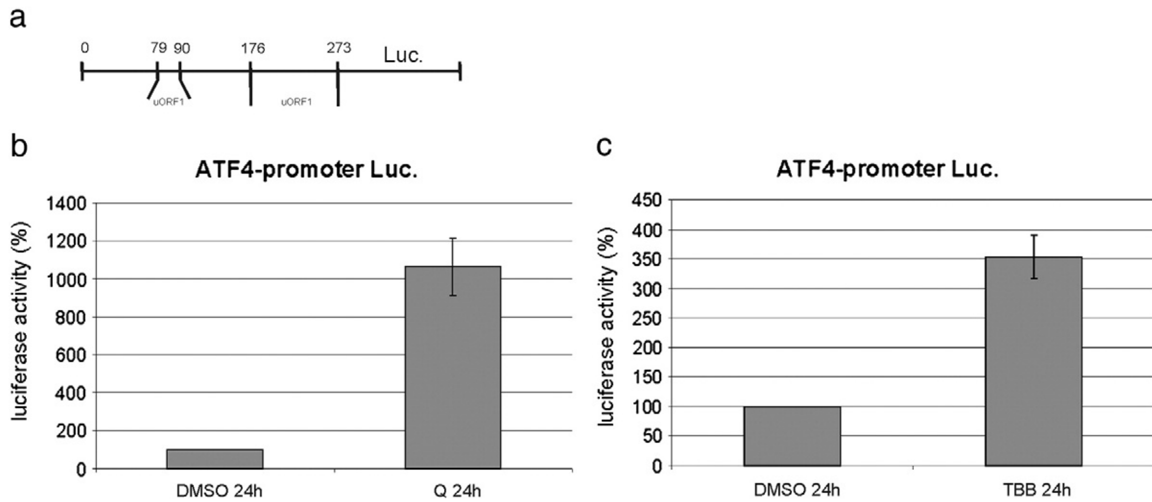


Fig. 8. Influence of the CK2 inhibition on *ATF4* gene expression. a) Schematic illustration of the ATF4 promoter region in front of the luc reporter gene. HeLa cells were transfected with a reporter plasmid expressing luciferase under the control of the ATF4 promoter for 24 h. Cells were incubated with fresh medium in the presence of b) quinalizarin (Q) and c) TBB for 24 h. Control cells were incubated in the presence of equal amounts of DMSO. Cell lysates were prepared and analyzed for luciferase activity. The luciferase activity of the mock (reporter plasmid expressing luciferase under the control of the ATF4 promoter without treatment) transfected cells was set 100%. The diagram represents 3 independent experiments.

new binding partners for CK2 were identified including the motor neuron protein KIF5C [10], the transcription factor Pdx-1 [8], the splice factor hPrp3p [7], the ribosomal proteins L41 [49], L5 [50], A-raf [51], mos [52], the Fas-associated protein FAF1 [53], the mitochondrial translation initiation factor 2 [54], propionyl CoA carboxylase β subunit [53] and the pleckstrin homology domain protein CKIP-1 [55] just to mention but few. These interactions with various cellular proteins strongly support the idea that CK2 is implicated in many different intracellular processes. Here, we present ATF4 as a new binding partner and substrate of CK2. This interaction was demonstrated by pull-down assays and by

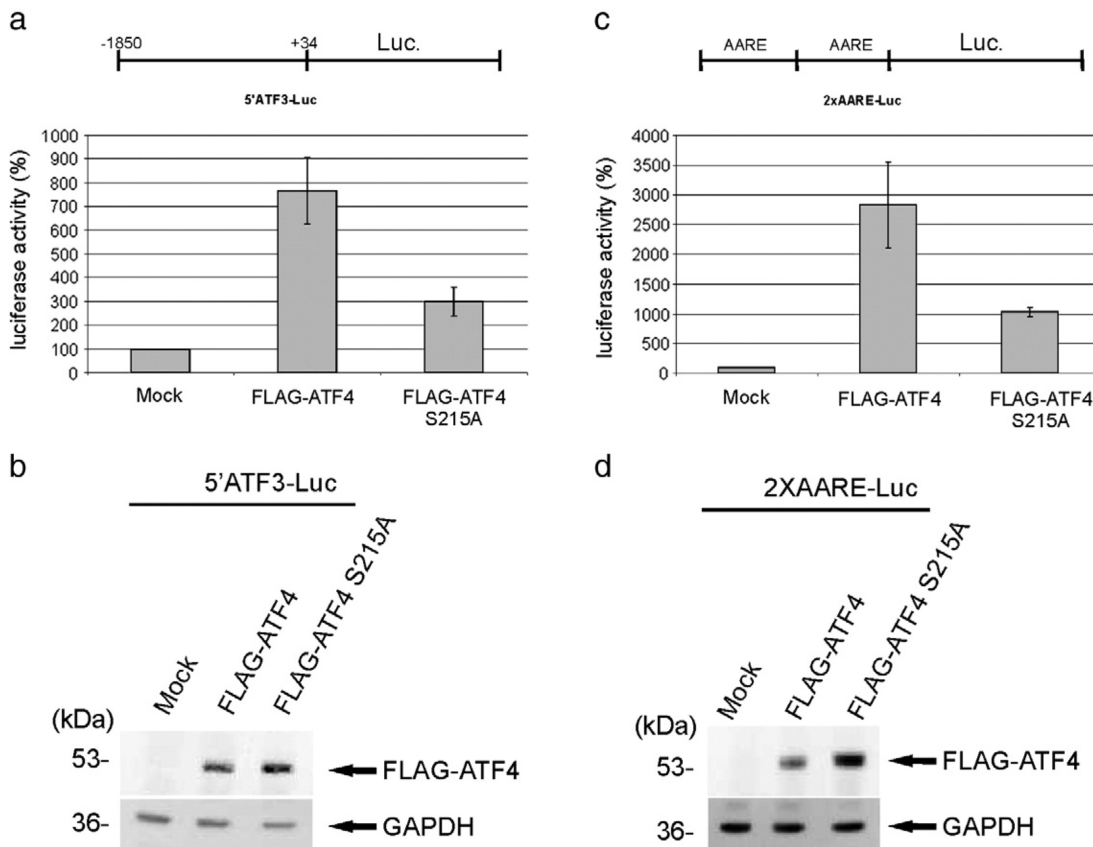


Fig. 9. ATF4 S215A represses promoter activity. a) HeLa cells were transfected with or without p3XFLAG-CMV ATF4, p3XFLAG-CMV ATF4 S215A and a reporter plasmid expressing luciferase under the control of an ATF4 target promoter (5′UTR ATF3-Luc) for 24 h. Cell lysates were prepared and analyzed for luciferase activity. The luciferase activity was calculated in relation to the corresponding protein amount. c) HeLa cells were transfected with or without p3XFLAG-CMV ATF4, p3XFLAG-CMV ATF4 S215A and a reporter plasmid expressing luciferase under the control of an ATF4 target promoter (AARE-Luc) for 24 h. Cell lysates were prepared and analyzed for luciferase activity. b) and d) The cell lysates from a) and c) were analyzed by Western blotting using anti-FLAG antibody and anti-GAPDH antibody. The relative intensities of FLAG protein contents were normalized first to GAPDH and then to luciferase activity.

co-immunoprecipitation assays. Both assays showed that ATF4 bound to CK2α whereas binding to CK2β was found only in the presence of the CK2 holoenzyme. Thus, one might speculate that CK2β was bound via its binding to the catalytic CK2α-subunits. This binding was confirmed by two other methods, namely an ELISA based binding assay as well as bimolecular fluorescent complementation (BiFC) experiments. These BiFC experiments showed a positive immunofluorescence signal only in the nucleus indicating that the interaction of CK2 with ATF4 is restricted to the nucleus.

ATF4 is a key transcriptional effector protein of the cellular stress response generally known as unfolded protein-response, which is induced by the accumulation of improperly folded proteins [56]. The ATF4 pathway is initiated by stress-activated protein kinases, e. g. the eukaryotic translation initiation factor 2-alpha kinase 3 (PERK), which phosphorylates eukaryotic translation initiation factor 2 (eIF2α) leading to a general inhibition of protein synthesis but selective translation of ATF4 [15]. Recently Manni et al. showed in multiple myeloma cells that CK2α silencing leads to an induction of the unfolded protein response (UPR) in the ER which leads to the activation of PERK and eIF2α (21213). One of the down-stream factors of eIF2α in the UPR is ATF4 whose expression was shown here to be upregulated by CK2 inhibition. Thus, with ATF4 we identified a new factor in the UPR which is activated by CK2 inhibition. ATF4 is important for cell proliferation, differentiation and long-term memory induction [17,57,58]. ATF4 is a phosphoprotein and it was already shown that it is regulated by phosphorylation at multiple sites [24]. It was an obvious question whether ATF4 is not only a binding partner of CK2 but also a substrate and whether the phosphorylation has any fundamental consequences for ATF4. Scanning the polypeptide chain of CK2 for putative CK2 phosphorylation sites revealed at least 12 sites. By using ATF4 fragments as well as by mutation of serine and threonine residues we identified serine 215 as the major CK2 phosphorylation site. Serine 215 is highly conserved in ATF4 from Xenopus, to rat, mouse and human, which suggests that this residue is important for the function of ATF4. Serine 215 is in close vicinity to the recognition motif for β-TrCP, which is the receptor component of the SCF E3 ubiquitin ligase. β-TrCP co-localizes with ATF4 in the nucleus and controls the stability and subsequently the transcriptional activity of ATF4 [23] as well. Thus, one might speculate that the CK2 phosphorylation at serine 215 might be also implicated in the regulation of the stability of ATF4. Working with wild-type ATF4 and the phosphorylation mutant of ATF4, where serine 215 was replaced by alanine, we always noticed a higher level of the phosphorylation mutant ATF4 protein. This elevated level can be due to a higher stability of the mutant protein or to an elevated synthesis of ATF4. Here, we showed that mutation of serine 215 to alanine resulted in an elevated stability of ATF4. A similar observation was made for serine 219 which represents a putative CK1 site [24]. Mutation of serine 219 also led to an altered stability of the protein. These data support the idea that the region around serine 215 and serine 219 is implicated in the regulation of the stability of ATF4. It has been previously reported that CK2 phosphorylation affects also the stability of other proteins. The bcl-2 family protein Bid is phosphorylated by CK2 which prevents its cleavage by caspase-8 [59]. The transcription factors max and myc and also PTEN are stabilized by a CK2 mediated phosphorylation [60–62]. On the other hand the PML tumor suppressor is phosphorylated by CK2, which results in an ubiquitin-mediated degradation [63]. Also the von Hippel Lindau protein VHL is rapidly degraded after CK2 phosphorylation [64]. These data together with our present data support the idea that CK2 might also regulate cellular processes by altering the stability of substrate proteins.

By using the ATF4 promoter we could further demonstrate that CK2 regulated the transcription of the ATF4 gene. Inhibition of CK2 kinase activity led to an up-regulation of the transcription of ATF4 indicating that the enzymatic activity of CK2 is necessary for the regulation. Thus, the level of ATF4 seems to be regulated by CK2 by two different mechanisms; first, by the regulation of the transcription of ATF4 and second, by the reduction of the ATF4 stability after CK2 phosphorylation at serine 215. Recently Schneider et al. also published an increase in the level of ATF4 after treatment of prostate cancer cells with CK2 inhibitors [43]. However, in these cells the elevated level of ATF4 was achieved without an upregulation of the transcription of ATF4. Furthermore, in this study it was shown that ATF4 is responsible for the transactivation of CHOP, which was earlier shown to be responsible for apoptosis induction in this particular prostate cancer cell [42]. A dominant negative mutant of ATF4 inactivated the transcriptional factor activity of ATF4 with respect to CHOP expression. The CK2 phosphorylation mutant of ATF4 exhibits the same effect demonstrating that the phosphorylation at serine 215 is required for the transcription factor activity of ATF4. As a transcriptional activator ATF4 regulates not only the expression of CHOP [66] but also the expression of RANKL (a receptor of nuclear factor-κB ligand) [65], osteocalcin [66], GADD34 [67], the adenoviral E1a inducible gene E4 [68] and several other genes involved in the cellular redox system, amino acid metabolism, mitochondrial function [14] and differentiation including ATF3. The phosphorylation of ATF4 by CK2 seems to be a major role in the transcriptional regulation of down-stream factors such as ATF3 and CHOP. ATF3 is induced in response to endoplasmic reticulum (ER) stress or amino acid starvation by ATF4 [69] and also contributes to the induction of CHOP. Here, we show that mutant ATF4 S215A showed a reduced transcriptional activity for the CHOP 2xAARE promoter and the ATF3 promoter. So we conclude that the CK2 protein kinase activity and phosphorylation of ATF4 by CK2 regulate the transcription factor activity of ATF4. Our present data fit perfectly into the gap which was left over by the work of Manni et al. showing the activation of up-stream factors of ATF4 [41] and Schneider et al. [43], demonstrating the activation of down-stream factors of ATF4 in the UPR signaling pathway.

In addition to ATF4, CHOP and other members of the stress activated cellular pathway, such as Nrf-2, are substrates for protein kinase CK2. CK2 phosphorylation of Nrf-2 is necessary for the shuttling of Nrf-2 from the cytoplasm to the nucleus [21]. Phosphorylation of CHOP by CK2 leads to down-regulation of the CHOP transcription factor activity. With the identification of ATF4 as a further factor in this signaling pathway whose stability and transcription factor activity are directly regulated by CK2, we could further demonstrate the important role of CK2 in the ER-stress-signaling pathway.

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