Crosstalk between the NF-κB activating IKK-complex and the CSN signalosome

Lukas Orel a, Hannah Neumeier b, Karin Hochrainer c, Bernd R. Binder a, Johannes A. Schmid a, *,

a Department of Vascular Biology and Thrombosis Research, Center for Biomolecular Medicine and Pharmacology, Medical Univ. of Vienna, Austria
b Institute of Molecular Biotechnology, Vienna, Austria
c Weill Cornell Medical College, Department of Neurology and Neuroscience, Division of Neurobiology, NY, USA

Received: December 22, 2008; Accepted: July 21, 2009

Abstract

A great variety of signalling pathways regulating inflammation, cell development and cell survival require NF-κB transcription factors, which are normally inactive due to binding to inhibitors, such as IκBα. The canonical activation pathway of NF-κB is initiated by phosphorylation of the inhibitor by an IκB kinase (IKK) complex triggering ubiquitination of IκB molecules by SCF-type E3-ligase complexes and rapid degradation by 26S-proteasomes. The ubiquitination machinery is regulated by the COP9 signalosome (CSN). We show that IκB kinases interact with the CSN-complex, as well as the SCF-ubiquitination machinery, providing an explanation for the rapid signalling-induced ubiquitination and degradation of IκBα. Furthermore, we reveal that IKK's phosphorylate not only IκBα, but also the CSN-subunit Csn5/JAB1 (c-Jun activation domain binding protein-1) and that IKK2 influences ubiquitination of Csn5/JAB1. Our observations imply that the CSN complex acts as an inhibitor of constitutive NF-κB activity in non-activated cells. Knock-down of Csn5/JAB1 clearly enhanced basal NF-κB activity and improved cell survival under stress. The inhibitory effect of Csn5/JAB1 requires a functional MPN+ metalloprotease domain, which is responsible for cleaving ubiquitin-like Nedd8-modifications. Upon activation of cells with tumour necrosis factor-α, the CSN complex dissociates from IKK's allowing full and rapid activation of the NF-κB pathway by the concerted action of interacting protein complexes.

Keywords: inflammation • NF-κB • IκB kinase • protein interactions • ubiquitination • CSN complex • JAB1

Introduction

NF-κB, a key transcription factor for many cellular processes, is activated by a wide variety of signalling pathways, which converge at the level of the IκB kinase (IKK) complex, a 700–900 kD protein complex responsible for the phosphorylation of inhibitory IκB molecules on two nearby serine-residues [1]. This phosphorylation induces rapid and efficient poly-ubiquitination of IκB by SCF-type E3 ligases and subsequent degradation by 26S proteasomes often within a few minutes [2–7]. It was our aim to identify molecular mechanisms explaining this rapid ubiquitination and degradation based on a search for interaction partners of the IKK-complex. The SCF-complex responsible for poly-ubiquitination of IκB after phosphorylation by IKK's comprises Skp1, Cullin-1 and a TrCP-transducin repeat containing protein (TrCP) as substrate-specific F-box protein [8, 9]. The ubiquitination activity of the SCFTrCP complex was shown to be up-regulated by covalent attachment of Nedd8, a ubiquitin-like molecule, to the Cullin-moiety of SCF [10]. This so called neddylation apparently represents a general mechanism of SCF regulation [11] and is controlled by a protein complex designated as COP9-signalosome (constitutive photomorphogenesis signalosome, CSN), which is highly conserved from plants to human beings. The CSN complex consists of eight proteins with homologies to subunits of the 19S proteasome regulator and was reported to regulate the degradation of various signalling molecules [12–16]. CSN mediates the cleavage of Nedd8-residues from SCF complexes [17, 18] and leads to an inhibition of ubiquitination reactions in vitro as well as a destabilization of SCF, which is in line with the reported requirement of...
Nedd8-conjugation for the activity of SCF complexes. However, genetic studies revealed that CSN is actually required for sustained SCF activity in vivo (for review see [12]). This apparent inconsistency, which was termed the CSN paradox is currently explained by a working model postulating that subsequent cycles of neddylation and deneddylation, resulting in assembly and disassembly of SCF complexes, are important for the overall functionality of the ubiquitination machinery and the ‘reloading’ of SCF complexes with fresh, non-ubiquitinated components. Auto-ubiquitination represents a mechanism of SCF-self-inactivation and this process has to be counteracted by de-ubiquitination processes involving accessory proteins, as well as by neddylation reactions controlling the disassembly and reloading of SCF [12, 19, 20]. The neddylation activity of the CSN complex could be attributed to the subunit Csn5 [12, 18], also named JAB1 (for c-Jun activation domain binding protein-1 [21]). It could be shown that the Nedd8-cleaving activity depends on a metalllopeptase domain within JAB1/Csn5, which is also capable of driving de-ubiquitination reactions [22], therefore allowing two distinct pathways of interference with the SCF complex. Interestingly, JAB1 can also occur in a small complex different from CSN (JAB1 containing small complex, JACS [23, 24]), which is involved in cell cycle regulation and anchorage-dependent signal transduction. Whether and how this complex is functionally related to the CSN complex is currently not clarified.

It was reported that CSN can associate not only with SCF complexes but also with proteasomes [25, 26] indicating that the functional correlation between ubiquitination and proteasomal degradation is also supported by direct molecular associations. These protein-super complexes might be regulated by mutual enzymatic activities. Interestingly, purified fractions of the CSN signalosome contain kinase activity, phosphorylating IκB molecules, c-Jun and p53 [27, 28]. It was reported that caskin kinase II and protein kinase D are associated with the CSN complex [29], as well as inositol 1,3,4-trisphosphate 5/6-kinase [30]. Our search for interaction partners of IKK2 revealed that components of the CSN signalosome, as well as the SCF complex, interact with IKK2 – and thereby identify IKK2 as another CSN-associated kinase. Based on this observation, we show that mutual regulatory mechanisms exist between the NF-κB signalling pathway and the ubiquitination-proteasome system including the CSN complex. A similar, but distinct cross-talk between the NF-κB pathway and the CSN-signalosome was recently reported by Schweitzer et al. [31]. In this report, it was postulated that a CSN-associated deubiquitylase (USP15) causes a deubiquitination of IκBα representing a negative feedback mechanism of IκBα degradation and NF-κB activation. Our results are in line with a negative regulatory role of the CSN complex in NF-κB activation and identify Csn5/JAB1 and its metalllopeptase domain as important constituent of the negative regulatory mechanism. Taken together, these findings imply that the CSN complex interferes at least in two different ways with the ubiquitination and degradation of IκBα. Studies on the role of Csn5/JAB1 in Drosophila support a model, in which CSN5 acts as a negative regulator of the constitutive NF-κB pathway, although it does not block signal induced activation [32].
Cell culture and transfections

293 cells were cultured as described [37] and transfected with Lipofectamine-Plus™ (Invitrogen) [38] or with calcium/DNA-precipitates [39]. Analysis of transiently transfected cells was generally done 1 day after transfection. Tumour necrosis factor (TNFα) was added to cells at a concentration of 50 ng/ml to activate IKK2 and the NF-κB pathway. MG132 was added to some samples at a concentration of 50 μM to block proteasome activity. Stable myc-JAB1 cells were generated by selection with G418 (500 μg/ml). JAB1-knockdown cells were selected by co-transfection of the suppression construct with a Neomycin-resistance containing plasmid and selection with G418.

Yeast 2-hybrid assays

Yeast 2-hybrid screening was performed as described [33] except that mating of yeast was used to combine the bait with a pre-transformed library from human liver (3 × 10^6 independent clones, BD Clontech, Mountain View, CA, USA). 5.9 million transformants were obtained and plated on SD-Leu-Trp-Ade selection plates followed by transfer of positive clones to high stringency selection plates (SD-Leu-Trp-Ade-His + 25 mmol/l 3-aminotriazole). Library inserts were amplified by PCR from yeast colonies and sequenced. Unspecific binding of preys to the Gal4BD of the bait vector was excluded by retransformation of yeast with the library insert and either empty or IKK2-containing pAS2–1 vector. Subsequently all eight CSN subunits as described in [35] were tested for interaction with the IKK2 bait.

Co-immunoprecipitations and immunoblots

Protein interactions were verified for mammalian cells (293 cells) by co-immunoprecipitation studies either after transient transfection with tagged constructs or after precipitation of endogenous proteins with appropriate antibodies. In the first case, flag-tagged IKK2 was transfected either alone or in combination with myc-tagged interaction candidates (Csn3 – Csn5) followed by precipitation with flag-M2-affinity matrix (Sigma) and immunoblotting with anti-myc antibodies (clone 9E10, Abcam, Cambridge, UK) and anti-flag (Sigma-Aldrich, Vienna, Austria). Verification of the interaction between the endogenous IKK-complex and the CSN signalosome was achieved by immunoprecipitation of the IKK-complex with anti-IKK2 beads (as described in [33]) followed by Western blot with anti-JAB1-antibodies (Santa Cruz, Heidelberg, Germany, sc-9074) – or by precipitation of the CSN complex with anti-Csn7 obtained from W. Dubiel (precipitating the whole complex – W. Dubiel, personal communication) followed by immunodetection of JAB1 and IKK2 (with anti-IKK2, IMG-129 from Imgenex). The interaction between IKK- and SCF-complexes was shown by immunoprecipitation of IKK-complexes with anti-IKK2 and anti-NEMO antibodies (from Santa Cruz) and immunodetecion of endogenous Cul-1 and βTrCP2 (HOS) antibodies (from Labvision-Neomarker and Santa Cruz, respectively). Ubiquitination of JAB1 was assessed by co-transfection of His-tagged ubiquitin and flag-JAB1, immunoprecipitation of JAB1 and detection of ubiquitin-chains with anti-His antibodies (Sigma, H-1029). The turnover of endogenous JAB1 and IKK2 was measured in the presence and absence of TNFα (50 ng/ml) after timed addition of cycloheximide (50 μg/ml) to HUVEC cells, immunoblot analysis of JAB1 or IKK2 levels and quantification of the specific chemiluminescence with Lumiligator equipment (Roche, Vienna, Austria). Loading controls for immunoblots were performed with anti-actin-antibodies (Santa Cruz, sc-1616).

Fluorescence resonance energy transfer (FRET) microscopy

Fluorescence resonance energy transfer (FRET) microscopy was done with the 3-Filter method [40] on a Zeiss Axiovert135 microscope equipped with filter sets for ECFP, EYFP and the raw FRET signal (CFP excitation and YFP emission) using a 63×-oil immersion objective. Images were taken with a cooled CCD camera (CoolSnap, Roper Scientific GmbH, Ottobrunn, Germany) and processed with the ImageJ plug-in PixFRET as described in [41].

Other assays

Kinase assays were done essentially as in [2] using either wild-type or K44M-mutant IKK2 with recombinant GST-IκBα (described in [33], c-Jun, p53 (BioMol, Hamburg, Germany) or co-expressed/co-precipitated flag-JAB1 as substrates.

Reporter gene assays were done by transfection of a firefly luciferase reporter construct with five NF-κB binding sites (Stratagene, La Jolla, CA, USA) and a normalization construct for constitutive expression of β-Galactosidase (pUB6/V5-His/lacZ, Invitrogen). The reporter enzyme activities were measured with a luciferase reporter substrate (Promega, Mannheim, Germany) on a 96-well luminometer in triplicates.

Apoptosis assays

Apopotic cells were stained with Annexin V-FITC and propidium iodide (BD Pharmingen, Schwechat, Austria) and determined by flow analysis. Ubiquitination assays were performed according to [42] with slight modifications. Briefly, 293 cells were transfected with pMT107 his-ubiquitin expression vector (from Dirk Bohman) in presence of the appropriate tags (myc or flag).

Sequence alignments of Csn subunits to determine amino acid similarities and consensus positions were done with VectorNTI (Invitrogen) using the default settings. Statistics: All data presented in the manuscript are representative of several independent experiments.
Results

IKK complexes interact with CSN- and SCF-complexes

Searching for proteins interacting with IKK2 using a yeast two-hybrid screen (as described in [33]) and a human liver library, we identified the CSN subunits Csn5 and Csn7 as potential interaction partners of IKK2. Further testing of all CSN subunits (described in [35]), revealed that Csn3, Csn4, Csn5/JAB1 and Csn7 interact with IKK2 in the yeast system indicating that IKK2 might associate not only with a single component of the CSN signalosome but with the complex in its functional entity (Fig. 1A). Sequence alignment of the subunits Csn3, Csn4, Csn5 and Csn7 shows 61% consensus positions, whereas a comparison of all eight subunits reveals a value of just 19% indicating distinct amino acid similarities between the CSN subunits that interact with IKK2. Three of these molecular interactions could be verified in mammalian cells by ectopic expression of flag-tagged IKK2 together with myc-tagged Csn3, Csn4 or Csn5/JAB1, followed by immunoprecipitation with anti-flag beads and immunoblotting against the myc-tag (Fig. 1B, C and D). Next, we aimed at identifying the localization of the interaction between IKK2 and Csn5/JAB1 by means of FRET microscopy. CFP-tagged IKK2 was co-expressed with YFP-tagged JAB1 and compared to negative and positive FRET controls. Furthermore, this approach was also applied to test, whether just part of JAB1 is capable of interacting with IKK2. The results clearly showed that IKK2 interacts with JAB1 in the cytosol and that the N-terminal half of JAB1 is sufficient for the interaction (Fig. 2). We also tested whether other components of the IKK complex interact with JAB1 or other proteins of the CSN complex. We found that IKK1 interacts with JAB1/Csn5 and that NEMO/IKKγ – the third molecular component of the IKK complex, associates with Csn3 (Fig. S1). The later observation is in line with a previous report showing an interaction between ectopically expressed IKKγ and Csn3 [43], an interaction which has not been studied functionally in more detail. The multitude of interactions that we identified in the various co-immunoprecipitation experiments obviously indicates a functional link between the IKK-complex and the CSN signalosome. Most importantly, we could confirm the interaction between these two protein-complexes for physiological protein levels in non-transfected cells. Immunoprecipitation of the whole IKK-complex from untreated 293 cells clearly co-precipitated endogenous Csn5/JAB1 (Fig. 3A). However, as this cannot rule out an interaction of IKK-molecules with monomeric JAB1 or with the small JAB1-containing complex JACS [23, 24], we also checked whether IKK2 can be co-precipitated with the CSN-signalosome under conditions that pull down the entire CSN-complex, this could be achieved by CSN-immunoprecipitation with anti-Csn7 antibodies that precipitate the whole complex (W. Dubiel, personal communication) and detection of IKK2, as well as JAB1/Csn5 in the immunoprecipitate (Fig. 3B). As Csn7 is not present in the smaller JAB1 containing complex (termed JACS), these data clearly indicate that IKK molecules interact with CSN. It has been reported that the CSN complex can form a supercomplex with SCF-type E3-ligases and proteasomes [26].
with CSN – the regulator of SCF-type E3-ligases, prompted us to test, whether the SCF\(^{\mathrm{TrCP}}\)-ubiquitination machinery for I\(\kappa\)B\(\alpha\) has also the capability of interacting with IKK molecules. For answering this question, we performed immunoprecipitation of endogenous IKK-complexes using antibodies against IKK2 and NEMO followed by Western blot analysis of SCF-components. Cullin-1 could be clearly detected in the immunoprecipitate. Furthermore, also \(\beta\)TrCP – the substrate specific F-box protein of the SCF complex binding phosphorylated I\(\kappa\)B\(\alpha\) was found (Fig. 3C). As expected, we could also detect an interaction between JAB1/Csn5 and the SCF-component Cul-1 in co-immunoprecipitation experiments (Fig. S2). Taken together, our observations indicate the occurrence of multiple protein interactions between IKK-complexes, SCF-type E3-ligases and CSN signalosomes. While just part of the complexes might be associated with each other under the in vitro conditions of co-immunoprecipitations, the observed mutual interactions suggest the possibility of a concerted process of inducible phosphorylation and ubiquitination of I\(\kappa\)B molecules, with the latter reaction being regulated by the CSN complex. It is evident that not all of these interactions have to occur simultaneously for a functional cooperativity, or in other words that the various interaction partners do not necessarily form a stable entity – but that the multiple mutual affinities provide a basis for rapid and collaborative enzymatic reactions. Furthermore, the affinities between the signalling molecules and protein complexes generate a signalling network with a high potential of mutual regulatory processes.
JAB1/Csn5 acts as negative regulator of NF-κB signalling

After identifying the interactions between the IKK complex, the SCF ubiquitination machinery and the CSN complex, we raised the question, whether they have a role in regulating NF-κB activity. Based on a report indicating that full ubiquitination activity of SCF complexes with JAB1/Csn5 as the active component, we assumed that CSN might represent a negative regulator of NF-κB ubiquitination in the context of the IKK/SCF-complex. This notion was supported by reporter gene assays for NF-κB activity, which revealed that ectopic expression of JAB1 significantly reduces basal NF-κB activity, while suppression of endogenous JAB1 by RNA interference leads to a prominent up-regulation (Fig. 4A). Control experiments with a non-targeting siRNA sequence verified the specificity of the gene suppression effect and proved that the vector backbone of the ectopic expression vector did not have any unspecific effect (Fig. S3). The influence of JAB1 on NF-κB activity was furthermore shown by electrophoretic mobility shift assays (Fig. S4) and by monitoring IκBα degradation in stable transfectants after addition of TNFα (Fig. S5). Inhibition of basal NF-κB activity by overexpression of JAB1 was dependent on a functional proteolytic motif within JAB1 (the MPN domain), since point mutations in this region essential for deneddylation activity, eliminated the inhibitory effect (Fig. 4A, 4th column). The important role of the proteolytic MPN domain of JAB1 was further strengthened by the observation that the JAB1 variant with the mutated MPN-domain could not interact with Cullin-1 (Fig. 4B). Testing the impact of ectopic expression of JAB1 on p53 activity revealed the opposite effect as compared to NF-κB activity in line with a counteractive role of these two transcription factors [44] (data not shown). Interestingly, inhibition of constitutive NF-κB activity by ectopic expression of JAB1 did not block a subsequent partial NF-κB-activation by TNFα (Fig. 4C).

The effects observed after ectopic expression or gene suppression of JAB1 on NF-κB activity in reporter gene assays implies a potential role of JAB1/CSN in regulating biological processes downstream of NF-κB such as cell survival under stress. We tested this possibility by measuring the degree of apoptosis in stable transfectants after serum withdrawal or treatment with TNFα. Stable JAB1 knock-down cells were protected from apoptosis induced by these treatments suggesting that the higher constitutive NF-κB activity that is observed after gene suppression of NF-κB activity.
JAB1 improves cell survival under stress (Fig. 5A). In transient transfection experiments, co-expression of IKK2 with JAB1 had a similar apoptosis-protecting effect as gene suppression of JAB1 (Fig. 5B). This indicates that an excess of IKK2 blocks the NF-κB inhibiting and pro-apoptotic effect of Csn5/JAB1 presumably via self-activation of IKK2 due to enforced expression. Another possible explanation is that IKK2 leads to enhanced degradation of JAB1 (as shown in Fig. 9E and Fig S7), which would then reduce its pro-apoptotic effect.

Interestingly, ectopic expression of JAB1 did not significantly increase apoptosis after addition of TNFα, presumably because it cannot fully block TNFα-mediated NF-κB activation (see also Fig. 4C). Although apoptosis might be regulated by other factors than NF-κB, it is well known that this transcription factor has a crucial role for cell survival by up-regulating anti-apoptotic genes and by counteracting p53 via induction of the p53 destabilizing molecule Mdm2 or competition for transcriptional cofactors [44].

Taken together, these observations suggest an important role of the IKK/SCF/CSN-axis for the regulation of apoptosis via NF-κB – and they indicate a biological role of JAB1/CSN as negative regulator of constitutive NF-κB activity and cell survival under stress.

The IKK/CSN interaction affects phosphorylation and ubiquitination processes

The observed interaction between IKK2 and JAB1/Csn5 prompted us to test whether JAB1 can serve as substrate of IKK2. To this end, we performed in vitro kinase assays after co-immunoprecipitation of IKK2 and JAB1, which clearly demonstrated phosphorylation of JAB1 by IKK2 (Fig. 6A). Immunoprecipitated IKK2 was also capable of phosphorylating c-Jun, a known substrate of the originally postulated CSN-associated kinase [27], while we could not detect significant phosphorylation of p53 (Fig. 6B) – another substrate of a CSN-associated kinase. This supports the notion that different kinases can associate with the CSN complex. Interestingly, JAB1 could be phosphorylated not only by IKK2, but also by IKK1 as kinase (Fig. 6C). In vivo, the interaction between JAB1 and IKK2 or IKK1 caused a strikingly increased autophosphorylation of the respective IKK molecule (Fig. 6D and E) pointing at a bidirectional regulation. These data suggested that IKK-dependent phosphorylation of JAB1 might represent a regulatory mechanism for the association of IKK-molecules, the CSN complex and the ubiquitination machinery. This view was supported by the observation that JAB1/CSN dissociates from IKK2 rapidly after activation of the IKK-complex by TNFα (Fig. 7). Addition of TNFα to cells transfected with flag-tagged IKK2 and myc-tagged JAB1 followed by immunoprecipitation of the IKK2/JAB1 complex after different time points revealed a distinct dissociation of the complex within 10 to 20 min, when maximal activity of IKK2 is reached [3] (as also shown by kinetics of IκBα degradation in Fig. S6). The amount of JAB1 co-precipitated with IKK2 decreased significantly, while precipitated IKK2, as well as total JAB1 in the extracts remained constant (Fig. 7A and C). When JAB1 was co-transfected with a mutant IKK2 lacking kinase activity, addition of TNFα did not result in a decrease of JAB1 co-precipitated with IKK2 indicating that TNF-mediated dissociation of JAB1 and IKK2 requires the kinase activity of IKK2 (Fig. 7B and C). As with ectopically expressed wild-type IKK2 and JAB1, a clear dissociation was also observed for endogenous protein levels of non-transfected cells.
Fig. 6 Phosphorylation events in the IKK-CSN-complex.

(A) Phosphorylation of Csn5/JAB1 by IKK2. Wild-type or mutant flag-IKK2 and flag-JAB1 were co-expressed in 293 cells, followed by immunoprecipitation with anti-flag beads and kinase assays as well as immunoblot analysis (anti-flag) of IKK2 and JAB1 expression.

(B) Phosphorylation of c-Jun and IκBα by IKK2. Flag-tagged wild-type (wt) or mutant (mut) IKK2 was expressed in 293 cells, immunoprecipitated by anti-flag beads and kinase assays were performed as described in the ‘Materials and methods’ section using recombinant c-Jun, p53 (both from Biomol International, Inc., Hamburg, Germany) or GST-IκBα as substrates. A distinct phosphorylated band is visible for IκBα and a weaker band for c-Jun.

(C) Phosphorylation of Csn5/JAB1 by IKK1. 293 cells were transiently transfected with flag-tagged IKK1 and JAB1. MG132 was added to some samples to prevent proteasomal degradation. Anti-flag immunoprecipitates of cell extracts were subject to kinase assays and immunoblots against the flag-tag. JAB1-phosphorylation is most prominently visible in presence of MG132.

(D) Auto-phosphorylation of IKK2 is enhanced by ectopically expressed JAB1. Flag-tagged wild-type or mutant IKK2 was co-expressed with flag-JAB1. Extracts were prepared 1 day after transfection and immunoblots were done for phospho-IKK2 (P-IKK2), flag and JAB1 as indicated.

(E) Auto-phosphorylation of IKK1 is enhanced by ectopically expressed JAB1. Flag-tagged IKK1 and JAB1 were co-expressed and analysed as in (D). MG132 was added in one sample to block proteasomal degradation.
These results are in line with our observation that ectopically expressed JAB1 cannot block TNFα-induced NF-κB activity in reporter gene assays (Fig. 4C) and they substantiate a model in which JAB1/CSN is a negative regulator of the SCF-ubiquitination activity that has to be released from the IKK/SCF-complex to achieve maximal ubiquitination and degradation of IκBα. This release might be triggered by the observed phosphorylation of Csn5/JAB1 by IKK2. Since phosphorylation of IκBα by IKK2 after TNFα-Induced IKK2-activation initiates subsequent ubiquitination, we tested whether JAB1 is ubiquitinated as well in a TNFα-dependent manner. Using co-expression of His-tagged ubiquitin with flag-tagged JAB1 or IKK2, followed by anti-flag immunoprecipitation and Western blot detection of ubiquitin, we could clearly demonstrate ubiquitination of JAB1, which was enhanced by TNFα (Fig. 8A). In the presence of the proteasome inhibitor MG132 we observed an accumulation of ubiquitinated JAB1 indicating that ubiquitinated JAB1 is degraded by proteasomes. Using flag-tagged IKK2 instead of JAB1 in combination with His-tagged ubiquitin revealed poly-ubiquitination of IKK2, which was just visible in the presence of the proteasome inhibitor MG132. However, this poly-ubiquitination was not enhanced by TNFα (Fig. 8A). Since TNFα triggers IKK2 activity, we next asked, whether ubiquitination of JAB1 depends on IKK2. To that end, we co-transfected JAB1 with wild-type IKK2 and His-tagged ubiquitin in absence or presence of MG132, followed by precipitation of His-tagged ubiquitin with NiNTA-resin and detection of JAB1 by Western Blotting. NiNTA-precipitation was performed in presence of the chaotropic agent guanidine hydrochloride, which prevents co-precipitation of non-covalently linked proteins. The subsequent immunoblot clearly revealed poly-ubiquitinated JAB1 (Fig. 8B). Moreover, polyubiquitination was enhanced in presence of IKK2, which was most clearly visible when proteasomal degradation was blocked by
This finding was further strengthened by an additional experiment including mutant IKK2 lacking kinase activity (K44M mutant) and constitutive active IKK2 (S177E, S181E mutant). The constitutive active IKK2 strongly enhanced poly-ubiquitination of JAB1, whereas the kinase deficient IKK2 mutant clearly reduced JAB1 ubiquitination (Fig. 8C). The role of IKK2 for ubiquitination of JAB1 was furthermore supported by experiments using siRNA to suppress endogenous IKK2 (Fig. S7). We conclude that the poly-ubiquitination of JAB1 is triggered by IKK2 presumably via IKK2-mediated phosphorylation, as this process requires an active kinase domain of IKK2.

In order to study whether the TNF-α/H9251-induced and IKK2-dependent poly-ubiquitination of JAB1 results in a reduced half life of the protein, we performed experiments in the presence of cycloheximide to block protein synthesis and to monitor protein turnover. This indicated that TNF-α/H9251 enhanced the degradation of endogenous JAB1 (Fig. 9A and B). Performing the experiment with ectopically expressed JAB1 revealed a similar result of enhanced degradation of JAB1 but not IKK2 after addition of TNF-α/H9251 (Fig. 9C and D). Next we wanted to answer the question, whether the regulation of the half-life of JAB1 involves IKK2 as TNF-α-activated kinase.

To answer this question, we transfected 293 cells with myc-tagged JAB1 in combination with either wild-type or kinase-deficient mutant IKK2 followed by addition of cycloheximide to block protein synthesis. Cell extracts were prepared after different time points and the level of JAB1 quantified by immunoblotting. While JAB1 decreased readily in presence of wild-type IKK2, it remained stable in presence of the kinase-deficient mutant IKK2 (Fig. 9E and F).

**Discussion**

The first characterization of the CSN complex in human cells showed that it is associated with a kinase activity phosphorylating IκBα and p105-NF-κB molecules, as well as c-Jun [27]. However, a direct association of IKK2 with the CSN complex has not been shown so far, and other kinases, such as casein kinase II, protein kinase D and inositol 1,3,4-trisphosphate 5/6-kinase have been postulated to be associated with the CSN complex [29, 30]. We have evidence that IKK2 represents another CSN associated kinase,
which is furthermore associating with the SCF/TCP E3 ligase ubiquitinating IκB molecules. An earlier hint for a potential physiological interaction between the IKK- and the CSN-complex was provided by a report showing that NEMO/IKKγ interacts with the CSN subunit Csn3 [43]. However, this interaction, which was identified in the yeast two-hybrid system, was not verified for endogenous, physiological protein levels and thus not for entire IKK- and CSN protein-complexes. Our findings that IKK2 interacts with at least three of the eight CSN components, Csn3, Csn4, and Csn5, leads us to the assumption that IKK2 associates with the whole CSN complex. This is further substantiated by immunoprecipitation experiments of endogenous IKK-signalosomes or CSN-complexes, which clearly show co-precipitation of components of the other complex. The CSN-complex is an important regulator of SCF-type E3-ligases and thus interacts with SCF-complexes. However, this regulation is intricate and still not completely understood. CSN inhibits SCF-ubiquitination activity in vitro by deneddylating and de-stabilizing SCF, while it is required for sustained activity of SCF-complexes in vivo. This is currently explained by a model, in which CSN mediates disassembly of SCF complexes containing inactive, auto-ubiquitinated components, such as F-box proteins, thereby allowing re-assembly and loading with new, active subunits [12, 20, 45]. The results of our experiments suggest the CSN complex as a negative regulator of NF-κB activation. Gene suppression of the CSN subunit JAB1/Csn5 augmented constitutive NF-κB activity, while ectopic expression of JAB1 reduced it. This is in line with a study of Csn5 function in Drosophila [32], where the authors showed that Csn5 null mutants had a constitutive nuclear localization of the NF-κB orthologue Dorsal although its activity was repressed by accumulated Cactus (the Drosophila IκB orthologue). The authors also showed an elevated basal NF-κB activity in human 293T cells after shRNA-mediated suppression of Csn5, while TNF-mediated activation was not significantly influenced, which is in line with our observations. It has to be noted though that the effect of Csn5/JAB1 on NF-κB seems to depend on the cell type and cellular context, as loss of JAB1 was also reported to reduce NF-κB activity, e.g. in thymocytes [46] or synovial fibroblasts of rheumatoid arthritis patients [47].

In our hands, mutation of the proteolytic MPN domain, which is capable of cleaving Nedd8 and perhaps also other ubiquitin-like moieties [22], prevented the NF-κB suppressive effect of JAB1, indicating that it acts either via deneddylation or via de-ubiquitination of the SCF-complex or via de-ubiquitination of IκBα, thereby allowing re-assembly and loading with new, active subunits.
different mechanism of a CSN-controlled deubiquitination of IκBα was recently postulated to be mediated by the CSN-associated deubiquitinase USP15 [31]. However, since knock down of endogenous JAB1/Csn5 increased basal activity of NF-κB in our experiments, and since mutation of the MPN domain of JAB1 prevented its inhibitory effect, we assume an important role of JAB1 itself in this regulatory process. 

Taken together, our observations point at functionally important interactions between IKK-, SCF- and CSN-complexes. The tendency of the complexes involved in phosphorylation, ubiquitination and degradation of IκBα to interact with each other would explain the fast and efficient degradation of IκBα within a few minutes after activation of cells by TNFα. In this model, CSN acts as negative regulator of constitutive NF-κB activity, inhibiting constitutive SCF-mediated degradation of IκBα that might be triggered by a low basal activity of IKK2. Upon activation of the TNFα-signalling pathway, IKK2 is fully activated, phosphorylating not only IκB molecules but also JAB1/Csn5 and probably other components of the protein complexes involved. As a result, CSN dissociates from the super-complex, allowing rapid ubiquitination and degradation of IκBα and release of NF-κB. In addition to IκBα also JAB1 is poly-ubiquitinated dependent on IKK2 and degraded by proteasomes although with slower kinetics. Signal-induced association processes are certainly important for several steps of the NF-κB activation pathway such as recruitment of adapter proteins to cell surface receptors or activation of signalling kinases due to association with adapter proteins [44]. Our observations indicate that also dissociation events comprising negative regulators might have an important role in fine tuning basal and induced NF-κB activity. Our data of molecular interactions between the IKK-complex, the SCF-ubiquitination machinery and CSN as a negative regulator provide a plausible explanation for the rapid and efficient degradation of IκBα in the course of TNFα-mediated NF-κB activation. Furthermore our concept of a functional super-complex between IKK's and the ubiquitination system would also account for the multifaceted mutual regulation that is found between the molecular machines that are responsible for phosphorylation, ubiquitination and degradation. It is evident that such a functional super-complex should not be seen as a rigid entity with a permanent association of the sub-components, but rather as a dynamic molecular machine subject to dissociation and re-assembly events and complex regulatory processes. The complexity of this system is also reflected by the diversity of roles that JAB1 or the CSN signalosome seem to have in a variety of diseases such as rheumatoid arthritis [47], inflammatory disorders in general [48] atherosclerosis [49] or neurodegenerative diseases [50]. Moreover, JAB1 alone or in combination with the CSN complex have been reported to exert diverse functions in various forms of cancer, where they are involved in the degradation or stabilization of effector molecules such as oncogenes, tumour suppressor genes or cell cycle regulators [51]. The crosstalk of the JAB1/CSN-system with the IKK/NF-κB pathway that we describe in this study adds another level of complexity and brings in additional aspects of mutual regulation.

Acknowledgements

We are grateful for the hospitality and the support by the Ghosh group and we also thank Wolfgang Dubiel for providing anti-Csn7 antibodies and Hongyong Fu for providing yeast two-hybrid constructs for all the CSN subunits. The project was mainly funded by the Competence Center Biomolecular Therapeutics, Vienna. Furthermore, we acknowledge the support by the Austrian Academy of Sciences and the Max Kade foundation for funding a sabbatical of J.A.S. at the Immunobiology Section of Yale University Medical School and the support by the Ludwig Boltzmann Institute for Cancer Research, Vienna.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Interaction of IKK1 with JAB1/Csn5 and of NEMO with Csn3. 293 cells were transfected with expression constructs as indicated in the header followed by immunoprecipitation (IP: anti-flag) and immunoblot analysis of flag- and myc-tags in immunoprecipitates and extracts.

Fig. S2 Interaction between JAB1/Csn5 and Cullin-1: flag-tagged JAB1 and/or myc-tagged Cul-1 were transiently transfected in 293 cells followed by anti-flag immunoprecipitation and immunoblot analysis of flag- and myc-tagged proteins in immunoprecipitates (IP) and extracts.

Fig. S3 Control experiments for vector backbones and specificity of the siRNA. (A) 293 cells were transfected with myc-JAB1 in combination with the siRNA construct against JAB1 (si-JAB1), a control siRNA construct with non-targeting sequence (si-vector). Myc-JAB1 expression was assessed by immunoblotting. (B) the effect of the JAB1-targeting siRNA construct (si-JAB1) on expression of endogenous JAB1 was compared to that of a non-targeting siRNA control (si-scrambled) and control extracts.

Fig. S4 Electrophoretic mobility shift assay (EMSA) showing the effect of JAB1 on NF-κB binding activity. 293 cells were transfected with JAB1 expression or suppression constructs as indicated and compared to cells transfected with p65 NF-κB. EMSA was performed as described in [33]. One lane was loaded with p65-transfected extracts containing a 50-fold molar excess of the non-labelled NF-κB binding oligo-nucleotide as competitor (p65 + comp.). B. Quantification of the specific NF-κB bands shown in (A).

Fig. S5 IκBα degradation in stable myc-JAB1 and siJAB1 transfectants. Stable 293 transfectants of myc-JAB1 and siJAB1 were
generated by G418 selection of clones as described in the ‘Materials and methods’ section. TNFα (50 ng/ml) was added and cells were extracted after different time points, followed by immunoblot analysis of IkBα degradation. The film is overexposed with respect to lanes 1 and 2 (0 and 10 min TNFα) so to monitor the difference in IkBα degradation between 20 and 40 min after TNF addition.

**Fig. S6** Kinetics of IkBα degradation as readout for the fast activation of IKK2 following TNFα treatment. 293 cells were treated for different periods of time with TNFα, followed by rapid cell extraction and Western blot analysis of IkBα. Degradation is already clearly visible at 10 min, indicating fast activation of the upstream kinase IKK2 by TNFα.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. Schmid JA, Birbach A. IkappaB kinase beta (IKKbeta/IKK2/IKKB)–a key molecule in signaling to the transcription factor NF-kappaB. *Cytokine Growth Factor Rev.* 2008; 19: 157–65.

2. Mercurio F, Zhu H, Murray BW, et al. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science.* 1997; 278: 860–6.

3. Zandi E, Rothwarf DM, Delhase M, et al. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell.* 1997; 91: 243–52.

4. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* 2004; 25: 280–8.

5. Ghosh S, May MJ, Kopp EB. Phosphorylation meets ubiquitination: the control of NF-kappaB activity. *Ann Rev Immunol.* 1996; 14: 225–60.

6. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Ann Rev Immunol.* 2000; 18: 621–63.

7. Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol.* 2002; 3: 221–7.

8. Yaron A, Hatzubai A, Davis M, et al. Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. *Nature.* 1998; 396: 590–4.

9. Fuchs SY, Chen A, Xiong Y, et al. HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkappaB and beta-catenin. *Oncogene.* 1999; 18: 2039–46.

10. Read MA, Brownell JE, Gladysheva TB, et al. Nedd8 modification of cul-1 activates SCF[beta(TrCP)]-dependent ubiquitination of IkappaBalpha. *Mol Cell Biol.* 2000; 20: 2326–33.

11. Kawakami T, Chiba T, Suzuki T, et al. Nedd8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J.* 2001; 20: 4003–12.

12. Cope GA, Deshaies RJ. COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. *Cell.* 2003; 114: 663–71.

13. Wei N, Deng XW. The COP9 signalosome. *Annu Rev Cell Dev Biol.* 2003; 19: 261–86.

14. Schwarzeheimer C, Deng X. COP9 signalosome revisited: a novel mediator of protein degradation. *Trends Cell Biol.* 2001; 11: 420–6.

15. Bech-Otschir D, Seeger M, Dubiel W. The COP9 signalosome: at the interface between signal transduction and ubiquitin-dependent proteolysis. *J Cell Sci.* 2002; 115: 467–73.

16. Tomoda K, Kubota Y, Kato J. Degradation of the cyclin-dependent-kinase inhibitor p27kip1 is instigated by Jab1. *Nature.* 1999; 398: 160–5.

17. Lyapina S, Cope G, Shevchenko A, et al. Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science.* 2001; 292: 1382–5.

18. Cope GA, Suh GS, Aravind L, et al. Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science.* 2002; 298: 608–11.

19. Hetfeld BK, Helfrich A, Kapelari B, et al. The zinc finger of the CSN-associated deubiquitinating enzyme USP15 is essential to rescue the E3 ligase Rbx1. *Curr Biol.* 2005; 15: 1217–21.

20. Wei S, Geyer RK, Toda T, et al. CSN facilitates Cullin-RING ubiquitin ligase function by counteracting autocatalytic adapter instability. *Nat Cell Biol.* 2005; 7: 387–91.

21. Claret FX, Hibi M, Dutt S, et al. A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. *Nature.* 1996; 383: 453–7.

22. Groisman R, Polanowska J, Kuraoka I, et al. The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell.* 2003; 113: 357–67.

23. Fukumoto A, Tomoda K, Kubota M, et al. Small Jab1-containing subcomplex is regulated in an anchorage- and cell-cycle-dependent manner, which is abrogated by ras transformation. *FEBS Lett.* 2005; 579: 1047–54.

24. Tomoda K, Kato JY, Tatsumi E, et al. The Jab1/COP9 signalosome subcomplex is a downstream mediator of Bcr-Abl kinase activity and facilitates cell-cycle progression. *Blood.* 2005; 105: 775–83.

25. Peng Z, Shen Y, Feng S, et al. Evidence for a physical association of the COP9 signalosome, the proteasome, and specific SCF E3 ligases in vivo. *Curr Biol.* 2003; 13: R504–5.

26. Huang X, Hetfeld BK, Seifert U, et al. Consequences of COP9 signalosome and 26S proteasome interaction. *FEBS J.* 2005; 272: 3909–17.

27. Seeger M, Kraft R, Ferrell K, et al. A novel protein complex involved in signal
transduction possessing similarities to 26S proteasome subunits. FASEB J. 1998; 12: 469–78.

28. Bech-Otschir D, Kraft R, Huang X, et al. COP9 signalosome-specific phosphorylation targets p53 to degradation by the ubiquitin system. EMBO J. 2001; 20: 1630–9.

29. Uhle S, Medalia O, Waldron R, et al. Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome. EMBO J. 2003; 22: 1302–12.

30. Sun Y, Wilson MP, Majerus PW. Inositol 1,3,4-trisphosphate 5/6-kinase associates with the COP9 signalosome by binding to CSN1. J Biol Chem. 2002; 277: 45759–64.

31. Schweitzer K, Bozko PM, Dubiel W, et al. CSN controls NF-kappaB by deubiquitination of IkappaBalpha. EMBO J. 2007; 26: 1532–41.

32. Harari-Steinberg O, Cantera R, Denti S, et al. COP9 signalosome subunit 5 (CSN5/Jab1) regulates the development of the Drosophila immune system: effects on Cactus, Dorsal and hematopoiesis. Genes Cells. 2007; 12: 183–95.

33. Ebner K, Bandion A, Binder BR, et al. GMCSF activates NF-kappaB via direct interaction of the GMCSF receptor with IkappaB kinase beta. Blood. 2003; 102: 192–9.

34. Oltzinger W, Hofer-Warbinek R, Schmid JA, et al. Adenovirus-mediated expression of a mutant IkappaB kinase 2 inhibits the response of endothelial cells to inflammatory stimuli. Blood. 2001; 97: 1611–7.

35. Fu H, Reis N, Lee Y, et al. Subunit interaction maps for the regulatory parti-