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CK2 phosphorylation of C/EBPδ regulates its transcription factor activity

Lisa Schwind, Andreas D. Zimmer, Claudia Götz, Mathias Montenarh *

Medical Biochemistry and Molecular Biology, Saarland University, Building 44, D-66424 Homburg, Germany

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ABSTRACT

Protein kinase CK2 plays an essential role in cell viability in lower and higher eukaryotes. As a global regulator it phosphorylates and thereby regulates a broad array of cellular targets including a large number of transcription factors. Here, we have identified the CCAAT/enhancer binding protein δ (C/EBPδ) as a new substrate for CK2. Using point mutants of C/EBPδ the major phosphorylation site for CK2 was mapped to serine 57, which is located within the transactivation domain of C/EBPδ. For proper functioning as a transcription factor C/EBPδ has to be translocated into the nucleus where it forms heterodimers with other members of the C/EBP family of proteins and ATF4. Here, we found that CK2 phosphorylation does neither influence the subcellular localization of C/EBPδ nor its interaction with C/EBPβ, but rather does CK2 phosphorylation modulate the transcriptional activity of C/EBPδ. Moreover, we found that CK2 bound to C/EBPδ, which might help to target CK2 to the transcriptional machinery where it can phosphorylate other transcription factors or co-activators.

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1. Introduction

A greater understanding of the regulation of gene expression is achieved by analysing the regulation of transcription factors. Transcription factors are absolutely required for accurate transcriptional response in every cell type. This transcriptional response enables gene expression in a specific cell type or at distinct development stages as well as during differentiation (Struhl, 1989; Abel and Maniatis, 1989). One key step in the regulation of transcription factors seems to be the translocation of the protein from the cytoplasm into the nucleus or back. Another process, that seems to be implicated in the regulation of transcriptional activity, is the interaction of transcription factors with other cellular proteins, which in many cases enables the correct interaction of the transcription factors with specific DNA sequences. In addition, post-translational modifications of transcription factors represent another level of regulation. Among the post-translational modifications reversible phosphorylation plays a prominent role. According to the mode of action, transcription factors can be classified as zinc finger proteins, basic helix loop helix containing proteins or basic-leucine zipper (bZIP) transcription factors to mention just but a few. The CCAAT/enhancer binding protein family (C/EBPs) is a family of transcription factors with a highly conserved bZIP domain. Six isoforms namely C/EBPα to C/EBPζ were identified to date (Williams et al., 1991; Lekstrom-Himes and Xanthopoulos, 1998). Numerous studies have attributed a role of C/EBPs in controlling and regulation of cellular proliferation, differentiation and metabolism (Ramji and Foka, 2002; Rosen, 2005). It has been demonstrated that isoforms of C/EBPs can regulate the transcription of genes which are important for fat metabolism or adipocyte differentiation. C/EBPδ is an important member of the C/EBP family which is expressed during adipogenesis and contributes to it by regulating the expression of other key transcription factors such as the peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα (Cao et al., 1991; Clarke et al., 1997). In addition, C/EBPδ interacts with C/EBPβ to induce PPARγ expression during adipogenesis (Wu et al., 1996).

One of the protein kinases that phosphorylates transcription factors is protein kinase CK2 formerly known as casein kinase II. Among the different classes of transcription factors a number of transcription factors were already identified as CK2 substrates (Meggio and Pinna, 2003) including the CCAAT/enhancer binding protein (C/EBP) family of proteins. A known CK2 substrate within this family is C/EBPζ also known as CHOP, GADD153 or DDIT3 (Ubeda and Habener, 2003). Phosphorylation of CHOP by CK2 inhibits its transcription activation function, which has implications for ER stress induced apoptosis (Zinszner et al., 1998; Yamaguchi and Wang, 2004) or differentiation (Tang and Lane, 2000). CHOP and other members of the C/EBP family of proteins can form heterodimers with members of the activating transcription factors (ATFs). Recently, we identified ATF4 as a new substrate and

* Corresponding author. Tel.: +49 6841 1626502; fax: +49 6841 1626027.
E-mail address: Mathias.Montenarh@uks.eu (M. Montenarh).

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binding partner of CK2 (Schneider et al., 2012; Ampofo et al., 2013). We have shown that an ATF4 phosphorylation mutant, which can no longer be phosphorylated by CK2, exhibits a reduced transcription factor activity (Ampofo et al., 2013). Thus, we hypothesize that probably other members of the C/EBP proteins might be substrates for CK2 and that CK2 might regulate these proteins by phosphorylation. Here, we describe C/EBPδ as a new substrate for CK2 and analyse the impact of this phosphorylation on C/EBPδ functions.

2. Materials and methods

2.1. Reagents and antibodies

Protease inhibitor cocktail Complete™ was obtained from Roche Diagnostics (Mannheim, Germany). DMSO was purchased from Merck (Darmstadt, Germany). CX-4945 was bought from Selleckchem (Munich, Germany) and quinalizarin from Labotest (Niederschöna, Germany). Glutathione agarose was from Pierce (Rockford, USA). Anti-α-tubulin and Anti-FLAG antibodies were obtained from Sigma–Aldrich (Munich, Germany). The antibody against C/EBPδ was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-GST antibody and protein A sepharose were obtained from Amersham Biosciences (Freiburg, Germany). Detection of CK2 was performed by using the rabbit antibody #26 against CK2α (Faust et al., 1999) and the mouse monoclonal antibody 6D5 (Nastainczyk et al., 1995) was used to detect CK2β. Goat, mouse and rabbit secondary antibodies were all bought from Dianova (Hamburg, Germany).

2.2. Cell culture and drug treatment

HCT116 cells (ATCC Number: CCL-247) were cultured at 37 °C and 5% CO_2 in McCoy's 5A medium (PromoCell, Heidelberg, Germany) containing 10% foetal calf serum (FCS). The CK2 inhibitors CX-4945 and quinalizarin were dissolved in dimethyl sulfoxide (DMSO) to a 10 mM stock solution. Six hours after transfection cells were treated with the CK2 inhibitors at final concentrations of 10 μM (CX-4945), 50 μM (quinalizarin) or the same volume of DMSO as control for an overall period of 24 h.

2.3. Plasmids and cloning of promoter constructs

The cDNA of human C/EBPδ was kindly provided by Dr. Esta Sterneck (Centre for Cancer Research NCI-Frederick, USA). The cDNA was amplified by PCR with the specific primers hC/EBPδ-for: 5′-AGA GAG GGA TCC ATG AGC GCC GCG-3′; hC/EBPδ-rev: 5′-AGA GAG GAA TTC TTA CCG GCA GTC TGC TGT-3′ and cloned into pGEX4T-1 vector (BamHI/EcoRI), which was from GE Healthcare (Munich, Germany). The creation of the GST-C/EBPδ mutants was performed by "splicing by overlap extension"-PCR (SOE-PCR). Aside from primers hC/EBPδ-forward and–reverse mutation specific primers C/EBPδ-S57A-SOE-for: 5′-TGT ACG ACG ACG AGG CCG CCA TCG ACT TCA-3′; C/EBPδ-S57A-SOE-rev: 5′-TGA AGT CGA TGG CGG CCT CGT CGT CGT ACA-3′; C/EBPδ-S57D-SOE-for: 5′-TGT ACG ACG ACG AGG ACG CCA TCG ACT TCA-3′; C/EBPδ-S57D-SOE-rev: 5′-TGA AGT CGA TGG CGT CCT CGT CGT CGT ACA-3′; C/EBPδ-T159A-SOE-for: 5′-ACC CCG CCC GCG TCG CCG GAG-3′; C/EBPδFLAG-T159A-SOE-rev: 5′-CTC CGG CGA CGC GGG CGG GGT-3′ were used.

The p3xFLAG-CMV-7.1 vector was from Sigma–Aldrich (Munich, Germany). To generate the expression plasmids FLAG-C/EBPδ_{WT}, FLAG-C/EBPδ_{S57A} and FLAG-C/EBPδ_{S57D}, the respective cDNA was subcloned from the pGEX4T-1 construct into p3xFLAG-CMV-7.1 vector using EcoRI and BamHI.

The human cDNA of C/EBPβ LAP2 was from Addgene Inc. (Cambridge, U.S.A). Using PCR, it was amplified with the primers hC/EBPβ-for: 5′-AGA GAG GGA TCC ATG GAA GTG GCC AAC-3′; hC/EBPβ-rev: 5′-AGA GAG GAA TTC CTA GCA GTG GCC GGA-3′ and cloned into pRSETA vector (BamHI/EcoRI), which was from Invitrogen (Carlsbad, USA).

The firefly luciferase reporter vector (pGL4.10) was from Promega (Mannheim, Germany). The mouse PPARγ2 promoter fragment, containing 1302 nucleotides upstream of the start codon, was obtained by PCR amplification from purified mouse genomic DNA with the primers mPPARγ2-prom-for: 5′-AGA GAG CTC GAG GGG GAG TCA TTA AAT GAT G-3′; mPPARγ2-prom-rev: 5′-AGA GAG CCA TGG ACA GCA TAA AAC AGA GAT TTG-3′ and cloned (XhoI/NcoI) into pGL4.10. All DNA constructs were verified by sequencing.

2.4. Expression and purification of bacterially expressed proteins

C/EBPδ wild-type and mutants were expressed in *Escherichia coli* BL21(DE3) after induction with 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) for 3 h at 37 °C. Proteins were extracted with GST extraction buffer (2 mM $MgCl_2$, 500 mM KCl, 5% (v/v) glycerol, 0.65% (v/v) Chaps in 1× PBS (pH 7.4)) and sonification, purified by affinity chromatography with glutathione agarose and eluted with 10 mM glutathione in GST extraction buffer.

Recombinant α- and β-subunits of the CK2 holoenzyme cloned in a pT7-7 vector were expressed in *E. coli* BL21(DE3), and the proteins were purified according to Grankowski et al. (1991).

2.5. In vitro phosphorylation with protein kinase CK2

Recombinant GST-tagged C/EBPδ proteins (1 μg) were mixed with CK2 holoenzyme in a volume of 20 μl of kinase buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 50 μM ATP, 1 mM DTT). To start the reaction, 5 μl of kinase buffer including 2 μCi [^{32}P]γATP were added and the samples were incubated for 15 min at 37 °C. The reaction was stopped by adding 10 μl of 3× SDS sample buffer (195 mM Tris–HCl, pH 6.8, 0.03% (w/v) bromophenol blue, 15% (v/v) β-mercaptoethanol, 30% (v/v) glycerol, 6% (w/v) SDS). Samples were separated through a 12.5% SDS polyacrylamide gel. The gel was stained with Coomassie blue (0.2% Coomassie R250, 0.01% Coomassie G250, 50% methanol, 10% acetic acid) to verify equal amounts of protein and vacuum-dried. Phosphorylation was detected by autoradiography.

For the competition experiments the peptide with the sequence AMTDDESAIDFSAC comprising the CK2 phosphorylation site was pre-incubated with 100 ng of CK2 holoenzyme at 37 °C for 10 min. Subsequently, 1 μg of C/EBPδ protein was added and the mixture was incubated for additional 5 min at 37 °C. Samples were analysed as described above.

2.6. In vitro transcription/translation

In vitro transcription and translation of His-C/EBPβ were performed with the one step kit TNT® T7 Coupled Reticulocyte Lysate System (Promega, Mannheim, Germany) according to the manufacturer's instructions.

Briefly, the pRSETA-C/EBPβ-LAP2 plasmid was incubated in the presence of [^{35}S]methionine for 90 min at 30 °C in a total reaction volume of 50 μl. Thereafter, 7 μl translation mixture per GST-C/EBPδ protein was used for pull-down assay or the whole reaction mixture was frozen at −80 °C.

2.7. Pull-down assay

The assay was performed as described by Hagemeier et al. (1993) with a few modifications. Briefly, purified GST-C/EBPδ proteins (2 μg) were immobilized on glutathione agarose and incubated with *in vitro* translated His-tagged C/EBPβ (7 μl, 1 h room temperature) or untagged CK2 holoenzyme (10 μg, 4 °C overnight) in EBC

buffer (50 mM Tris–HCl, pH 8.0, 140 mM NaCl, 100 mM NaF, 200 μM sodium orthovanadate, 0.5% (v/v) NP40). After washing with NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% (v/v) NP40), bound proteins were eluted and separated by SDS polyacrylamide gel electrophoresis. Detection of bound proteins was accomplished either by Western blot (CK2) or by staining the gel with Coomassie blue, drying and autoradiography (C/EBPβ).

2.8. Transient transfection, cell lysis and luciferase assay

Transfection of cells was performed by using the FuGENE® HD transfection reagent (Promega, Mannheim, Germany) according to the manufacturer's instructions.

For the luciferase reporter assay, HCT116 cells were seeded into a 24-well plate (62,500 cells per well) in a total volume of 0.5 ml/well of cell culture medium and cultured overnight. Cells were then transfected with FuGENE® HD transfection reagent using a total of 0.5 μg of plasmid DNA (transfection mixture per well: 0.05 μg of promoter reporter plasmid, 0.45 μg of expression plasmid(s), 1.25 μl FuGENE® HD transfection reagent and 25 μl cell culture medium without FCS).

To measure luciferase activity, cells were collected at given time points after transfection by lysing in cell culture lysis reagent (Promega) and measured with the Luciferase Assay System (Promega) following the manufacturer's recommendations.

For Western blot analysis, cell lysates were centrifuged at 13,000 × g to remove cell debris. The protein content was determined with the Bradford method using the BioRad protein assay reagent (BioRad, Munich, Germany). Protein extracts were immediately used for Western blot analysis or stored at −20 °C.

2.9. SDS polyacrylamide gel electrophoresis and Western blot analysis

Proteins were separated on a 12.5% sodium dodecylsulfate-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (PVDF) by tank blotting using a transfer buffer containing 20 mM Tris–HCl, pH 8.3 and 150 mM glycine. The membrane was blocked with 5% dry milk in PBS containing 0.05% Tween-20 for 1 h at room temperature and then incubated with the specific antibody, which was diluted in PBS with 0.05% Tween-20 containing 1% dry milk powder for 1 h at room temperature or overnight at 4 °C. The membrane was washed with PBS Tween-20 containing 1% skimmed milk (2× 10 min), before being incubated with a peroxidase-coupled secondary antibody (anti-rabbit 1:30,000, anti-mouse 1:10,000 or anti-goat 1:5000) for 1 h at room temperature. The membrane was washed again in PBS Tween-20 (2× 10 min). Signals were developed and visualized by the Lumi-light system from Roche Diagnostic (Mannheim, Germany).

2.10. Immunofluorescence analysis

For immunofluorescence analysis HCT116 cells were seeded in 6-well plates on coverslips (300,000 cells per well) and cultured overnight. Cells were then transfected with FuGENE® HD transfection reagent using a total of 2.5 μg of plasmid DNA per well as indicated. Afterwards, cells were incubated for 24 h. Immunofluorescence analysis was performed as described (Faust et al., 2002). For the identification of transfected FLAG-C/EBPδ, the mouse monoclonal antibody clone M2 (1:600) against the FLAG-tag was used.

2.11. Co-immunoprecipitation

For co-immunoprecipitation experiments, HCT116 cells were seeded in 100 mm cell culture dishes and cultured overnight. Cells were then transfected with FuGENE® HD transfection reagent

(A)

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        10         20         30         40         50         60
MSAALFSLDG PARGAPWPAE PAPFYEPGRA GKPGRGAEPG ALGEPGAAAP AMYDDESAID
        70         80         90        100        110        120
FSAYIDSMAA VPTLELCHDE LFADLFNSNH KAGGAGPLEL LPGGPARPLG PGPAAPRLLK
       130        140        150        160        170        180
REPDWGDGDA PGSLLPAQVA ACAQTVVSLA AAGQPTPPTS PEPPRSSPRQ TPAPGPAREK
       190        200        210        220        230        240
SAGKRGPDRG SPEYRQRRER NNIAVRKSRD KAKRRNQEMQ QKLVELSAEN EKLHQRVEQL
       250        260
TRDLAGLRQF FKQLPSPPFL PAAGTADCR
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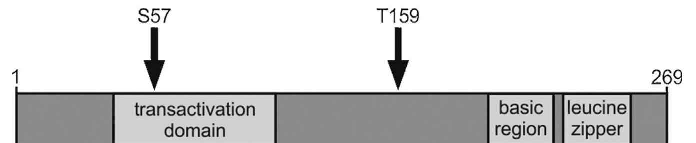

(B)

```
 41  --ASAMYDDESAIDFSAYIDSMAAVP   64  CEBPD_BOVIN
 40  --APAMYDDESAIDFSAYIDSMAAVP   63  CEBPD_SHEEP
 47  STTPAMYDDESAIDFSAYIDSMAAVP   72  CEBPD_MOUSE
 47  STTPAMYDDESAIDFSAYIDSMAAVP   72  CEBPD_RAT
 47  AAAPAMYDDESAIDFSAYIDSMAAVP   72  CEBPD_HUMAN
 17  SAAPAIYDDESAIDFSSYIDSMASVP   42  Q5BKL6_XENTR
 61  SNAPAIYDDESAIDFSAYIESMSTVP   86  Q6P612_DANRE

138  AAAAQPTPPASPEPPRRSPA-PPAPG  162  CEBPD_BOVIN
137  APAAQPTPPASPDPPRRSPA-PPAPG  161  CEBPD_SHEEP
150  AAAAQPTPPTSPEPPRGSPGPSLAPG  175  CEBPD_MOUSE
150  AAAAQPTPPTSPEPPRGSPGPSLAPG  175  CEBPD_RAT
150  AAAGQPTPPTSPEPPRSSPRQTPAPG  175  CEBPD_HUMAN
122  ----QPTPPTSPEPSTSACPSPASPS  143  Q5BKL6_XENTR
167  MHTGQPTPPTTPEPEPVAHRR-----  187  Q6P612_DANRE
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Fig. 1. C/EBPδ contains two putative CK2 phosphorylation sites with high conservation. (A) Amino acid (aa) sequence of human C/EBPδ protein and localization of the two putative phosphorylation sites for CK2 (boxes, arrows). Transactivation domain: aa 41–108 (Ji et al., 2003; Svotelis et al., 2005), basic region: aa 195–222 and leucine zipper: aa 226–254 [www.uniprot.org]. (B) Analysis of conservation among different species with respect to the putative phosphoacceptor sites (boxes).

using a total of 8 μg of plasmid DNA (4 μg FLAG-C/EBPδ and 4 μg FLAG-C/EBPβ) and cultured for additional 20 h. Proteins were extracted 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)) and 2 mg of total protein were subjected to immunoprecipitation.

For co-immunoprecipitation of CK2, nuclear proteins were extracted. Therefore, cells were resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, Complete™) and incubated on ice for 20 min. After centrifugation the supernatant containing cytoplasmic proteins was removed and the pellet was resolved in buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, Complete™). Nuclear proteins were isolated by pressing the suspension through a 21G and 23G cannula and centrifugation. 500 μg of nuclear proteins were used for immunoprecipitation. The cell lysate was pre-cleared once with protein A sepharose beads over a period of 2 h at 4 °C in EBC buffer. The supernatant was incubated with a rabbit anti-C/EBPδ (C-17) antibody for 3 h or over night. Beads were washed three times with NETN buffer. Bound proteins were eluted with 3× SDS sample buffer and analysed by Western blot with the mouse monoclonal antibody M2 (1:2000) against the FLAG-tag and CK2α and CK2β specific antibodies.

2.12. Staining of phosphoproteins

Detection of phosphoproteins was accomplished with the Pro-Q® Diamond Phosphoprotein Blot Stain Kit (P33356) from Molecular Probes (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Briefly, proteins were fixed on the PVDF membrane, washed, stained with Pro-Q® Diamond Blot Stain Reagent and destained. After drying the blot, fluorescence was detected with the Typhoon-Trio imaging system (GE-Healthcare) using the 580-nm band-pass filter (580 BP 30) in combination with the Image Quant TL software 7.0 (GE-Healthcare).

After staining of phosphoproteins, total proteins were stained with Ponceau S solution (0.2% (w/v) Ponceau S, 15% (v/v) acetic acid, 40% (v/v) methanol). After washing, the blot was blocked and incubated with antibodies as described in 2.9.

2.13. Statistics

Microsoft Excel 2010 software was used to analyse the data. Results were expressed as arithmetic mean ± SEM. Differences between the experimental groups were analysed using Student's *t*-test (two-tail, unpaired), statistical significant differences were shown as follows: $^{***}p < 0.001$, $^{**}p < 0.01$ or $^{*}p < 0.05$.

3. Results

The C/EBP transcription factor family of proteins is known to be modified by post-translational modifications which are important mechanisms to regulate them. C/EBPα, β and δ are phosphorylated by different kinases such as p38 kinase or GSK3-kinase (Terragni et al., 2011; Galibert et al., 2001). In 1996 Osada reported that C/EBPδ was phosphorylated by protein kinase CK2. A phosphorylation site was, however, not identified in this study (Osada et al., 1996). We started an *in silico* analysis of C/EBPδ for the presence of CK2 phosphorylation sites on the polypeptide chain of human C/EBPδ. As shown in Fig. 1A we found two putative CK2 phosphorylation sites on the polypeptide chain of C/EBPδ (two boxes). Both sites fulfil the requirements for a minimal CK2 consensus sequence, which requires an acidic residue at position +3 downstream from the phosphoacceptor site (S/T-x-x-D/E/pS/pY) (Salvi et al., 2009). Further analysis revealed that both sites are highly conserved among different species (Fig. 1B). In the next step we

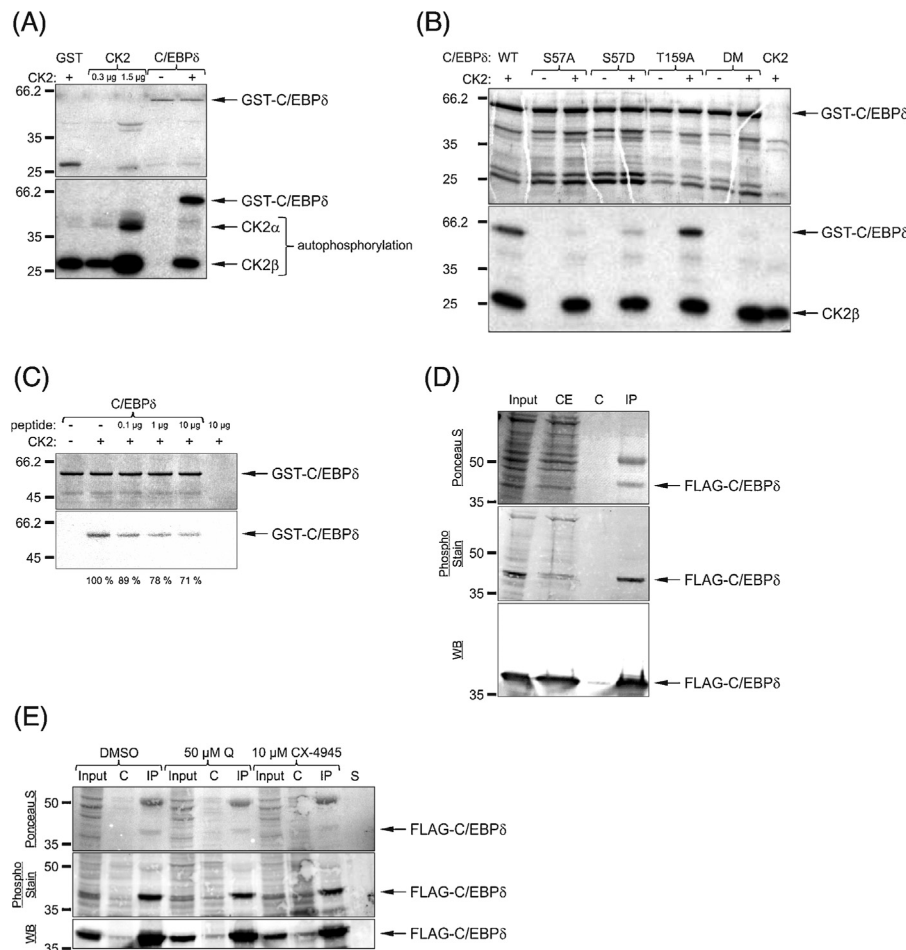

Fig. 2. C/EBPδ is a substrate of CK2 and is phosphorylated at position serine 57 on the polypeptide chain. (A) Phosphorylation of GST-C/EBPδ by CK2 *in vitro* kinase assay. The recombinant GST-C/EBPδ was incubated with or without recombinant CK2 holoenzyme in the presence of kinase buffer and 2 µCi of [^{32}P]γATP for 15 min at 37 °C. The samples were separated on a 12.5% SDS-polyacrylamide gel and the radioactivity was detected by autoradiography (lower part). The upper part shows Coomassie blue staining of the gel. The GST-tag and the CK2 holoenzyme alone were used as controls. (B) The phosphorylation of the recombinant GST-C/EBPδ mutants S57A, S57D, T159A, and double mutant (DM) was performed as described in (A). (C) *In vitro* phosphorylation of GST-C/EBPδ with a competitive peptide containing serine 57. GST-C/EBPδ was added to the reaction mix after a 10 min pre-incubation of CK2 holoenzyme and peptide. The reaction was stopped 5 min after further incubation. (D and E) Immunoprecipitation and phosphoprotein staining of FLAG-C/EBPδ. Transfection of FLAG-C/EBPδ into HCT116 cells was performed for 24 h (D) or 6 h after transfection cells were treated with DMSO, 50 µM quinalizarin (Q) or 10 µM CX-4945 and incubated for additional 18 h (E). FLAG-C/EBPδ was precipitated from nuclear extracts with the C/EBPδ-specific antibody M-17 and stained with Phosphostain reagent. Afterwards, C/EBPδ was detected by Western blot with an anti-FLAG antibody. 5% of nuclear extract was used as input. C: pre-precipitate; IP: immunoprecipitate; S: sepharose control.

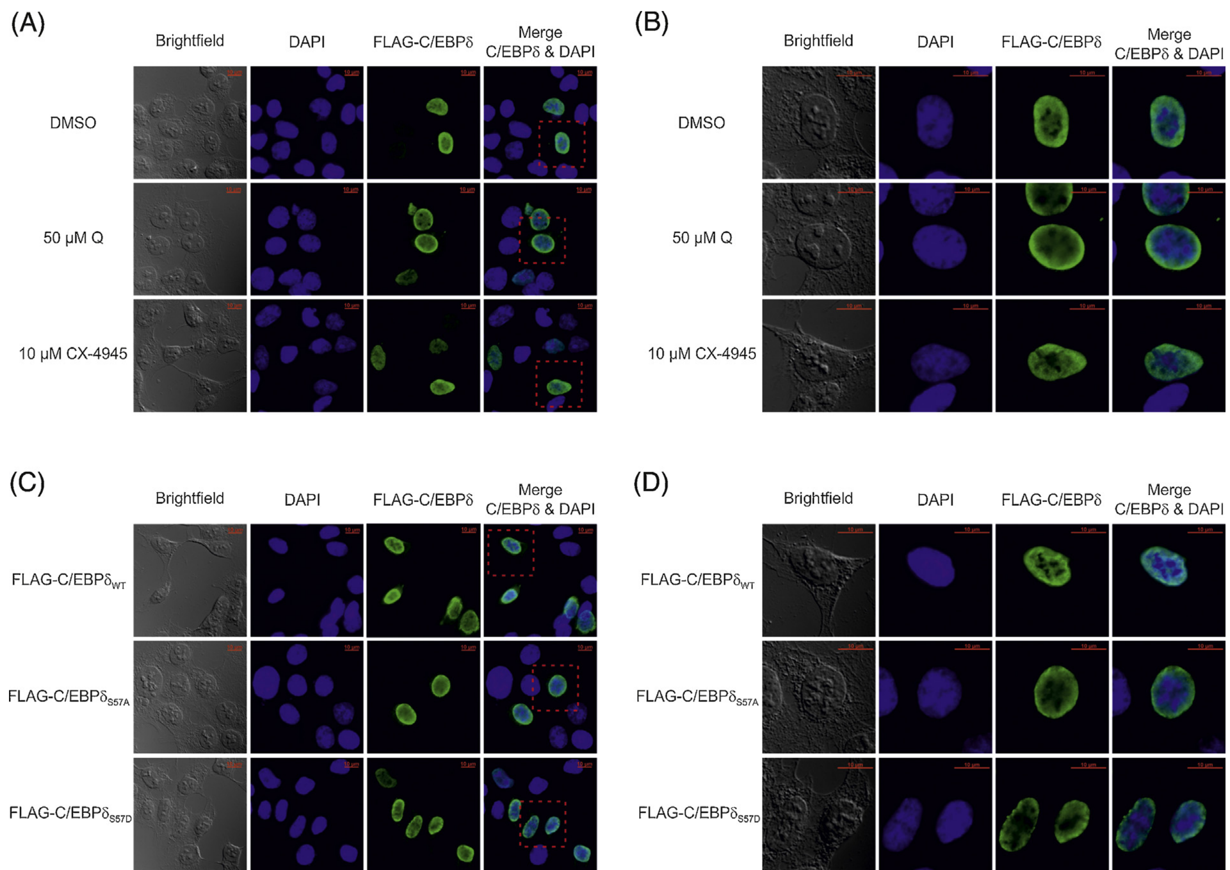

Fig. 3. Subcellular localization of C/EBPδ is unaffected after CK2 inhibition or mutation of Ser57. (A and B) Immunofluorescence analysis of HCT116 cells transfected with FLAG-C/EBPδ_{WT}. Six hours after transfection cells were treated with DMSO, 10 μM CX-4945 or 50 μM quinalizarin and incubated for additional 18 h. (C and D) Immunofluorescence analysis of HCT116 cells transfected for 24 h with FLAG-C/EBPδ_{WT}, -$_{S57A}$ or -$_{S57D}$. FLAG-C/EBPδ was detected with the FLAG antibody M2 and labelled by Alexa Fluor 488-coupled secondary antibody (green). The nucleus was stained with DAPI. One representative of at least 3 experiments is shown here. B and D are higher magnifications of A and B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

attempted to prove that C/EBPδ is a substrate for protein kinase CK2. Therefore, we expressed a GST-tagged C/EBPδ protein in bacteria, purified the protein and incubated it with CK2 and [^{32}P]γATP. As controls we used the GST-tag alone with [^{32}P]γATP, CK2 with [^{32}P]γATP and C/EBPδ without CK2 but with [^{32}P]γATP. Samples were analysed on an SDS polyacrylamide gel. As shown in Fig. 2A lower panel, we found a strongly phosphorylated protein band for GST-tagged C/EBPδ which cannot be observed in the absence of CK2 and also in the CK2 control. The GST-tag itself shows no phosphorylation. The other strong phosphorylated protein-band belongs to CK2β which is autophosphorylated. The upper panel shows the Coomassie blue stained gel which belongs to the autoradiography shown in the lower panel. The stained gel shows the proteins present in the different samples and depicts that similar amounts of protein were used for the assay. Thus, we show that C/EBPδ is indeed a substrate for protein kinase CK2.

In order to define which of the two putative sites identified by the *in silico* analysis or whether both sites are phosphorylated by CK2 we generated alanine mutants at position 57 or 159 and a mutant where both sites were mutated to alanine. Wild-type C/EBPδ and the phosphorylation mutants were phosphorylated by CK2 and [^{32}P]γATP and analysed on an SDS polyacrylamide gel. As shown in Fig. 2B wild-type C/EBPδ (WT) and the mutant T159A were phosphorylated by CK2 whereas there was no band for the phosphorylated S57A mutant of C/EBPδ. There was also no phosphorylated protein band for the double mutant (DM). Since wild-type and mutants of C/EBPδ contain a GST-tag and there is no phosphorylated protein band, these results also exclude that the GST-tag is phosphorylated by CK2. We also constructed a C/EBPδ mutant where serine 57 had been replaced by glutamic acid in order to mimic a negative charge at position 57. There is a faint band for this mutant protein after phosphorylation with [^{32}P]γATP which might indicate the generation of a new weak phosphorylation site (Fig. 2B). The upper panel shows the corresponding Coomassie blue stained gel, demonstrating that we used equal amounts of C/EBPδ protein for the phosphorylation reactions. These data provided strong evidence that serine 57 is the major CK2 phosphorylation site.

In order to support this conclusion we phosphorylated C/EBPδ with CK2 in the absence or presence of increasing concentrations of a peptide with the sequence AMTDDESAIDFSAC representing the sequence around serine 57. As shown in Fig. 2C in the presence of increasing amounts of this peptide we found a reduction in the phosphorylation of C/EBPδ compared to the control which further supports the idea that serine 57 is the major CK2 phosphorylation site. The upper part of Fig. 2C shows the Coomassie blue stained gel corresponding to the autoradiogram in the lower part of Fig. 2C showing that we used the same amount of GST-C/EBPδ protein for the phosphorylation experiments.

To demonstrate that C/EBPδ is indeed a phosphoprotein in cells we transfected FLAG-C/EBPδ into HCT116 cells. 24 h after transfection C/EBPδ was precipitated with a C/EBPδ specific antibody. The input control, the pre-precipitate and the immunoprecipitate were analysed on a 12.5% SDS polyacrylamide gel. Proteins were transferred onto a PVDF membrane which was first incubated with Phos-phostain (Fig. 2D, middle panel) to detect phosphorylated proteins. Afterwards, all proteins were stained with Ponceau S (upper panel) and the precipitated C/EBPδ was detected with a FLAG-antibody

(lower panel). These results demonstrated that C/EBPδ is a phosphoprotein detectable with the Phosphostain. In the next step we repeated the experiment but cells were additionally incubated with the CK2 inhibitors quinalizarin (Cozza et al., 2009) or CX-4945 (Siddiqui-Jain et al., 2010). Fig. 2E shows a reduction of the intensity of the C/EBPδ protein band labelled with Phosphostain in the presence of either quinalizarin or CX-4945 (middle panel) supporting the idea that it is phosphorylated by endogenous CK2. After normalization of the phosphorylated bands (Phosphostain, middle panel) from three independent experiments to the respective total C/EBPδ (lower panel, WB) the phosphorylation was reduced to 84% by the quinalizarin treatment and to 90% by the CX-4945 treatment.

It is known for several CK2 substrates that their sub-cellular localization is regulated by CK2 phosphorylation (Hübner et al., 1997; Jans and Jans, 1994; Miyata et al., 2011; Wilson et al., 2002). Therefore, we wanted to know whether CK2 phosphorylation of C/EBPδ would influence the subcellular localization of C/EBPδ. Cells were transfected with FLAG-C/EBPδ_{WT} and then treated with quinalizarin (Q) or CX-4945 or the solvent control DMSO for 18 h. The immunofluorescence analysis revealed that C/EBPδ was found exclusively in the nucleus (Fig. 3A and B). As a further prove we transfected either FLAG-C/EBPδ_{WT} or FLAG-C/EBPδ_{S57A} or FLAG-C/EBPδ_{S57D} into HCT116 cells and analysed the localization of C/EBPδ by immunofluorescence. As shown in Fig. 3C and D, wild-type and the two mutant C/EBPδs were found exclusively in the nucleus. C/EBPδ is evenly distributed over the nucleus with a slightly more perinuclear intensity. From these two experiments we conclude that CK2 phosphorylation of C/EBPδ has no influence on the nuclear localization of C/EBPδ.

C/EBPδ forms stable complexes with another member of the C/EBP family namely C/EBPβ (Newman and Keating, 2003). In order to analyse whether phosphorylation of C/EBPδ by CK2 might influence this interaction we incubated highly purified GST-C/EBPδ_{WT} or the two mutants C/EBPδ_{S57A} and C/EBPδ_{S57D} with [^{35}S]methionine labelled *in vitro* translated C/EBPβ. Complexes were precipitated with GSH-sepharose and then analysed on an SDS polyacrylamide gel. Fig. 4A upper part shows a Coomassie blue stained gel demonstrating that we used equal amounts of GST-C/EBPδ for the pull-down assay. The lower part shows the bound C/EBPβ demonstrating that equal amounts of C/EBPβ were bound to C/EBPδ. Thus, CK2 phosphorylation of C/EBPδ does not influence binding to C/EBPβ.

In order to confirm these results under *in vivo* conditions we transfected FLAG-C/EBPδ_{WT} or FLAG-C/EBPδ_{S57A} or FLAG-C/EBPδ_{S57D} together with FLAG-C/EBPβ into HCT116 cells. C/EBPδ was precipitated with a specific antibody and complexes were analysed on an SDS polyacrylamide gel. Protein bands were visualized with an antibody against the FLAG-tag. As shown in Fig. 4B we found equal amounts of C/EBPβ in the co-immunoprecipitates for C/EBPδ_{WT} as well as for the CK2 phosphorylation mutants. This result confirms the observation shown in Fig. 4A that CK2 phosphorylation does not influence complex formation between C/EBPδ and C/EBPβ.

An increasing number of proteins bind to CK2 and these protein–protein interactions provide another level of regulation for CK2 in the cell (Montenarh and Götz, 2013). Therefore, in the next step we asked whether C/EBPδ might bind to CK2. We incubated GST-C/EBPδ or the GST-tag alone with the CK2 holoenzyme. As shown in Fig. 5A GST-C/EBPδ co-precipitated with the CK2 holoenzyme. There was no binding of CK2 to the GST-tag or to GSH-agarose. In order to support the finding about binding of CK2 to C/EBPδ we attempted to co-immunoprecipitate CK2 and C/EBPδ from HCT116 cells. Cells were transfected with the FLAG-tagged C/EBPδ construct. 24 h after transfection cells were lysed and FLAG-C/EBPδ was precipitated from the cell extract with a C/EBPδ-specific antibody. The precipitate was analysed on an SDS-polyacrylamide

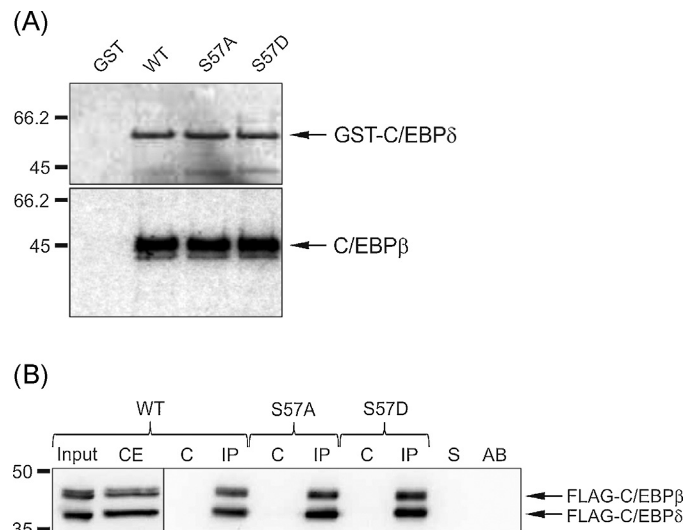

Fig. 4. Binding of C/EBPδ to C/EBPβ is not affected by phosphorylation of C/EBPδ by CK2. (A) GST pull-down assay. Bacterially expressed and purified GST-tagged C/EBPδ_{WT} and mutant proteins were bound to glutathione (GSH) agarose and then incubated with *in vitro* translated His-C/EBPβ for 1 h. Proteins were separated on a 12.5% SDS-polyacrylamide gel followed by staining with Coomassie blue (upper panel). Binding of His-tagged-C/EBPβ was detected by autoradiography (lower panel). One representative of at least 3 experiments is shown here. (B) Co-immunoprecipitation of FLAG-C/EBPδ and FLAG-C/EBPβ. HCT116 cells were transfected with FLAG-C/EBPδ and FLAG-C/EBPβ. Lysates were incubated with C/EBPδ specific antibody M-17. Eluted proteins from immunoprecipitates were detected with an anti-FLAG antibody M2. Input: 1% of cell extract used in the experiment; CE: cell extract after IP; C: pre-precipitate; IP: immunoprecipitate; S: sepharose control; AB: antibody control.

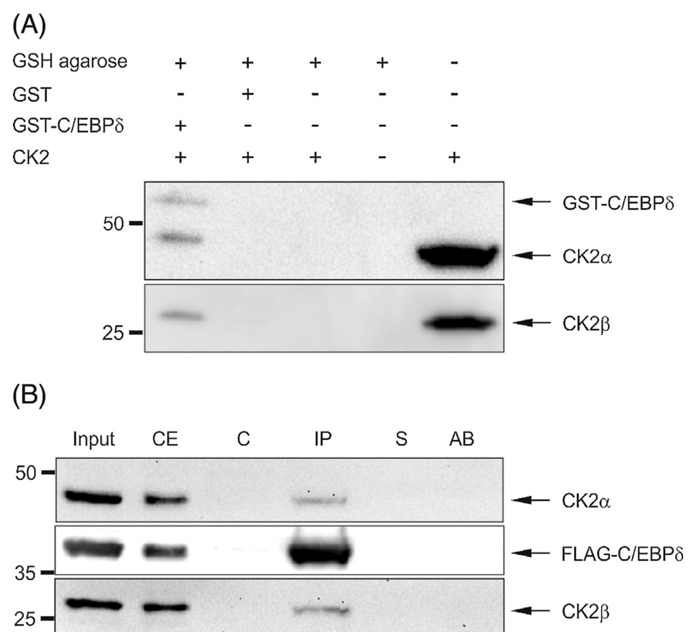

Fig. 5. Protein kinase CK2 binds to C/EBPδ *in vitro* and *in vivo*. (A) GST-C/EBPδ_{WT} was immobilized on glutathione agarose and incubated with recombinant CK2 holoenzyme overnight. After washing, proteins were separated on a 12.5% SDS-polyacrylamide gel followed by Western blot. C/EBPδ, CK2α and CK2β were detected with specific antibodies. GST-tag and CK2 alone were used as controls. (B) Co-immunoprecipitation of FLAG-C/EBPδ and CK2. HCT116 cells were transfected with FLAG-C/EBPδ. The nuclear extract was incubated with C/EBPδ specific antibody M-17 for 3 h. Input: 5% of cell extract used in the experiment; CE: cell extract after IP; C: pre-precipitate; IP: immunoprecipitate; S: sepharose control; AB: antibody control.

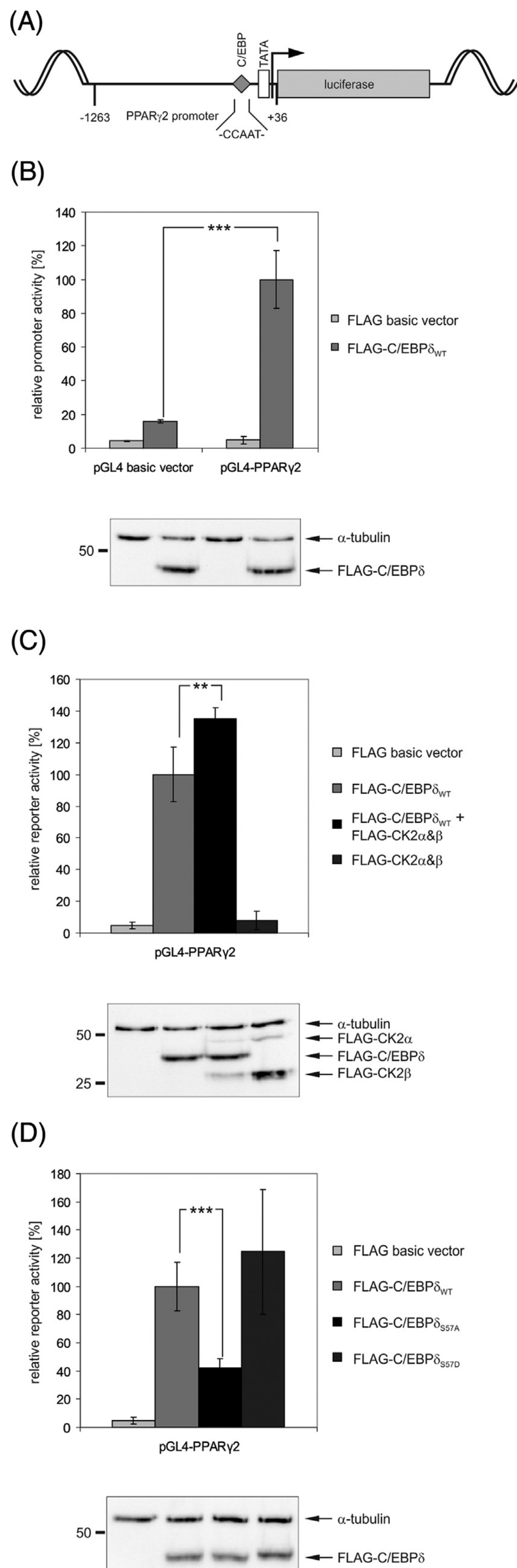

Fig. 6. Mutation of CK2 phosphorylation site Ser57 reduces transactivation capability of C/EBPδ. (A) Schematic representation of the reporter gene construct for the murine PPARγ2 promoter with depicted C/EBP binding site and TATA-Box. Luciferase reporter gene assays were carried out in HCT116 cells after transient co-transfection of expression plasmids FLAG-C/EBPδ_{WT} alone (B) or FLAG-C/EBPδ_{WT}, FLAG-CK2α and FLAG-CK2β (C) or FLAG-C/EBPδ_{WT}, $_{S57A}$ and $_{S57D}$ (D) together with pGL4-PPARγ2 promoter construct (B-D) or empty reporter plasmid (pGL4 basic vector) as negative control (B). The empty expression plasmid p3xFLAG-CMV-7.1 served as vector control in each case. Relative promoter activities measured 24 h after transient transfection are presented here. PPARγ2 promoter activity measured after co-transfection with C/EBPδ_{WT} was set to 100%. Means and standard deviations of three independent experiments are shown. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Western blot analyses of co-transfected HCT116 cells were analysed in parallel and illustrate expression of FLAG-C/EBPδ_{WT} (B), FLAG-C/EBPδ_{WT}, FLAG-CK2α and FLAG-CK2β (C) and FLAG-C/EBPδ_{WT}, $_{S57A}$ and $_{S57D}$ (D). Anti-FLAG antibody was used at a dilution of 1:2000. Detection of α-tubulin protein by anti-α-tubulin antibody (1:1000) was used as a loading control for the extracts.

gel followed by Western blot with CK2α and CKβ specific antibodies. As a control precipitate FLAG-C/EBPδ was detected with a FLAG-antibody. As shown in Fig. 5B both CK2α and CK2β were co-immunoprecipitated with C/EBPδ confirming the data obtained with the pull-down experiment shown in Fig. 5A. From these results we conclude that C/EBPδ binds to CK2.

It is well known that many transcription factors are regulated by phosphorylation (Bohmann, 1990). Moreover, protein kinase CK2 phosphorylation of transcription factors is known to stimulate or to abrogate transcriptional activity (Meggio and Pinna, 2003). Therefore, we analysed whether CK2 phosphorylation of C/EBPδ might also influence its transactivation functions. It has been shown that the PPARγ2 promoter is regulated by C/EBPδ (Moore et al., 2003) and therefore this promoter was cloned in front of a luciferase reporter construct (pGL4-PPARγ2, Fig. 6A). This reporter construct was transfected into HCT116 cells together with the FLAG-C/EBPδ_{WT} construct. As a control we used the corresponding reporter construct without the PPARγ2 promoter (pGL4 basic vector). Fig. 6B shows that the PPARγ2 promoter but not the basic vector construct was activated by C/EBPδ.

Next, we tested whether CK2 might modulate C/EBPδ transcriptional activity. Therefore, FLAG-C/EBPδ was transfected either alone or together with CK2α and CK2β and the PPARγ2 reporter construct. The result of this reporter assay is shown in Fig. 6C. C/EBPδ stimulated transcription of the PPARγ2 promoter. In the presence of CK2 there was an even higher level of the luciferase activity. The CK2 holoenzyme alone had no effect on the reporter construct. Thus, these results indicate that CK2 phosphorylation of C/EBPδ increases the transcriptional activity of C/EBPδ.

To further validate this finding we used the phosphorylation mutants C/EBPδ_{S57A} and C/EBPδ_{S57D} to analyse an influence of CK2 phosphorylation on the C/EBPδ transactivation. As shown in Fig. 6D we found an increase in the luciferase activity for FLAG-C/EBPδ_{WT} in the presence of the PPARγ2 promoter compared to the basic vector construct. The phosphorylation mutant C/EBPδ_{S57A} showed a reduced transcriptional activity compared to wild-type C/EBPδ. The phosphorylation mutant C/EBPδ_{S57D} which mimics a CK2 phosphorylation by providing a negative charge showed an elevated luciferase activity compared to the alanine mutant and to wild-type C/EBPδ. These results confirm the data obtained with an overexpression of the CK2 holoenzyme (Fig. 6C).

4. Discussion

The transcription factor C/EBPδ is a member of the family of CCAAT/enhancer binding protein transcription factors which modulate many biological processes such as cell differentiation, proliferation, mobility, cell death, inflammation and metastasis (Balamurugan and Sterneck, 2013). Like all other members of this family of transcription factors C/EBPδ contains a basic region-leucine zipper DNA binding domain in the C-terminus and an N-terminal transactivation domain. C/EBP transcription factors can bind to DNA only as dimers by virtue of the leucine zipper domain. Binding partners are all members of the C/EBP family including C/EBPζ which is also known as CHOP and also ATF4 (Grigoryan

et al., 2009). Both, CHOP and ATF4 were found to be substrates for protein kinase CK2 (Ubeda and Habener, 2003; Ampofo et al., 2013) and the interaction between CK2 and both transcription factors plays a role in the ER stress response regulation (Schneider et al., 2012). In the present study we identified C/EBPδ as a substrate for protein kinase CK2 by an *in silico* analysis with the CK2 phospho acceptor site with the consensus sequence ST-x-x-D/E/pS/pY (Salvi et al., 2009). The result of this *in silico* analysis was confirmed by an *in vitro* phosphorylation of C/EBPδ with CK2. Since the *in silico* analysis revealed two putative CK2 phosphorylation sites we created point mutations to map down the major phosphorylation site to serine 57. Serine 57 is located within an acidic environment which fits perfectly to the requirements for CK2 phosphorylation. The other putative CK2 phosphorylation site is surrounded by a proline-rich region which is a negative selection for CK2 phosphorylation sites (Marin et al., 1992). The sequence around serine 57 is highly conserved among different species which hints to a functional relevant region. By staining C/EBPδ from HCT116 cells with Phosphostain we have shown that it is a phosphoprotein. The inhibition of CK2 with quinalizarin or with CX-4945 resulted in slightly but reproducible reduced staining, implying that the CK2 is one among different other kinases that phosphorylate C/EBPδ.

Since C/EBPs form heterodimers with other members of the C/EBP family of proteins it was an obvious question whether the CK2 phosphorylation might interfere with the heterodimerization. However, as presented here, CK2 phosphorylation does not influence heterodimerization with C/EBPβ. The leucine zipper region, which is implicated in binding of the other C/EBPs, is located in the C-terminus of C/EBPs. The CK2 phosphorylation site is located in the N-terminus which might somehow exclude a direct influence of the CK2 phosphorylation on the heterodimerization and is consistent with the present results.

The nuclear localization of many proteins is regulated by phosphorylation (An et al., 2010; Lam et al., 1999; Li et al., 1997; Krempler et al., 2005) and in particular by CK2 phosphorylation (Kawamura et al., 1981; Xiao et al., 1998; Hübner et al., 1997; Jans and Jans, 1994; Wilson et al., 2002). In some cases the CK2 phosphorylation sites are in close vicinity of the nuclear localization signal (NLS). As shown here by using inhibitors of the protein kinase activity of CK2 or by using CK2 phospho mutants of C/EBPδ it turned out that CK2 phosphorylation of C/EBPδ does not influence its subcellular localization. We found strong evidence that besides being a substrate for CK2, C/EBPδ also binds to CK2. This is an interesting observation because in addition to the transcriptional function C/EBPδ has a transport function for the DNA repair protein FANCD2 into the nucleus (Wang et al., 2010). Thus, it might be possible that C/EBPδ targets CK2 in the nucleus to phosphorylate other C/EBP family members such as CHOP or the transcription factor and binding partner of C/EBPδ ATF4.

Protein phosphorylation is a versatile tool for the regulation of the transcription factor activity. It has been shown that protein phosphorylation of transcription factors can either enhance DNA binding activity and transcriptional efficacy (Molkentin et al., 1996) or disrupt DNA binding and transcription factor activity (Ramsay et al., 1995; Grigoryan et al., 2009). The major CK2 phosphorylation site serine 57 is located in the transactivation domain of C/EBPδ. Here, we show that co-expression of C/EBPδ with CK2 enhances the transcription factor activity of C/EBPδ, concerning the PPARγ2-promoter. An elevated phosphorylation of C/EBPδ might alter the conformation of the polypeptide chain in order to enhance DNA binding or the recruitment of other proteins of the transcriptional machinery. Alternatively phosphorylation of C/EBPδ might directly help to recruit other cellular co-activators or co-factors to enhance transcription.

In summary, we have identified C/EBPδ as a new substrate for CK2; we localized the major phosphorylation site to serine 57. CK2 phosphorylation of C/EBPδ did neither influence its subcellular localization nor the interaction with C/EBPβ. However, we show that the transcription factor activity of C/EBPδ is regulated by CK2 phosphorylation. In addition we demonstrate that CK2 binds to C/EBPδ which may reflect a substrate/enzyme interaction or which might indicate targeting of CK2 to other members of the transcription machinery.

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