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## ***Identification of lysine residues critical for the transcriptional activity and poly-ubiquitination of the NF- $\kappa$ B family member RelB***

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**Running title:** Identification of lysine residues critical for RelB-activity.

### ***Synopsis***

RelB is the key component of the alternative NF- $\kappa$ B signalling pathway. However, RelB exerts also a negative effect via the recruitment of a DNMT1-Daxx complex to NF- $\kappa$ B target genes. Importantly, the molecular mechanisms, which determine the functions of RelB are still largely unknown. Here, we aimed to analyze whether ubiquitination of RelB might be involved in the regulation of RelB. Indeed, RelB is constitutively poly-ubiquitinated in the B cell lines Namalwa and 70Z/3. While a PMA+Ionomycin-induced augmentation of RelB poly-ubiquitination was linked to its proteasomal degradation in B cells, the constitutive RelB poly-ubiquitination seems to affect non-proteasomal functions. Consistently, a significant RelB-poly-ubiquitination in 293 HEK cells correlated with an augmentation of the transcriptional activity of RelB. Yet, neither nuclear localization nor DNA-binding was enhanced by RelB-poly-ubiquitination. Interestingly, basal RelB-poly-ubiquitination depends neither on Lys<sup>48</sup>- nor on Lys<sup>63</sup>-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<sup>273/274</sup> and Lys<sup>305/308</sup> significantly decreased the basal RelB-activity and the ubiquitin-induced augmentation of the RelB-activity. Collectively, these data imply a dual role of RelB-poly-ubiquitination for the stability and activity of this transcription factor.

### ***Introduction***

The transcription factor NF- $\kappa$ B 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- $\kappa$ B dimers, composed of the subunits RelA, RelB, c-Rel, NF- $\kappa$ B1/p50, and NF- $\kappa$ B2/p52. Inactive NF- $\kappa$ B is restrained in the cytoplasm of resting cells by the members of the I $\kappa$ B family which become site-specific phosphorylated upon cell stimulation by the multi-subunit I $\kappa$ B-kinase-complex –composed of the kinases IKK1 and IKK2, and the adaptor protein NEMO- and subsequently degraded by the proteasome [1, 2]. Free NF- $\kappa$ B translocates to the nucleus where it supports the expression of various NF- $\kappa$ B target genes. Besides this canonical NF- $\kappa$ B signaling pathway, an alternative IKK1-dependent pathway was described which is characterized by the activation of RelB-p52 heterodimers and the expression of a specific subset of NF- $\kappa$ B target genes [3]. In addition to its role in the alternative NF- $\kappa$ B pathway, RelB is also linked to various other functions in the NF- $\kappa$ B system. For example, RelB is part of a second wave of the NF- $\kappa$ B 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- $\kappa$ B 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-DNMT1 co-repressor complex has recently been described [5]. Post-translational modifications like phosphorylation, ubiquitination or acetylation are crucial for the diverse functions of the NF- $\kappa$ B transcription factors [6-8]. For instance, the recruitment of the co-activator CBP to the NF- $\kappa$ B 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 point to a prominent role of non-proteasomal ubiquitination for the regulation of transcription factors. The transcriptional activity of the transcription factor c-myc, for instance, is distinctively augmented by the ubiquitination-mediated recruitment of the coactivator p300 [9]. The HectH9 mediated ubiquitination of c-myc occurs in a NLS 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. Here, we aimed to determine whether NF- $\kappa$ B family members, especially RelB, are also subject of an ubiquitination-based regulatory mechanism. Recently, we reported that the NF- $\kappa$ B family member RelB is degraded during the initiation phase of the T cell activation via the proteasomal pathway [10]. The RelB-proteolysis is regulated by a specific phosphorylation at Thr<sup>84</sup> and Ser<sup>552</sup> of RelB. Here, we demonstrate that the poly-ubiquitination of the NF- $\kappa$ B 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 poly-ubiquitination. Remarkably, the poly-ubiquitination 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 here that the ubiquitination of NF- $\kappa$ B subunits might be a novel regulatory mechanism in the control of the NF- $\kappa$ B dependent gene expression.

## **Experimental**

### *Cells, reagents and antibodies*

Antibodies for RelB, NEMO, IKK2, NIK, I $\kappa$ B $\alpha$  and the HA-tag were purchased from Santa Cruz Biotechnology. An antibody specific for poly-ubiquitinated proteins was obtained from Biomol, and the anti-FLAG antibody and anti-FLAG-antibody coupled sepharose was from Sigma. 293 HEK cells were propagated in DMEM + 10%FCS containing streptomycin and penicillin. For the cultivation of the B cell lines Namalwa, 70Z/3 and S107 RPMI-media + 10% FCS including streptomycin and penicillin was used. PMA was purchased from Sigma-Aldirch, 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 carboxy-terminal RelB-deletion mutants were described previously [11, 12]. The different Flag-RelB CT and Flag-RelB<sub>240-320</sub> 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 K48/63R-ubiquitin have been described previously [13]. The NF- $\kappa$ B-dependent luciferase reporter construct and the renilla luciferase reporter construct under the control of the ubiquitin-promoter have been described elsewhere [14]. For the *in vitro* mutagenesis the QuickChange *in vitro* mutagenesis kit was used following the manufacturers protocol. Sequences of the oligonucleotides used for cloning or for *in vitro* mutagenesis are available upon request.

### *In vivo ubiquitination analysis*

HEK 293 cells were transiently transfected with expression vectors coding for HA-ubiquitin and Flag-tagged RelB and lysed after 48 hours in TNT-buffer (200 mM NaCl, 20 mM HEPES pH 7.6, 1 % Triton X-100, 1 mM DTT, 20 mM NaF, 20 mM Na-glycerophosphate, 2  $\mu$ M leupeptin, 1 mM PMSF) including 0.5 % SDS or in RIPA-buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0,5% Na-deoxycholat, 0,1% SDS, 20 mM NaF, 20 mM Na-glycerophosphate, 2  $\mu$ M leupeptin, 1 mM PMSF) as indicated and were heated for 5 minutes 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+0.5% SDS or RIPA-buffer and PBS, and were boiled for 5 minutes 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 HA-tag, Flag-tag or RelB, as indicated.

### *Immunoprecipitation and immunoblot analysis*

For immunoprecipitation of ectopically expressed or endogenous proteins 0.5-1 mg protein extract was incubated with the appropriate antibody for one hour on ice prior to the addition of 25  $\mu$ l of 50% slurry of ProteinA-coupled Sepharose (Santa Cruz Biotechnology) and an additional incubation for one hour under agitation. The precipitates were subsequently washed with TNT+0.5%SDS or RIPA-buffer and PBS and subjected to immunoblot analysis. For anti-FLAG immunoprecipitations 10  $\mu$ l anti-FLAG antibody coupled sepharose (Sigma) was used. Immunoblots were performed according to standard protocols. In brief, the proteins were separated by SDS-PAGE on 8-10% polyacryl amid gels, transferred on nitrocellulose membrane (Whatman) and the resulting membrane was blocked for one hour in TBS+Tween20 with 5% dry milk. Subsequently, the membrane was incubated o. n. with the appropriate primary antibody in TBS+Tween20 with 5% dry milk, washed extensively, incubated for one hour in the appropriate horse-raddish peroxidase coupled secondary antibody prior to the detection with ECL substrate (Pierce).

### *Transfection and Luciferase reporter assay*

For the transfection of 293 HEK cells we used the CaPO<sub>4</sub>-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 molar CaCl<sub>2</sub> solution for five minutes prior to the addition of an appropriate volume of 2xHeBS (280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM HEPES, pH 7.04). After another five minutes of incubation the sample was added to the cells. For the electroporation of S107 pre B cells, 1x10<sup>7</sup> cells were mixed with DNA, incubated on ice for 5 minutes prior to a pulse with 250 V and 950  $\mu$ F. The cells were subsequently transferred to a culture dish with the appropriate media and incubated for 24-48 hours. For determination of the luciferase activity, the cells were lysed in TNT-buffer and the activity of the renilla luciferase as well 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 and mean deviation are stated.

## **Results**

Since RelB is able to act either as a positive or as a negative effector of the NF- $\kappa$ B-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 293 HEK cells as indicated in Figure 1A. 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* poly-ubiquitination of RelB under these experimental conditions. Consistent with the hypothesis that poly-ubiquitination alters the function of RelB, we observed a significant augmentation of the RelB-activity in NF- $\kappa$ B reporter gene assays using 293 HEK 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- $\kappa$ B-dependent reporter gene expression. Similar results were obtained with S107 pre B cells which display a very low basal NF- $\kappa$ B activity due to a loss of RelB-expression [15] and which are defective in various NF- $\kappa$ B signal pathways (Figure 1C).

Next, we determined whether the observed positive effect of ubiquitin coexpression on the RelB-activity is 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 NF- $\kappa$ B factors, like p50 or p52 [12]. Consistently, S368A-RelB was found to be inactive in NF- $\kappa$ B reporter gene assays (Figure 2A). Furthermore, in contrast to 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 poly-ubiquitination was indistinguishable between wild type and S368A-RelB (Figures 2A, lanes 4+6, and 2B, lanes 5+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- $\kappa$ B reporter gene assay, we analyzed the potential effect of poly-ubiquitination on nuclear localization and DNA-binding of RelB. As shown in Figure 3A, neither the basal NF- $\kappa$ B DNA-binding activity in 293 HEK 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 likely not the basis for the increased NF- $\kappa$ B activity since the RelB-mediated NF- $\kappa$ B-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 poly-ubiquitination of the endogenous RelB in Namalwa B cells revealed a moderate basal RelB-poly-ubiquitination in unstimulated Namalwa cells (Figure 4A, lane 1). This moderate RelB-poly-ubiquitination remained stable upon stimulation with TNF $\alpha$  (Figure 4A, lanes 4+5). In contrast, the stimulation with PMA+ionomycin induced a significant increase in RelB-poly-ubiquitination (Figure 4A, compare lanes 1+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 poly-ubiquitination of RelB (Figure 4A). Consistent 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-poly-ubiquitination suggesting that the augmented RelB-poly-ubiquitination observed upon PMA+ionomycin stimulation is linked to a proteasomal-dependent degradation of RelB.

Lipopolysaccharide has been demonstrated to induce the alternative NF- $\kappa$ B pathway, which depends on the activity of RelB. Thus, we analyzed whether a LPS-stimulation for 8 or 24 hours had an effect on RelB-poly-ubiquitination levels. As shown in Figure 4C, we indeed observed an increased ubiquitin signal after RelB-immunoprecipitation. Yet, also the RelB-levels were augmented suggesting that there is no net increase in RelB-poly-ubiquitination by LPS-stimulation. To further analyze whether RelB-poly-ubiquitination might be rendered triggering the alternative NF- $\kappa$ B signaling 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- $\kappa$ B pathway [3]. Again, the levels of RelB-poly-ubiquitination remained largely unaltered upon NIK-coexpression. However, we observed a slight change in the pattern of the RelB-poly-ubiquitination by NIK coexpression (Figure 4D, compare lanes 9 and 11).

Based on these results, the possibility remained that the RelB-poly-ubiquitination 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<sup>84</sup> and Ser<sup>552</sup> [10]. Yet, RelB-mutants with either inactivating alanine substitutions (Flag-RelB-ST/A) or phospho-mimetic glutamic acid substitutions (-ST/E) at Thr<sup>84</sup> and Ser<sup>552</sup> revealed no significant differences regarding their *in vivo* poly-ubiquitination levels (Figure 5B). These results collectively imply that the basal poly-ubiquitination 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 in ubiquitin used for poly-ubiquitin chain conjugation determines the biological function of the poly-ubiquitination, with a Lys<sup>48</sup>-conjugated poly-ubiquitin chain subjecting the target protein to proteasomal degradation and a Lys<sup>63</sup>-conjugated poly-ubiquitin chain responsible for most non-proteasomal functions. Therefore, we next compared wild type HA-ubiquitin with HA-ubiquitin mutants harboring either a K48R- or a K63R-substitution in an *in vivo* RelB-poly-ubiquitination 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 positive control (Figure 6C). In addition, both ubiquitin mutants, K48R- and K63R-ubiquitin, augmented the RelB-activity in NF- $\kappa$ B reporter gene assays (Figure 6B). Surprisingly, an efficient RelB-poly-ubiquitination in HEK 293 cells was also observed with a HA-tagged ubiquitin-mutant harboring combined arginine substitutions of lysine 48 and 63 (Figure 6D) regardless of the presence or absence of MG132 suggesting that RelB-poly-ubiquitination involves unconventional poly-ubiquitin conjugation.

In order to map the RelB-domain(s) containing the ubiquitin-acceptor site(s) we performed *in vivo* ubiquitination assays with different carboxy-terminal Flag-tagged RelB-deletion mutants (for a schematic representation of the mutants used, see Figure 7A). Surprisingly, all Flag-RelB mutants used were poly-ubiquitinated using either wild type or K48/63R-ubiquitin (Figure 7B, upper panel, lanes 8-12 and 14-18; please note that the low poly-ubiquitination level of full length RelB is due to a lower expression level of this protein), although the poly-ubiquitination levels were lower in case of the RelB-deletion mutant spanning the amino acids 348-552 (RelB<sub>CT</sub>). 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 by 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- $\kappa$ B reporter gene assays (Figure 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). Interestingly, the same three RelB-mutants showed also 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 lysines within M7, M9, and M11 are located in the Rel Homology Domain (RHD) required for dimerization and DNA-binding of RelB. To exclude the possibility that the substitution of Lys<sup>220</sup>, Lys<sup>273/274</sup>, or Lys<sup>305/308</sup> affect the DNA-binding of the M7-, M9-, and M11-mutants, we tested these RelB-mutants in a gelretardation assay with a NF- $\kappa$ B-specific probe and included the M10-mutant as control. Yet, 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-poly-ubiquitination observed with the different deletion mutants, the *in vivo* poly-ubiquitination of the M7-, M9-, M10-, and M11-mutants remained unaltered regardless whether wild type HA-ubiquitin (Figure 9B, lanes 6-11) or the HA-K48/63R-ubiquitin mutant (Figure 9B, lanes 12-17) was used. Indeed, a significant effect on the *in vivo* poly-ubiquitination was generally only detectable upon combined substitution of various lysine residues as shown for the substitution of all lysine residues in the carboxy-terminus of RelB (K386: Lys<sup>386</sup>, Lys<sup>387</sup> and Lys<sup>389</sup>; K410: Lys<sup>410</sup>, Lys<sup>413</sup>, Lys<sup>414</sup>, Lys<sup>415</sup>, Figure 10A, lanes 7-10 and Figure 10B, lanes 2-4). Taken together, these results collectively suggest that among the various ubiquitination-target sites only the poly-ubiquitination at specific lysine residues of RelB mediate the positive effect on its transcriptional activity.

### Discussion

The NF- $\kappa$ B 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- $\kappa$ B include post-translational modifications of the different NF- $\kappa$ B subunits. For example, a tightly regulated recruitment of co-activators or co-repressors –like CBP or HDAC1, 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. Here, we aimed to analyze whether a specific ubiquitination of RelB might be involved in the regulation of RelB-functions. Indeed, we observed a distinct *in vivo* poly-ubiquitination 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-poly-ubiquitination was further increased by stimulation of the cells with PMA+ionomycin. However, this increase in RelB-poly-ubiquitination is most likely linked to the proteasomal degradation of RelB since we observed a correlation between an augmented RelB-poly-ubiquitination and a partial RelB-stabilization upon pre-treatment with the proteasome inhibitor MG132. Proteasomal degradation of various NF- $\kappa$ B subunits, like RelA/p65, RelB or NF- $\kappa$ B1/p50 has been described previously [10, 19, 20]. In contrast, the unchanged half-life time of RelB upon ubiquitin coexpression (Figure 2), the constitutive RelB-poly-ubiquitination by K48R-ubiquitin (Figure 5), and the normal poly-ubiquitination of the Ser<sup>84</sup>- and Thr<sup>552</sup>-mutants of RelB (Figure 2) pointed collectively to additional non-proteasomal functions of the basal RelB-poly-ubiquitination detected in unstimulated 293 HEK, 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 poly-ubiquitination 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-poly-ubiquitination causes a reduced Daxx-interaction of this transcription factor is currently unknown. The occasionally observed increased RelB levels contribute most likely not to the augmented RelB-activity as transfection of increasing amounts of a RelB-expression vector led to similar results in NF- $\kappa$ B reporter assays probably due to low levels of the RelB-interaction partners p50 or p52. Usually, the Lys<sup>48</sup>-conjugated poly-ubiquitin chains assign the ubiquitinated protein for a proteasomal degradation, whereas the formation of Lys<sup>63</sup>-conjugated poly-ubiquitin chains renders the protein-interaction pattern of the target protein [21]. However, Lys<sup>48</sup> and Lys<sup>63</sup> are not the only lysine residues in ubiquitin used for conjugation. Furthermore, also mixed ubiquitin-conjugates have been identified [22]. Thus, the efficient RelB-poly-ubiquitination seen with K48/63R-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<sup>48</sup> and Lys<sup>63</sup> [23]. NEMO seems to be another protein modified by differently conjugated poly-ubiquitin chains since we obtained similar results using NEMO regarding the poly-ubiquitination with K48R- and K63R-ubiquitin. Indeed, besides the Lys<sup>63</sup>-conjugated NEMO-poly-ubiquitination also a Lys<sup>6</sup>-conjugated NEMO-poly-ubiquitination has been described [23]. Mapping of the RelB domains containing the poly-ubiquitination sites revealed the existence of various poly-ubiquitination sites in all RelB-domains analyzed. Although the low specificity of protein ubiquitination has been reported for other ubiquitination targets, for instance c-Myc or MALT1 [9, 13], the high degree of redundancy of poly-ubiquitination 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<sup>220</sup> (M7) and more pronounced of Lys<sup>273/274</sup> (M9) or Lys<sup>305/308</sup> (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<sup>273/274</sup> (M9) and Lys<sup>305/308</sup> (M11) is furthermore highlighted by the fact that the activity of the M15-mutant with a complete inactivation of the lysine residues in the carboxy-terminal TAD of RelB remained unaltered. Thus, it is possible that the poly-ubiquitination at these particular lysine residues (Lys<sup>273/274</sup> (M9) or Lys<sup>305/308</sup> (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- $\kappa$ B-subunit RelA [7], seems to be not responsible for the ubiquitin-effect as an interaction of CBP with RelB was not detectable regardless of the absence or presence of ubiquitin (data not shown). Interestingly, poly-ubiquitination could be a general regulatory mechanism to control the activity of nuclear NF- $\kappa$ B, 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 poly-ubiquitination of RelB. Alternatively, also other post-translational modifications occurring at lysine residues, for instance an acetylation or the attachment of Sumo moieties could be involved. Indeed, we already observed an *in vivo* acetylation and sumoylation of RelB in 293 HEK cells (data not shown).

Conclusively, we demonstrate here that the NF- $\kappa$ B subunit RelB is poly-ubiquitinated 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 poly-ubiquitin chain conjugation involved in RelB-poly-ubiquitination. Another important question pertains the identity of the ubiquitin ligases or deubiquitinases involved in the poly-ubiquitination of RelB. One feasible candidate for an E3-ligase involved in the proteolysis-linked RelB-poly-ubiquitination might be COMMD1, which mediates the ubiquitination and subsequent degradation of the NF- $\kappa$ B 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, like Daxx. Finally, we want to determine whether also other NF- $\kappa$ B 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- $\kappa$ B subunit RelA (J. L. and R.M., unpublished observations) suggesting that this kind of post-translational modification could be a general regulatory mechanism for the control of the NF- $\kappa$ B system.

### Footnotes

### Abbreviations

IB: immunoblot; IP: immunoprecipitation; Lys: lysine residue; LPS; lipopolysaccharide, PMA: Phorbol 12-Myristate 13-Acetate; TNF $\alpha$ : tumor necrosis factor; Thr: threonine residue; Ser: serine residue; CT: carboxy terminus; EMSA: electrophoretic mobility shift assay.

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### Figure legends

**Figure 1: RelB poly-ubiquitination in vivo correlates with an augmentation of its transcriptional activity.** A, For an *in vivo* ubiquitination assay 293 HEK cells were transiently transfected with 1  $\mu$ g 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 IP. The resulting immunoprecipitates were boiled in TNT-buffer with 0.5% SDS. 50% of the sample was used for western blot analysis (IP 1, upper part) and 50% for a second anti-FLAG IP (IP 2, middle part). To control the expression of the proteins 10% of the cell lysates were subjected to an additional western blot (input, lower part). B, 293 HEK cells were transiently transfected with a NF- $\kappa$ B dependent luciferase reporter construct together with a ubiquitin-promoter driven Renilla luciferase reporter either alone or in conjunction with increasing amounts of a 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 hours, lysed in TNT buffer, and the dual luciferase analysis was performed as described under experimental procedures. C, A similar NF- $\kappa$ B-dependent luciferase reporter analysis was performed with S107 pre B-cells transiently transfected by electroporation with expression vectors for RelB (5  $\mu$ g) or HA-ubiquitin (5 and 10  $\mu$ g) as indicated.

**Figure 2: The ubiquitin-effect on RelB-activity depends on the DNA-binding potential of RelB.** A, NF- $\kappa$ B luciferase reporter assay with HEK 293 cells expressing either HA-ubiquitin, wild type Flag-RelB or Flag-S368A-RelB alone or in combination, as indicated. Twenty-four hours 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 or Flag-S368A-RelB. Ectopically expressed Flag-RelB was immunoprecipitated under denaturing conditions (RIPA-buffer) with anti-Flag antibodies and probed in a subsequent immunoblot for HA-ubiquitin (upper panel) and RelB (lower panel). A fraction of the samples was subjected to anti-HA and anti-RelB immunoblots to ensure an efficient expression of the different proteins (lower part).

**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  $\mu$ g) and HA-ubiquitin (0.5 and 1  $\mu$ g) as indicated. After 48 hours, the cells were lysed and the resulting whole cell extracts were subjected to gel retardation studies with either a  $\kappa$ B-specific (upper panel) or an Oct-specific oligonucleotide. To ensure the identity of the RelB-containing complex (I) supershift analysis was performed with antibodies specific for RelA or RelB using extracts from cells transfected with RelB and HA-ubiquitin (lanes 7+8). \* and \*\* mark unspecific bands. Western blot analysis was performed to control for the expression of Flag-RelB and HA-ubiquitin (right part). B, For the determination of the nuclear localization of RelB, nuclear and cytoplasmic extracts from 293 HEK cells which ectopically express either RelB (lanes 4+10), increasing amounts of HA-ubiquitin (lanes 2+3 and 8+9) or both proteins (lanes 5+6 and 11+12) were generated and subjected to western blot analyses with the indicated antibodies. C, A NF- $\kappa$ B-reporter assay was performed after transient transfection of 293 HEK cells with the indicated amounts of a Flag-RelB-expression vector.

**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 TNF $\alpha$  for the indicated periods of time, lysed with RIPA-buffer and the resulting whole cell extracts were subjected to an anti-RelB IP analysis. Anti-RelB and anti-I $\kappa$ B $\alpha$  immunoblots were additionally performed to control the efficiency of the stimulation. B, Namalwa cells were either left untreated or were pretreated with MG132 (25  $\mu$ M) for 30 minutes prior to

stimulation with PMA+ionomycin for the indicated time points. The cells were lysed in RIPA buffer, the resulting whole cell extracts were used for anti-RelB IPs prior to immunoblot analysis with the indicated antibodies. *C*, 70Z/3 pre B cells were either left untreated or were stimulated with 1  $\mu$ g/ml LPS for 8 or 24 hours prior to a lysis in RIPA buffer. The resulting whole cell extracts were subjected to an anti-RelB IP analysis. *D*, *In vivo* ubiquitination analysis with whole cell extracts from 293 cells transiently transfected with expression vectors for Flag-RelB, HA-ubiquitin without or with an additional NIK expression vector.

**Figure 5: RelB stability remains unaltered by ubiquitin coexpression.** *A*, To analyze 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 (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. *B*, *In vivo* ubiquitination assay with either wild type FLAG-RelB (lanes 2+6), with a FLAG-RelB<sub>TS/A</sub>-mutant (T84A/S552A, lanes 3+7) or with a FLAG-RelB<sub>TS/E</sub>-mutant (T84/S552E, lanes 4+8).

**Figure 6: Characterization of the poly-ubiquitin conjugates attached to RelB.** For an *in vivo* ubiquitination assay of Flag-RelB (*A*) or Flag-NEMO (*C*) 293 HEK cells were transiently transfected with 1  $\mu$ g Flag-RelB or Flag-NEMO alone or in conjunction with 1  $\mu$ g of expression vectors for wild type HA-ubiquitin or HA-ubiquitin mutants with an arginine substitution of either Lys<sup>48</sup> (K48R) or Lys<sup>63</sup> (K63R) as indicated. The cells were lysed in RIPA-buffer and the resulting whole cell extracts were subjected to an anti-FLAG IP under denaturing conditions prior to immunoblot analyses with the indicated antibodies. *B*, NF- $\kappa$ B reporter gene assay after transient transfection of 293 HEK 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-K48/63R-ubiquitin mutant. The cells were either left untreated (upper part) or were pretreated with the proteasome inhibitor MG132 (lower part).

**Figure 7: Mapping of the RelB-domain modified by ubiquitin-attachment.** *A*, Schematic representation of the Flag-tagged RelB-deletion mutants used. *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 K48/63R-ubiquitin (lanes 13-18). 90% of the whole cell extracts were used for an anti-FLAG IP under denaturing conditions (upper part) and 10% for control western blot analysis (input, lower panel). The anti-RelB immunoblot was included to monitor the expression levels of full length RelB.

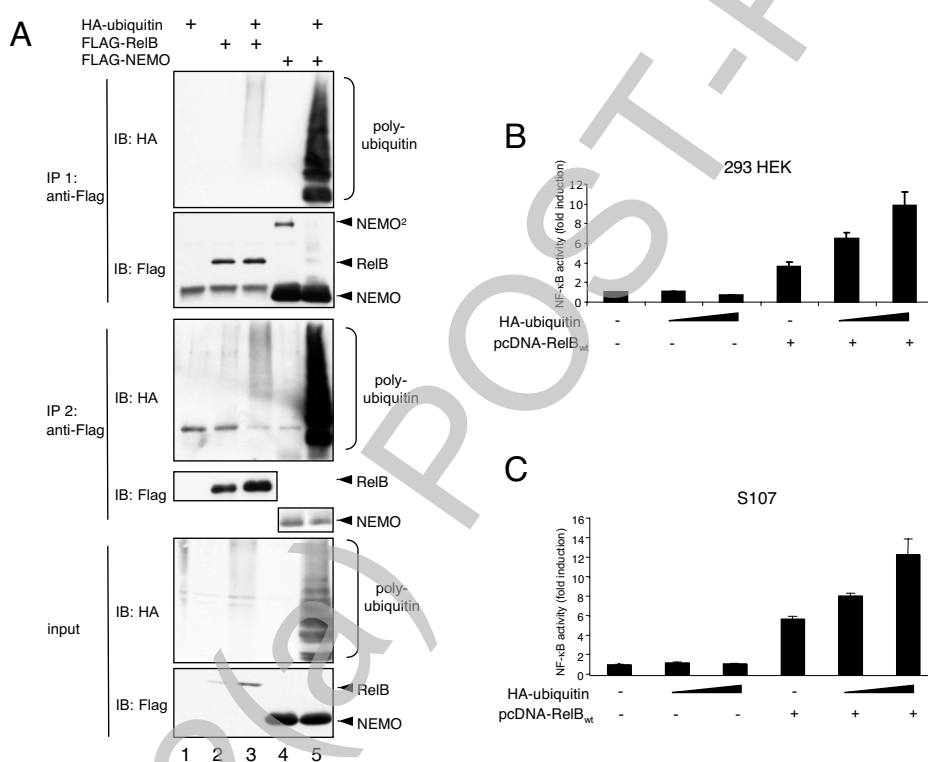
**Figure 8: Identification of the lysine residues critical for the basal activity of RelB and the ubiquitin-induced augmentation of RelB-activity.** *A*, Schematic representation of the RelB-mutants with lysine to arginine substitutions. Note: Some of the mutants harbor a single mutation and several mutants contain the combined substitutions of closely located lysine residues. *B*, NF- $\kappa$ B reporter gene assays. The relative basal activity of the various RelB mutants in comparison to wild type RelB is depicted. The increase in NF- $\kappa$ B activity mediated by wild type 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- $\kappa$ B reporter gene assays. Here, the fold increase in activity upon HA-ubiquitin co-expression in comparison to the activity of the respective RelB-variant alone is depicted in relation to wild type RelB (arbitrarily set to 100%). *D*, NF- $\kappa$ B reporter gene analysis with the indicated RelB-variants alone or in combination with p50 or p52 expression vectors.



**Figure 9: Characterization of selected RelB mutants with lysine to arginine substitutions.** A, Gelretardation analysis of selected RelB-mutants ectopically expressed in conjunction with HA-ubiquitin. For the gelretardation assay either a  $^{32}\text{P}$ -labelled  $\kappa\text{B}$ -specific probe (upper panel) or Oct-specific probe (second panel) was used. Immunoblot analyses with the indicated antibodies were performed to ensure similar expression levels of the RelB-variants or HA-ubiquitin (lower part). B, *In vivo* ubiquitination analysis under denaturing conditions of the indicated RelB-variants using either wild type HA-ubiquitin (lanes 6-11) or HA-K48/63R-ubiquitin (lanes 12-17).

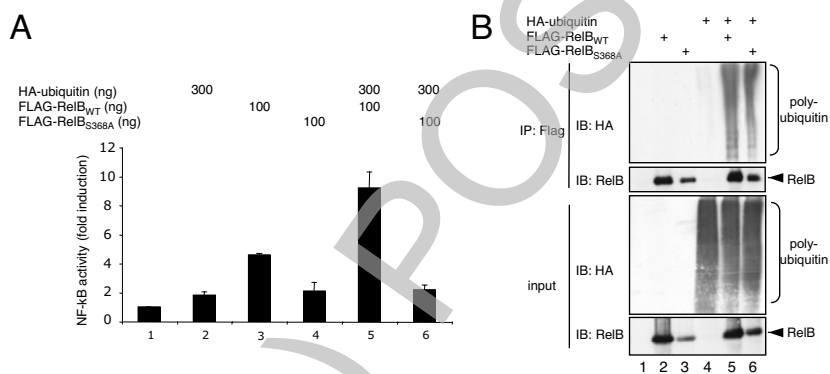
**Figure 10: Analysis of the poly-ubiquitination of the carboxy-terminus of RelB.** A, *In vivo* ubiquitination analysis of the carboxy-terminal Flag-tagged RelB deletion mutants carrying either asparagine substitutions at the positions Lys<sup>386</sup>, Lys<sup>387</sup>, Lys<sup>389</sup> (K386N, lanes 4 and 8), at positions Lys<sup>410</sup>, Lys<sup>413</sup>, Lys<sup>414</sup> and Lys<sup>415</sup> (K410N, lanes 5 and 9) or at all positions (CT-DM), in comparison with the wild type counterpart (lanes 6 and 10). B, A similar *in vivo* ubiquitination analysis of wild type FLAG-RelB or the FLAG-RelB M15-mutant harboring lysine to arginine substitutions at the positions 386, 387, 389, 410, 413 and 415.

Figure 1



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Figure 2



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Figure 3

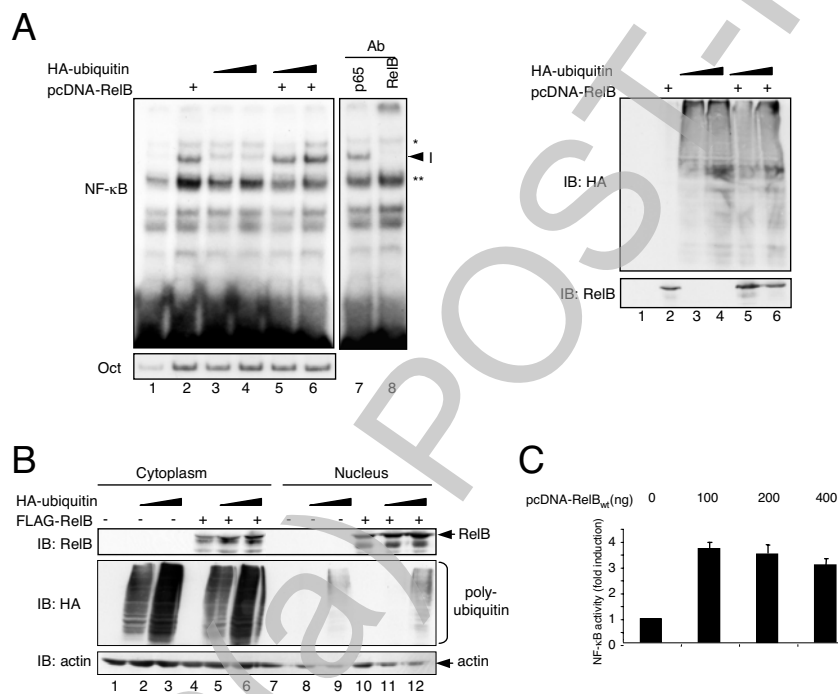
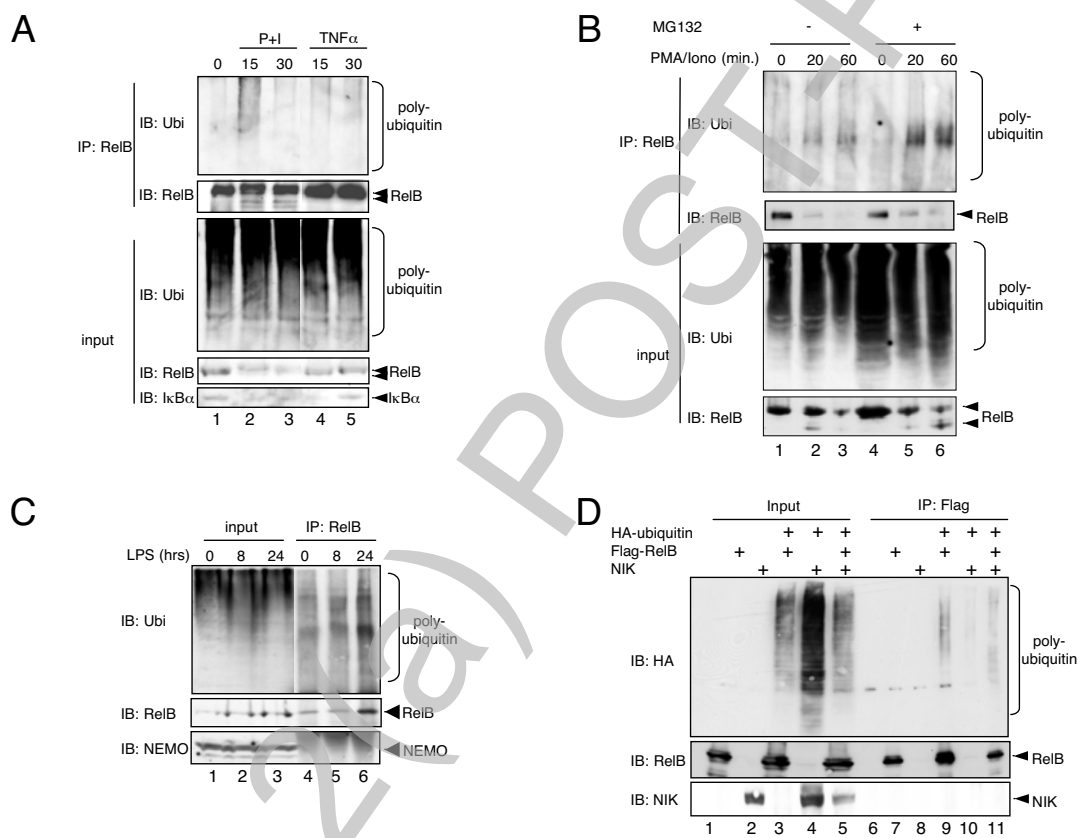


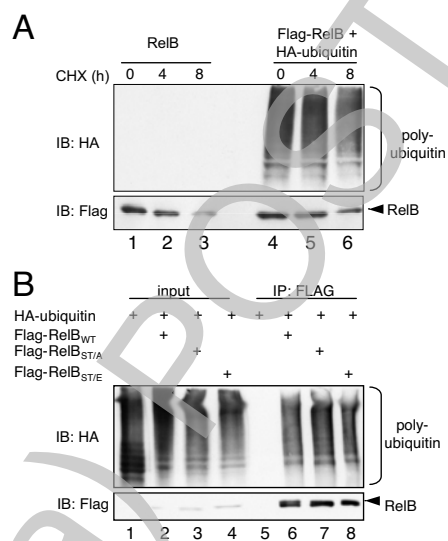


Figure 4



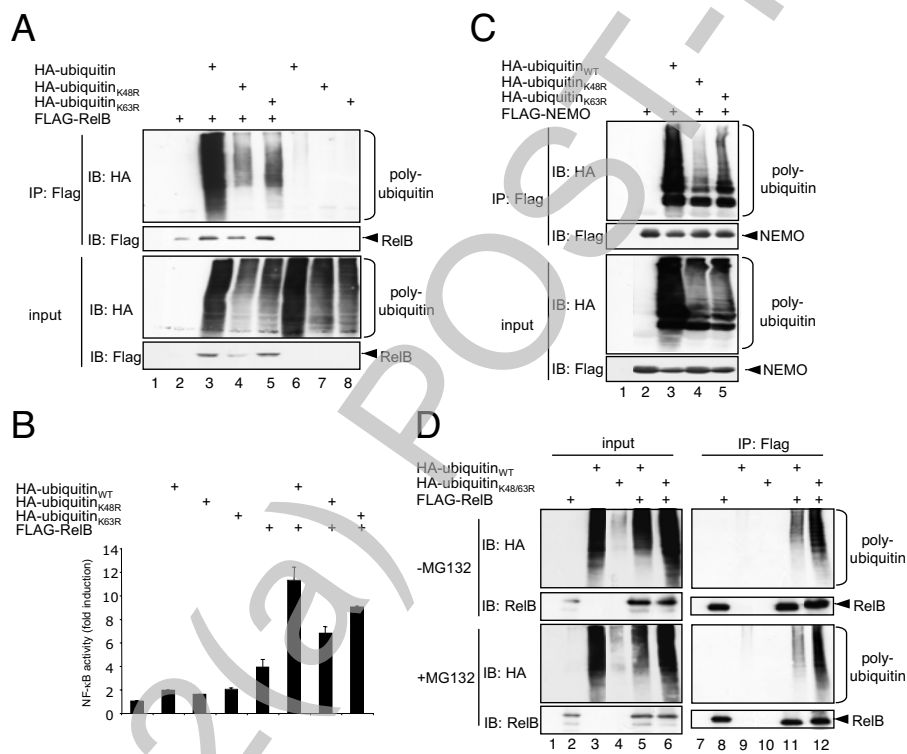
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Figure 5



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Figure 6

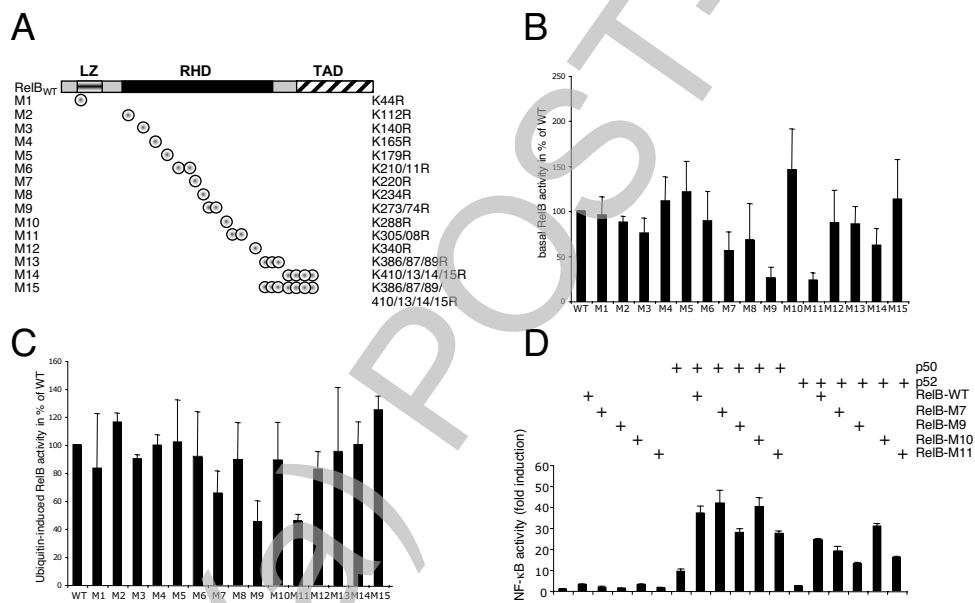


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Figure 8



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Figure 10

