Identification of lysine residues critical for the transcriptional activity and polyubiquitination of the NF-κB family member RelB

Julia LEIDNER*, Lysann PALKOWITSCH*, Uta MARIENFELD*, Dietmar FISCHER† and Ralf MARIENFELD*¹

*Institute of Physiological Chemistry, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany, and †Institute of Experimental Neurology, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany

RelB is the key component of the alternative NF-κB (nuclear factor κB) signalling pathway. However, RelB exerts also a negative effect via the recruitment of a DNMT1 (DNA methyltransferase 1)–Daxx (death domain-associated protein) complex to NF-κB target genes. Importantly, the molecular mechanisms which determine the functions of RelB are still largely unknown. In the present study, we aimed to analyse whether ubiquitination of RelB might be involved in the regulation of RelB. Indeed, RelB is constitutively polyubiquitinated in the B-cell lines Namalwa and 70Z/3. Although a PMA+ionomycin-induced augmentation of RelB polyubiquitination was linked to its proteasomal degradation in B-cells, the constitutive RelB polyubiquitination seems to affect non-proteasomal functions. Consistently, a significant RelB polyubiquitination in HEK (human emryonic kidney)-293 cells correlated with an augmentation of the transcriptional activity of RelB. Yet, neither nuclear localization nor DNA binding was enhanced by RelB polyubiquitination. Interestingly, basal RelB polyubiquitination depends neither on Lys^{48} nor on Lys^{63} conjugates but might involve unconventional ubiquitin conjugates. Mapping of the ubiquitination target sites in RelB revealed the existence of various lysine residues, which serve as ubiquitination acceptors. However, only the substitution of $Lys^{273/274}$ and $Lys^{305/308}$ significantly decreased the basal RelB activity and the ubiquitin-induced augmentation of the RelB activity. Collectively, these results imply a dual role of RelB polyubiquitination for the stability and activity of this transcription factor.

Key words: co-factor, gene expression, nuclear factor κB (NF-κB), protein stability, RelB, ubiquitination.

INTRODUCTION

The transcription factor NF-κB (nuclear factor κB) plays an essential role for the regulation of immune and inflammatory responses, development and cancer. The basis for the pleiotropic effects of this transcription factor is the functional divergence of the different NF-κB dimers, composed of the subunits RelA, RelB, c-Rel, NF-κB1/p50 and NF-κB2/p52. Inactive NF-κB is restrained in the cytoplasm of resting cells by the members of the IκB (inhibitor of NF-κB) family which become site-specific phosphorylated upon cell stimulation by the multi-subunit IKK (IκB kinase) complex, composed of the kinases IKK1 and IKK2, and the adaptor protein NEMO (NF-κB essential modulator), and it is then subsequently degraded by the proteasome [1,2]. Free NF-κB translocates to the nucleus where it supports the expression of various NF-κB target genes. Besides this canonical NF-κB signalling pathway, an alternative IKK1-dependent pathway has been described which is characterized by the activation of RelB–p52 heterodimers and the expression of a specific subset of NF-κB target genes [3]. In addition to its role in the alternative NF-κB pathway, RelB is also linked to various other functions in the NF-κB system. For example, RelB is part of a second wave of the NF-κB response, either supporting the expression of a defined set of genes or terminating the expression of another set of genes [4]. Thus RelB acts either as an activator or a repressor of NF-κB target gene expression by mainly unknown mechanisms. One feasible model is the recruitment of co-activators or co-repressors based on specific post-translational modifications of RelB. Indeed, an interaction of RelB with the Daxx (death domain associated-protein)–DNMT1 (DNA methyltransferase 1) co-repressor complex has recently been described [5]. Post-translational modifications such as phosphorylation, ubiquitination or acetylation are crucial for the diverse functions of the NF-κB transcription factors [6–8]. For instance, the recruitment of the co-activator CBP [CREB (cAMP-response-element-binding protein)-binding protein] to the NF-κB subunit RelA depends on either site-specific phosphorylation or acetylation of RelA [6,7]. In contrast, the molecular mechanisms underlying the ambivalent functions of RelB are currently unknown. In addition to acetylation and phosphorylation steps, growing evidence points to a prominent role of non-proteasomal ubiquitination for the regulation of transcription factors. The transcriptional activity of the trancription factor c-Myc, for instance, is distinctively augmented by the ubiquitination-mediated recruitment of the co-activator p300 [9]. The HectH9 mediated ubiquitination of c-Myc occurs in a NLS (nuclear localization sequence) spanning region at various lysine residues and is crucial for the expression of c-Myc target genes, the interaction with p300 and induction of cell proliferation. In the present study, we aimed to determine whether NF-κB family members, especially RelB, are also the subject of an ubiquitination-based regulatory mechanism. Previously, we reported that the NF-κB family member RelB is degraded during the initiation phase of T-cell activation via the proteasomal pathway [10]. The RelB proteolysis is regulated by a specific phosphorylation at Thr^{84} and Ser^{552} of RelB. In the present study,
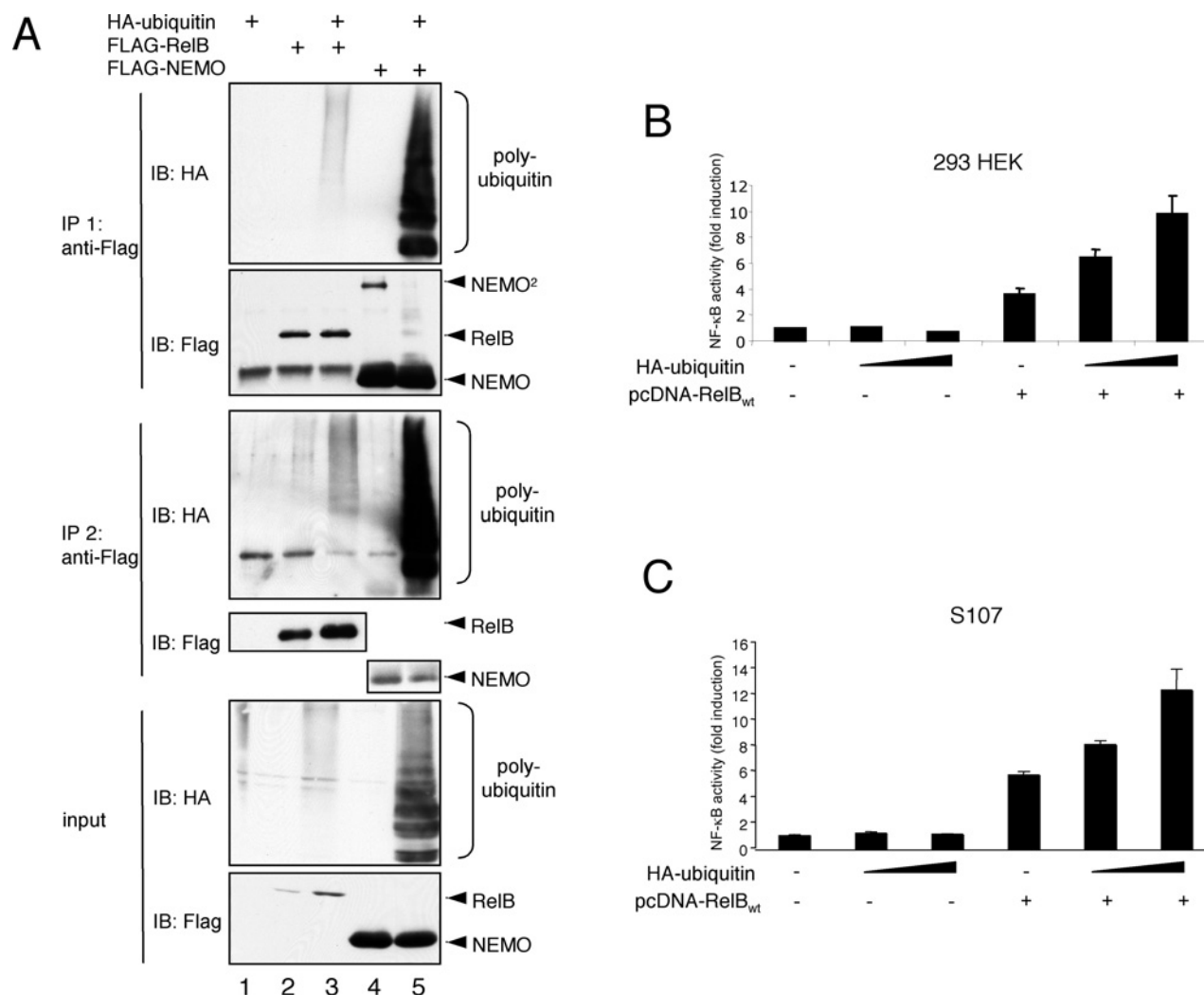

Figure 1   RelB polyubiquitination _in vivo_ correlates with an augmentation of its transcriptional activity

(**A**) For an _in vivo_ ubiquitination assay HEK-293 cells were transiently transfected with 1 μg of either FLAG–RelB or HA–ubiquitin as indicated. The cells were lysed in TNT buffer and 90 % of the cell lysates were subjected to an anti-FLAG immunoprecipitation (IP). The resulting immunoprecipitates were boiled in TNT buffer with 0.5 % SDS. For Western blot analysis 50 % of the sample was used (IP 1, top panel) and 50 % for a second anti-FLAG immunoprecipitation (IP 2, middle panel). To control the expression of the proteins, 10 % of the cell lysates were subjected to additional Western blot analysis (input, bottom panel).IB, immunoblot. (**B**) HEK-293 cells were transiently transfected with an NF-κB-dependent luciferase reporter construct together with a ubiquitin-promoter driven _Renilla_ luciferase reporter either alone or in conjunction with increasing amounts of an HA–ubiquitin expression vector (200 ng and 400 ng), an expression vector for FLAG–RelB (100 ng), or a combination of both expression vectors. The cells were incubated for 24 h, lysed in TNT buffer, and the dual luciferase analysis was performed as described in the Experimental section. (**C**) A similar NF-κB-dependent luciferase reporter analysis was performed with S107 pre-B-cells transiently transfected by electroporation with expression vectors for RelB (5 μg) or HA–ubiquitin (5 and 10 μg) as indicated.

we demonstrate that the polyubiquitination of the NF-κB subunit RelB is not only a required trigger for its proteolysis, but also an important signal to enhance its transactivation potential. Yet, the nuclear localization or the DNA-binding activity of RelB remained unaltered upon polyubiquitination. Remarkably, the polyubiquitination of RelB is highly variable, taking place at several lysine residues throughout the RelB protein. Yet, we identified lysine residues at positions 273, 274, 305 and 308 in RelB, which are critical for both the basal transcriptional activity and the ubiquitination-induced increase in the activity of RelB. Conclusively, we demonstrate in the present study that the ubiquitination of NF-κB subunits might be a novel regulatory mechanism in the control of the NF-κB-dependent gene expression.

EXPERIMENTAL

Cells, reagents and antibodies

Antibodies for RelB, NEMO, IKK2, NIK (Nck-interacting kinase), IκBα and the HA (haemagglutinin)-tag were purchased from Santa Cruz Biotechnology. An antibody specific for polyubiquitinated proteins was obtained from Biomol, and the anti-FLAG antibody and anti-FLAG-antibody-coupled sepharose was from Sigma. HEK (human embryonic kidney)-293 cells were propagated in DMEM (Dulbecco's modified Eagle's medium) plus 10 % FCS (foetal calf serum) containing streptomycin and penicillin. For the cultivation of the B-cell lines Namalwa, 70Z/3 and S107, RPMI medium plus 10 % FCS including streptomycin and penicillin was used. PMA was purchased **Q2**

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from Sigma–Aldirch, and ionomycin and MG132 were from Calbiochem.

Expression vectors and *in vitro* mutagenesis

The expression vectors encoding p50, p52, NIK, full-length FLAG–RelB or the various C-terminal RelB-deletion mutants have been described previously [11,12]. The different FLAG–RelB C-terminal and FLAG–RelB$_{240-320}$ vectors were cloned by inserting PCR-generated RelB fragments in-frame into the EcoRI and BamHI restriction sites of the pFLAG–CMV2 vector. The expression vectors for HA–ubiquitin, K48R-, K63R- or K48R/K63R-ubiquitin have been described previously [13]. The NF-κB-dependent luciferase reporter construct and the *Renilla* luciferase reporter construct under the control of the ubiquitin promoter have been described previously [14]. For the *in vitro* mutagenesis the QuikChange® *in vitro* mutagenesis kit was used following the manufacturer's protocol. Sequences of the oligonucleotides used for cloning or for *in vitro* mutagenesis are available upon request from the authors.

In vivo ubiquitination analysis

HEK-293 cells were transiently transfected with expression vectors coding for HA–ubiquitin and FLAG-tagged RelB and were lysed after 48 h in TNT buffer [200 mM NaCl, 20 mM Hepes (pH 7.6), 1 % (v/v) Triton X-100, 1 mM DTT (dithiothreitol), 20 mM NaF, 20 mM sodium glycerophophate, 2 μM leupeptin and 1 mM PMSF] including 0.5 % SDS or in RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 0,1 % SDS, 20 mM NaF, 20 mM sodium glycerophophate, 2 μM leupeptin and 1 mM PMSF] as indicated and were heated for 5 min at 95 °C to ensure the denaturation of the cellular proteins. The resulting lysates were used for anti-FLAG immunoprecipitations. The precipitates were washed extensively with TNT plus 0.5 % SDS or RIPA buffer and PBS, and were boiled for 5 min at 95 °C prior to the separation of the precipitated proteins by standard SDS/PAGE. The resulting membrane was subjected to immunoblot analyses with antibodies specific for the HA tag, the FLAG tag or RelB, as indicated.

Immunoprecipitation and immunoblot analysis

For immunoprecipitation of ectopically expressed or endogenous proteins 0.5–1 mg of protein extract was incubated with the appropriate antibody for 1 h on ice prior to the addition of 25 μl of 50 % slurry of Protein A–Sepharose (Santa Cruz Biotechnology) and an additional incubation for 1 h under agitation. The precipitates were subsequently washed with TNT plus 0.5 % SDS or RIPA buffer and PBS, and subjected to immunoblot analysis. For anti-FLAG immunoprecipitations, 10 μl of anti-FLAG-antibody-coupled sepharose (Sigma) was used. Immunoblots were performed according to standard protocols. In brief, the proteins were separated by SDS/PAGE (8–10 % gels), transferred on to nitrocellulose membrane (Whatman) and the resulting membrane was blocked for 1 h in TBS [Tris-buffered saline (???)] plus ??? Tween20 with 5 % (w/v) non-fat dried skimmed milk. Subsequently, the membrane was incubated overnight with the appropriate primary antibody in TBS plus Tween 20 with 5 % (w/v) non-fat dried skimmed milk, washed extensively, incubated for 1h in the appropriate HRP (horseradish peroxidase)-coupled secondary antibody prior to the detection with ECL (enhanced chemiluminescence) substrate (Pierce).
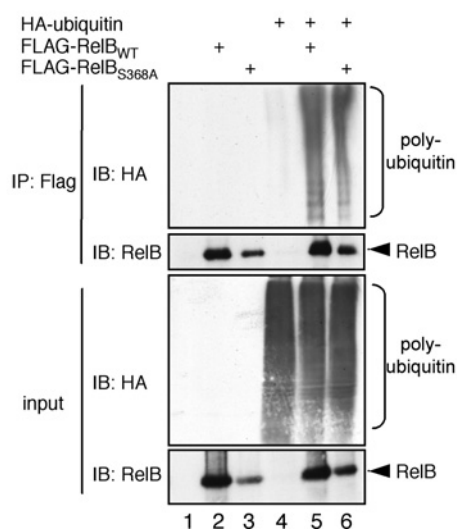
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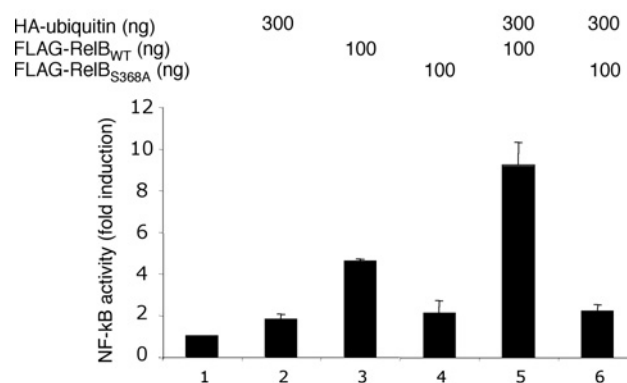
Figure 2   The ubiquitin effect on RelB activity depends on the DNA-binding potential of RelB

(**A**) NF-κB luciferase reporter assay with HEK-293 cells expressing either HA–ubiquitin, wild-type FLAG–RelB (FLAG-RelB$_{WT}$) or FLAG–S368A-RelB (FLAG-RelB$_{S368A}$) alone or in combination, as indicated. At 24 h post-transfection, the cells were harvested and the activity of the firefly as well as the *Renilla* luciferase was determined. (**B**) *In vivo* ubiquitination analysis of wild-type FLAG-RelB (FLAG-RelB$_{WT}$) or FLAG-S368A-RelB (FLAG-RelB$_{S368A}$). Ectopically expressed FLAG–RelB was immunoprecipitated (IP) under denaturing conditions (RIPA buffer) with anti-FLAG antibodies and probed in a subsequent immunoblot (IB) for HA–ubiquitin (top panel) and RelB (bottom panel). A fraction of the samples was subjected to anti-HA and anti-RelB immunoblots to ensure an efficient expression of the different proteins (input; lower panel).

Transfection and luciferase reporter assay

For the transfection of HEK-293 cells we used the calcium phosphate method. Briefly, on the day before transfection the cells were plated with a density of 50 % on an appropriate tissue culture dish. On the day of transfection the DNA was incubated with a 0.2 M CaCl$_2$ solution for 5 min prior to the addition of an appropriate volume of 2 × HeBS [280 mM NaCl, 1.5 mM Na$_2$HPO$_4$ and 50 mM Hepes (pH 7.04)]. After a further 5 min of incubation, the sample was added to the cells. For the electroporation of S107 pre-B-cells, 1×10^7 cells were mixed with DNA, incubated on ice for 5 min prior to a pulse with 250 V and 950 μF. The cells were subsequently transferred to a culture dish with the appropriate medium and incubated for 24–48 h. For the determination of the luciferase activity, the cells were lysed in TNT buffer and the activity of the *Renilla* luciferase, as well
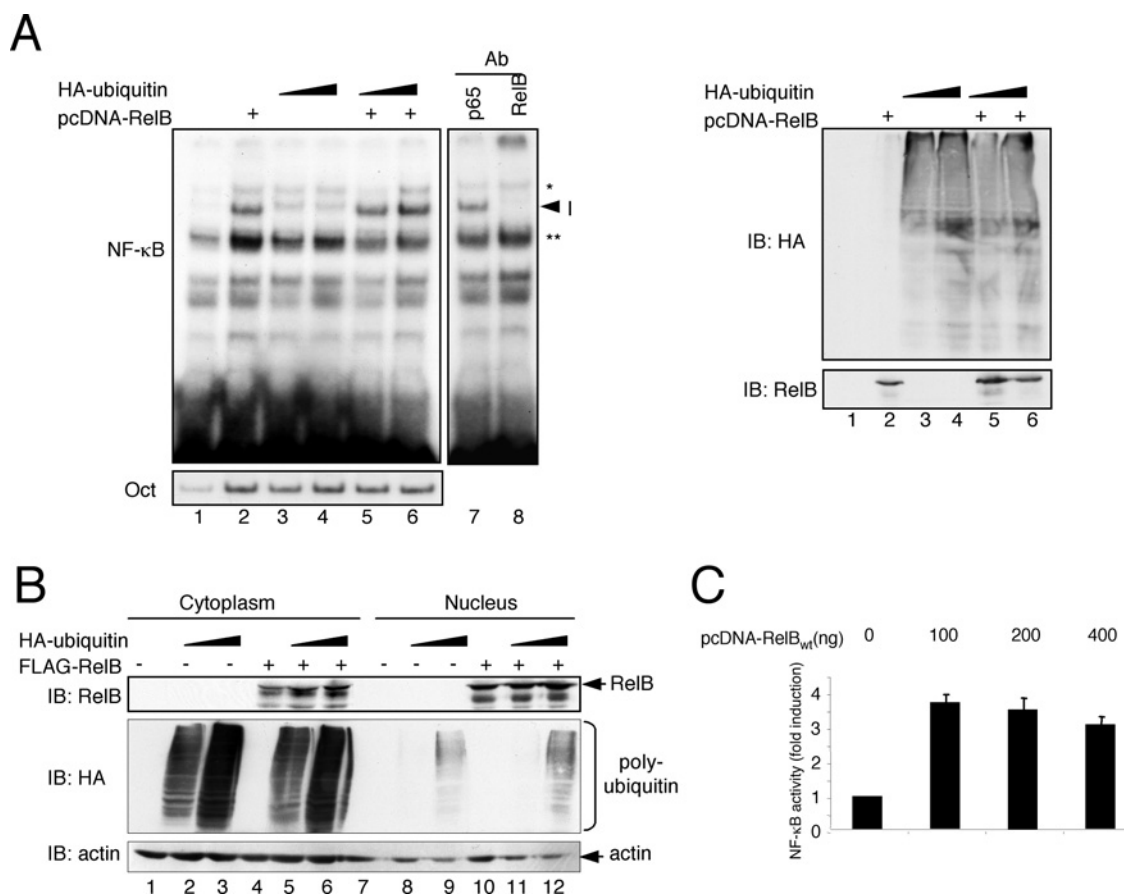
Figure 3 **DNA binding and nuclear expression of RelB remains unaltered upon ubiquitin co-transfection**

(**A**) Cells were transfected with expression vectors for RelB (1 μg) and HA–ubiquitin (0.5 and 1 μg) as indicated. After 48 h, the cells were lysed and the resulting whole cell extracts were subjected to gel-retardation studies with either a κB-specific (top panel) or an Oct-specific oligonucleotide. To ensure the identity of the RelB-containing complex (indicated as I) supershift analysis was performed with antibodies specific for RelA or RelB using extracts from cells transfected with RelB and HA–ubiquitin (lanes 7 and 8). * and ** indicate unspecific bands. Western blot analysis was performed to control for the expression of FLAG–RelB and HA–ubiquitin (right-hand panel). Ab, antibody; IB, immunoblot. (**B**) For the determination of the nuclear localization of RelB, nuclear and cytoplasmic extracts from HEK-293 cells which ectopically express either RelB (lanes 4 and 10), increasing amounts of HA–ubiquitin (lanes 2 and 3, and lanes 8 and 9) or both proteins (lanes 5 and 6, and lanes 11 and 12) were generated and subjected to Western blot analyses with the indicated antibodies. IB, immunoblot. (**C**) An NF-κB-reporter assay was performed after transient transfection of HEK-293 cells with the indicated amounts of a FLAG–RelB expression vector.

as the firefly luciferase, was measured according to the Dual-Glo luciferase assay protocol (Promega). For the calculation of the basal transcriptional activity the increase in activity obtained with wild-type RelB was set arbitrarily to 100%. For the calculation of the ubiquitin-induced augmentation the increase in fold-induction in samples including ubiquitin was divided by the value without ubiquitin, the resulting value for wild-type RelB was set arbitrarily to 100%. Both calculations are based on at least four independent experiments and the mean value $\pm$ S.D. are stated.

RESULTS

Since RelB is able to act either as a positive or as a negative effector of NF-κB-dependent gene expression we set out to investigate whether a non-proteasomal ubiquitination might be involved in the regulation of RelB functions. To determine whether RelB could be ubiquitinated *in vivo*, we established an *in vivo* ubiquitination assay using ectopical expression of HA–ubiquitin, FLAG–RelB or, as positive control, FLAG–NEMO, in HEK-293 cells as indicated in Figure 1(A). With the resulting

whole cell extracts we performed two successive rounds of anti-FLAG immunoprecipitations, using first mild and in the second round more stringent conditions including the addition of 0.5% SDS. An anti-HA signal was detected after both rounds of immunoprecipitation, implying a constitutive *in vivo* polyubiquitination of RelB under these experimental conditions. Consistent with the hypothesis that polyubiquitination alters the function of RelB, we observed a significant augmentation of the RelB activity in NF-κB reporter-gene assays using HEK-293 cells (Figure 1B) including either increasing amounts of an ubiquitin-expression vector alone or in combination with a RelB-encoding vector. Ectopical expression of HA–ubiquitin alone had no effect on the NF-κB-dependent reporter gene expression. Similar results were obtained with S107 pre-B-cells which displayed a very low basal NF-κB activity due to a loss of RelB-expression [15] and which are defective in various NF-κB signalling pathways (Figure 1C).

Next, we determined whether the observed positive effect of ubiquitin co-expression on the RelB activity was based on the DNA-binding capability of RelB. A RelB mutant with a serine-to-alanine substitution at position 368 (S368A-RelB) is defective in DNA binding and heterodimer formation with other
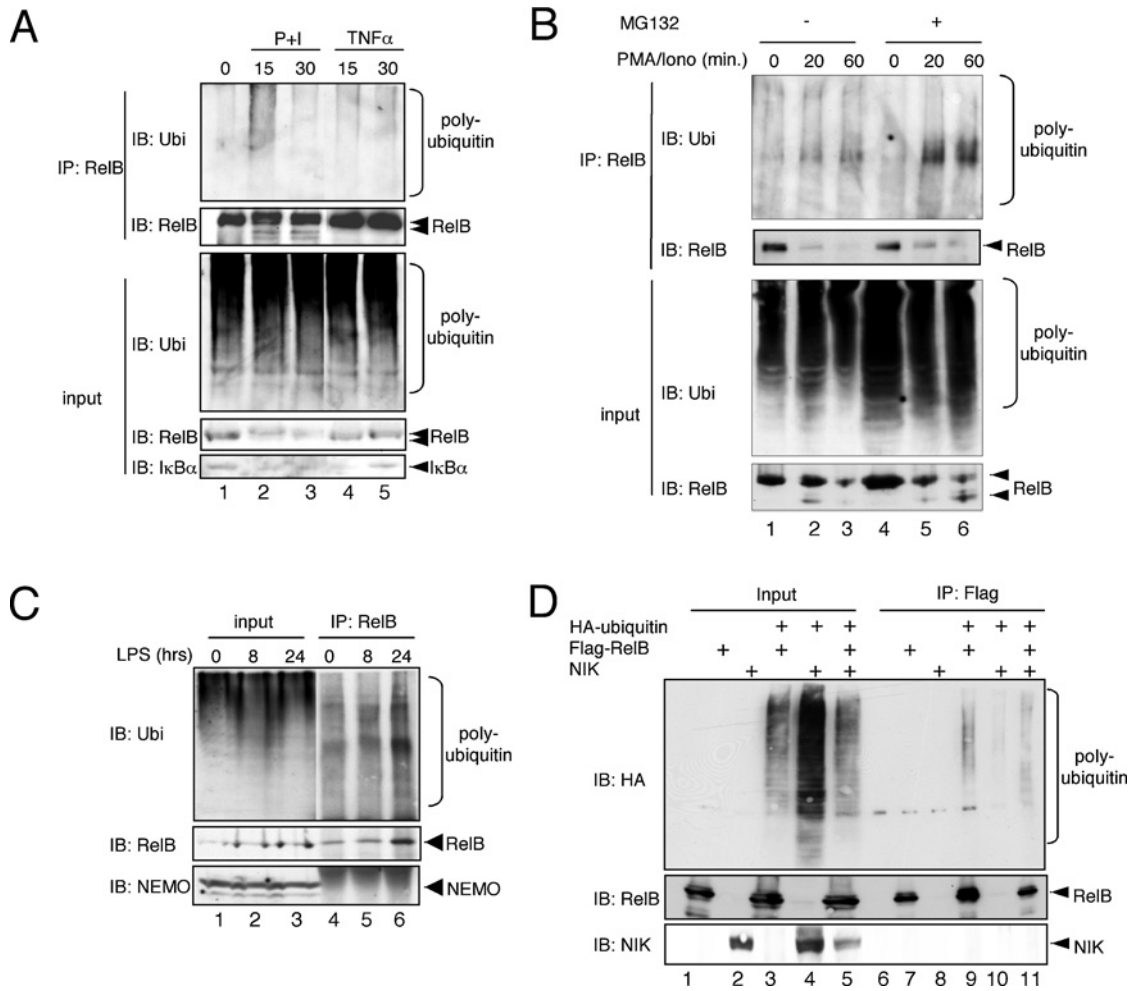
Figure 4   Ubiquitination analysis of endogenous RelB B cell lines

(**A**) Namalwa B-cells were either left untreated or were stimulated with PMA+ionomycin (P+I) or TNFα for the indicated periods of time, lysed with RIPA buffer and the resulting whole cell extracts were subjected to an anti-RelB immunoprecipitation (IP) analysis. Anti-RelB and anti-IκBα immunoblots were additionally performed to control the efficiency of the stimulation. IB, immunoblot; Ubi, ubiquitin. (**B**) Namalwa cells were either left untreated or were pretreated with MG132 (25 μM) for 30 min prior to stimulation with PMA + ionomycin (PMA/Iono) for the indicated time points. The cells were lysed in RIPA buffer, the resulting whole cell extracts were used for anti-RelB immunoprecipitation (IP) prior to immunoblot (IB) analysis with the indicated antibodies. Ubi, ubiquitin. (**C**) 70Z/3 pre-B-cells were either left untreated or were stimulated with 1 μg/ml LPS for 8 or 24 h prior to lysis in RIPA buffer. The resulting whole cell extracts were subjected to an anti-RelB immunoprecipitation (IP) analysis. IB, immunoblot. (**D**) *In vivo* ubiquitination analysis with whole cell extracts from HEK-293 cells transiently transfected with expression vectors for FLAG–RelB, HA–ubiquitin without or with an additional NIK expression vector. IP, immunoprecipitation; IB, immunoblot.

NF-κB factors, such as p50 or p52 [12]. Consistently, S368A-RelB was found to be inactive in NF-κB reporter-gene assays (Figure 2A). Furthermore, in contrast with the situation using wild-type RelB, we observed no positive effect on the activity of S368A-RelB upon addition of ubiquitin whereas the level of polyubiquitination was indistinguishable between wild-type and S368A-RelB (Figures 2A, lanes 4 and 6, and 2B, lanes 5 and 6) suggesting that the DNA binding and/or the heterodimer formation is necessary for the observed ubiquitin effect on the activity of RelB.

To determine the molecular mechanism underlying the increased RelB activity observed in the NF-κB reporter-gene assay, we analysed the potential effect of polyubiquitination on nuclear localization and DNA binding of RelB. As shown in Figure 3(A), neither the basal NF-κB DNA-binding activity in HEK-293 cells nor the binding of the ectopically expressed RelB was affected by the addition of HA–ubiquitin, suggesting that the ubiquitination-dependent enhancement of the RelB

activity is not caused by an increased DNA binding of RelB. Moreover, the analysis of the subcellular distribution of the ectopically expressed FLAG–RelB revealed no differences in the nuclear RelB levels regardless of the presence or absence of HA–ubiquitin (Figure 3B, lanes 10–12). In addition, also an ubiquitin-induced moderate increase of RelB levels, which we occasionally observed, is most probably not the basis for the increased NF-κB activity since the RelB-mediated NF-κB activity remained stable even with high amounts of RelB expression vector (Figure 3C), probably due to the lack of sufficient amounts of endogenous p50 or p52 required for active RelB complexes.

RelB plays a key role in B-cell biology and is highly expressed in different B-cell lines [16–18]. An analysis of the polyubiquitination of the endogenous RelB in Namalwa B cells revealed a moderate basal RelB polyubiquitination in unstimulated Namalwa cells (Figure 4A, lane 1). This moderate RelB polyubiquitination remained stable upon stimulation with TNFα (tumour necrosis factor α; Figure 4A, lanes 4 and
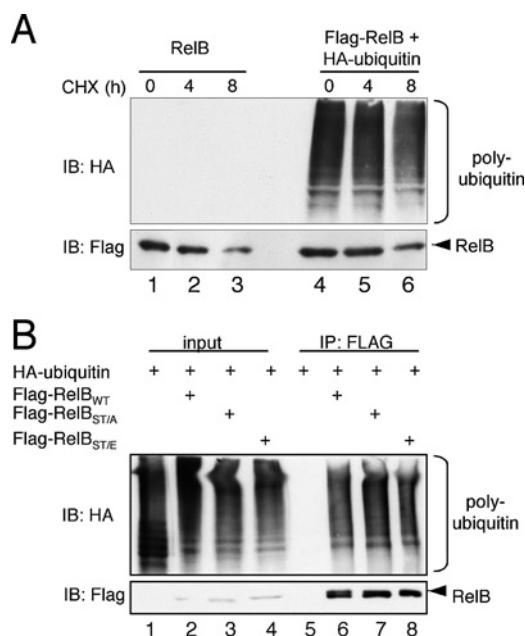
Figure 5   RelB stability remains unaltered by ubiquitin coexpression

(**A**) To analyse the impact of ubiquitin on the half-life of RelB, cells ectopically expressing either FLAG–RelB alone (lanes 1–3) or in conjunction with HA–ubiquitin (lanes 4–6) were either left untreated or were treated with cycloheximide (CHX; 50 ng/ml) for the indicated times prior to cell lysis. The resulting whole cell extracts were used for Western blot analyses with the indicated antibodies. IB, immunoblot. (**B**) *In vivo* ubiquitination assay with wild-type FLAG–RelB (lanes 2 and 6), with a FLAG-RelB$_{ST/A}$ mutant (T84A/S552A, lanes 3 and 7) or with a FLAG-RelB$_{ST/E}$-mutant (T84E/S552E, lanes 4 and 8). IB, immunoblot.

5). In contrast, the stimulation with PMA+ionomycin induced a significant increase in RelB polyubiquitination (Figure 4A, compare lanes 1 and 2). Previously, we reported a proteasomal-dependent degradation of RelB upon PMA + ionomycin stimulation of T-cells. Indeed, we also observed a decrease of cellular RelB in PMA + ionomycin-stimulated Namalwa B cells (Figure 4A, compare lanes 1,2 and 3), which correlated with the appearance of a faster-migrating RelB signal and an increased polyubiquitination of RelB (Figure 4A). Consistent **Q5** with our previously published results [??], the pretreatment of Namalwa cells with the proteasome inhibitor MG132 led to a partial stabilization of the faster-migrating RelB-degradation products (Figure 4B, compare lanes 1–3 with lanes 4–6) which correlated with an increased RelB polyubiquitination suggesting that the augmented RelB polyubiquitination observed upon PMA + ionomycin stimulation is linked to a proteasomal-dependent degradation of RelB.

LPS (lipopolysaccharide) has been demonstrated to induce the alternative NF-κB pathway, which depends on the activity of RelB. Thus we analysed whether an LPS stimulation for 8 or 24 h had an effect on RelB polyubiquitination levels. As shown in Figure 4(C), we indeed observed an increased ubiquitin signal after RelB immunoprecipitation. The RelB levels also were augmented, suggesting that there is no net increase in RelB polyubiquitination by LPS stimulation. To further analyse whether RelB polyubiquitination might be rendered triggering the alternative NF-κB signalling pathway, we performed an *in vivo* RelB-ubiquitination analysis without or with the addition of the protein kinase NIK, a crucial upstream component of the alternative NF-κB pathway [3]. Again, the levels of RelB polyubiquitination remained largely unaltered upon NIK co-

expression. However, we observed a slight change in the pattern of the RelB polyubiquitination by NIK co-expression (Figure 4D, compare lanes 9 and 11).

Based on these results, the possibility remained that the RelB polyubiquitination in HEK-293 cells is simply a part of the proteasomal degradation process of RelB. However, we observed no significant reduction of the half-life time of ectopically expressed RelB with or without the additional expression of HA–ubiquitin (Figure 5A). A site-specific phosphorylation often triggers the subsequent ubiquitination of a target protein, and the degradation of RelB is linked to its phosphorylation at Thr[84] and Ser[552] [10]. Yet RelB mutants with either inactivating alanine substitutions (FLAG–RelB-ST/A) or phosphomimetic glutamic acid substitutions (-ST/E) at Thr[84] and Ser[552] revealed no significant differences regarding their *in vivo* polyubiquitination levels (Figure 5B). These results collectively imply that the basal polyubiquitination of RelB is not linked to the proteolysis of this transcription factor but might be involved in the regulation of the transcriptional activity of RelB.

The lysine residue in ubiquitin used for polyubiquitin chain conjugation determines the biological function of the polyubiquitination, with a Lys[48]-conjugated polyubiquitin chain subjecting the target protein to proteasomal degradation and a Lys[63]-conjugated polyubiquitin chain responsible for most non-proteasomal functions. Therefore we next compared wild-type HA–ubiquitin with HA–ubiquitin mutants harbouring either a K48R or a K63R substitution in an *in vivo* RelB polyubiquitination assay. Surprisingly, both ubiquitin mutants were efficiently attached to RelB *in vivo* (Figure 6A, lanes 3–5). Similar results were obtained with NEMO, another known ubiquitination target protein, which we used as a positive control (Figure 6C). In addition, both ubiquitin mutants, K48R- and K63R-ubiquitin, augmented the RelB activity in NF-κB reporter-gene assays (Figure 6B). Surprisingly, an efficient RelB polyubiquitination in HEK-293 cells was also observed with a HA-tagged ubiquitin mutant harbouring combined arginine substitutions of Lys[48] and Lys[63] (Figure 6D) regardless of the presence or absence of MG132, suggesting that RelB polyubiquitination involves unconventional polyubiquitin conjugation.

In order to map the RelB domain(s) containing the ubiquitin-acceptor site(s) we performed *in vivo* ubiquitination assays with different C-terminal FLAG-tagged RelB-deletion mutants (for a schematic representation of the mutants used, see Figure 7A). Surprisingly, all FLAG–RelB mutants used were polyubiquitinated using either wild-type or K48R/K63R-ubiquitin (Figure 7B, upper panel, lanes 8–12 and 14–18; please note that the low polyubiquitination level of full-length RelB is due to a lower expression level of this protein), although the polyubiquitination levels were lower in the case of the RelB-deletion mutant spanning the amino acids 348–552 (RelB$_{CT}$). Thus we concluded from these results that several ubiquitination acceptor sites must exist which are located throughout the entire RelB protein.

To define the lysine residues critical for the activity of RelB, we performed a mutational-scanning analysis substituting the 21 lysine residues in the RelB protein with arginine residues as indicated (Figure 8A). Subsequently, the various RelB mutants with single or combined lysine-to-arginine substitutions were tested regarding their basal activity and ubiquitination-induced activity increase in NF-κB reporter-gene assays (Figures 8B and 8C). Here, only the mutants M7 (K220R), and more pronounced M9 (K273/274R) and M11 (K305/308R), displayed a significantly reduced basal activity (Figure 8B), which could not be fully restored by the addition of p50 or p52 expression vectors, with the exception of the M7-p50 combination (Figure 8D).
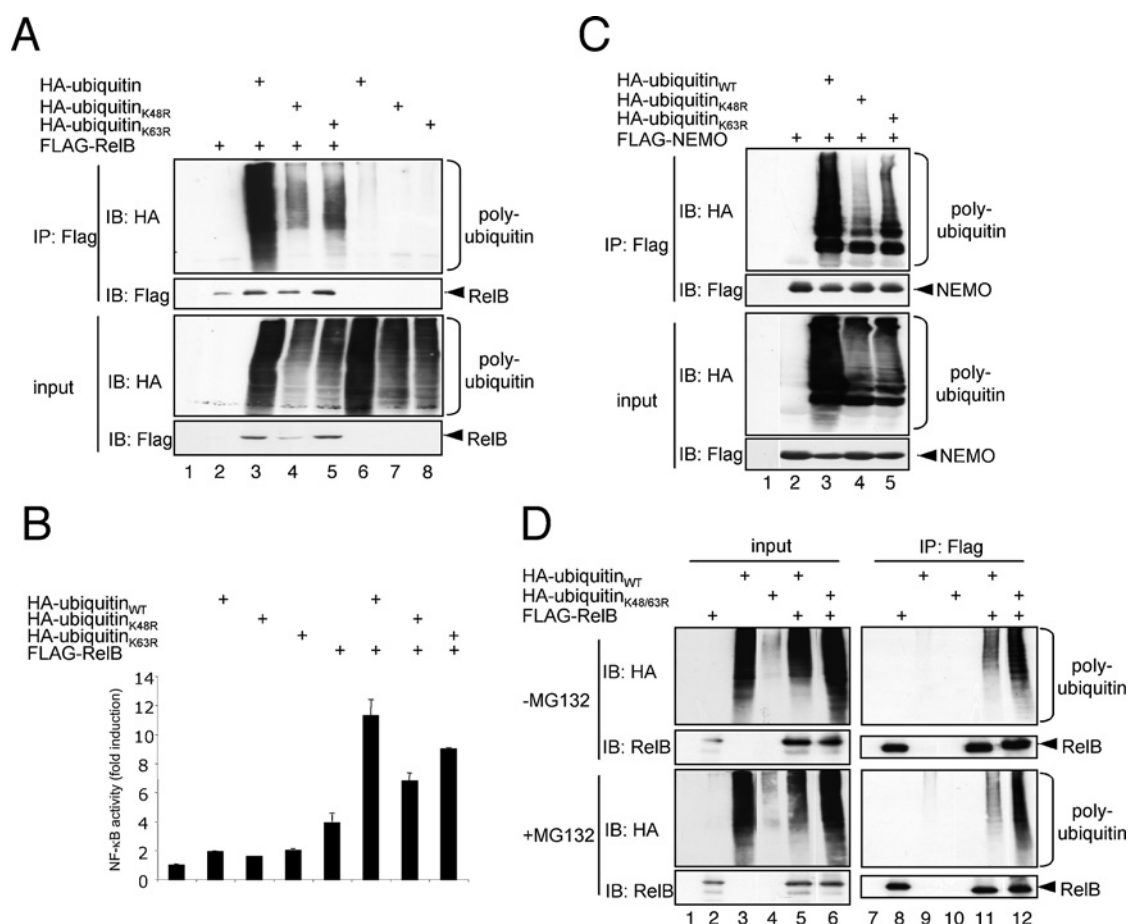
Figure 6   Characterization of the polyubiquitin conjugates attached to RelB

For an *in vivo* ubiquitination assay of FLAG–RelB (**A**) or FLAG–NEMO (**C**), HEK-293 cells were transiently transfected with 1 μg of FLAG–RelB or FLAG–NEMO alone or in conjunction with 1 μg of expression vectors for wild-type HA–ubiquitin or HA–ubiquitin mutants with an arginine substitution of either Lys^{48} (K48R) or Lys^{63} (K63R) as indicated. The cells were lysed in RIPA buffer and the resulting whole cell extracts were subjected to an anti-FLAG immunoprecipitation (IP) under denaturing conditions prior to immunoblot (IB) analyses with the indicated antibodies. (**B**) NF-κB reporter-gene assay after transient transfection of HEK-293 cells with 100 ng of the FLAG–RelB vector alone or in combination with 400 ng of the different HA–ubiquitin variants as indicated. (**D**) *In vivo* ubiquitination analysis of FLAG–RelB in conjunction with a HA–K48R/K63R-ubiquitin mutant (HA–ubiquitin$_{K48/63R}$). The cells were either left untreated (top panels) or were pretreated with the proteasome inhibitor MG132 (bottom panels). IB, immunoblot; IP, immunoprecipitation.

Interestingly, the same three RelB mutants also showed a less pronounced ubiquitin-induced activity increase (Figure 8C), implicating a critical role of these lysine residues for the ubiquitination-based augmentation of RelB activity. However, all of the lysine residues within M7, M9 and M11 are located in the RHD (Rel homology domain) required for dimerization and DNA binding of RelB. To exclude the possibility that the substitution of Lys^{220}, Lys$^{273/274}$ or Lys$^{305/308}$ affect the DNA binding of the M7, M9 and M11 mutants, we tested these RelB mutants in a gel-retardation assay with a NF-κB-specific probe and included the M10 mutant as a control. Neither of the different RelB variants tested revealed alterations in their DNA-binding capacity (Figure 9A, upper panel, lanes 3–7). Consistent with the high degree of variability of RelB polyubiquitination observed with the different deletion mutants, the *in vivo* polyubiquitination of the M7, M9, M10 and M11 mutants remained unaltered, regardless of whether wild-type HA–ubiquitin (Figure 9B, lanes 6–11) or the HA–K48R/K63R-ubiquitin mutant (Figure 9B, lanes 12–17) was used. Indeed, a significant effect on the *in vivo* polyubiquitination was generally only detectable upon combined substitution of various lysine residues as shown for the substitution of all lysine residues in the C-terminus of RelB (K386: Lys^{386}, Lys^{387} and

Lys^{389}; K410: Lys^{410}, Lys^{413}, Lys^{414}, Lys^{415}; Figure 10A, lanes 7–10 and Figure 10B, lanes 2–4). Taken together, these results suggest that among the various ubiquitination-target sites only the polyubiquitination at specific lysine residues of RelB mediate the positive effect on its transcriptional activity.

DISCUSSION

The NF-κB transcription factors regulate a wide variety of cellular processes, including inflammatory and immune responses [2]. Several of the control mechanisms ensuring the temporal and spatial activity of NF-κB include post-translational modifications of the different NF-κB subunits. For example, a tightly regulated recruitment of co-activators or co-repressors, such as CBP or HDAC1 (histone deacetylase 1) respectively, depends on site-specific phosphorylations or acetylations of RelA [6,7]. In contrast, the molecular mechanisms which determine whether RelB acts as either a gene-specific transcriptional activator or repressor are currently unknown. In the present study, we aimed to analyse whether a specific ubiquitination of RelB might be involved in the regulation of RelB functions. Indeed, we
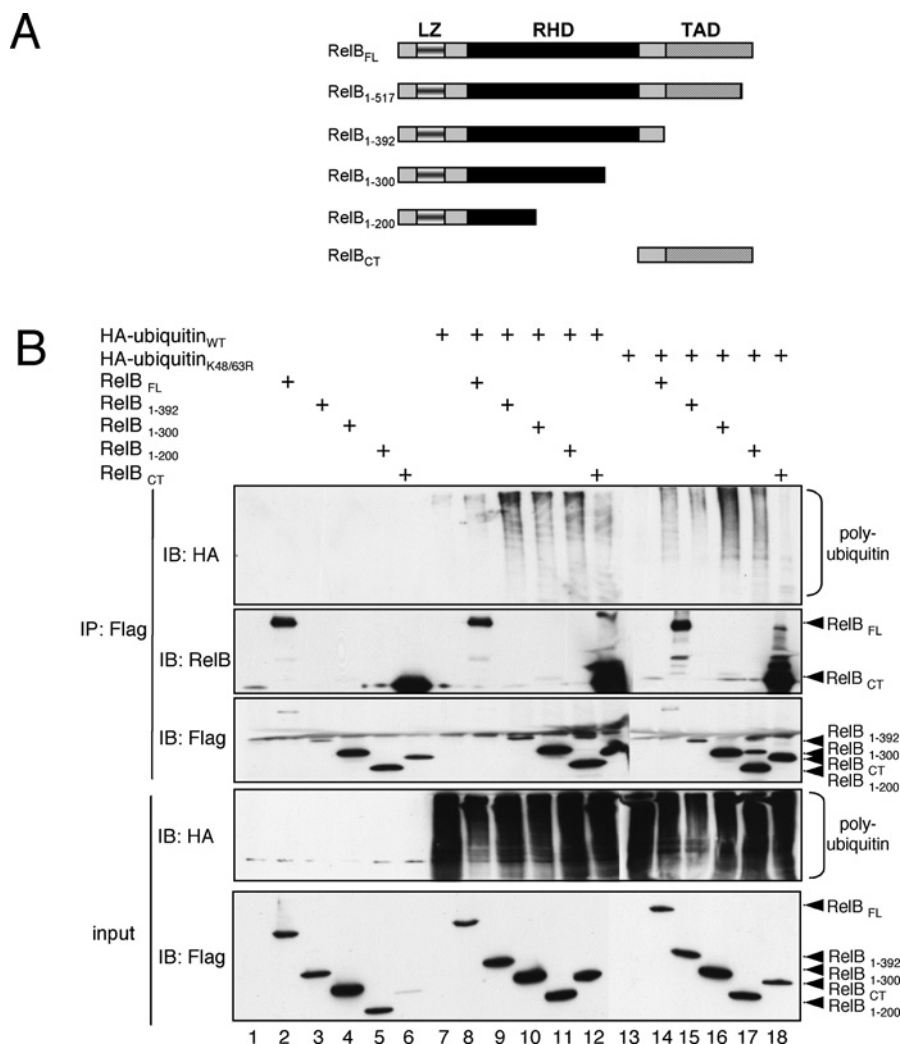
Figure 7   Mapping of the RelB domain modified by ubiquitin attachment

Q6   (**A**) Schematic representation of the FLAG-tagged RelB deletion mutants used. LZ, ?????. (**B**) *In vivo* ubiquitination assay of ectopically expressed FLAG-tagged RelB deletion mutants in combination with either wild-type HA–ubiquitin (lanes 7–12) or K48R/K63R-ubiquitin (lanes 13–18). A 90 % proportion of whole cell extracts were used for an anti-FLAG immunoprecipitation (IP) under denaturing conditions (IP: Flag; top panels) and 10 % was used for control Western blot analysis (input; lower panel). The anti-RelB immunoblot was included to monitor the expression levels of full-length RelB. IB, immunoblot.

observed a distinct *in vivo* polyubiquitination of RelB upon ectopic co-expression of FLAG–RelB and HA–ubiquitin as well as in unstimulated Namalwa or 70Z/3 B cells. In addition, RelB polyubiquitination was further increased by stimulation of the cells with PMA+ionomycin. However, this increase in RelB polyubiquitination is most probably linked to the proteasomal degradation of RelB since we observed a correlation between an augmented RelB polyubiquitination and a partial RelB stabilization upon pre-treatment with the proteasome inhibitor MG132. Proteasomal degradation of various NF-κB subunits, such as RelA/p65, RelB or NF-κB1/p50 has been described previously [10,19,20]. In contrast, the unchanged half-life time of RelB upon ubiquitin co-expression (Figure 2), the constitutive RelB polyubiquitination by K48R-ubiquitin (Figure 5), and the normal polyubiquitination of the Ser[84] and Thr[552] mutants of RelB (Figure 2) pointed collectively to additional non-proteasomal functions of the basal RelB polyubiquitination detected in unstimulated HEK-293, 70Z/3 and Namalwa cells (Figures 1 and 4). The increased RelB activity observed by the addition of ubiquitin, as shown by reporter-gene assays, suggests that the augmentation of the transactivational potential of RelB might be one of these additional non-proteasomal functions. As an ubiquitin-induced increase in nuclear expression or DNA binding of RelB was not observed, we would like to speculate that polyubiquitination of RelB might affect its interaction with transcriptional co-activators or co-repressors similar to the reported ubiquitin-dependent interaction of c-Myc with p300 [9]. This notion is also supported by the fact that the ubiquitin effect was not observed using the S368A-RelB mutant, which is incapable of interacting with p50 or p52, and thus incapable of interacting with DNA. Indeed, RelB has been shown to interact with the repressor protein Daxx [4,5]. However, whether the RelB polyubiquitination causes a reduced Daxx interaction of this transcription factor is currently unknown. The occasionally observed increased RelB levels do not probably contribute to the augmented RelB activity, as transfection of increasing amounts of a RelB expression vector led to similar results in NF-κB reporter assays, probably due to low levels of the RelB interaction partners p50 or p52. Usually, the Lys[48]-conjugated polyubiquitin chains assign the ubiquitinated protein for a proteasomal degradation,
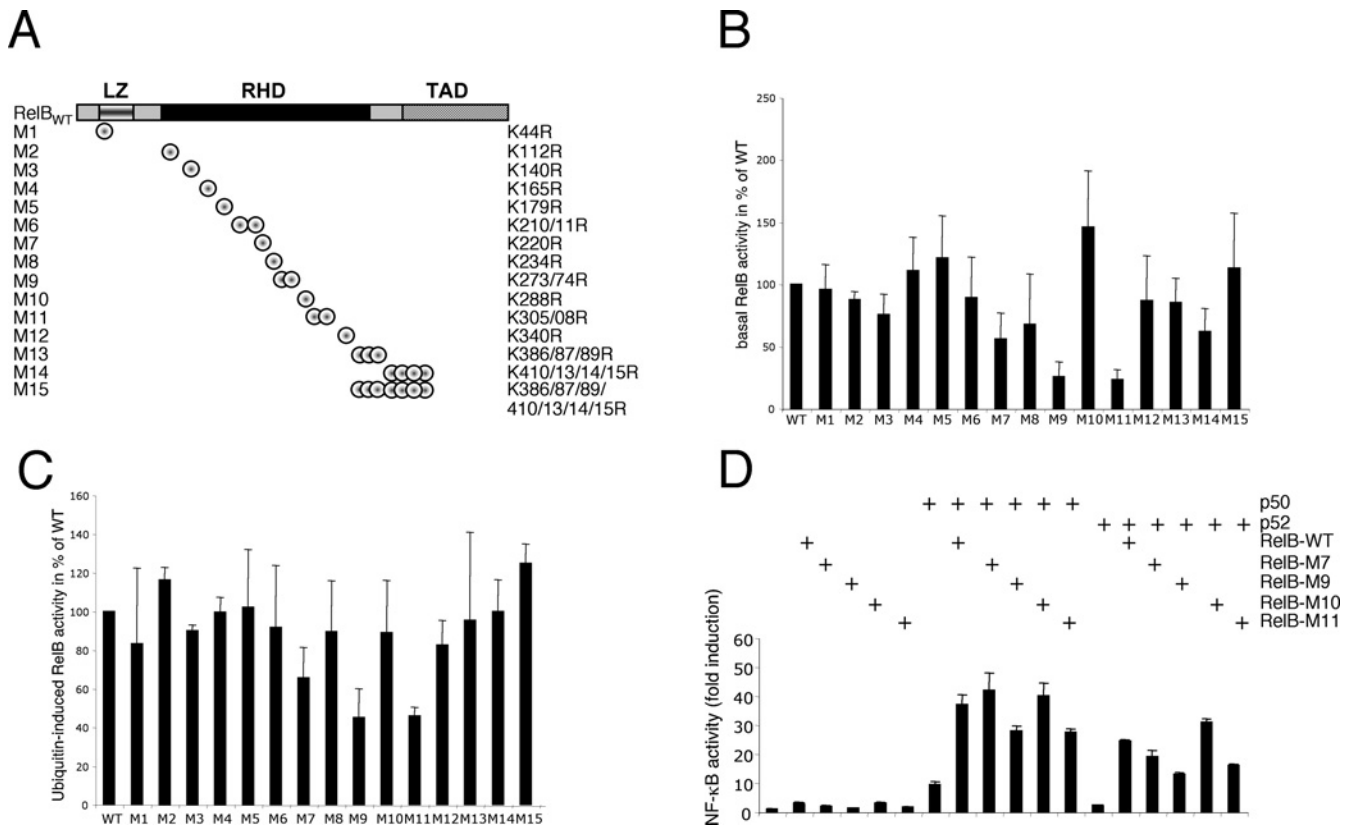
Figure 8   Identification of the lysine residues critical for the basal activity of RelB and the ubiquitin-induced augmentation of RelB-activity

Q6

(**A**) Schematic representation of the RelB mutants with lysine-to-arginine substitutions. Note: some of the mutants harbour a single mutation and several mutants contain the combined substitutions of closely located lysine residues. LZ, ????. (**B**) NF-κB reporter-gene assays. The relative basal activity of the various RelB mutants in comparison with wild-type RelB is depicted. The increase in NF-κB activity mediated by wild-type (WT) RelB was arbitrarily set to 100%, the results from at least four independent experiments were included. (**C**) Comparison of the ubiquitin-induced activity increase of the different RelB variants as monitored by NF-κB reporter-gene assays. Here, the fold-increase in activity upon HA–ubiquitin co-expression in comparison with the activity of the respective RelB variant alone is depicted in relation to wild-type (WT) RelB (arbitrarily set to 100%). (**D**) NF-κB reporter gene analysis with the indicated RelB variants alone or in combination with p50 or p52 expression vectors. WT, wild-type.
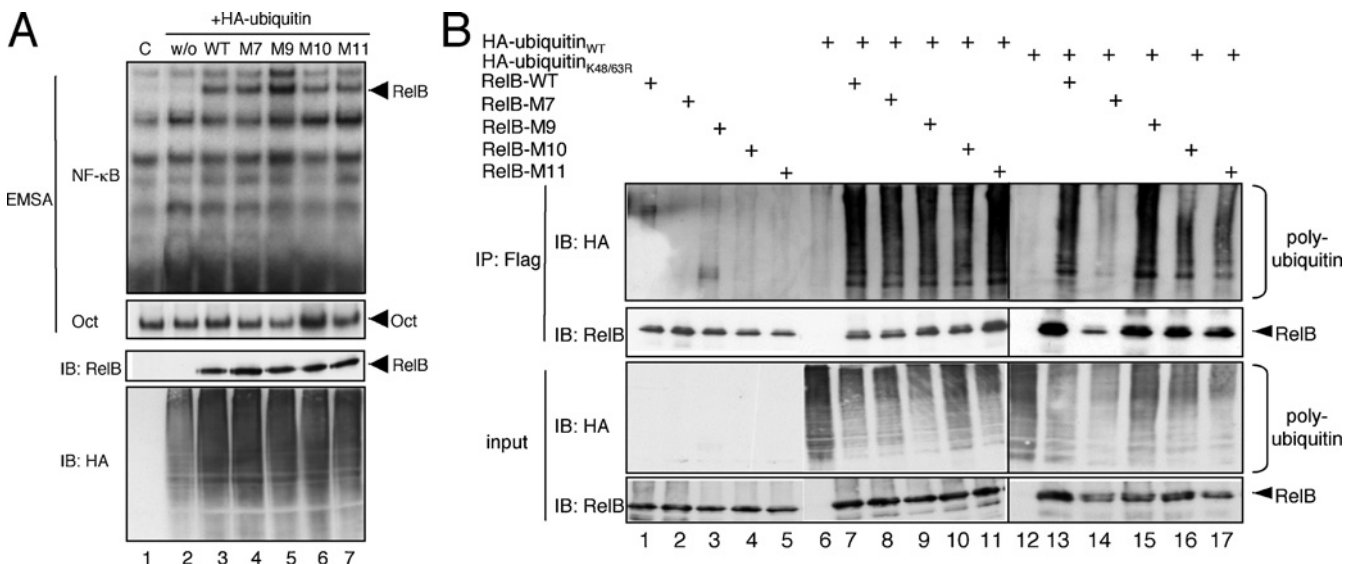

Figure 9   Characterization of selected RelB mutants with lysine-to-arginine substitutions

Q7

(**A**) Gel-retardation analysis of selected RelB mutants ectopically expressed in conjunction with HA–ubiquitin. For the gel-retardation assay either a ^{32}P-labelled κB-specific probe (EMSA; top panel) or Oct-specific probe (EMSA; bottom panel) was used. Immunoblot (IB) analyses with the indicated antibodies were performed to ensure similar expression levels of the RelB variants or HA–ubiquitin (IB: RelB and IB: HA). w/o, without; WT, wild-type. (**B**) *In vivo* ubiquitination analysis under denaturing conditions of the indicated RelB variants using either wild-type HA–ubiquitin (lanes 6–11) or HA–K48R/K63R-ubiquitin (lanes 12–17). IB, immunoblot; IP, immunoprecipitation; WT, wild-type.
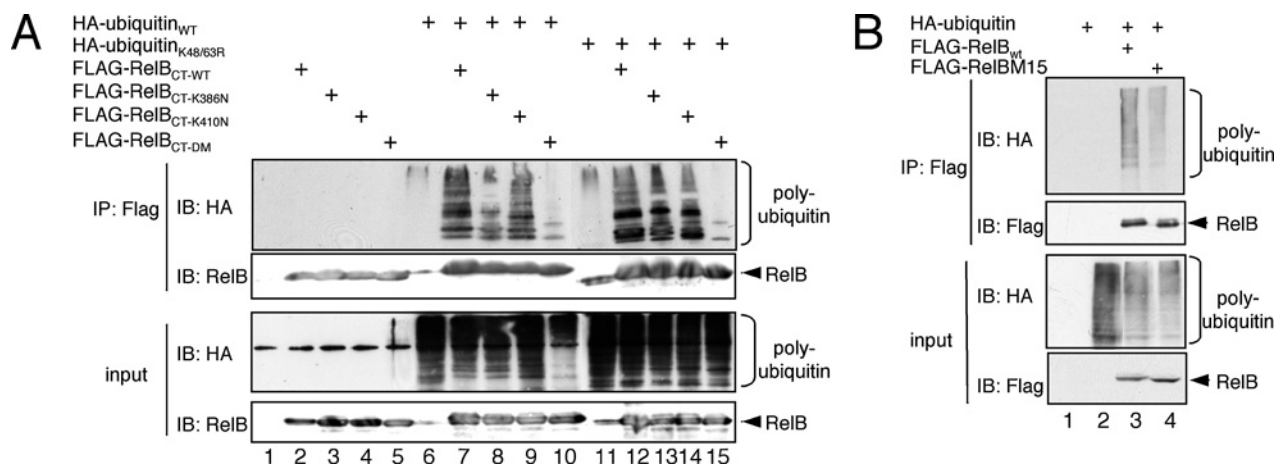
Figure 10   Analysis of the polyubiquitination of the C-terminus of RelB

(**A**) *In vivo* ubiquitination analysis of the C-terminal FLAG-tagged RelB deletion mutants carrying either asparagine substitutions at the positions Lys[386], Lys[387], Lys[389] (K386N, lanes 4 and 8), at positions Lys[410], Lys[413], Lys[414] and Lys[415] (K410N, lanes 5 and 9) or at all positions (CT-DM), in comparison with the wild-type (WT) counterpart (lanes 6 and 10). (**B**) A similar *in vivo* ubiquitination analysis of wild-type FLAG–RelB or the FLAG–RelB M15 mutant harbouring lysine-to-arginine substitutions at positions 386, 387, 389, 410, 413 and 415. IB, immunoblot; IP, immunoprecipitation.

whereas the formation of Lys[63]-conjugated polyubiquitin chains renders the protein-interaction pattern of the target protein [21]. However, Lys[48] and Lys[63] are not the only lysine residues in ubiquitin used for conjugation. Furthermore, mixed ubiquitin conjugates have been identified [22]. Thus the efficient RelB polyubiquitination seen with the K48R/K63R-ubiquitin double mutant could either be caused by the formation of mixed ubiquitin conjugates, probably including endogenous ubiquitin moieties, or by lysine residues other than Lys[48] and Lys[63] [23]. NEMO seems to be another protein modified by differently conjugated polyubiquitin chains, since we obtained similar results using NEMO with regards to the polyubiquitination with K48R- and K63R-ubiquitin. Indeed, besides the Lys[63]-conjugated NEMO polyubiquitination a Lys[6]-conjugated NEMO polyubiquitination has also been described [23]. Mapping of the RelB domains containing the polyubiquitination sites revealed the existence of various polyubiquitination sites in all RelB-domains analysed. Although the low specificity of protein ubiquitination has been reported for other ubiquitination targets, for instance c-Myc or MALT1 (mucosa-associated lymphoid tissue translocation gene 1) [9,13], the high degree of redundancy of polyubiquitination target sites in RelB is still surprising. However, the ubiquitination of the different lysine residues in RelB is not equally important since only the inactivation of Lys[220] (M7) and more pronounced of Lys[273/274] (M9) or Lys[305/308] (M11) had a profound effect on their basal activity as well as the ubiquitination-based activity increase, which cannot be explained by an affected DNA binding. The specific role of Lys[273/274] (M9) and Lys[305/308] (M11) is furthermore highlighted by the fact that the activity of the M15 mutant with a complete inactivation of the lysine residues in the C-terminal TAD of RelB remained unaltered. Thus it is possible that the **Q8** polyubiquitination at these particular lysine residues [Lys[273/274] (M9) or Lys[305/308] (M11)] affects the recruitment of transcriptional co-activators or the release of co-repressors of unknown identity. The co-activator CBP, known to interact with the NF-κB subunit RelA [7], seems to not be responsible for the ubiquitin effect, as an interaction of CBP with RelB was not detectable regardless of the absence or presence of ubiquitin (results not shown). Interestingly, polyubiquitination could be a general regulatory mechanism to control the activity of nuclear NF-κB, probably independent of the activation of the IKK complex. Therefore the reason for the reduced basal transcriptional activity of the M9 and

M11 RelB mutants might be the requirement of a constitutive but moderate polyubiquitination of RelB. Alternatively, other post-translational modifications occurring at lysine residues, for instance an acetylation or the attachment of SUMO moieties, could also be involved. Indeed, we already observed an *in vivo* acetylation and SUMOylation of RelB in HEK-293 cells (results not shown).

Conclusively, we demonstrate in the present study, that the NF-κB subunit RelB is polyubiquitinated at various lysine residues *in vivo* and the inactivation of specific lysine residues in RelB leads to a decreased transcriptional activity of this transcription factor. However, several important questions remain to be answered in future experiments. First, it will be necessary to define the nature of polyubiquitin chain conjugation involved in RelB polyubiquitination. Another important question pertains to the identity of the ubiquitin ligases or deubiquitinases involved in the polyubiquitination of RelB. One feasible candidate for an E3 ligase involved in the proteolysis-linked RelB polyubiquitination might be COMMD1 [copper metabolism (Murr1) domain containing 1], which mediates the ubiquitination and subsequent degradation of the NF-κB family member RelA [24]. Furthermore, it will be interesting to explore the molecular mechanism by which the activity of RelB is altered, especially the identity of the recruited co-activators or released co-repressors, such as Daxx. Finally, we also want to determine whether other NF-κB family members are modified by non-proteosomal ubiquitination. Indeed, we obtained similar results regarding an *in vivo* ubiquitination and augmentation of the transcriptional activity using the NF-κB subunit RelA (J. Leidner and R. Marienfeld, unpublished work), suggesting that this kind of post-translational modification could be a general regulatory mechanism for the control of the NF-κB system.   **Q9**

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Identification of lysine residues critical for the transcriptional activity and polyubiquitination of the NF-κB family member RelB

Julia LEIDNER*, Lysann PALKOWITSCH*, Uta MARIENFELD*, Dietmar FISCHER† and Ralf MARIENFELD*[1]

*Institute of Physiological Chemistry, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany, and †Institute of Experimental Neurology, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany
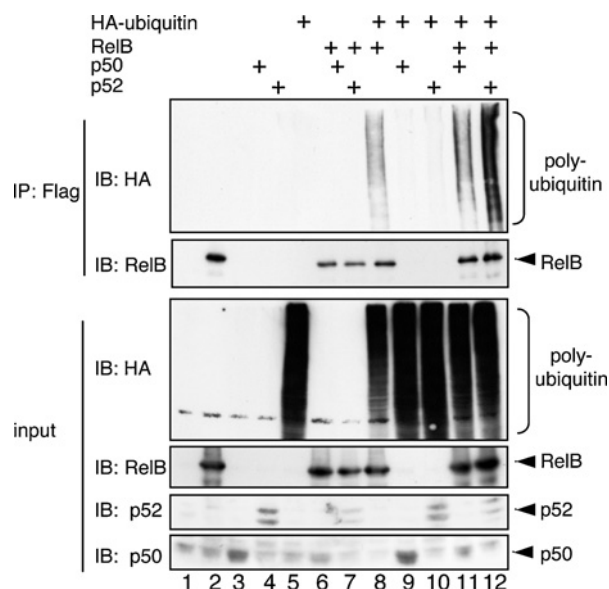

Figure S1   Influence of p50 or p52 co-expression on the *in vivo* polyubiquitination of RelB

HEK-293 cells were transiently transfected with expression vectors for FLAG-tagged RelB, HA–ubiquitin, p50 or p52, as indicated. The cells were lysed 48 h post-transfection with RIPA buffer and the resulting whole cell extracts were subjected to an anti-FLAG immunoprecipitation (IP) experiment under denaturing conditions (IP: Flag, top panels). A fraction of the samples was used for immunblot (IB) analyses with the indicated antibodies (input, lower panels) to ensure the expression of the different exogenous proteins.

[1]   To whom correspondence should be addressed (email ralf.marienfeld@uni-ulm.de).