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IKK-mediated Regulation of the COP9 Signalosome *via* Phosphorylation of CSN5

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Abstract

The COP9 signalosome (CSN) is an evolutionarily conserved multi-subunit protein complex, which controls protein degradation through deneddylation and inactivation of cullin-RING ubiquitin E3 ligases (CRLs). Recently, the CSN complex has been linked to the NF-κB signaling pathway due to its association with the IKK complex. However, how the CSN complex is regulated in this signaling pathway remains unclear. Here, we have carried out biochemical experiments and confirmed the interaction between the CSN and IKK complexes. In addition, we have determined that overexpression of IKKα or IKKβ leads to enhanced phosphorylation of CSN5, the catalytic subunit for CSN deneddylase activity. Mutational analyses have revealed that phosphorylation at serine 201 and threonine 205 of CSN5 impairs CSN-mediated deneddylation activity *in vitro*. Interestingly, TNF-α treatment not only enhances the interaction between CSN and IKK, but also induces an IKK-dependent phosphorylation of CSN5 at serine 201, linking CSN to TNF-α signaling through IKK. Moreover, TNF-α treatment affects CSN interaction network globally, especially the associations of CSN with the proteasome complex, eukaryotic translation

*Correspondence should be addressed to: Dr. Lei Fang (njfanglei@nju.edu.cn), 22 Hankou Road, Gulou District, Nanjing University, Nanjing, 210093, People's Republic of China. Phone: +86-25-83596845, Fax: +86-25-83596845. Dr. Chaojun Li (licj@nju.edu.cn), 22 Hankou Road, Gulou District, Nanjing University, Nanjing, 210093, People's Republic of China. Phone: +86-25-83596845, Fax: +86-25-83596845. Dr. Lan Huang (lanhuang@uci.edu), D233 Medical Science I, Department of Physiology & Biophysics, University of California, Irvine, CA 92697-4560, USA. Phone: (949) 824-8548, Fax: (949) 824-8540.

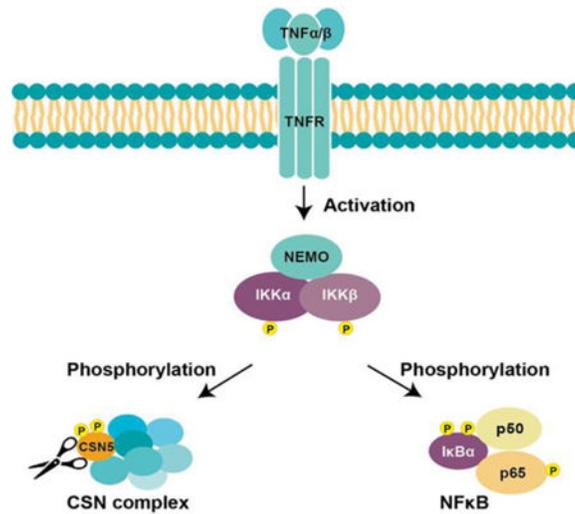initiation factor complex and CRL components. Collectively, our results provide new insights into IKK-mediated regulation of CSN associated with NF-κB signaling pathway.

Graphical Abstract

Keywords

The COP9 signalosome; CSN5; TNF-α; NF-κB signaling pathway; IKK complex; phosphorylation; deneddylation

Introduction

The COP9 signalosome (CSN) complex is a highly conserved multifunctional protein complex, traditionally consisting of eight subunits (CSN1–8) and best known for its deneddylase activity[1, 2]. It is well established that the CSN complex associates with cullin-RING ubiquitin E3 ligases (CRLs) and inactivates them through the removal of covalently attached ubiquitin-like Nedd8 modification[1, 2]. Recently, CSN9, a.k.a. CSNAP, was identified as the ninth integral CSN subunit, which is associated with the regulation of CRLs by CSN[3, 4]. CRLs represent the largest evolutionarily conserved superfamily of multi-subunit E3s, which embody ~30% of all human E3 proteins and coordinate degradation of ~20% of the proteins processed by the proteasome[5, 6]. It has been shown that the dynamic cycle of neddylation and deneddylation is essential for the proper assembly and function of CRLs[1, 2]. It has been reported that CSN can inactivate CRLs by enzymatic and non-catalytic regulations[4, 7–9]. Therefore, the CSN complex plays an important role in regulating proteasomal degradation and is vital to a diverse array of biological processes, including proliferation, apoptosis, signal transduction, and maintenance of DNA fidelity[10–12]. Aberration in the CSN complex, either through mutation or altered expression of its subunits, has been linked to developmental defects and diseases in mouse models and to the initiation and progression of cancer in human[13, 14].

The CSN complex has been associated with multiple signaling pathways including the NF-κB (nuclear factor-kappa B) pathway[15–18]. The NF-κB pathway is activated in response to acute cellular stresses and cytokines such as TNF-α, resulting in the rapid induction of NF-κB-mediated transcription of target genes. Prior to activation, NF-κB remains sequestered and inactive in the cytoplasm due to its interaction with the inhibitory IκB proteins. After IκBs are phosphorylated by IKKα and/or IKKβ, they are subsequently ubiquitinated by CRLs and degraded by proteasomes[19, 20]. Thus, freed NF-κB is able to translocate into the nucleus and activate transcription of its target genes. Prolonged NF-κB activity can be harmful to a cell or organism, therefore, the magnitude and duration of the NF-κB response is tightly regulated, through inhibitory auto-phosphorylation of IKKs and NF-κB-mediated upregulated transcription of IκBs[19, 20].

Although the exact role of the CSN complex in NF-κB signaling is still being defined, CSN has been shown to play a supportive role in NF-κB activation, shifting cells towards NF-κB-dependent cell survival and away from TNF-α-induced apoptosis in fibroblast-like synoviocytes of patients with rheumatoid arthritis[15]. In addition, it was reported that down-regulation of individual CSN subunits by RNAi in stimulated epithelial cells results in sustained NF-κB nuclear accumulation and transcription of target genes[16]. It appears that CSN interacts with IκBα conditionally and controls its stability in part through the lesser described CSN-associated deubiquitinylase activity[16]. Moreover, an interaction between IKKs and the CSN complex has been reported, leading to phosphorylation and subsequent ubiquitination of the CSN5 subunit [17]. The authors asserted that the CSN complex acts as an inhibitor of constitutive NF-κB activity in untreated cells and dissociates from IKK complexes following activation[17]. However, the precise phosphorylation sites of CSN5 directly regulated by IKKs were not determined. Together, these reports suggest an important role for CSN in the intricate and self-regulated NF-κB signaling pathway.

Due to the critical importance of the NF-κB pathway in cellular and organismal responses to stress, further understanding of the molecular mechanisms underlying the regulation of the CSN complex in this signaling pathway would help define CSN biology. To this end, we first performed biochemical experiments to confirm the interaction between IKKs and the CSN complex. We then determined that overexpression of IKKα or IKKβ induced phosphorylation of CSN5 at nine sites by mass spectrometric analysis. Among them, S201 and T205 located in the sequence region of CSN5 that aligned well with phosphorylation consensus sequences of IKK substrates. *In vitro* kinase assay has demonstrated that IKK phosphorylated CSN5 directly at these two specific sites. Mutagenesis results have indicated that phospho-mimetic mutations of CSN5 led to a statistically significant decrease in CSN deneddylase activity *in vitro*, whereas double phospho-resistant mutations appeared to have little effect. Moreover, TNF-α treatment not only altered IKK-CSN interaction and the interaction network of CSN, but also induced IKK-dependent phosphorylation of CSN5. Collectively, our results suggest that the activation of IKKs leads to CSN5 phosphorylation and attenuate CSN deneddylase activity, underlying the regulation of the CSN complex in TNF-α and NF-κB signaling pathways.

Material and Methods

Chemicals and Reagents –

The following antibodies were used in these studies: RGS-His (Invitrogen), β-Actin (Abcam), FLAG (Sigma), Cul1 (Abcam), and Jab1/CSN5 (Cell Signaling Technology). Streptavidin agarose resin was purchased from Thermo Scientific (Waltham, MA). SuperSignal West Pico chemiluminescent substrate was purchased from Pierce Biotechnology (Rockford, IL). Transfection reagents used were Lipofectamine 2000 (Invitrogen; New York, NY) and TurboFect (Thermo Scientific; Waltham, MA). Cell culture media was purchased from Corning (Manassas, VA). Sequencing grade trypsin was purchased from Promega Corp. (Madison, WI). All other general chemicals for buffers and culture media were from Fisher Scientific and/or VWR International.

Cells and Plasmids –

Generation of the 293[HBTH-CSN5] stable cell line was described previously [21]. 293[HBTH-CSN5] cells were grown to 70–80% confluence in DMEM medium containing 10% FBS, 1% penicillin/streptomycin, and 1 μM biotin, then were transected with pcDNA3.0 control, FLAG-IKKα, or FLAG-IKKβ plasmids (generous gifts from Dr. Joan Steffan, UC Irvine) using Lipofectamine 2000 *per* the manufacturer's protocol (Invitrogen). 24 hrs after transfection, cells were treated with 20 nM calyculin for 30 min before collecting cells. The collected cells were washed 3 times with PBS and stored in −80 °C.

For phosphorylation site studies, CSN5 S201A/T205A (MutA) and CSN5 S201D/T205D (MutD) mutations were introduced by PCR using a HBTH-CSN5-pQCXIP [21] as a template with the following primers:

ST-A primer 1: GGAAT*AGC*CTGGTACTC*AGC*AGGTCCTTCATCAGGAGGTTTG

ST-A primer 2: GGACCT*GCT*GAGTACCAG*GCT*ATTCCACTTAATAAAATAGAAG

ST-D primer 1: GGAAT*ATC*CTGGTACTC*ATC*AGGTCCTTCATCAGGAGGTTTG

ST-D primer 2: GGACCT*GAT*GAGTACCAG*GAT*ATTCCACTTAATAAAATAGAAG

and CSN5 forward primer, 5'-TATA TTAATTAA C ATG GCGGCGTCCGGGAGC-3'; and CSN5 reverse primer, 5'-GTCA GATATC TTAAGAGATGTTAATTTG-3'. Briefly, CSN5 forward primer and ST-A primer 1 were used to amplify the first fragment of the CSN5 S201A/ T205A gene, ST-A primer 2 and CSN5 reverse primer were used to amplify the second fragment. The full length CSN5 S201A/T205A was obtained by annealing both fragments and amplifying using the CSN5 forward primer and CSN5 reverse primer. The generated CSN5 S201A/T205A containing a PacI site at the 5' end and an EcoRV site at the 3' end was cloned into HBTH-pQCXIP, a derivative of pQCXIP (Clontech, Mountain View, CA) [22] to produce HBTH-CSN5-MutA-pQCXIP. HBTH-CSN5-MutD-pQCXIP was generated using the same strategy. Plasmids and mutations were verified *via* DNA sequencing. The constructs were transiently transfected into 293 cells as described above. After 24 hrs, cells were collected, rinsed with PBS, and stored at - 80 °C.

Affinity Purifications of the CSN Complex –

Under native condition, 293[HBTH-CSN5] cell pellets were lysed in lysis buffer (100 mM NaCl, 25 mM Tris-HCl, 10% glycerol, 0.35% NP-40, 5 mM ATP, 1 mM DTT, 5 mM MgCl$_2$, 1X protease inhibitor cocktail (Roche), 1X phosphatase inhibitor, pH 7.5). Lysates were passed 10–15 times through a 22G needle and were centrifuged at 13,000 rpm for 15 min to remove cell debris. The supernatant was then incubated with streptavidin agarose resin for 2 hrs at 4 °C. The streptavidin beads were then washed with 50 bed volumes of the lysis buffer, followed by a final wash with 30 bed volumes of TEB buffer (50 mM Tris-HCl, pH 7.5). Beads were incubated in 2 bed volumes of TEB buffer with 1% TEV at 4 °C overnight and then passed through a column to collect the eluate. When necessary, eluates were stored in 10% glycerol at - 80 °C. For *in vitro* deneddylase experiments, purifications were performed as described above but the elution step was performed in buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% Glycerol.

SDS-PAGE and Immunoblotting –

Cell lysates and eluates were separated by 12% SDS-PAGE. Proteins were transferred to a PVDF membrane and analyzed by standard immunoblotting. HBTH-CSN5 containing a three amino acids "RGS" sequence before His-tag in HBTH was detected using an RGS-His antibody; IKKα and IKKβ were detected with a FLAG antibody. Cul1 and neddylated-Cul1 were detected by Cul1 antibody. β-Actin was used as loading control. HRP-conjugated secondary antibodies were visualized with chemiluminescent substrate *per* the manufacturer's instruction.

LC-MS/MS –

The TEV eluates containing CSN5 from FLAG-IKKα, FLAG-IKKβ expressing cells were in-solution digested by adding 1% Trypsin and incubating at 37 °C overnight as described[21]. The digested peptides were subsequently desalted using Vivapure C18 microspin columns (Vivascience) prior to mass spectrometry analysis. LC-MS/MS was carried out by nanoflow reverse phase liquid chromatography (RPLC) (Eksigent, CA) coupled on-line to a Linear Ion Trap (LTQ)-Orbitrap XL mass spectrometer (Thermo-Electron Corp). The LC analysis was performed using a capillary column (100 μm ID x 150 mm long) packed with C18 resin (GL Sciences) and the peptides were eluted using a linear gradient of 2–35% B in 105 min; (solvent A: 100% H$_2$O/0.1% formic acid; solvent B: 100% acetonitrile/0.1% formic acid). A cycle of one full FT scan mass spectrum (350–2000 m/z, resolution of 60,000 at m/z 400) was followed by ten data-dependent MS/MS acquired in the linear ion trap with normalized collision energy (setting of 35%). Target ions selected for MS/MS were dynamically excluded for 30s.

Database Searching for Peptide Identification and Quantification –

Monoisotopic masses of parent ions and corresponding fragment ions, parent ion charge states and ion intensities from the tandem mass spectra (MS/MS) were obtained by using in-house software with Raw_Extract script from Xcalibur v2.4. Following automated data extraction, the resultant peak lists for each LC-MS/MS experiment were submitted to the development version (5.19.1) of Protein Prospector (UCSF) for database searching against

SwissProt random.concat database (04/08/2019, 20419/559228 entries searched). Because the samples were purified from human cell lines, *Homo sapiens* were selected as the restricted species. Trypsin was set as the enzyme with a maximum of two missed cleavage sites. The mass tolerance for parent ion was set at ± 20 ppm, whereas ± 0.6 Da tolerance was chosen for the fragment ions. Chemical modifications such as protein N-terminal acetylation, methionine oxidation, N-terminal pyroglutamine, deamidation of asparagine, phosphorylation on serine and threonine were selected as variable modifications. Cysteine carbamidomethylation were selected as fixed modifications. The Search Compare program in Protein Prospector was used for summarization, validation and comparison of results. To determine the expectation value cutoff that corresponds to a percent false positive (% FP) rate, each project was searched against a normal database concatenated with the reversed form of the database. An algorithm in Search Compare automatically plots the expectation values versus % FP rate for each search result. Based on these results, we chose an expectation value cutoff corresponding to 0.01% FP for all peptides. At this false positive rate, false protein hits from decoy database were not observed. The identified phosphorylated peptides have been further validated by manual inspection of their MS/MS.

Amino acid sequences of CSN5 protein from several model species including HUMAN (*Homo sapiens*), RAT (*Rattus norvegicus*), MOUSE (*Mus musculus*), XENLA (*Xenopus laevis*), DANRE (*Danio rerio*), DROME (*Drosophila melanogaster*), CAEEL (*Caenorhabditis elegans*), ARATH (*Arabidopsis thaliana*) and YEAST (*Saccharomyces cerevisiae*) were downloaded from Uniprot database (https://www.uniprot.org) and were automatically multiple aligned and phylogenetically analyzed using ClustalW[23]. Alignment of the phosphorylated CSN5 peptide sequence to known IKK substrates was performed by inputting the CSN5 peptide sequence and the regions surrounding previously characterized IKK-mediated phosphorylation sites [24] into the ClustalW program [23] using standard parameters.

TNF-α Treatment and QTAX Experiments –

293[HBTH-CSN5] cells were treated with TNF-α for 0 or 30 min, washed 3 times with 1x PBS, and then crosslinked with 1% formaldehyde for 10 min similarly as described[25]. The CSN complex and its associating proteins were purified under denaturing condition using the QTAX strategy (Quantitative analysis of tandem affinity-purified *in vivo* cross-linked protein complexes) as previously described[25], and the resulting digests were analyzed by LC-MS/MS for protein identification as mentioned above. A label-free QTAX approach based on spectral counting was employed for comparing protein abundance between treated and untreated samples[25]. Database searching was performed using ProteinPospector (version 5.24.0) as described above.

Analysis of CSN interacting proteins induced by TNF-α treatment –

Only proteins identified with 2 unique peptides were selected for comparison between TNF-α and untreated samples. Label-free quantitation was performed based on spectral counting. CSN interacting proteins with treated/untreated ratios 2 were considered as up-regulated proteins. These proteins were classified by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation using DAVID online tools (http://

david.abcc.ncifcrf.gov). For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the up-regulated protein against all identified proteins (Prism6 software); KEGG database was used to map the up-regulated protein at concerned metabolic pathways (https://www.kegg.jp); protein-protein interaction network analysis (PPI) was performed *via* STRING online tools (http://string-db.org), and manually reorganized; Heatmap of up-regulated proteins in several concerned protein complexes was generated using the R package "pheatmap".

In Vitro Deneddylation Activity Assay –

In vitro deneddylation activity assay was performed using two independent methods. In the first method, cleavage of neddylated-Cul1 was measured as previously described[26]. Briefly, purified CSN complexes were incubated with purified Cul1 conjugated to FLAG-Nedd8[26] in buffer that contains 40 mM Tris-HCl, pH 7.4, 0.6 mM DTT, and 0.2 mg/ml BSA at 37 °C for 15 min. Deneddylation reactions as well as larger volumes of purified CSN complexes were separated by 4–20% SDS-PAGE and proteins were transferred to PVDF membranes. HBTH-CSN5 was detected using a CSN5 antibody and Cul1 was detected using a Cul1 antibody. Levels of CSN5, neddylated Cul1, and deneddylated Cul1 were quantified using a Li-Cor Odyssey scanner (Lincoln, NE). In the other method, deneddylation activity of different CSN complexes was measured using NEDD8-CHOP-Reporter DeNEDDylation Assay Kit (LifeSensors) following the user manual.

In Vitro Kinase Assay –

IKKα and IKKβ were purified from 293T cells transiently transfected with FLAG-IKKα and FLAG-IKKβ plasmids, respectively. CSN5 was purified from 293[HBTH-CSN5] stable cells and *E. Coli* B121 (DE3), respectively. The *in vitro* kinase assay was performed as described previously[27] using IKKα and IKKβ as the kinases, and CSN5 as substrate. The resulted CSN5 from the *in vitro* kinase assay was separated by 12% SDS-PAGE, Coomassie blue stained and subjected to in gel digestion to identify and quantify specific CSN5 phosphorylation sites at S201 and T205 using SRM method (selected reaction monitoring) by LC-MS/MS.

Results

CSN interacts with IKKα and IKKβ

To study the regulation of CSN during NF-κB activation, we first attempted to validate interactions between the CSN and IKKs by immunoprecipitation (IP) and immunoblotting using our previously generated 293 cell line stably expressing HBTH-CSN5[21]. The HBTH tag contains two hexahistidine tags flanking a signal sequence for *in vivo* biotinylation and a Tobacco Etch Virus (TEV) protease cleavage site, allowing for effective purification of the functional CSN complex. To isolate IKK complexes, constructs encoding FLAG-IKKα or FLAG-IKKβ were transiently transfected into 293[HBTH-CSN5] cells. Empty vector plasmid was used as the control (The upper panel of Fig. 1A). IKK complexes from these cells were purified with anti-FLAG conjugated beads and were then separated by SDS-PAGE. When probing HBTH-CSN5 with an RGS-His antibody, immunoblotting analysis has revealed that HBTH-CSN5 was co-purified with IKKα and IKKβ respectively (The middle panel, Fig.

1A). This result indicates that CSN can associate with complexes containing both FLAG-IKKα and FLAG-IKKβ.

To further evaluate the interaction between the CSN and IKKs, we also performed a reciprocal purification using HBTH-CSN5 as the bait to isolate CSN5-containing complexes from the same cell types described above. After binding to streptavidin resins, CSN complexes were eluted by TEV cleavage and separated by SDS-PAGE. Immunoblots probed with a FLAG antibody indicate co-purification of both FLAG-IKKα and FLAG-IKKβ with CSN complexes (The lower panel, Fig. 1A). Together, our results confirmed the interactions between the CSN and IKKs, thus establishing the basis for us to further explore the CSN complex in the context of NF-κB activation.

Overexpression of IKKα/β leads to phosphorylation of CSN5

To understand the biological significance of the interactions between the CSN complex and IKKs, we wanted to determine whether overexpression of the IKKs would result in enhanced phosphorylation of CSN5. To this end, the CSN complex was affinity purified respectively from $293^{HBTH-CSN5}$ cells transiently transfected with FLAG-IKKα, FLAG-IKKβ, or an empty vector control using the same procedure as described[21]. The isolated CSN complexes were then digested by trypsin and analyzed by LC-MS/MS to characterize CSN5 phosphorylation induced by IKK overexpression. Figure 1B illustrates the MS/MS of a tryptic peptide (MH_2^{2+} 1107.02) from the CSN peptide digest. A fragment ion at m/z 1058.39^{2+} was obtained due to the loss of H_3PO_4 (−98Da) from the parent ion (MH_2^{2+} 1107.02), indicating that the peptide is phosphorylated. Based on the series of b and y ions, the peptide was determined as the phosphorylated form of a CSN5 peptide with the sequence of ^{192}GYKPPDEGPSEYQTIPLNK^{210}. There are four potential phosphorylation sites (tyrosine 193, serine 201, tyrosine 203 and threonine 205) in this sequence. Since b_6, b_7, b_9 and y_4~y_7 ions were detected as non-phosphorylated forms, it was suspected that S201 or Y203 was most likely phosphorylated. However, detection of phosphorylated forms of b_{10} and the neutral loss of H_3PO_4 ions of b_{10} supports that S201 was phosphorylated. The phosphorylation of S201 was confirmed by the detection of phosphorylated forms of y_{12-13}, y_{15-17}, b_{10}, b_{15}, the neutral loss of H_3PO_4 ions of b_{10} and b_{15}, which was further validated by comparing to the MS/MS of the non-phosphorylated form of the same peptide (Figure 1C). In addition, our analysis has identified another phosphorylated peptide with the same m/z but yielding a different MS/MS as displayed in Figure 1D. In comparison to the upper panel of Figure 1B, the detection of phosphorylated forms of y_6~y_8 ions and non-phosphorylated forms of b_{11} and b_{12} indicated that T205 was phosphorylated, which was further supported by the observation of neutral loss H_3PO_4 ions of y_6 and y_8. Similarly, seven additional phosphorylated peptides have been identified unambiguously by peptide sequencing and the results are summarized in Table 1. It is noted that all nine unique phosphorylation sites identified here have not been reported before. The corresponding MS/MS of the other identified phosphorylated peptides are shown in Supplementary Figure 1.

Phylogenetic analysis demonstrated that the two phosphorylation sites S201 and T205 of CSN5 were highly conserved in evolution from plant to mammal (Fig. 2A), suggesting that

these sites may be biologically important. To understand whether these sites are potential IKK substrates, we compared the identified CSN5 sequences to known IKK substrates such as IκBα, IκBβ, IκBε and NF-κB[24], and found that only the sequence in the region of 192–210 of CSN5 closely aligns with the IKK conserved consensus sequences, with both S201 and T205 residues converged well with the highly conserved IKK-induced phosphorylation sites (Fig. 2B).

To determine whether IKK overexpression induces CSN5 phosphorylation at these two sites, we employed a label-free based approach to quantify the relative abundance of non-phosphorylated and phosphorylated forms of [192]GYKPPDEGPSEYQTIPLNK[210] in the presence or absence of IKK expression based on extracted ion chromatograms (XICs). As shown in Figure 3, although the peptide containing phosphorylated T205 was identified in all samples, its abundance was significantly increased in samples with IKK overexpression whereas its non-phosphorylated form did not markedly change. Interestingly, the peptide containing phosphorylated S201 was only observed in IKK transfected samples (Fig. 3B, 3C and Table 1), indicating that it was directly induced by IKK overexpression. Notably, the peak corresponding to the phosphorylated S201 peptide was more prominent in the sample transfected with IKKα than IKKβ (Fig. 3B, 3C), which implies a direct phosphorylation by IKKα or may also be related to the stronger interaction between IKKβ and the CSN as was observed in our purifications. Therefore, these sites may be important in regulating the activity of CSN5 and the CSN complex during NF-κB activation.

IKKα/β directly phosphorylates CSN5 at S201 and T205 *in vitro*

To investigate whether IKKα could directly phosphorylate CSN5, we performed *in vitro* kinase assays using purified IKKα, IKKβ and CSN5. The resulting samples were digested and analyzed by the SRM method to specifically quantify changes in CSN5 phosphorylation at S201 and T205. Specifically, phosphopeptides [192]GYKPPDEGP(pS)EYQTIPLNK[210] (MH_3^{3+} 738.34) and [192]GYKPPDEGPSEYQ(pT)IPLNK[210] (MH_3^{3+} 738.34) as well as their non-phosphorylated form [192]GYKPPDEGPSEYQTIPLNK[210] (MH_3^{3+} 711.69) were selected for targeted analysis. The results showed that both IKKα and IKKβ could phosphorylate CSN5 purified from 293[HBTH-CSN5] cells at S201 and T205 residues *in vitro* (Fig. 4 A–C). Similarly, IKKα and IKKβ could also phosphorylate recombinant CSN5 purified from *E. coli* at these two specific sites (Fig. 4D, 4E). Therefore, these results have demonstrated that S201 and T205 residues of CSN5 are IKK substrates, providing a link between the CSN complex and NF-κB signaling.

Phosphorylation of CSN5 at S201/T205 attenuates CSN deneddylase activity

To determine whether phosphorylation at S201 or T205 of CSN5 could in fact regulate CSN function, we generated HBTH-CSN5 constructs with either double S201A/T205A phospho-resistant mutations (MutA) or double S201D/T205D phospho-mimetic mutations (MutD). The mutant and wild-type HBTH-CSN5 constructs were transiently transfected into 293 cells respectively to affinity purify CSN5-, CSN5MutA-, or CSN5MutD-containing complexes as described[21]. The subunit composition and abundance of isolated CSN complexes from each of these samples appeared similar as seen *via* SDS-PAGE (Fig. 5A) and MS analysis (Supplementary Table 1), indicating the mutations did not affect the

formation of the CSN complex. To examine CSN function, the isolated CSN complexes were then subjected to *in vitro* deneddylation assay designed to measure the deconjugation of Nedd8 from neddylated FLAG-Cul1[26].

As seen in Fig. 5A by immunoblotting analysis, the wild-type CSN complex was able to carry out deneddylation as indicated by a molecular weight shift consistent with the removal of a Nedd8 molecule from neddylated FLAG-Cul1. Interestingly, CSN5MutA complexes appear to have slightly up-regulated deneddylation activity, while CSN5MutD complexes display reduced deneddylation activity. To better quantify the activity change of CSN mutants, a more sensitive deneddylase activity assay using NEDD8-CHOP-Reporter Kit (LifeSensors) was performed with three biological replicates. Although the CSN5MutA complex appeared to have a bit higher activity than the wild-type CSN complex, the difference was negligible and statistically insignificant ($p = 0.4695$, based on Student's *t*-test; Fig. 5B). In contrast, the CSN5MutD complex displayed 72% decrease in deneddylase activity compared to the wild-type CSN with statistical significance ($p < 0.01$, Student's *t*-test; Fig. 5B). These results suggest that phosphorylation of CSN5 at S201 and/or T205 are biologically important and have an inhibitory effect on CSN deneddylase activity.

TNF-α modulates CSN interaction network

TNF-α is an important cytokine that induces IKK activation and downstream NF-κB signaling pathway[9, 20, 24]. To investigate whether TNF-α plays a role in regulating the CSN interaction dynamics and function, we employed the QTAX strategy[25] to compare CSN interacting proteins with or without TNF-α treatment (Fig. 6A). The QTAX strategy has been demonstrated advantageous for capturing all types of interactions including stable, dynamic and/or transient interactions in a single experiment[25, 28]. Briefly, *in vivo* formaldehyde cross-linking of treated or untreated 293 cells stably expressing HTBH-CSN5 was performed respectively. The cross-linked cells were lysed under denaturing conditions and subjected to tandem affinity purification[25]. This process was evaluated by immunoblotting as illustrated in Fig. 6B, which showed efficient cross-linking and purification. The isolated cross-linked protein complexes were digested and analyzed by LC-MS/MS. As a result, IKK-CSN interaction was only identified after TNF-α treatment (Supplementary Table 2), which also led to the detection of CSN5 phosphorylation at S201 (Supplementary Figure 2). Among the total of 977 proteins identified, label-free based quantitation determined that 210 proteins showed increased interactions with CSN after TNF-α treatment and 63 of them were only found in TNF-α treated sample (Supplementary Table 3). These results suggest that TNF-α signaling modulates CSN interaction network. To understand TNF-α induced changes, we carried out several bioinformatics analyses to infer the associations of CSN interactors with cellular pathways and biological processes (Fig. 6 C–F). KEGG pathway analysis showed that CSN associating proteins enhanced by TNF-α treatment were mainly enriched in proteasome, splicesome and various cell metabolism pathways (Fig. 6C). The top 5 enriched biological processes were cell-cell adhesion, NIK/NF-κB signaling, regulation of translational initiation, ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle and proteasome-mediated ubiquitin-dependent protein catabolic process (Fig. 6D). Cellular component analysis showed significant enrichment of these proteins in cell-cell adhesion and proteasome

complex (Fig. 6E). Notably, translational initiation factor activity and other important activities required for ubiquitin-proteasome degradation pathway, such as proteasome-activating ATPase activity, protein disulfide isomerase activity, cysteine-type endopeptidase activity and ubiquitin-protein transferase activity, were enriched in molecular function analysis (Fig. 6F). Further protein-protein interaction network analysis revealed that the other 2 PCI containing complexes, proteasome and eIF3, were recruited to the CSN complex (Fig. 6G and 6H). Taken together, our results suggest that TNF-α activates IKKα and mediates its interaction with CSN to phosphorylate CSN5. In addition, TNF-α signaling modulates CSN interaction network globally, especially the components involved in the UPS.

Discussion

In this report, we have confirmed IKK-CSN interactions and showed that overexpression of IKKs leads to phosphorylation of CSN5, corroborating very well with a previous report[17]. Importantly, our findings have shed new insights on the regulation of the CSN complex by IKKs as we have identified specific CSN5 phosphorylation sites that occur in response to overexpression of either IKKα or IKKβ. While it is possible that the observed phosphorylation of CSN5 is a result of kinases downstream of IKK activation, we have found that the phosphorylation sites appear specific to IKK overexpression and also align well with the consensus sequence of known IKK substrates. More importantly, *in vitro* kinase assay has determined that IKK directly phosphorylates CSN5. Therefore, our results strongly suggest that CSN5 is an IKK substrate and activation of IKK leads to the phosphorylation of CSN5 at S201 and T205 residues. Moreover, we have determined that phospho-mimetic mutations of S201/T205 significantly attenuated CSN deneddylase activity *in vitro*, whereas phosphor-resistant mutants did not seem to have much effect. This indicates that IKK-dependent phosphorylation of CSN5 is biologically relevant and may constitute an important mode of the regulation of CSN function in IKK-associated signaling pathways.

Since CSN has been implicated in regulating the NF-κB pathway[15–18], we next explored whether TNF-α treatment would lead to IKK-mediated phosphorylation of CSN5. Indeed, TNF-α signaling not only induced CSN5 phosphorylation at S201, but also triggered IKK binding to the CSN complex. The enhanced IKK-CSN interaction is suspected to be directly associated with CSN5 phosphorylation. Additionally, TNF-α appears to have a more profound impact on CSN interaction network as it increased associations of CSN with multiple protein complexes involved in a diverse array of cellular process, including the proteasome and eIF3 complex. Interestingly, CSN shares a common architecture with the lid subcomplex of the 26S proteasome by consisting of six subunits harbouring PCI domain (proteasome-CSN-eIF3) at their C-terminus, plus two subunits containing MPN domains (Mpr1/Pad1 N-terminal)[29, 30]. The translation initiation complex eIF3 also contains PCI and MPN domain proteins, but seems to deviate from the 6+2 stoichiometry[31, 32]. Although it is generally assumed that evolutionarily conserved protein domains participate in similar biochemical processes, the CSN complex, the 26S proteasome lid and eIF3 appear to have distinct functions. Nonetheless, some subunits of the three complexes have been shown to interact[29, 33–36]. For example, the mammalian Int-6 protein, a subunit of the eIF3 translation

initiation factor, has been confirmed to interact with Rpt4, CSN3 and CSN6[37]. Later studies have suggested that Int-6 binds to the CSN holocomplex, and is also associated with the 26S proteasome[37]. In addition, the CSN complex has been reported to interact with the 26S proteasome and compete with the 19S lid[38, 39]. Therefore, we speculate that the three functionally different but structurally homologous protein complexes possess intrinsic yet unraveled connections, and may potentially be able to form a supercomplex or transiently assemble into different complexes *via* subunit exchange to coordinate complicate biological functions in TNF-α induced activation of NF-κB pathway.

Based on our results, we propose a reciprocal regulation between the CSN complex and the NF-κB signaling pathway as depicted in Fig. 7. TNF-α stimulates IKK activation, which phosphorylates IκBs, and also targets CSN5 at S201/T205 residues. IKK-induced phosphorylation of CSN5 then attenuates CSN deneddylase function. As cullin neddylation is essential to the formation and activity of CRL complexes that enables substrate binding and protein ubiquitination[5, 40, 41], decreased CSN-mediated deneddylation of cullins potentially elongates CRL ubiquitination activity, thus prolonging the ubiquitination of NF-κB inhibitory proteins IκBs. This would ensure rapid degradation of IκBs by the proteasome, allowing the freed NF-κB to translocate into the nucleus to initiate transcription and translation. Intriguingly, TNF-α treatment enhanced CSN interaction with CRL5 components and several proteasome-associated deubiquitinases, suggesting that CSN phosphorylation may affect their interactions. CSN-CRL interactions are known to be critical to co-regulation of both complexes through interaction-induced conformational changes[9, 42, 43], and post-translational modifications such as phosphorylation would likely influence CSN-CRL complex formation by disrupting the interaction interface[9]. Therefore, we suspect that phosphorylation-dependent CSN-CRL interactions may be involved in modulating protein ubiquitination/deubiquitinating in the NF-κB pathway. In summary, we have identified novel and biologically relevant CSN5 phosphorylation event induced by IKK activation, which establishes a strong basis for us to further study the functional crosstalk and co-regulation between CSN and the NF-κB pathway *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

Abbreviations

|	
CRL	cullin-RING ubiquitin E3 ligase
CSN	COP9 Signalosome
TNF-α	tumor necrosis factor alpha
IKK	inhibitor of nuclear factor kappa-B kinase
eIF	eukaryotic translation initiation factor complex
IP	immunoprecipitation
LC-MS/MS	liquid chromatography tandem mass spectrometry
MPN	Mpr1-Pad1-N-Terminal
RPLC	reverse phase liquid chromatography
SCF	Skp1-Cul1-F-box
TEV	tobacco etch virus
XIC	extracted ion chromatogram
QTAX	quantitative analysis of tandem affinity-purified *in vivo* cross-linked protein complexes
SRM	selected reaction monitoring

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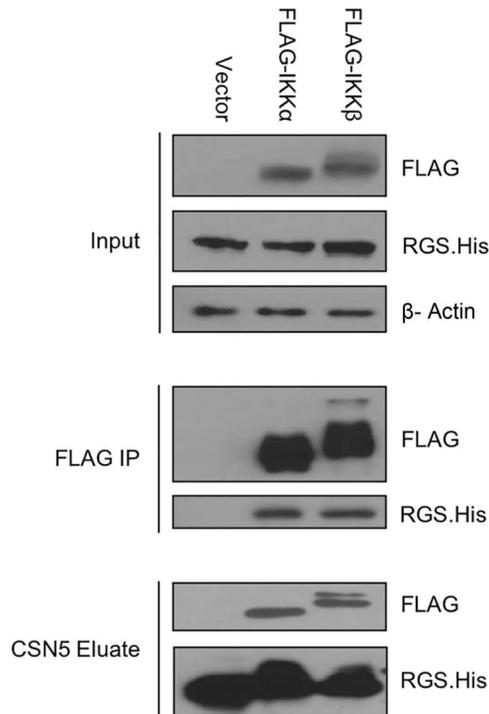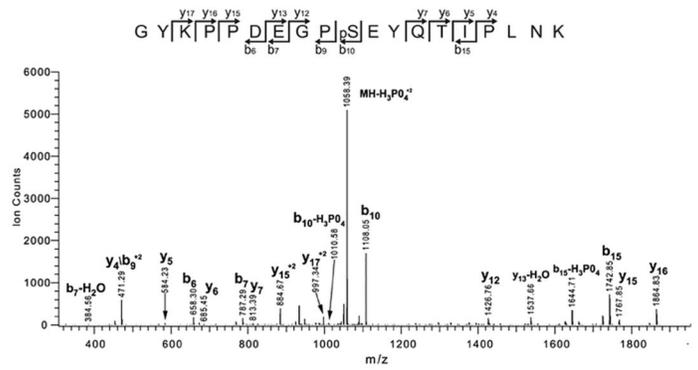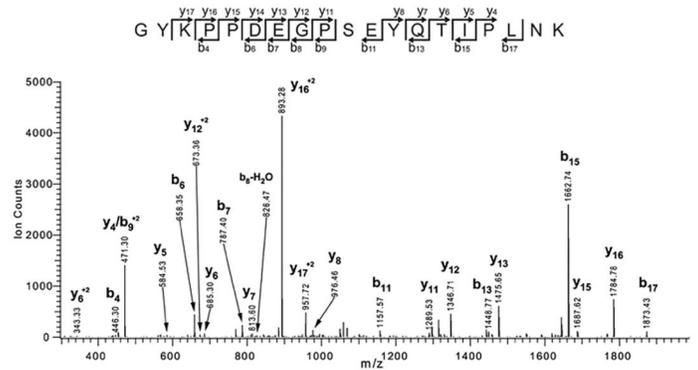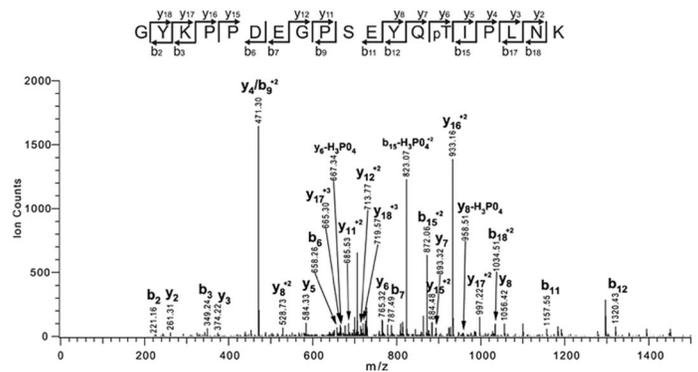
Figure 1. Ectopically expressed IKKα/β interact with HBTH-CSN5 and induce its phosphorylation at S201 and T205.
(A) Ectopically expressed IKKα and IKKβ interact with HBTH-CSN5. The upper panel: immunoblot of cell lysates from 293[HBTH-CSN5] cells transiently transfected with either FLAG-IKKα or FLAG-IKKβ shows expression of HBTH-CSN5, FLAG-IKKα or FLAG-IKKβ. The middle panel: immunoblot showing that an anti-FLAG IP co-purifies HBTH-CSN5, as visualized with an anti-RGS-His antibody. The lower panel: immunoblot of a streptavidin purification performed shows FLAG-IKKα or FLAG-IKKβ is co-purified with HBTH-CSN5. Actin is shown as a loading control. (B-D) Representative MS/MS of non-phosphorylated and phosphorylated peptides identified for CSN5. (B) Peptide corresponding to phosphorylated S201 induced by IKKα, GYKPPDEGPS(Phospho)EYQTIPLNK (MH$_2^{+2}$ 1107.02). (C) The non-phosphorylated peptide GYKPPDEGPSEYQTIPLNK (MH$_2^{+2}$

1067.04). (D) Peptide corresponding to phosphorylated T205 induced by IKK, GYKPPDEGPSEYQT(Phospho)IPLNK (MH$_3^{+3}$ 738.35).

Figure 2. Phosphorylation sites S201 and T205 of CSN5 are evolutionarily conserved and align with the consensus sequence of known IKK substrates.

(A) Dendrogram and intercepted multiple sequence alignment of CSN5 protein in several common model species based on ClustalW. (B) The identified CSN5 phospho-peptide sequence was aligned with domains surrounding known phosphorylation sites of IKK substrates using ClustalW. Conserved phosphorylation sites S201 and T205 of CSN5 were highlighted. *Per* the ClustalW site, the asterisk indicates positions which have a single, fully conserved residue; the colon indicates conservation between groups of strongly similar properties; and the period indicates conservation between groups of weakly similar properties.
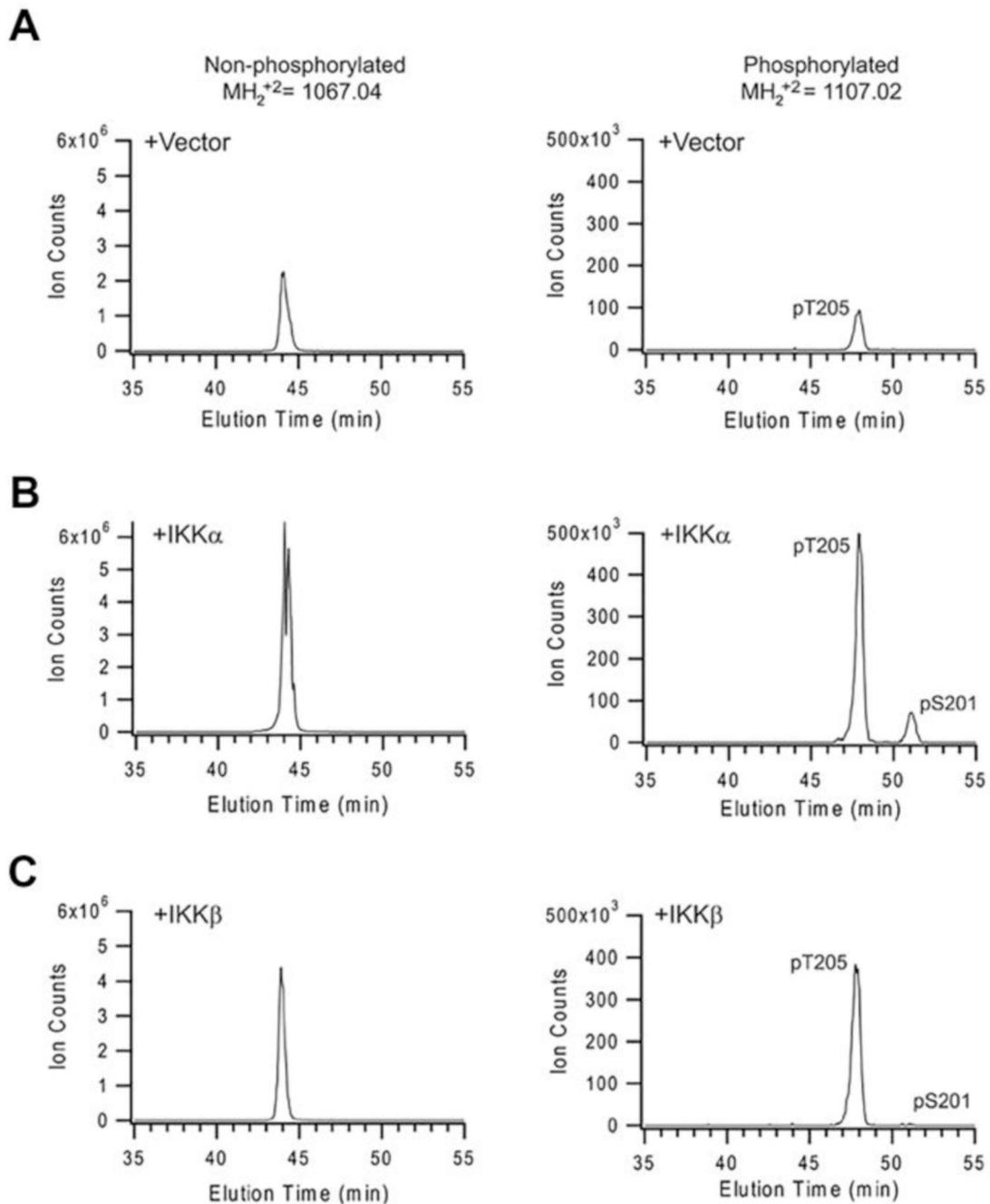
Figure 3. Extracted ion chromatograms for S201 or T205 phosphorylation of CSN5 induced by IKKα/β *in vivo*.
Extracted ion chromatograms for non-phosphorylated GYKPPDEGPSEYQTIPLNK (MH_2^{+2} 1067.04) peptide and phosphorylated GYKPPDEGPS(Phospho)EYQTIPLNK (MH_2^{+2} 1107.03) or GYKPPDEGPSEYQT(Phospho)IPLNK (MH_2^{+2} 1107.00) peptides, as indicated, from cells transfected with vector only (A), IKKα (B), and IKKβ (C). Peaks corresponding to phosphorylation of either S201 or T205 are indicated.
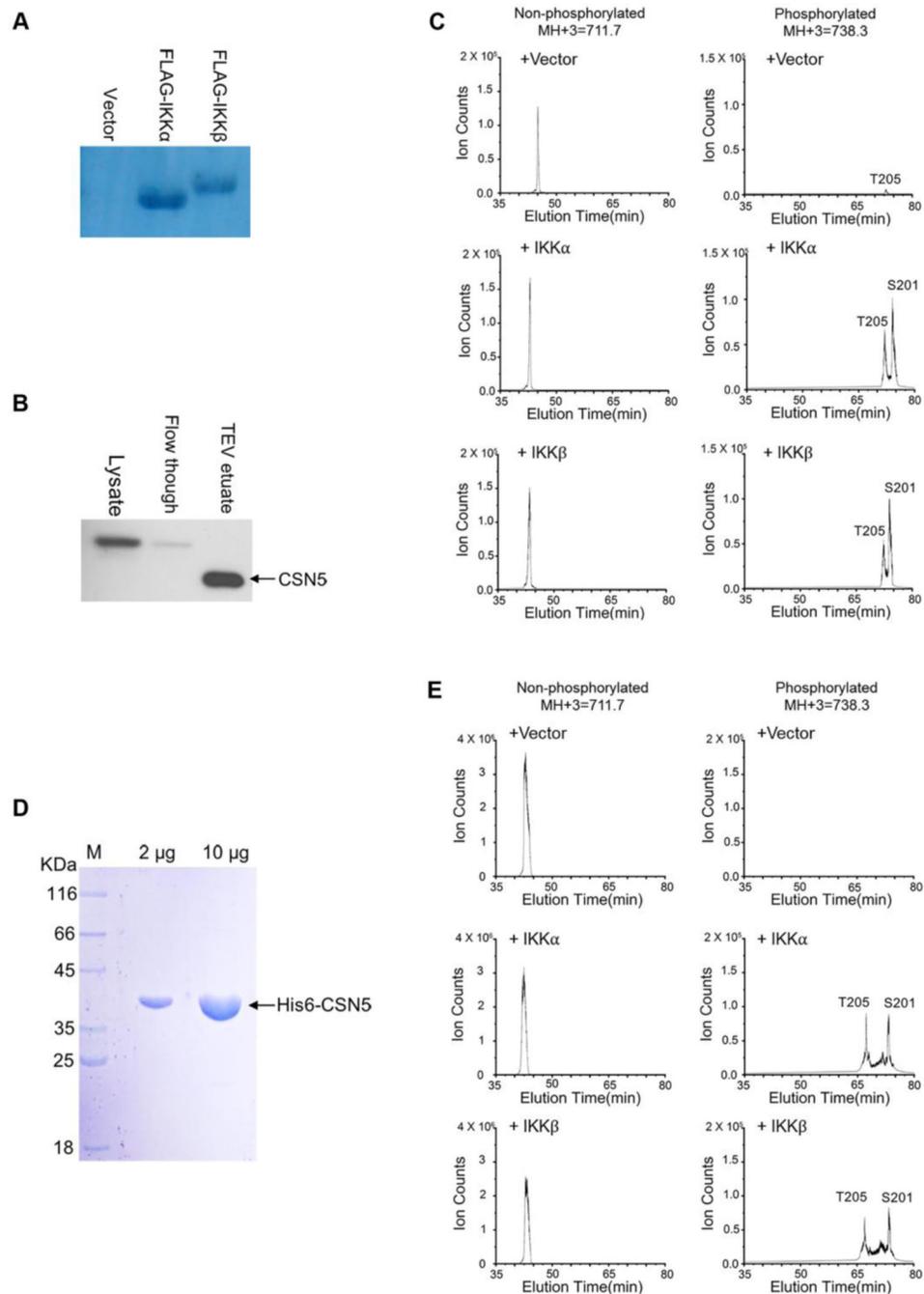
Figure 4. IKKα/β directly phosphorylates CSN5 at S201 and T205 *in vitro*.
(A) IKKα and IKKβ were purified from 293T cells transiently transfected with FLAG-IKKα and FLAG-IKKβ plasmids, respectively. CSN5 was purified from 293[HBTH-CSN5] stable cells (B) and *E. Coli* B121 (DE3) (D), respectively. The *in vitro* kinase assay was performed using IKKα and IKKβ as the kinases, and CSN5 as substrate. The resulted CSN5 from the *in vitro* kinase assay was separated by 12% SDS-PAGE, Coomassie blue stained and subjected to in gel digestion to identify and quantify specific CSN5 phosphorylation sites at S201 and T205 using SRM method by LC-MS/MS. In the SRM experiments,

peptides GYKPPDEGPS(Phospho)EYQTIPLNK[210] (MH_3^{3+} 738.34) and GYKPPDEGPSEYQT(Phospho)IPLNK (MH_3^{3+} 738.34) were selected as phosphorylated form, and GYKPPDEGPSEYQTIPLNK (MH_3^{3+} 711.69) was used as non-phosphorylated control. (C) Both IKKα and IKKβ could phosphorylate CSN5 from $293^{HBTH-CSN5}$ cells at S201 and T205 residues *in vitro*. (E) IKKα and IKKβ could also phosphorylate recombinant CSN5 from *E. coli* at S201 and T205 residues *in vitro*. Peaks corresponding to phosphorylation of either S201 or T205 are indicated.
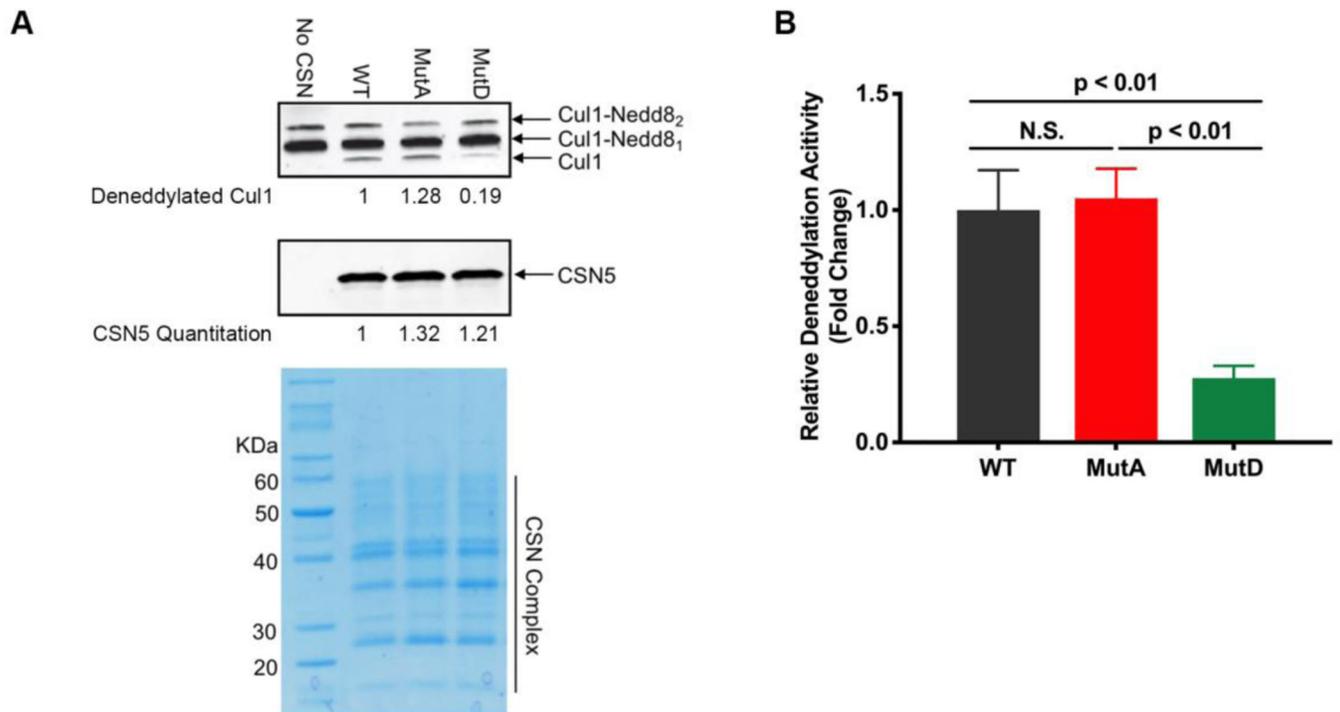
Figure 5. Effects of CSN5 phospho-mutants on CSN-mediated deneddylation activity.
(A) Representative immunoblots of *in vitro* deneddylation assay. Upper arrow indicates neddylated-Cul1 and lower arrow indicates Cul1 after removal of Nedd8. Deneddylated-Cul1 and CSN5 levels were quantified and shown as indicated. Coomassie blue staining of CSN complex purified from CSN5-WT, MutA and MutD was used as a loading control of CSN used for deneddylation activity assay. (B) CSN5MutD markedly inhibited CSN-mediated deneddylation activity. The deneddylation activity of CSN5-WT, MutA and MutD were measured and quantified using NEDD8-CHOP-Reporter DeNEDDylation Assay Kit. The figure showed averaged results from three biological repeats. Error bars indicate the standard deviation between three experiments. $P<0.01$ indicates the statistically significant decrease of CSN5MutD deneddylation activity compared to CSN5WT and CSN5MutA.
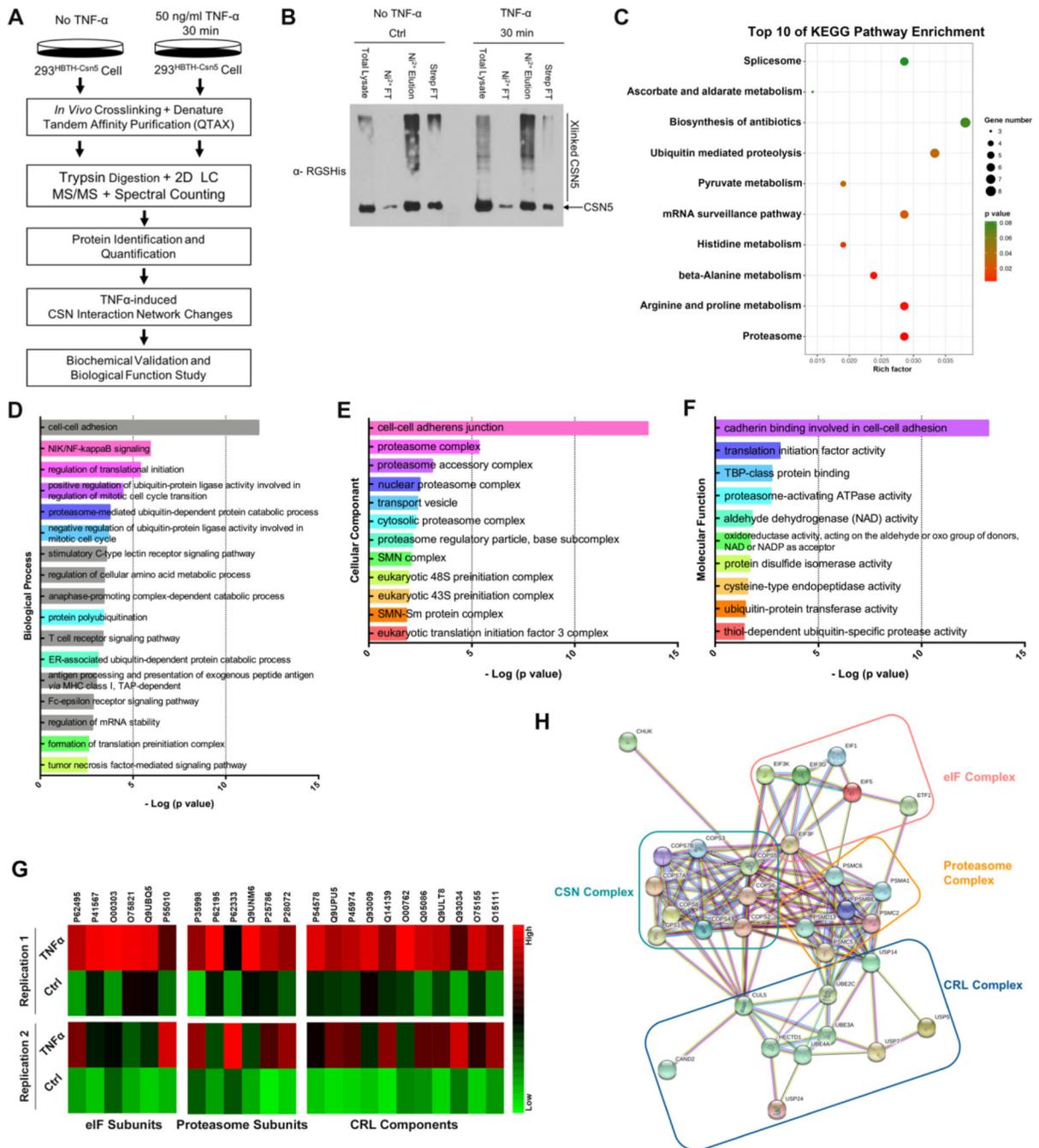
Figure 6. Mapping the dynamic changes of CSN interaction network induced by TNF-α using QTAX strategy.

(A) Work flow of mapping the dynamic changes of CSN interaction network induced by TNF-α using QTAX strategy. (B) Immunoblots showing the crosslinking and tandem purification efficiency with an anti-RGS-His antibody. (C) KEGG pathway analysis of CSN associating proteins enhanced by TNF-α stimulation. (D-F) GO analysis of CSN associating proteins enhanced by TNF-α stimulation. D-F show biological process, cellular component and molecular function analysis, respectively. (G) Interaction network analysis of TNF-α enhanced CSN association with proteasome, CRLs components and eIF3 complex. (H) A

heatmap showing enhanced association of proteasome, CRLs components and eIF3 complex with CSN induced by TNF-α. The proteins with a TNF/No TNF ratio > 2 and peptide count > 2 were considered as up-regulated CSN5 associating proteins.
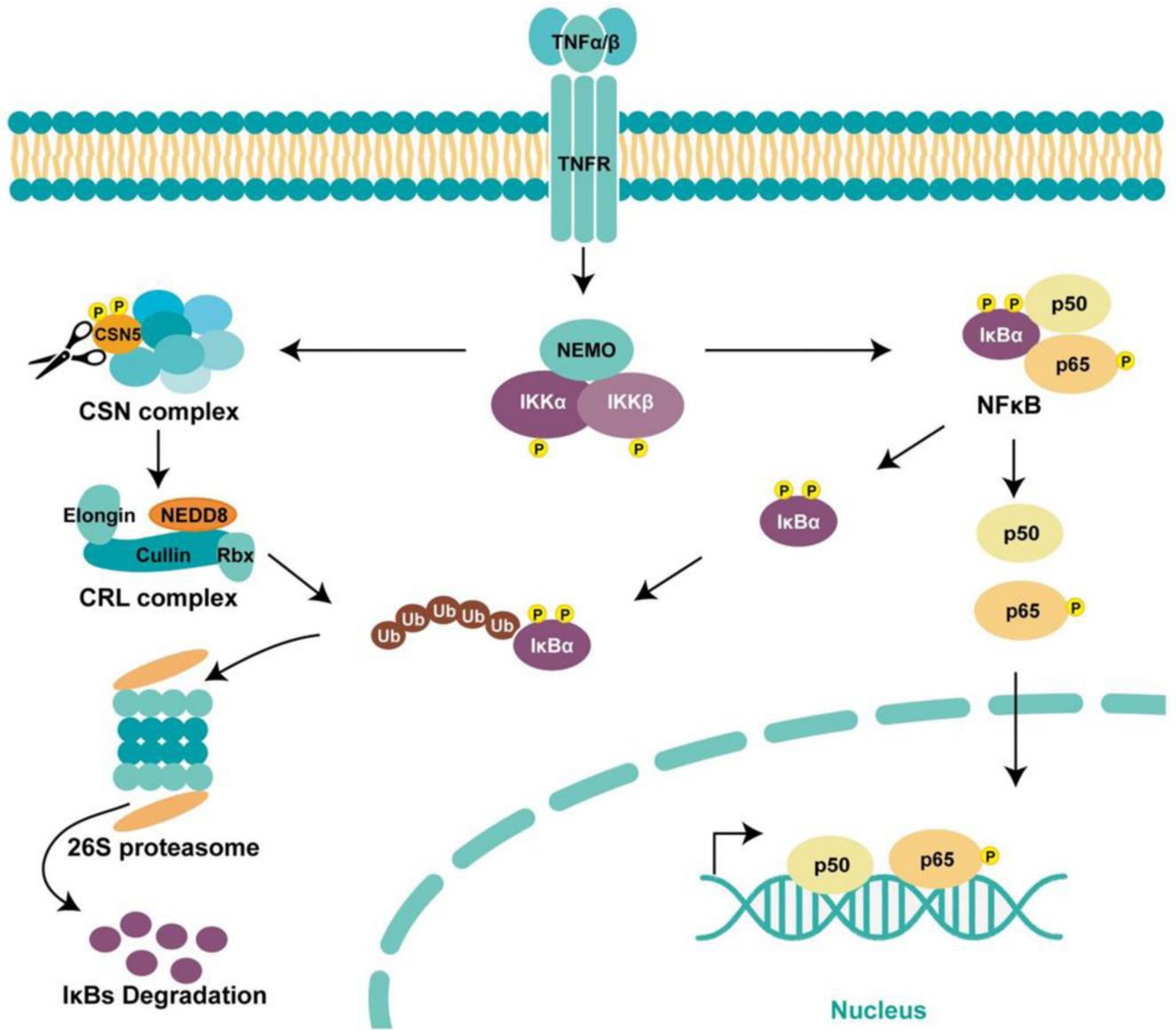
Figure 7.
Working model for reciprocal regulation between the CSN complex and the NF-κB signaling pathway.

Table 1.

Identification of potential IKK-targeted CSN5 phosphorylation sites by LC-MS/MS.

pS: phosphorylated serine; pT: phosphorylated threonine.

Phosphorylated Peptides Identified by MS/MS	IKKα Sample	IKKβ Sample
[1]MAA(pS)GSGMAQK[11]	Y	N
[12]TWELANNMQEAQ(pS)IDEIYK[30]	Y	Y
[192]GYKPPDEGP(pS)EYQTIPLNK[210]	Y	Y
[192]GYKPPDEGPSEYQ(pT)IPLNK[210]	Y	Y
[273]LEQ(pS)EAQLGR[282]	Y	N
[283]GSFMLGLE(pT)HDR[294]	Y	Y
[303] A(pT)RDSCK(pT)TI EAIHGLMSQVIK[324] * [303]A(pT)RDSCKT{pT)IEAIHGLMSQVIK[324] [303]ATRD(pS)CK(pT)TIEAIHGLMSQVIK[324] [303]ATRD(pS)CKT{pT)IEAIHGLMSQVIK[324]	Y	Y
[310] (pT)TI EAI HGLM(pS)QVI K[324] * [310]T(pT)IEAIHGLM{pS)QVIK[324]	Y	Y
[310]TTIEAIHGLM(pS)QVIK[324]	Y	Y

*MS/MS analysis can't distinguish these identified sequences (See Supplemental Figure 1). "Y" indicates Yes; "N" indicates No.