Published in final edited form as:

Cell Mol Life Sci. 2012 June ; 69(12): 2057–2073. doi:10.1007/s00018-011-0912-2.

Monoubiquitination of nuclear RelA negatively regulates NF- κ B activity independent of proteasomal degradation

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Abstract

Termination and resolution of inflammation is tightly linked to the inactivation of one of its strongest inducers, NF- κ B. While canonical post-stimulus inactivation is achieved by upregulation of inhibitory molecules that relocate NF- κ B complexes to the cytoplasm, termination of the NF- κ B response can also be accomplished directly in the nucleus by posttranslational modifications e.g. ubiquitination of the RelA subunit. Here we reveal a functional role for RelA monoubiquitination in regulating NF- κ B activity. By employing serine-to-alanine mutants we found that hypo-phosphorylated nuclear RelA is monoubiquitinated on multiple lysine residues. Ubiquitination was reversed by I κ B α expression and was reduced when nuclear translocation was inhibited. RelA monoubiquitination decreased NF- κ B transcriptional activity despite prolonged nuclear presence and independently of RelA degradation, possibly through decreased CREB-binding protein (CBP) co-activator binding. Polyubiquitin-triggered proteasomal degradation has been proposed as a model for RelA inactivation. However, here we show that proteasomal inhibition, similar to RelA hypo-phosphorylation, resulted in nuclear translocation and monoubiquitination of RelA. These findings indicate a degradation-independent mechanism for regulating the activity of nuclear RelA by ubiquitination.

Keywords

NF- κ B; I κ B α ; ubiquitination; transcription

Introduction

The NF- κ B/Rel family of dimeric transcription factors is involved in the immediate early transcription of a large array of genes induced by mitogenic or pathogen-associated stimuli. The canonical and most abundant form of NF- κ B is composed of a 50 kDa (p50; NF κ B1) and a 65 kDa (p65; RelA) subunit. In the resting state NF- κ B is retained in the cytoplasm by inhibitory proteins of the I κ B family, namely I κ B α , I κ B β , I κ B ϵ , p100 (I κ B δ) and p105 (I κ B γ) [1]. Formation of NF- κ B/I κ B complexes masks the nuclear localization signals present in NF- κ B dimers, which prevent nuclear translocation and transcriptional activity. One of the key events in the activation of NF- κ B is the liberation of functional NF- κ B dimers from I κ Bs which is achieved by signal-induced complete (e.g. I κ B α , I κ B β , I κ B ϵ) or

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partial (e.g. p100 and p105) proteolytic degradation of the inhibitor involving several pathway specific protein kinase modules [2]. The transcriptional activator RelA/NF- κ B is targeted by multiple post-translational modifications including phosphorylation [3–5], acetylation [6,7], proline isomerization [8], methylation [9], and ubiquitination [10–12] controlling DNA binding, transcriptional activity, interaction with co-activators and protein stability.

Ubiquitin is covalently linked to proteins due to the sequential action of 3 types of enzymes known as E1, E2, and E3 with the respective ability to activate, conjugate and transfer the ubiquitin moiety to the ϵ -amino group of a lysine residue within the target protein. To become eligible for degradation by the 26S proteasome, ubiquitin chains consisting of at least four ubiquitin peptides that are branching of lysine 48 (K48) must be attached to the substrate [13]. Degradation of transcriptional activators by the proteasome machinery is an efficient way to limit transcription to a specific time window. Several mammalian transcription factors, c-Jun [14], c-myc [15] and p53 [16], for example, are short-lived proteins that are destroyed by ubiquitin-mediated proteolysis. While many transactivators are regulated by this way, the traditional view has linked termination of NF- κ B-mediated transcription to nuclear expression of I κ B α . It is believed that after stimulus-induced degradation newly synthesized I κ B proteins enter the nucleus and bind to DNA-resident NF- κ B molecules removing them from their binding sites and resulting in Crm-1-dependent relocation to the cytoplasm [17,18]. However, this classical model of I κ B-dependent termination of NF- κ B signaling has been challenged by the fact that NF- κ B transcriptional activity is efficiently terminated in the absence of I κ B α once the stimulating agent is removed [11]. In this model, nuclear RelA is proposed to be ubiquitinated and degraded, and therefore inhibition of the 26S proteasome results in higher transcriptional activity. The nature of RelA ubiquitination however has not been revealed.

In this study we investigated the functional impact of RelA ubiquitination elicited by either hypo-phosphorylation or proteasomal inhibition on NF- κ B activity. We found that mutation of previously described phosphorylation sites within its Rel Homology Domain (RHD) leads to multiple monoubiquitin additions. RelA monoubiquitination inhibited its transcriptional activity without affecting protein stability. Instead we find that RelA monoubiquitination results in decreased binding of the CBP transcriptional co-activator providing a possible mechanism for its reduced transcriptional potential. We further show that nuclear localization is essential for RelA ubiquitination and that proteasomal inhibition leads to RelA monoubiquitination by prolonging RelA nuclear retention. These findings raise the possibility that nuclear activity of RelA is controlled by monoubiquitination.

Materials and Methods

Antibodies

The following antibodies were used in this study: β -actin (AC-15, Sigma), CBP (A-22, Santa Cruz Biotechnology), c-myc (9E10), Eps15 (Covance), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon), hemagglutinin (HA) (12CA5, Roche Applied Science and F-7, Santa Cruz Biotechnology), I κ B α (6A920, Imgenex), lamin B (M-20), RelA (C-20, both Santa Cruz Biotechnology), mono/polyubiquitin (P4D1, Covance and Ubi-1, Invitrogen) and polyubiquitin (FK1, Biomol).

Plasmid constructs

pcDNA3 and pLXI vectors expressing human myc-RelA wt, S205A, S276A and S281A have been described [19]. HA-RelA wt and S276A were cloned into pcDNA3-HA vector (Invitrogen) by standard cloning procedures. pcDNA3-myc-RelA S205/276/281A was

generated by one-step mutagenesis of S276/281A using myc-RelA S205A as a template. pcDNA-myc-RelA S276A was used as template for mutation of all 18 K located in human RelA to R. pcDNA-myc-RelA S276A NLS mutant was obtained by mutagenesis of aa 301–304 from KRKR to DQNQ [20]. pMT107 encoding for wt polyhistidine-tagged ubiquitin was obtained from D. Bohmann (URMC, Rochester, NY). pcDNA3-HA-ubiquitin wt, K29R, K48R, K63R, and K29/48/63R (K3xR) were from I. Dikic (Goethe University, Frankfurt am Main, Germany). These constructs were used to clone ubiquitin variants by replacing the HA-tag with a His₆-tag via unique *Bam*HI/*Nhe*I cloning sites. pcDNA3-his-ubiquitin K7xR was generated out of pcDNA3-his-ubiquitin K3xR by sequentially mutating K6, K11, K27 and K33 to R. To obtain pLXI-H-Ub-RelA and pcDNA3-Ub-RelA, HA-tagged ubiquitin was cloned into pLXI-H (Clontech) and pcDNA3 (Invitrogen) by PCR amplification with *Hpa*I/*Xho*I and *Hind*III restriction sites, respectively. Myc-RelA was placed down-stream of the ubiquitin coding sequence by standard cloning techniques. To prevent isopeptidases from cleaving the ubiquitin tag from RelA the carboxyl-terminal GG motif of ubiquitin was mutated to AV [21]. For pLXI-H-pLacZ-RelA wt, aa 756–829 of the bacterial *LacZ* gene were cloned upstream of RelA wt. This stretch was chosen due to its similarity to human ubiquitin in terms of aa composition resulting in a comparable isoelectric point of pLacZ (pI 7.01) and Ub (pI 7.02). The construct was cloned by 2-fragment cloning into *Xho*I/*Bam*HI-digested pLXI-H. pLacZ was amplified from pLacZi (Clontech) with primers containing *Xho*I and *Hind*III restriction sites and placed up-stream of a myc-RelA wt fragment featuring *Hind*III/*Bam*HI overhangs. HA-I κ B α was obtained by transferring a *Bam*HI/*Eco*RI fragment from pKSII/ECI-6 (kind gift from R. de Martin, Medical University of Vienna, Vienna, Austria) to a modified pcDNA3 vector harboring an amino-terminal HA-tag. Human p50 was obtained through PCR amplification from HeLa cDNA with primers harboring *Eco*RI/*Xho*I restriction sites and cloned into pCMV-myc vector (Clontech). Mutations were performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to manufacturers instructions. Plasmid constructs were verified by automated DNA sequencing.

Cell culture reagents and transfections

Human embryonic kidney (HEK) 293T, HeLa, RelA^{-/-} and I κ B α ^{-/-} 3T3 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μ g/ml streptomycin B. Retrovirally transduced 3T3 stably expressing myc-RelA wt or mutants, Ub-RelA wt or pLacZ-RelA wt were generated as described [19]. Positive clone pools were cultured in medium containing 250 μ g/ml hygromycin B (InvivoGen). Transfection of HEK293T was carried out with calcium phosphate. Where indicated, cells were treated with 5 μ M Clasto-Lactacystin β -lactone (Cayman), 50 μ M MG132, 1 μ M MG262, 100 μ g/ml CHX (all Calbiochem), 1 μ g/ml LPS (Sigma), 10 ng/ml human recombinant TNF, 100 ng/ml epidermal growth factor (EGF) (both Invitrogen) or 20 ng/ml LMB (Alexis Biochemicals).

Histidine-pull down ubiquitination assay

Cells were harvested in phosphate-buffered saline (PBS) and 1/20 of samples was removed for expression control. Residual cells were pelleted, lysed in buffer A (6 M guanidine-hydrochloride, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM imidazole, pH 8) and sonicated for 15 sec at 25% amplitude. 400 μ g of each protein lysate were incubated with Ni-NTA agarose resin (Qiagen). Beads were washed twice in buffer A, twice in buffer A/TI, once in buffer TI (25 mM Tris-HCl, 20 mM imidazole, pH 6.8) and suspended in SDS sample buffer. Ubiquitinated RelA was detected in pull-downs with RelA-specific antibody.

RelA pull-down ubiquitination assay

Cells were harvested in PBS, resuspended in 50 mM Tris-HCl pH 8, 1% SDS and 5mM DTT and boiled for 10 min to denature all proteins. For immunoprecipitation, lysates were

diluted with 10 vol of 50 mM Tris-HCl pH 8, 150 mM sodium chloride, 1 mM EDTA, 1% Igepal CA-630 and 0.5% sodium-deoxycholate. All buffers were supplemented with protease inhibitors (Roche Applied Science) and 20 mM N-ethylmaleimide (Calbiochem). For endogenous RelA, after pre-clearing, 1/20 of lysates was removed for expression control. RelA was then pulled down with RelA-specific antibody coupled to protein-A sepharose. Over-expressed RelA from HEK293T was pulled down with anti-c-myc antibody. Precipitates were examined for the presence of ubiquitinated RelA with respective anti-ubiquitin antibodies.

Co-immunoprecipitation assay

For RelA-CBP assay, 3T3 cells were lysed in 50 mM HEPES pH 7.9, 250 mM sodium chloride, 1% Igepal CA-630, 1 mM EDTA, protease inhibitors. For RelA-p50 assay, HEK293T cells co-transfected with HA-RelA and myc-p50 expression plasmids were harvested in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 1% Igepal CA-630, 0.5 M sodium-deoxycholate, 0.1% SDS, 1 mM EDTA, supplemented with protease inhibitor. After incubation for 30 min in lysis buffer, lysates were clarified by centrifugation and supernatants were incubated with CBP-specific antibody coupled to protein-A sepharose or HA-coupled affinity agarose (Sigma). 1/20 of lysates was kept for expression control. Samples were washed and immunoprecipitated proteins were eluted by addition of SDS-sample buffer. Lysates and precipitated proteins were analyzed by SDS-PAGE and immunoblotting.

Identification of ubiquitinated RelA by nanoLC/MS/MS analysis

Samples obtained by histidine-ubiquitin-pull down were separated on SDS-PAGE, visualized in gel with SYPRO Ruby (Molecular Probes) and the positive gel protein bands in the range from 80 to 150 kDa found in a separate immuno blotting assay were excised. The SDS-gel slices were subjected to in-gel digestion by trypsin and subsequent extraction as reported previously [22]. The digest was reconstituted in 10 µl of 2% acetonitrile with 0.5% formic acid for nanoLC-ESI-MS/MS analysis, which was carried out using a LTQ-Orbitrap Velos (Thermo-Fisher Scientific) mass spectrometer equipped with “Plug and Play” nano ion source device (CorSolutions LLC). The nanoLC was carried out by UltiMate3000 MDLC system (Dionex). A detailed protocol of the MS analysis can be found in Supplementary Information.

RelA and IκBα immunohistochemistry

Cells seeded on glass cover slips were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked in PBS/0.05% Tween20/0.1% bovine serum albumin (Sigma-Aldrich). Cells were incubated with rabbit polyclonal RelA, mouse monoclonal HA (isotype IgG_{2b}) or mouse monoclonal c-myc (isotype IgG₁) antibodies in blocking buffer. After incubation with Alexa488-labeled anti-rabbit IgG, Alexa488-labeled anti-mouse IgG₁, or Alexa568-labeled anti-mouse IgG_{2b} (all Molecular Probes) in PBS/0.05% Tween20, cover slips were mounted on microscope slides with SlowFade Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Subcellular distribution of RelA and IκBα was analyzed using a Nikon Eclipse TE2000 microscope equipped with a 40× objective. Pictures were acquired with a charge-coupled device using identical acquisition parameters.

RelA protein stability

Cells were treated with CHX for the indicated time points. Total cell lysates were prepared by lysis in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM sodium chloride, 1 mM EDTA, 1% Igepal CA-630, 0.5% sodium-deoxycholate, 0.1% SDS, protease inhibitors). For

cytosolic extracts cells were harvested in PBS, pelleted and lysed in 10 mM Tris-HCl pH 8, 320 mM sucrose, 3 mM CaCl₂, 2 mM MgOAc, 0.5% Triton X-100, 1mM DTT, protease inhibitors). Nuclei were spun down, washed once and sonicated in Laemmli buffer for 10 sec at 25% amplitude. Separation of cytosol and nuclei was ensured by exclusive detection of GAPDH in cytosolic and lamin B in nuclear fractions. For pulse chase metabolic labeling cells were washed twice with warm PBS, starved in methionine/cysteine-free DMEM (Invitrogen)/0.5% dialyzed FBS for 30 min and labeled with 200 μ Ci/ml [³⁵S]-methionine (Perkin Elmer) for 1 hour. Cells were washed twice in warm PBS and DMEM supplemented with 10% FBS/2 mM methionine/2 mM cysteine was added with or without TNF and MG132. Total cell lysates as well as cytosolic/nuclear extracts were essentially obtained as described above. For immunoprecipitation nuclei were lysed in RIPA buffer and cytosolic fractions were diluted in RIPA at a ratio of 1/10. RelA was pulled down from lysates with RelA-specific antibody coupled to protein-A sepharose. Radioactive labeled RelA in precipitates was visualized by SDS-PAGE and autoradiography.

Quantitative real time-polymerase chain reaction (qPCR)

qPCR was performed as described [19]. For a complete list of primer sequences used see the Online Supplement.

Results

RelA phospho-serine 205, 276 and 281 mutants are constitutively ubiquitinated

We have shown earlier that inhibition of RelA phosphorylation in the RHD at serines 205, 276 and 281 negatively influences NF- κ B transcriptional activity [19]. In our efforts to identify the mechanism responsible for this regulation we have found that transcriptionally impaired RelA phospho-serine to alanine (SA) mutants exhibit increased and prolonged nuclear localization when compared to wild type (wt) RelA [23]. Recent reports describe a key role for ubiquitination of nuclear RelA in down-regulating NF- κ B transcriptional activity [11,12,24]; therefore we wanted to investigate if ubiquitination could contribute to the negative regulation of RelA S205A, S276A and S281A activity. To examine the ubiquitination status of RelA we ectopically expressed wt and mutant proteins together with 6 $\times$ histidine-ubiquitin and detected ubiquitinated RelA in Ni-NTA pull downs. Under these conditions ubiquitination of RelA wt was barely detectable while RelA S205A, S276A and S281A were ubiquitinated (Fig 1a). In contrast ubiquitination levels of RelA S311A, another RHD phospho-serine mutant whose transcriptional activity was in our hands unaffected by the mutation [19], was found to be comparable to RelA wt. Similar results were obtained when expressing RelA proteins in combination with HA-ubiquitin (Fig S1). Next we detected ubiquitination of endogenous RelA wt and S276A. For this study we employed RelA deficient mouse embryonic fibroblasts (RelA^{-/-} 3T3) [25] retrovirally reconstituted with RelA wt or S276A. After pull down of RelA from cell lysates under denaturing conditions, ubiquitinated RelA was detected with an anti-ubiquitin antibody. As shown in Fig 1b, comparable to HEK293T, resting 3T3 cells had only low amounts of ubiquitinated RelA wt, but substantial amounts of ubiquitinated RelA S276A. We have previously established that RelA S205A, S276A and S281A are predominantly localized to the nuclear compartment of 3T3 cells [23] and therefore thought that the nuclear localization of these mutants compared to RelA wt could trigger RelA ubiquitination. This raises the question if nuclear translocation of RelA wt after tumor necrosis factor (TNF) stimulation could induce RelA wt ubiquitination. However, the ubiquitination levels of RelA wt as well as S276A remained unchanged after TNF stimulation for 30 and 120 min (Fig 1b) indicating that transient nuclear localization as seen after TNF stimulation is not sufficient to trigger RelA ubiquitination. Therefore we reason that ubiquitination of RelA is specifically induced when its cytosolic redistribution is defective.

Ubiquitination of hypo-phosphorylated RelA does not enhance RelA protein degradation

It was suggested that ubiquitination of RelA limits its transcriptional activity by enhancing its degradation [8,11,12]; therefore we wished to test whether RelA SA mutants due to their increased ubiquitination would be less stable than RelA wt. First we over-expressed RelA wt and each mutant and examined RelA protein levels compared to β -actin in whole cell extracts in the presence of the protein synthesis inhibitor cycloheximide (CHX) for up to 16 hours in HEK293T cells. RelA wt as well as SA mutant levels remained stable at all time points tested (Fig 2a). Next we repeated the experiment in RelA^{-/-} 3T3 reconstituted with RelA wt or mutants stimulated with lipopolysaccharide (LPS) prior to CHX for 30 min to induce NF- κ B nuclear translocation and activity. Whereas the presence of CHX inhibited I κ B α resynthesis and thus confirmed the translational block, we did not observe reduced RelA SA protein stability when compared to RelA wt (Fig 2b). Stimulation of fibroblasts with TNF in conjunction with protein synthesis inhibition leads to apoptotic cell death [26,27]. Therefore we determined the effect of TNF induction on RelA protein stability in 3T3 by [³⁵S]-pulse chase experiments. Similar to LPS, pre-stimulation with TNF for 30 min did not significantly affect the half-life of RelA S276A compared to RelA wt (Fig 2c). We have shown so far that the increased ubiquitination does not affect turnover of total cellular RelA SA proteins. Because ubiquitination might specifically target nuclear RelA we next investigated RelA wt and RelA S276A stabilities in nuclear fractions of LPS-stimulated 3T3. As shown in Fig 2d relative amounts of both RelA wt and S276A were gradually increased in the nucleus after CHX addition. At the same time RelA levels in corresponding cytosolic extracts were decreased. This pattern reflects the accumulation of RelA in the nuclear compartment after consecutive LPS/CHX treatment due to blockage of I κ B α resynthesis [28]. In summary, extensively ubiquitinated RelA SA proteins are as stable as RelA wt.

Hypo-phosphorylated RelA is monoubiquitinated on multiple lysine residues

The functional consequence of ubiquitination on the fate of a target protein is highly dependent on the molecular nature of ubiquitin polymers attached to a substrate. Our data thus far indicate that ubiquitination of RelA SA proteins does not trigger RelA degradation possibly implying that RelA SA mutants might not be targeted by K48-linked polyubiquitination. We therefore studied RelA ubiquitination by pull down using a K48R ubiquitin mutant deficient in K48-anchored branch formation. Whereas no ubiquitinated RelA wt could be detected in any of the pull downs, RelA SA proteins were readily precipitated with both wt and K48R ubiquitin (Fig 3a). This result implies that RelA is not targeted by K48-linked polyubiquitination and hence is in accordance with a non-degradative effect of ubiquitin on RelA. Out of the remaining 6 lysines of ubiquitin used for chain formation K29 and K63 were most prominently shown to regulate protein activities independently of proteolysis [29]. Therefore we tested next whether K29R and K63R ubiquitins could be conjugated to RelA. Deficiency of neither K29 nor K63 had any influence on RelA SA ubiquitination (Fig 3a). Finally we employed an ubiquitin mutant where all 7 lysines were substituted by arginines (K7xR) thus rendering it unable to form ubiquitin chains. Interestingly, ubiquitination of RelA S276A was still evident with K7xR ubiquitin (Fig 3b), suggesting RelA to be monoubiquitinated. One limitation of our experimental setup could be that the endogenous ubiquitin present in cells could form mixed chains with the introduced K7xR ubiquitin mutant resulting in similar ubiquitin conjugation patterns as ubiquitin wt. Therefore we investigated the ubiquitination status of two proteins known to be polyubiquitinated, I κ B α after proteasomal inhibition [30] and RelA wt with co-expression of the ubiquitin ligase PDLIM2 [12], in presence of ubiquitin wt and K7xR. In contrast to RelA S276A ubiquitination, both MG132-mediated I κ B α and PDLIM2-triggered RelA wt ubiquitination were clearly reduced with the ubiquitin mutant (Fig S2), thus showing the validity of our approach. To further confirm observed data we analyzed RelA

ubiquitination by pulling down RelA from HEK293T cell extracts over-expressing either RelA wt or mutants and detecting ubiquitinated RelA in precipitates with 2 different anti-ubiquitin antibodies specific for mono- and polyubiquitin (P4D1) and polyubiquitin only (FK1) [31]. Before executing the assay we tested both antibodies positively for their functionality by detection of polyubiquitinated I κ B α with FK1 as well as P4D1 and exclusive detection of monoubiquitinated Eps15 with P4D1 (Fig S3). In line with data from the pull downs, ubiquitinated RelA was only detectable with P4D1 antibody but not with the polyubiquitin-specific antibody FK1 (Fig 3c). The same result was obtained for endogenous RelA in 3T3 cells (Fig 3d). Since ubiquitinated RelA is detected as multiple bands or higher molecular weight smear we assume that RelA is monoubiquitinated on more than one lysine residue. This is also reflected by the fact that no single or double mutation of any of the 18 lysines encoded in human RelA S276A resulted in inhibition of RelA ubiquitination (Fig 3e). In summary, our data support that RelA is multiply monoubiquitinated, a modification that does not lead to proteasomal degradation.

Nuclear localization and absence of I κ B α are a prerequisite for RelA ubiquitination

Next we thought to determine circumstances under which RelA ubiquitination occurs. On one hand RelA de-phosphorylation could directly regulate RelA ubiquitination, however we considered this scenario as unlikely, because all mutants are differentially phosphorylated [19] while exhibiting the same ubiquitination pattern. On the other hand the prolonged nuclear localization of SA mutants due to lack of I κ B α [23] could trigger ubiquitination in order to regulate NF- κ B-dependent transcription in absence of I κ B α . Since RelA mutants shared both extended nuclear localization and ubiquitination, and nuclear localization had been suggested earlier as a possible regulator of NF- κ B ubiquitination [11,12] we further tested this possibility. The subcellular localization of RelA mutants was investigated so far only in 3T3 stimulated with TNF [23], therefore we first determined RelA localization in HEK293T where most ubiquitination assays were performed. We observed that extensively ubiquitinated RelA proteins S205A, S276A and S281A were predominantly nuclear whereas non-ubiquitinated RelA wt and S311A were largely cytosolic (Fig S4a). The nuclear retention of RelA SA mutants was correlated with lower I κ B α protein levels in RelA S205A, S276A and S281A cells compared to RelA wt and S311A cells (Fig 1). To investigate a possible function for I κ B α in RelA ubiquitination we tested if co-expression of I κ B α in RelA SA mutant cells would have any impact on RelA ubiquitination. Interestingly re-introduction of I κ B α completely abolished RelA ubiquitination (Fig 4a). Since introduction of I κ B α into RelA SA cells results in increased I κ B α -RelA binding as well as cytosolic relocation of the NF- κ B/I κ B complex [23], the decrease in RelA ubiquitination as a result of I κ B α expression could be attributed to both. To test if I κ B α would inhibit RelA ubiquitination when cytosolic relocation is blocked we introduced I κ B α into RelA S276A cells in presence of the nuclear export inhibitor Leptomycin B (LMB). Addition of LMB largely inhibited I κ B α -mediated cytosolic translocation of RelA S276A (Fig S4b), but did not re-establish RelA ubiquitination (Fig 4b). Next we treated RelA wt expressing cells with LMB for up to 4 hours. This treatment led to sustained nuclear accumulation of RelA wt (Fig S4c), but was not sufficient to trigger RelA ubiquitination (Fig 4c). In contrast RelA S276A was ubiquitinated at all time points in absence of exogenous I κ B α . Together these results reveal that I κ B α blocks RelA ubiquitination independently of RelA subcellular localization, but does not answer whether nuclear localization is a prerequisite for RelA ubiquitination. To this end we constructed a RelA S276A nuclear localization signal mutant (RelA S276A mNLS) and confirmed by immunofluorescence that nuclear translocation of this mutant was blocked (Fig S4d). When comparing the ubiquitination status of RelA S276A and S276A mNLS we found RelA S276A was substantially ubiquitinated whereas ubiquitination of the NLS mutant was only marginal (Fig 4d). Hence nuclear translocation is required for RelA to be efficiently ubiquitinated. Altogether our data indicate that hypo-

phosphorylated RelA is ubiquitinated in the nucleus when not bound to I κ B α . We showed earlier that RelA SA mutation did not alter NF- κ B complex formation and p50 heterodimerization [19]. However, due to the overrepresentation of exogenous RelA in our experimental setup it appears likely that RelA homodimer formation is favored in these cells over RelA-p50 heterodimer formation. To investigate if p50 association has an impact on RelA ubiquitination, we co-expressed p50 in equimolar concentration to RelA and tested for RelA ubiquitination levels. As shown in Fig S5a, RelA ubiquitination remained constant in presence of p50, indicating that the dimer composition plays no role in RelA ubiquitination.

Proteasomal blockage in I κ B α ^{-/-} cells leads to nuclear translocation of RelA

Ubiquitination of RelA was recently demonstrated to play a key role in regulating the NF- κ B response in I κ B α ^{-/-} cells [11]. Whereas in our hands RelA protein stability was not affected by ubiquitination (Fig 2) it was suggested that termination of NF- κ B-dependent transcription in I κ B α ^{-/-} cells is achieved by ubiquitin-dependent degradation of RelA. Therefore we decided to monitor RelA stability and subcellular localization in I κ B α ^{-/-} cells. According to published protocols [11] we stimulated cells with TNF for 15 min, followed by wash out and incubation for 6 hours with proteasomal inhibitors. After confirming ubiquitination of RelA (Fig 5a) as prior seen also by Saccani et al [11] we examined RelA protein levels in nuclear and corresponding cytosolic fractions of metabolically labeled cells under the same treatment paradigm with and without MG132. RelA protein levels decreased in both cytosolic and nuclear fractions over a time course of 6 hours after TNF stimulation independently of MG132 (Fig 5b), indicating that a certain amount of total cellular RelA protein is degraded in a proteasome-independent manner. However, the relative rate of RelA decay in both compartments differed depending on MG132 addition. Without MG132 we observed a more pronounced decrease in nuclear RelA levels compared to MG132-treated cells. This was however compensated by a smaller decline in cytosolic RelA in cells not treated with MG132 (Fig 5b). These data indicate a shift of cytosolic to nuclear RelA after proteasomal inhibition. To test this hypothesis we detected RelA in TNF-stimulated I κ B α ^{-/-} cells by indirect immunofluorescence after 1, 3 and 6 hours MG132 treatment. In spite of I κ B α absence, nuclear RelA was completely cleared over the time course of 6 hours medium after a pulse of 15 min TNF. Addition of MG132, however, led to nuclear retention of RelA with simultaneous loss of cytoplasmic immunoreactivity (Fig 5c). Taken together our data imply that the increase of nuclear RelA after proteasomal blockage is a result of nuclear retention and is not due to increased RelA protein stability.

Inhibition of the proteasome leads to monoubiquitination of nuclear RelA

After establishing that inhibition of the proteasome in I κ B α ^{-/-} cells leads to ubiquitination and nuclear translocation of RelA, we wanted to investigate whether proteasomal inhibition leads to monoubiquitination as we had shown earlier for RelA SA mutants. Accordingly we analyzed RelA wt ubiquitination in presence of proteasome inhibitors. RelA wt was only minimally ubiquitinated while ubiquitination of a RelA SA triple mutant was abundant (Fig 6a). Treatment of cells with proteasome inhibitors MG132 or MG262 for 3 hours increased RelA wt ubiquitination to a level comparable to RelA 3xSA. This augmentation was not altered by TNF treatment. In contrast, proteasomal inhibition did not further enhance RelA 3xSA ubiquitination. Next we determined RelA wt and 3xSA subcellular localizations in presence or absence of MG132. RelA 3xSA was located in the nucleus regardless of MG132, which is consistent with proteasomal inhibition independent ubiquitination of RelA 3xSA (Fig 6b). RelA wt, in contrast, was mainly cytosolic in absence of MG132, but translocated to the nucleus after addition of the proteasome inhibitor. Next we probed the molecular structure of RelA ubiquitination induced by proteasomal inhibition by ectopically expressing RelA wt together with wt-, K29R-, K48R-, K63R- or K3xR-ubiquitin in presence

of MG132 and assessing RelA ubiquitination by pull down assay. Like for RelA S276A we observed that all ubiquitin variants were efficiently conjugated to RelA (Fig 6c). In addition we determined if RelA wt would be ubiquitinated in presence of K7xR-ubiquitin when the proteasome is blocked. As shown in Fig 6d, RelA wt was equally conjugated to wt and K7xR-ubiquitin. Finally we tested the nature of endogenous ubiquitinated RelA obtained by proteasomal inhibition with ubiquitin conjugation-specific antibodies. Similarly to RelA S276A (Fig 3d), ubiquitinated RelA was not detectable with the poly-ubiquitin-specific FK1 antibody, but was highly reactive with the P4D1 antibody recognizing also monoubiquitin (Fig 6e). Consequently we propose that proteasomal inhibition-induced nuclear translocation of RelA leads to RelA monoubiquitination.

Fusion of a single ubiquitin to RelA wt alters NF- κ B transcriptional activity in a similar way than S276A mutation

Thus far we have demonstrated that RelA nuclear translocation caused either by hypo-phosphorylation or proteasomal inhibition results in RelA monoubiquitination. However, we did not address whether monoubiquitination changes RelA transcriptional activity. The best approach to determine the physiological consequences of RelA ubiquitination would be the transcriptional characterization of a constitutively ubiquitinated or non-ubiquitinated RelA mutant protein, which requires the identification of RelA ubiquitination sites. However we had failed to identify ubiquitin acceptor sites responsible for RelA S276A ubiquitination by a mutagenesis approach targeting all 18 RelA lysine residues by single or double KR substitutions (Fig 3e). In another effort to identify RelA ubiquitin acceptor sites after proteasomal inhibition we applied tandem mass spectrometry (MS) for determining ubiquitinated lysine residues. After tryptic digest of ubiquitinated RelA pulled down from MG132-treated HEK293T cells we identified 3 ubiquitinated RelA lysine residues, including K62, K123 and K315 (Table 1, Fig S6). This MS result provides direct evidence that RelA is ubiquitinated at more than one position after proteasomal inhibition, however a RelA mutant lacking these 3 residues still underwent extensive ubiquitination (data not shown). A very recently published article by Li et al also addressed the location of RelA ubiquitination sites [32]. While MS led to the identification of seven ubiquitinated RelA lysines, among them also K62, K123 and K315, it was necessary to eliminate all 18 lysines contained in RelA to abolish RelA ubiquitination. Since such an extensive alteration of RelA leads to a non-functional protein [32] it cannot be used for further studies.

Therefore, as an alternative approach to mimic constitutive monoubiquitination of RelA we fused a single ubiquitin moiety to the amino terminus of RelA wt (Ub-RelA) (Fig 7a). This method was previously established to reveal the role of monoubiquitination in regulating p53 and FOXO4 transcriptional activities [33,34]. We characterized the fusion protein by testing its ability to interact with p50 and I κ B α by co-immunoprecipitation and found that the Ub-RelA fusion associated efficiently with both proteins (Fig S5b and data not shown). To ascertain that the effects on RelA transcriptional activity were not a result of the amino-terminal tagging of RelA we used as a control a portion of the LacZ gene (pLacZ) with similar biochemical features to ubiquitin (Fig 7a). After ensuring comparable protein expression levels of RelA wt, RelA S276A, Ub-RelA and pLacZ-RelA stably introduced into RelA^{-/-} 3T3 (Fig 7b) we studied their transcriptional activity in TNF-stimulated cells by monitoring mRNA levels of four NF- κ B-dependent genes. As shown in Fig 7c, RelA wt and pLacZ-RelA efficiently induced transcription of all genes tested. In contrast RelA S276A and Ub-RelA showed gene-specific transcriptional impairment. While both severely reduced transcription from IL-6 and ICAM-1 genes, there was no effect on IP-10 and only minor effects on MHC-I mRNA expression (Fig 7c). The similarity in transcriptional potencies between RelA S276A and Ub-RelA indicates that the increased monoubiquitination of RelA SA mutants could indeed account for the decrease in RelA

transcriptional activity. Supportive for this conclusion is also the subcellular localization of Ub-RelA at different time points after TNF stimulation. Like shown earlier for RelA S276A [23], Ub-RelA translocated to the nucleus 30 min after TNF addition, but did not relocate to the cytoplasm after 2 and 4 hours of TNF stimulation (Fig 7d). The control fusion protein pLacZ-RelA, on the other hand, readily translocated back to the cytoplasm 2 hours after TNF was added.

Hypo-phosphorylation of RelA at S276 was shown to inhibit NF- κ B activity by abrogating the interaction with the transcriptional co-activator CBP [35]. Since hypo-phosphorylated RelA resembled the transcriptional profile of monoubiquitinated RelA, we wanted to examine if monoubiquitin attachment to RelA is affecting CBP binding. Indeed we found that both RelA S276A and to a larger extent Ub-RelA exhibited a decreased affinity for CBP when compared to RelA wt (Fig 7e). Thus impaired recruitment of CBP to the transcriptional machinery could be responsible for the decreased expression of NF- κ B target genes by RelA S276A and Ub-RelA. Taken together, our data indicate that monoubiquitination plays an important role in regulating RelA transcriptional activity in a gene-specific manner by influencing the interaction between RelA and CBP.

Discussion

Strength and duration of NF- κ B activity is controlled at multiple levels. Whereas binding of its inhibitor I κ B constitutes the main mechanism for silencing of NF- κ B, post-translational modification of the DNA-binding subunits by phosphorylation, acetylation and ubiquitination has been described to fine-tune the activity of nuclear NF- κ B [36]. While previous reports have linked RelA ubiquitination to nuclear degradation, here we provide evidence that nuclear sequestered RelA is targeted by multiple monoubiquitination resulting in RelA transcriptional silencing without affecting its cellular half-life.

Here we show that inhibition of phosphorylation at three serine residues within the RHD as well as inhibition of the proteasome lead to monoubiquitination of RelA. The unexpected finding that RelA also undergoes monoubiquitination is in contrast to previously reported RelA polyubiquitination, which was mainly triggered by addition of proteasome inhibitors and the ubiquitin ligases SOCS1/COMMD1 and PDLIM2 [8,10–12,32]. By MS analysis it has been determined that polyubiquitin chains linked via K29, K33, K48 and K63 can be attached to RelA after proteasomal blockage [32], indicating that inhibition of the proteasome also induces degradation-independent RelA modification [37]. On the other hand the nature of ubiquitin additions conjugated by co-expression of SOCS1 and PDLIM2 ubiquitin ligase components has not been addressed, but it has been described that it results in destabilization of RelA [8,10,12,38,39], pointing at RelA to be polyubiquitinated.

The observation that RelA is polyubiquitinated is often based on the detection of a high molecular weight ubiquitinated RelA species in immunoblotting. This alone, however, is not a conclusive evidence for RelA polyubiquitination, since also multiple monoubiquitin attachments would lead to the same ubiquitination pattern. The fact that mutation of all lysines in RelA is necessary to prevent its ubiquitination after addition of MG132 [32] provides basic evidence that enough monoubiquitin moieties could be conjugated to RelA lysine residues to shift a substantial portion of ubiquitinated RelA past a molecular weight of 100 kDa.

In our study we detect mainly monoubiquitin additions to RelA. A slight decrease in ubiquitinated RelA species with addition of KR ubiquitin variants (Figs. 3a, 3b, 6c, 6d) however indicates that RelA is not exclusively modified with monoubiquitin. The amount of polyubiquitinated RelA however lies below the detection range of the polyubiquitin-specific

FK1 antibody, which could easily detect polyubiquitinated I κ B α (Figs. 3c, 3d, 6e, S3). In conclusion, contemplating all available published data on RelA ubiquitination it becomes apparent that RelA is not exclusively targeted by one type of ubiquitin modification, but is likely to be conjugated to the full spectrum of possible ubiquitin variants on multiple ubiquitin acceptor residues.

Two paradigms led to RelA ubiquitination. First we found that ubiquitination of RelA was markedly enhanced by inhibiting phosphorylation at S205, S276 and S281. While in most cases phosphorylation induces ubiquitination as reported for p53 [40] and c-myc [41], phosphorylation can also inhibit ubiquitination as seen in c-Jun [42] or ATF-2 [43]. Both circumstances have been shown for RelA. Phosphorylation at S468 by the IKK complex induces its ubiquitination by enabling the association with the COMMD1/SOCS-1 E3 ligase complex [38,39], whereas, confirmative of our result, phosphorylation at S276 by the kinase Pim-1 inhibits RelA ubiquitination [44]. If inhibition of RelA phosphorylation at S205, S276 and S281 directly contributes to RelA ubiquitination by e.g. permitting the binding of an ubiquitin ligase, remains to be determined. However, we propose a different mechanism where phosphorylation deficiency at these discrete sites only indirectly induces RelA ubiquitination. Inhibition of RelA phosphorylation at S205, S276 and S281 reduces NF- κ B transcriptional activity resulting in decreased cellular I κ B α levels [19,23]. This is reflective of I κ B α ^{-/-} cells, where prolonged nuclear presence of NF- κ B leads to RelA ubiquitination [11]. Therefore we believe that RelA S205A, S276A and S281A display an elevated ubiquitination level due to their extended nuclear localization caused by insufficient I κ B α induction. Data presented in our work are in support of this conclusion. First, inhibition of phosphorylation at S311, which neither results in decreased cellular I κ B α levels nor nuclear accumulation of NF- κ B [23], did not enhance RelA ubiquitination. Second, reintroduction of I κ B α into RelA mutant cells completely abolished RelA ubiquitination, suggesting that RelA cannot be ubiquitinated when bound by I κ B α . In line with this we find that nuclear accumulation of RelA caused by LMB, a specific inhibitor of Crm1-dependent nuclear export [45], was not sufficient to induce RelA ubiquitination (Fig 4c). Apart from nuclear retention of RelA, LMB treatment also leads to nuclear sequestration of I κ B α resulting in RelA-I κ B α complex formation and transcriptional silencing [46–48]. Interestingly and similar to RelA-I κ B α interactions, ubiquitination of NF- κ B p50 is negatively regulated by its binding to the I κ B family member Bcl-3 [49]. Third, inhibition of nuclear translocation of RelA S276A diminished its ubiquitination (Fig 4d), underlining the importance of nuclear transfer for RelA to be ubiquitinated.

The second inducer of RelA ubiquitination was proteasomal inhibition. The most prevalent and immediate interpretation for this phenomenon is that proteasomal inhibition blocks RelA degradation resulting in accumulation of ubiquitinated RelA species [8,11,12]. However, this explanation is not entirely satisfactory. In contrast to short-lived transcription factors like p53, c-myc or c-Jun, whose activities are primarily regulated by degradation and resynthesis [14,16,50], RelA protein was reported to be comparably stable [28,51,52]. This coheres with the fact that NF- κ B activation does not require *de novo* protein synthesis [53,54] and its termination is mainly regulated by an auto-regulatory feedback loop involving proteins produced in response to NF- κ B activation [55,56]. In line with a proteasome-independent regulatory function of RelA ubiquitination we found in this study that ubiquitinated RelA phospho-mutants were stable under all examined conditions. Although it cannot be entirely ruled out that RelA is degraded by the proteasome under certain conditions we show evidence herein that the impact of proteasomal inhibition on RelA ubiquitination is the result of nuclear retention rather than stabilization of RelA. This is based on the observation that nuclear localization, as evidenced by RelA phospho-mutants, was sufficient to induce ubiquitination and ubiquitination was not further increased by treatment with MG132 (Fig 6a). In addition we observed in I κ B α ^{-/-} cells that increase in

nuclear RelA after MG132 treatment was paralleled by a decrease in cytosolic RelA (Fig 5b and 5c) supporting the hypothesis that proteasomal inhibition leads to nuclear translocation and retention of RelA. The mechanism for RelA nuclear translocation induced by proteasomal inhibition warrants further investigation. On the one hand proteasomal inhibition was shown to inhibit NF- κ B activity by blocking I κ B α degradation [30,57], but on the other hand it has also been shown to result in NF- κ B induction due to IKK activation [58–60]. In the latter case I κ B α is degraded independently of the proteasome, a mechanism, which has been shown to regulate inducible as well as constitutive NF- κ B activation [61,62]. The lack of I κ B α then consequently leads to nuclear retention and enhanced DNA-binding of NF- κ B [58,60].

Finally we provide evidence that monoubiquitination negatively regulates RelA transcriptional activity in a gene-specific manner. Fusion of a single ubiquitin to RelA was sufficient to cause prolonged nuclear retention after TNF stimulation while at the same time inhibiting its transcriptional activity on some genes, but not on others (Fig 7c). The transcriptional profile was similar to the RelA S276A mutant, which, comparable to the ubiquitin-RelA fusion, was also monoubiquitinated (Fig 3) and predominantly nuclear localized [23]. While an increase in NF- κ B-dependent transcription has been described after proteasomal inhibition in I κ B α ^{−/−} cells [11], it is not clear to what extent this effect is RelA-dependent. We observed substantial induction of NF- κ B-dependent genes in TNF-stimulated RelA^{−/−} 3T3 after MG132 treatment (Fig S7). It is therefore likely that proteasomal inhibition can increase transcription at least of some NF- κ B-dependent genes without the involvement of RelA.

How RelA multiple monoubiquitination influences NF- κ B activity remains to be largely elusive, but our data indicate that ubiquitinated RelA loses its ability to bind to CBP (Fig 7e). This could be either the result of lower binding affinity due to changes in protein properties by ubiquitin attachment or due to an induced spatial separation of RelA from CBP. For example, ubiquitination could translocate RelA to transcriptionally selective nuclear compartments such as PML-bodies [12] or lead to nucleolar sequestration of RelA shown to be preceded by ubiquitination after proteasomal inhibition [24]. Another regulatory mechanism could involve competition of ubiquitin and acetyl-groups for RelA lysine residues. Interestingly, acetylation of K123, an ubiquitin-acceptor site identified in this study and by others [32], promotes I κ B α -mediated nuclear export [7]. Thus, it is possible that ubiquitination at this site leads to nuclear retention of RelA by inhibiting acetyl-lysine-mediated I κ B α interaction.

In summary, we present RelA monoubiquitination as a novel regulatory modification mediating gene-specific silencing of NF- κ B activity. In future studies it will be important to identify proteins involved in the RelA monoubiquitination process. The identification and characterization of these new mediators in the NF- κ B pathway will not only improve our knowledge and understanding of mechanisms involved in inflammation and associated diseases, but will also provide new targets for therapy and drug discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the following persons for providing materials used in this study: Drs. Amer A. Beg (RelA^{−/−} 3T3), Dirk Bohmann (pMT107), Rainer De Martin (pKSII/ECI-6), Ivan Dikic (pcDNA3-HA-ubiquitin) and Alexander Hoffmann (I κ B α ^{−/−} 3T3). We also thank Dr. Wei Chen for performing the MS analysis. This work was

supported by National Institute of Health Grant [HL077308 to J.A.] and American Heart Association Scientist Development Grant [10SDG2600298 to K.H.].

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Fig 1. Mutation of NF- κ B RelA serines 205, 276, and 281 leads to increased RelA ubiquitination
(a) HEK293T cells were transfected with myc-RelA wt, S205A, S276A, S281A or S311A together with his-ubiquitin. His-ubiquitin-coupled proteins were pulled down under denaturing conditions with nickel-conjugated beads. Cell lysates and precipitates were probed with RelA, I κ B α and β -actin-specific antibodies. **(b)** RelA^{-/-} 3T3 were retrovirally reconstituted with either RelA wt or S276A mutant and stimulated with TNF for 0, 0.5, and 2 hours. After lysis endogenous RelA was pulled down with anti-RelA antibody. Precipitates were examined for the presence of ubiquitinated RelA with anti-ubiquitin

antibody. RelA, I κ B α and β -actin protein levels were detected in input cell lysates. IB, immunoblot; IP, immunoprecipitation; PD, pull down.

Fig 2. Protein stability of RelA is not influenced by its ubiquitination status

(a) HEK293T cells were transiently transfected with RelA wt, S205A, S276A, or S281A and treated with cycloheximide (CHX) for indicated time points. Expression of RelA and β -actin was monitored. (b) RelA^{-/-} 3T3 retrovirally reconstituted with RelA wt or mutants were stimulated for 30 min with LPS and treated with CHX for indicated time points. Immunoblots were probed with RelA, $\text{I}\kappa\text{B}\alpha$ and β -actin-specific antibodies. 0*, w/o addition of CHX, but harvested with 16 hour time points. (c) RelA^{-/-} 3T3 reconstituted with RelA wt or S276A were stimulated with TNF for 30 min in presence of [³⁵S]-methionine and chased for 0, 0.5, 1, 2 and 4 hours in presence of TNF. Immunoprecipitated RelA protein was detected by autoradiography and RelA protein levels from four experiments were quantified (lower panel = representative autoradiograph). (d) RelA^{-/-} 3T3 reconstituted with RelA wt or S276A were stimulated for 30 min with LPS and CHX for indicated time points. Cell lysates were separated into cytosolic and nuclear fractions. Presence of RelA was detected with RelA-specific antibody. Separation of cytosol and

nuclei was ensured by exclusive detection of GAPDH in cytosolic and lamin B in nuclear fractions. Relative cytosolic and nuclear RelA from 2 experiments were quantified by normalization to GAPDH and lamin B protein levels, respectively. IB, immunoblot.

Fig 3. NF- κ B RelA phospho-mutants are monoubiquitinated on multiple lysine residues
(a) His-ubiquitin without lysines conventionally used for chain formation is still efficiently conjugated to RelA. HEK293T expressing his-ubiquitin wt, K29R, K48R, K63R or K29/48/63R (K3xR) together with myc-RelA proteins were lysed and coupled proteins were pulled down with nickel-conjugated beads. Precipitates were probed with RelA-specific antibody. **(b)** Monoubiquitin is readily conjugated to RelA S276A. HEK293T cells were transfected with RelA S276A and ubiquitin wt, K3xR or K7xR. His-tagged proteins were precipitated from whole cell extracts using Ni-agarose beads and RelA was detected in immunoblots. **(c)** RelA is immunoreactive with P4D1 antibody that recognizes mono- and

polyubiquitin but not with FK1 antibody specific for polyubiquitin. HEK293T cells were transfected with his-ubiquitin and myc-RelA wt, S205A, S276A, or S281A. Lysates were precipitated with myc-affinity agarose followed by detection of RelA in immunoblot with anti-myc, anti-ubiquitin P4D1 and anti-polyubiquitin FK1 antibodies. **(d)** Ubiquitin attached to endogenous RelA S276A in 3T3 is not detectable with a polyubiquitin-specific antibody. RelA^{-/-} 3T3 reconstituted with either RelA wt or S276A were left untreated or treated with TNF for 30 min. Extracts were subject to immunoprecipitation with anti-RelA antibody and ubiquitinated RelA was detected in precipitates with either P4D1 or FK1 anti-ubiquitin antibodies. Equal precipitation of RelA was ensured by staining of precipitates with RelA. **(e)** All RelA S276A lysine-to-arginine mutants are ubiquitinated. HEK293T were transfected with his-ubiquitin and indicated myc-RelA KR mutants. Assay was carried out as described above. IB, immunoblot; IP, immunoprecipitation; PD, pull down.

Fig 4. Extended nuclear localization and absence of IκBα are required for RelA ubiquitination
(a) IκBα inhibits RelA ubiquitination. HEK293T cells were transfected with his-ubiquitin, myc-RelA, and either empty vector (–) or HA-IκBα. Ubiquitination assay was performed under denaturing conditions. **(b)** Nuclear RelA S276A is not ubiquitinated in presence of IκBα. HEK293T were transfected with myc-RelA S276A in absence or presence of HA-IκBα. To maintain RelA nuclear with co-expression of IκBα LMB was added for 2 hours. Assay was performed as in (A). **(c)** Nuclear accumulation of RelA wt in presence of IκBα does not lead to an increase of RelA ubiquitination. HEK293T cells transfected with myc-RelA and his-ubiquitin were treated with Leptomycin B (LMB) for indicated time points. Cells were harvested in guanidine hydrochloride-containing buffer and his-pull down was

carried out. **(d)** Reduced nuclear translocation of RelA S276A results in diminished ubiquitination. HEK293T cells were transfected with his-ubiquitin and either empty vector control (–), RelA wt, RelA S276A or RelA S276A NLS mutant (mNLS). Ubiquitination of RelA was determined by his-ubiquitin pull down and RelA antibody staining. IB, immunoblot; PD, pull down.

Fig 5. Proteasomal inhibition results in nuclear accumulation rather than stabilization of RelA in $I\kappa B\alpha^{-/-}$ cells

(a) Treatment of $I\kappa B\alpha$ knockout cells with β -lactone and MG132 leads to an increase in RelA ubiquitination. $I\kappa B\alpha^{-/-}$ 3T3 were stimulated with a pulse of TNF for 15 min, then either left untreated or incubated with β -lactone or MG132 for 6 hours. Ubiquitinated RelA was pulled down with RelA antibody and detected with anti-ubiquitin antibody. (b) Addition of MG132 causes a shift in cytosolic to nuclear RelA, but does not stabilize RelA protein. $I\kappa B\alpha^{-/-}$ fibroblasts were pulse-labeled with [35 S]-methionine and during chase treated as in (A). Cells were lysed to obtain cytosolic and nuclear extracts. Radiolabeled RelA was detected in autoradiography. Graph shows results obtained from 3 experiments. Percent

levels of RelA after 6 hours with or without MG132 treatment were compared to 100% RelA at time point 0 (represents RelA levels observed after 15 min TNF). Lower panel shows representative autoradiograph (non-relevant lanes were digitally removed). **(c)** MG132 treatment prolongs RelA nuclear localization. After $\text{I}\kappa\text{B}\alpha^{-/-}$ 3T3 were treated with TNF for 15 min and then either left untreated or stimulated with MG132 for 1, 3, and 6 hours, cells were processed for immunofluorescence with RelA antibody. Nuclei were stained with DAPI. Bar = 10 μm . The graph shows ratios of cytosolic and nuclear fluorescence obtained by automatic quantification of RelA compared to DAPI staining (n=195–244 cells, derived from 3 experiments). Values N/C >1 indicate predominantly nuclear RelA. IB, immunoblot; IP, immunoprecipitation.

Fig 6. Proteasomal inhibition results in nuclear accumulation and monoubiquitination of RelA
(a) Blockage of the proteasome leads to increased RelA wt ubiquitination levels. HEK293T cells were transfected with myc-RelA wt or mutant S205/276/281A (RelA 3xSA) together with his-ubiquitin. Where indicated cells were treated for 3 hours with 50 μ M MG132 or 1 μ M MG262 to block proteasomal protein degradation. Ubiquitinated RelA was detected in his-ubiquitin pull down fractions. Expression of myc-RelA, endogenous $\text{I}\kappa\text{B}\alpha$ and β -actin in cell lysates was monitored by immunoblotting. **(b)** Addition of MG132 leads to nuclear retention of RelA wt. HEK293T cells transfected with myc-RelA wt or 3xSA were left untreated or exposed to MG132 for 3 hours. RelA proteins were detected in immunostaining with myc-specific antibody. Nuclei were visualized with DAPI staining. Size bar = 10 μ m. **(c)** MG132 does not trigger conventional RelA K29, K48 or K63 ubiquitination. HEK293T cells were transfected with myc-RelA wt together with his-ubiquitin variants. Cells were treated with MG132 for 3 hours and processed to determine RelA ubiquitination. **(d)** K7xR ubiquitin is efficiently conjugated to RelA after proteasomal inhibition. HEK293T cells were transfected with myc-RelA, his-ubiquitin wt, K3xR and K7xR. After treatment with MG132 for 3 hours cells were lysed and ubiquitinated RelA was pulled down with Ni-agarose. **(e)** Endogenous ubiquitinated RelA detected after proteasomal inhibition of 3T3 cells is not reactive with a polyubiquitin-specific antibody. $\text{I}\kappa\text{B}\alpha^{-/-}$ 3T3 cells were treated with TNF for 30 min followed by MG132 for 3 hours and then harvested to immunoprecipitate endogenous RelA with RelA-specific antibody. Ubiquitinated RelA species were detected in immunoblotting with anti-ubiquitin P4D1, but not with anti-ubiquitin FK1 antibody. IB, immunoblot; PD, pull down.

Fig 7. Conjugation of monoubiquitin to RelA wt leads to a similar alteration of NF- κ B-dependent transcription as RelA S276A mutation

(a) Schematic representation of RelA constructs used in this study. (b) Equal expression levels of RelA proteins in 3T3. RelA^{-/-} 3T3 were retrovirally reconstituted with indicated constructs. Expression levels of RelA proteins were monitored by immunoblot with RelA-specific antibody. (c) Ubiquitin-conjugation to RelA wt leads to a similar change in NF- κ B transcriptional activity as seen for RelA S276A mutation. RelA^{-/-} 3T3 expressing empty vector (control), RelA wt, RelA S276A, Ub-RelA and pLacZ-RelA were stimulated with TNF for 0.5, 1.5, 3 and 6 hours, total RNA was isolated, and mRNA levels of IL-6, ICAM-I, IP-10 and MHC-I were analyzed by qPCR. Fold induction of experimental groups was

calculated relative to expression level of unstimulated cells, which was set to 1. Error bars represent mean + SEM of triplicates derived from 3 independent experiments. **(d)** Attachment of ubiquitin but not pLacZ results in prolonged nuclear localization of RelA. RelA^{-/-} 3T3 stably expressing Ub-RelA and pLacZ-RelA were stimulated with TNF for indicated time points and processed for immunostaining with RelA antibody. Nuclei were stained with DAPI. Size bar = 10 μ m. **(e)** RelA S276A and Ub-RelA bind less efficiently to CBP than RelA wt. Lysates from RelA^{-/-} 3T3 expressing empty vector (control), RelA wt, RelA S276A or Ub-RelA were subjected to immunoprecipitation with CBP antibody. Presence of RelA in precipitates was tested with RelA-specific antibody. IB, immunoblot; IP, immunoprecipitation.

Table 1
Identification of ubiquitinated peptides of RelA protein by nanoLC-MS/MS analysis.

Sequence	Modification	XCorr	Δ Score	Intensity	Charge	m/z [Da]	MH+ [Da]	Δ M [ppm]
57-THPTIKInGYTGPGTVR-73	K62 (GlyGly), N64 (Deamidated)	3.33	0.47	7.70E+04	3	643.0068	1927.0047	0.78
123-KRDLEQAISQR-133	K123 (GlyGly)	4.23	0.07	8.47E+04	3	486.5989	1457.7823	0.19
315-KSPFSGPTDPPPPR-329	K315 (GlyGly)	3.02	0.26	4.35E+04	3	583.9725	1749.9029	-0.18