2 Results

2.1 Functional role of Malt1 in IKK/NF-κB signaling

2.1.1 CBM complex formation upon T cell activation

The proteins Carma1, Bcl10 and Malt1 were recently identified as crucial mediators of T cell receptor dependent NF-κB activation [154, 155, 156, 157, 158]. When this project was started, it was known that Carma1 and Bcl10, as well as Malt1 and Bcl10 interact upon overexpression of the proteins [161, 162, 178, 179]. First studies had indicated a stimulus-induced and Carma1 dependent recruitment of Bcl10 to the TCR complex [154, 168], as well as constitutive association of Bcl10 and Malt1 in B and T cells [179]. Thus, stimulus-induced formation of a Carma1/Bcl10/Malt1 complex was suggested to be crucial for IKK activation upon TCR triggering. However, direct evidence for CBM complex formation under physiological conditions had not yet been provided. Therefore, CBM complex formation in T cells was investigated under stimulatory conditions. To confirm the postulated interactions and to identify further potential binding partners or protein modifications critical for T cell activation, complex formation was examined using gelfiltration analysis combined with co-immunoprecipitations.

First, lysate of unstimulated Jurkat T cells was analyzed on a superose 6 column. Western Blot analysis of the collected fractions revealed elution profiles for Bcl10, Malt1, Carma1 and the cellular IKK complex (Fig. 2.1). Elution volumes of marker proteins are depicted by arrows, allowing an estimation of the apparent molecular weight.

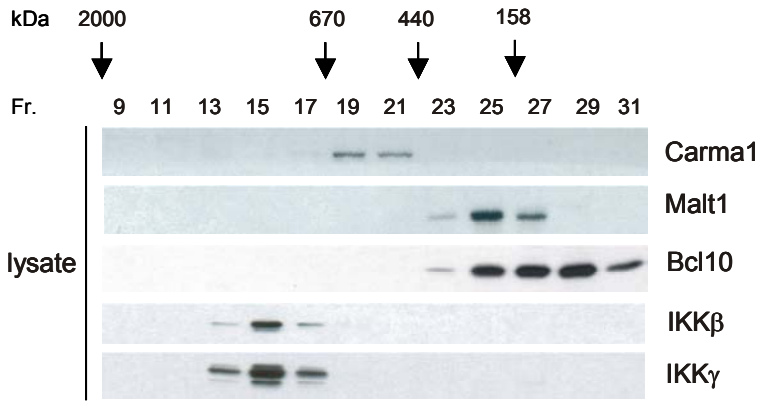


Figure 2.1: Gelfiltration elution profiles of endogenous proteins from unstimulated Jurkat T cells. 1 x 10^8 Jurkat cells were lysed in CoIP buffer without glycerol and subjected to size-exclusion chromatography on a superose 6 column. 0.5 ml fractions were collected and analyzed by Western blotting using the indicated antibodies. Elution volumes of marker proteins are given as apparent molecular weight standard (kDa).

IKKβ and IKKγ were both detected at an apparent molecular weight of 700 to 900 kDa, the size of the cellular IKK complex [75]. Carma1 eluted in fractions 19 to 21, which correspond

to a much higher molecular weight than that of a Carma1 monomer (132 kDa). This observation could be due to a non globular conformation of Carma1, oligomerization, or interaction with unknown binding partners. Malt1 (91 kDa) and Bcl10 (26 kDa) co-eluted in fractions 23 to 27 hinting at an association of these two proteins. Bcl10 elution continued until fraction 31, indicating a Malt1-unassociated Bcl10 pool.

To examine stimulus-dependent CBM complex formation, Jurkat T cells were left untreated or stimulated with phorbol-12-myristate-13-actetate (PMA) and ionomycin for 20 min. PMA possesses structural similarity to diacylglycerol and can therefore directly activate PKCθ. Ionomycin induces Ca^{2+} influx from intracellular calcium storage compartments, thereby activating the transcription factor NFAT and increasing NF-κB activation [144]. Hence, PMA/ionomycin is used to mimic TCR/CD28 induced NF-κB activation in T cells. After stimulation, cellular extracts were fractionated by size-exclusion chromatography and the collected fractions subjected to Bcl10 co-immunoprecipitation and Western blot analysis (Fig. 2.2). In unstimulated Jurkat T cells a pre-formed complex of Bcl10 and Malt1 eluted in the low molecular weight fractions, proving the constitutive interaction of the two proteins. Upon PMA/ionomycin (P/I) stimulation, association of Bcl10-Malt1 with Carma1 could be detected, providing physiological evidence for complex formation of these three proteins upon T cell activation.

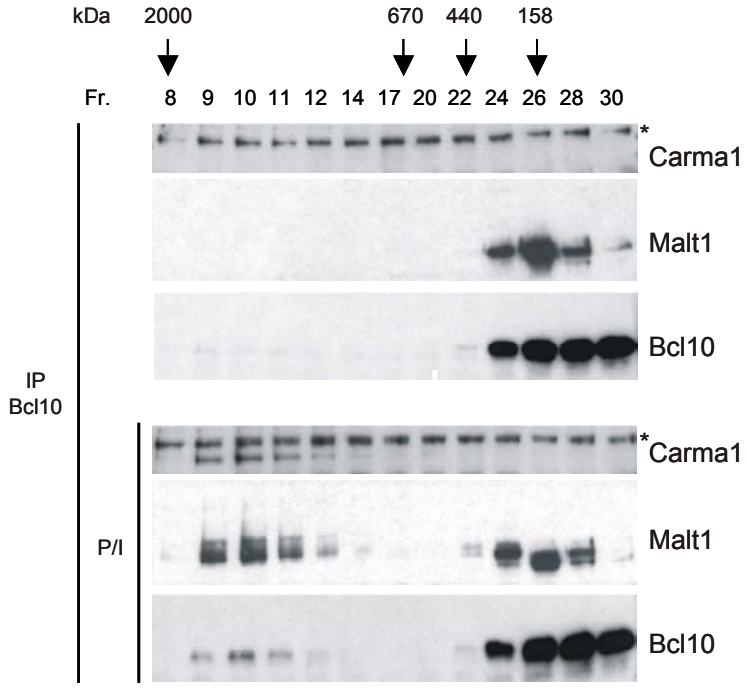


Figure 2.2: Gelfiltration analysis of stimulus-induced CBM complex formation. Jurkat cell lysates were fractionated by size-exclusion chromatography followed by Bcl10 immunoprecipitation. Precipitates were analyzed by Western blotting. Upon PMA/ionomycin (P/I) stimulation (lower panels) pre-associated Bcl10 and Malt1 are recruited to Carma1. The complex eluted at an apparent molecular weight of more than 1500 kDa. Carma1-associated Malt1 showed higher molecular weight species indicative for protein modification. Elution volumes of maker proteins are given as apparent molecular weight standards. An asterisk marks an unspecific band.

The elution volume of the stimulus-dependent CBM-complex (peak fraction 10) corresponds to an apparent molecular weight of more than 1500 kDa. This exceeds the expected molecular weight of a heterotrimer (approximately 260 kDa), which could be due to oligomerization and/or additional proteins binding to the CBM complex.

In addition, a smeary appearance of Carma1-associated Malt1 was noticed caused by higher molecular weight species of Malt1. This could be an indication for posttranslational modification of Malt1 upon stimulation e.g. by ubiquitination.

To investigate if Malt1 was modified by ubiquitin-conjugation, fractions were pooled and denatured after gelfiltration by the addition of SDS (1 % final concentration) to disrupt any protein-protein interactions. Subsequently, Malt1 was precipitated and analyzed by Western blotting (Fig. 2.3). Indeed, the higher molecular weight species of Malt1 (fraction I) could also be detected with an anti-ubiquitin-antibody, showing that Carma1-associated Malt1 is posttranslationally modified by attachment of ubiquitin.

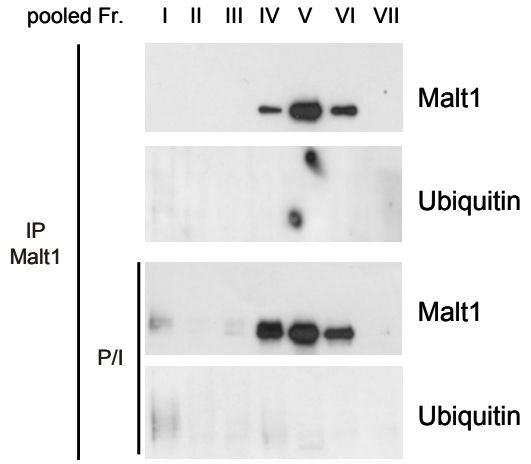


Figure 2.3: Carma1-associated Malt1 is ubiquitinated. After gelfiltration, fractions were pooled (I: 9-11, II: 12-14, III: 16-18, IV: 20-22, V: 24-26, VI: 27-29, VII: 30-32), denatured by 1% SDS and subjected to Malt1 immunoprecipitation. Precipitates were analyzed by Western blotting. Higher molecular weight species of Malt1 in fraction I could be detected by an anti-ubiquitin antibody, showing that Malt1 is modified by ubiquitination upon P/I stimulation.

2.1.2 Stimulus-dependent Malt1 ubiquitination in Jurkat T cells

To further investigate Malt1 ubiquitination in the course of T cell activation, Jurkat T cells were stimulated with PMA/ionomycin for different time periods up to 2 hours. Subsequent Malt1 immunoprecipitation and Western blot analysis showed that ubiquitination of Malt1 is transient. It peaks at approximately 30 min and vanishes after 1 h of stimulation (Fig. 2.4). This coincides with the degradation and resynthesis of IκBα, which was used to monitor activation of the IKK/NF-κB pathway. All ubiquitination experiments were performed under denaturing conditions (1% SDS, see 6.4.6) to ensure that Malt1 specific modification was detected (see also control experiment Fig. 2.6). Malt1 ubiquitination could also be observed upon stimulation of Jurkat T cells through CD3/28 receptor co-ligation, representing the more

physiological stimulus for TCR dependent NF-κB activation (Fig. 2.4 B). Thus, these results demonstrate that Malt1 is transiently modified by attachment of ubiquitin during TCR-induced activation of NF-κB.

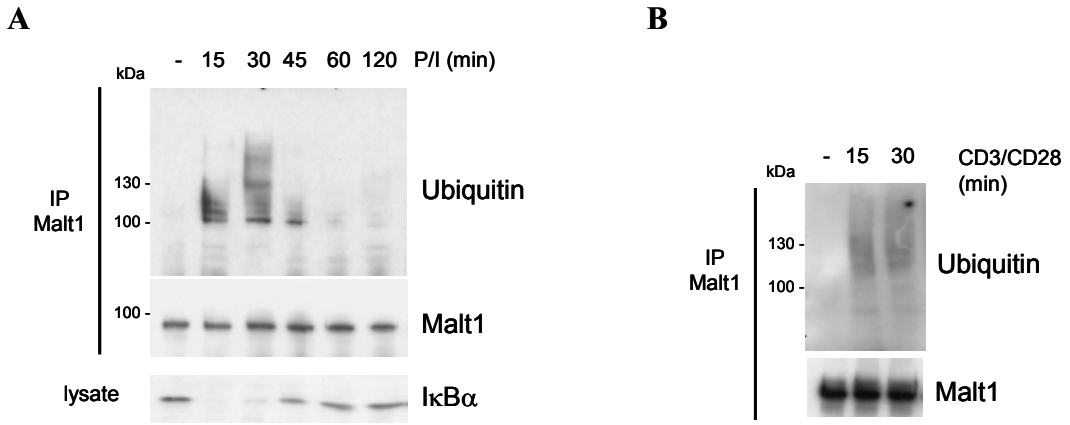


Figure 2.4: Malt1 ubiquitination in Jurkat T cells. (**A**) Kinetic analysis of Malt1 ubiquitination. Jurkat T cells were stimulated with PMA/ionomycin (P/I) for the indicated time periods, lysed (1% SDS) and subjected to Malt1 IP. (**B**) Malt1 ubiquitination in response to CD3/28 receptor co-ligation. Jurkat cells were stimulated with CD3 and CD28 antibodies and analyzed as in (A).

Since no decrease of Malt1 protein level could be observed after stimulation with PMA/ionomycin or CD3/28 antibodies and Malt1 ubiquitination kinetics coincided with IKK activation, it was conceivable that ubiquitination of Malt1 could rather be involved in signal propagation than protein degradation.

2.1.3 TRAF6 functions as an E3 ligase for Malt1.

Since the E3 ubiquitin ligase TRAF6 was shown to interact with the C-terminus of Malt1 in overexpression experiments and *in vitro* [183], it was next examined if TRAF6 could be involved in Malt1 ubiquitination. Therefore, Malt1 was co-expressed in HEK293 cells with TRAF6, TRAF6Δ (an N-terminal deletion construct (aa 289-522) missing the catalytically active RING domain), and the TRAF6 homologue TRAF2 (Fig. 2.5). Cells were either lysed in CoIP buffer to detect protein-protein interactions (Fig. 2.5 A), or in ubiquitination buffer (1% SDS) for analysis of Malt1 ubiquitination after MycMalt1 IP (Fig. 2.5 B). Indeed, co-expressed TRAF6 bound to Malt1 as had been published before (Fig. 2.5 A). In addition, TRAF6 induced Malt1 ubiquitination upon co-expression. TRAF6Δ failed to do so, although it was still able to associate with Malt1 (Fig. 2.5 A and B). This shows that the C-terminal part of TRAF6 mediates interaction with Malt1 and that catalytical activity of the TRAF6 RING

domain is needed to induce Malt1 ubiquitination. Furthermore, TRAF2 did not associate with Malt1 or promote Malt1 ubiquitination, proving specificity of the Malt1-TRAF6 interaction.

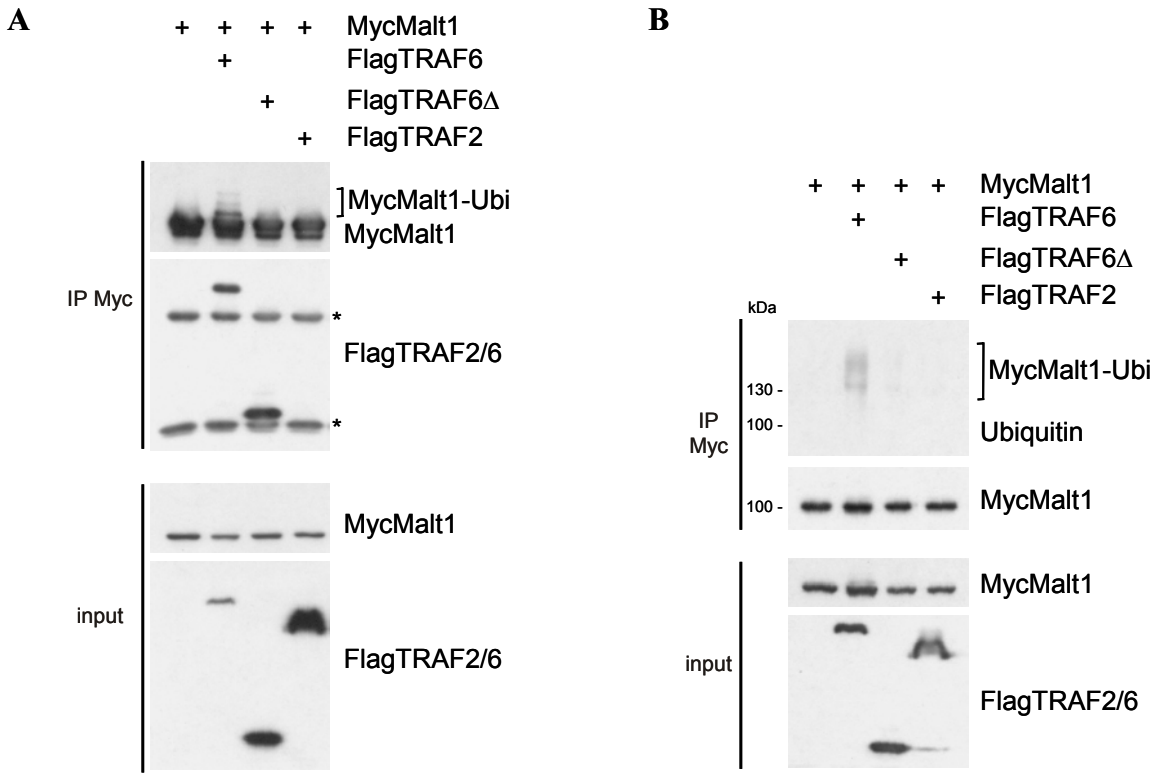


Figure 2.5: TRAF6 induces Malt1 ubiquitination. HEK293 cells were transfected with MycMalt1, FlagTRAF6, FlagTRAF6Δ (aa 289-522), and FlagTRAF2 and either lysed in (**A**) CoIP buffer for detection of bound proteins after MycMalt1 IP or in (**B**) ubiquitination buffer for detection of Malt1 ubiquitination after MycMalt1 IP. Precipitates were analyzed by Western blotting with the indicated antibodies. Cross-reaction bands of antibody heavy and light chains after IP are marked by asterisks.

Additionally, a control experiment was performed to prove the efficient disruption of protein-protein interactions after lysis in ubiquitination buffer. HEK293 cells were transfected with MycMalt1, FlagTRAF6, and FlagBcl10 and cells were lysed either in CoIP or ubiquitination buffer. After MycMalt1 IP, precipitates were analyzed for binding of TRAF6 or Bcl10 to Malt1 (Fig. 2.6). Western blot results clearly showed that association of TRAF6 and Bcl10 with Malt1 could be detected after lysis of cells in CoIP buffer, but interaction was completely abolished in ubiquitination buffer. This proves that lysis of cells in ubiquitination buffer prevents protein-protein interactions. The control experiment thus ensures that upon Malt1 IP under these conditions specifically ubiquitination of Malt1 and not of an associated factor, e.g. TRAF6, is detected. Furthermore, simultaneous co-expression of Bcl10 and TRAF6 with Malt1 showed that Bcl10/Malt1 interaction did not interfere with TRAF6 binding to Malt1. Thus, Bcl10 and TRAF6 can associate independently with Malt1.

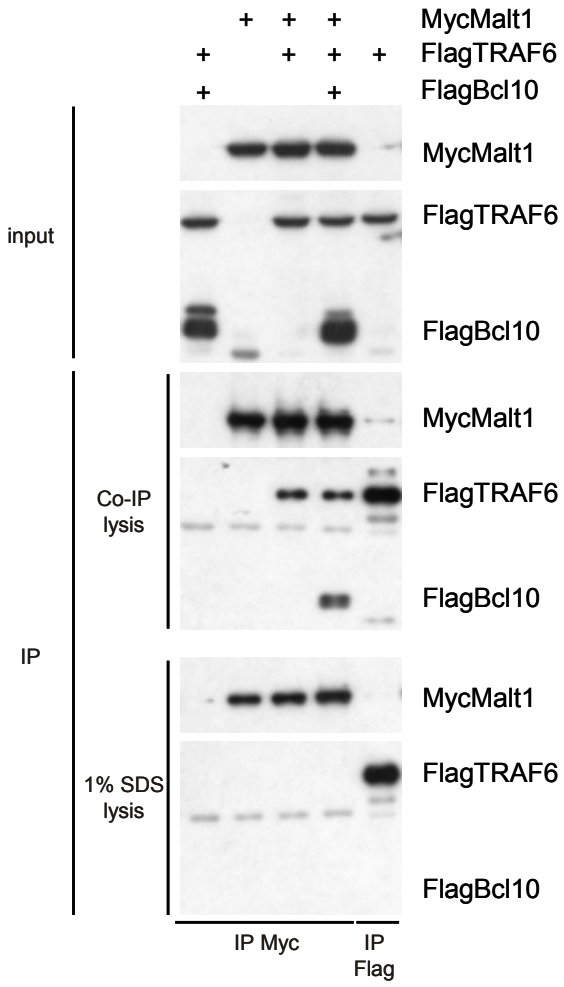


Figure 2.6: Lysis under denaturing conditions prevents protein-protein interactions. HEK293 cells were transfected with MycMalt1, FlagTRAF6 and FlagBcl10, lysed in CoIP or ubiquitination buffer (1% SDS) and subjected to MycMalt1 or control Flag IP. Malt1 precipitates were analyzed for bound TRAF6 and Bcl10 by Western blotting as indicated. Control Flag IPs demonstrate that eqivalent amounts of FlagTRAF6 could be directly precipitated under both lysis conditions. However, co-immunoprecipitation of TRAF6 or Bcl10 with MycMalt1 was completely abolished after lysis in ubiquitination buffer. Futhermore, Bcl10/Malt1 interaction did not affect TRAF6 binding to Malt1 as can be seen by equivalent amounts of TRAF6 co-precipitated with Malt1 in lanes 3 and 4 (CoIP buffer lysis).

Since TRAF6 was shown to associate with the C-terminus of Malt1 [183], it was next examined, whether it is also the Malt1 C-terminus that is targeted by TRAF6-induced ubiquitination. Therefore, consecutive N-terminal deletions of Malt1 were performed to narrow down the region of ubiquitination. The respective Malt1 constructs were transfected in HEK293 cells together with TRAF6 and analyzed for ubiquitination (Fig. 2.7 A). Western blot results demonstrated that the last 200 amino acids of Malt1 (612-813) still became ubiquitinated upon overexpression of TRAF6. Further N-terminal deletion, however, lead to complete abrogation of Malt1 ubiquitination (aa 684–813). Since the TRAF6 binding motifs are also found in the C-terminal part of Malt1, all constructs were analyzed for their ability to associate with TRAF6 to ensure that defective Malt1 ubiquitination was not due to impaired Malt1-TRAF6 interaction (Fig. 2.7 B). In CoIP experiments, Malt1 684-813 revealed a slightly reduced affinity for TRAF6. Hence, although the deletion analysis suggested that the region from amino acid 612 to 684 contains the acceptor lysines, a contribution of further C-terminal lysine residues could not be completely excluded.

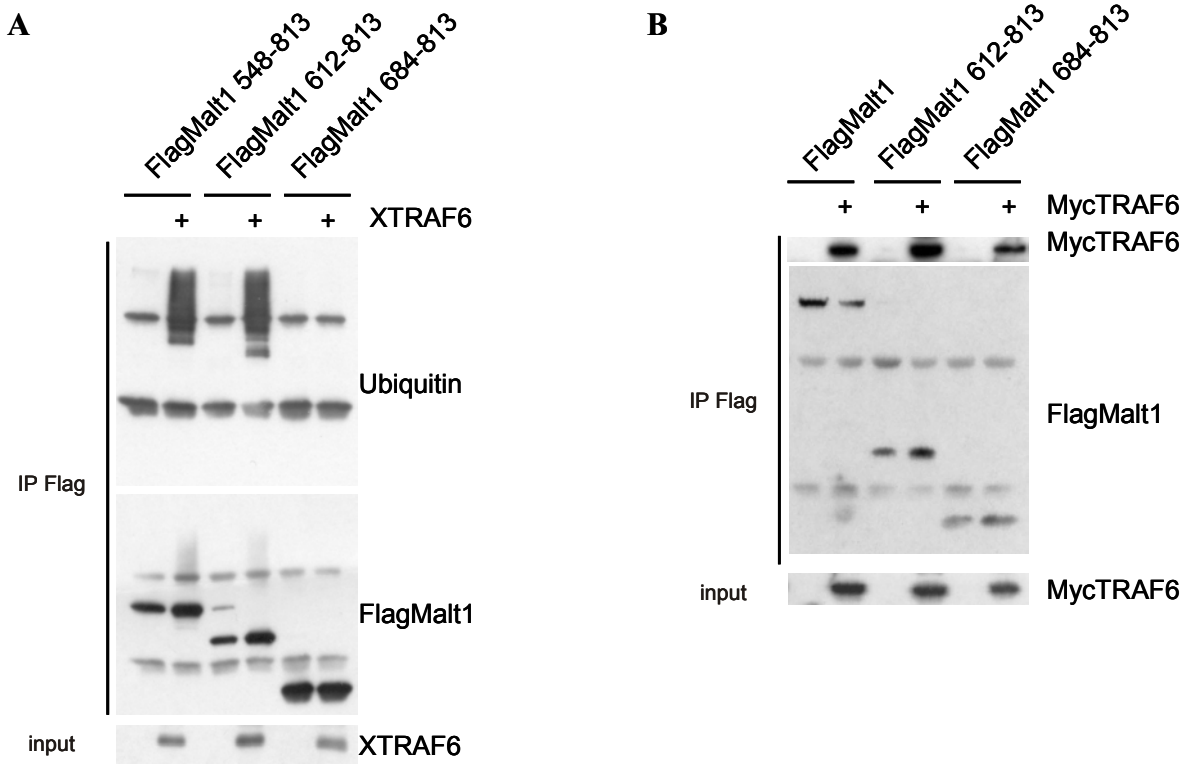


Figure 2.7: TRAF6-dependent Malt1 ubiquitination occurs in the C-terminus. HEK293 cells were transfected with FlagMalt1 548-813, 612-813, 684-813, and XTRAF6 or MycTRAF6. Cells were lysed in ubiquitination buffer for detection of Malt1 ubiquitination (**A**) or CoIP buffer for detection of TRAF6 interaction after FlagMalt1 IP (**B**). Deletion analysis suggested that the lysine acceptor residues are located between amino acids 612 and 684.

To further support the finding that TRAF6 can act as E3 ligase for Malt1 and to determine the linkage of Malt1-conjugated ubiquitin chains, an *in vitro* ubiquitination assay for Malt1 was established. For that purpose GSTMalt1 482-813 and GSTTRAF6 were purified from *E. coli* as described in chapter 6.6.1 (Fig. 2.8 A). GSTMalt1, bound to glutathione sepharose beads, was incubated with GSTTRAF6, E1 enzyme, E2 enzyme (Ubc13/Uev1a heterocomplex), ubiquitin or ubiquitin mutants, and Energy Regeneration Solution (ERS) containing ATP, magnesium, and ATP regenerating enzymes at 30 °C for 2 h. Ubiquitination was detected by Western blotting after boiling of the ubiquitination reactions in ubiquitination buffer (1% SDS) to abolish any non-covalent protein-protein interactions and Malt1 immunoprecipitation (Fig. 2.8 B). Also in this *in vitro* system Malt1 could successfully be ubiquitinated by TRAF6. To investigate whether ubiquitin chains attached to Malt1 are linked through lysine 48 or lysine 63, ubiquitin proteins were employed, in which one of these two lysine residues was mutated to an arginine. Mutation of lysine 63 to arginine (ubiquitin K63R) led to complete abolishment of TRAF6 dependent Malt1 ubiquitination, whereas exchange of lysine 48 (ubiquitin K48R) did not effect Malt1 ubiquitination (Fig. 2.8 B).

Taken together these data clearly show that TRAF6 can serve as an E3 ligase for Malt1, mediating the assembly of K63-linked ubiquitin chains to the Malt1 C-terminus. In general, K63 linkages are involved in regulation of protein-protein interactions, protein localization, or protein activity and do not mediate protein degradation. Therefore, the determination of K63 linkage of Malt1 attached ubiquitin chains further strengthened the hypothesis that Malt1 ubiquitination could be involved in signal propagation and does not represent a degradation signal.

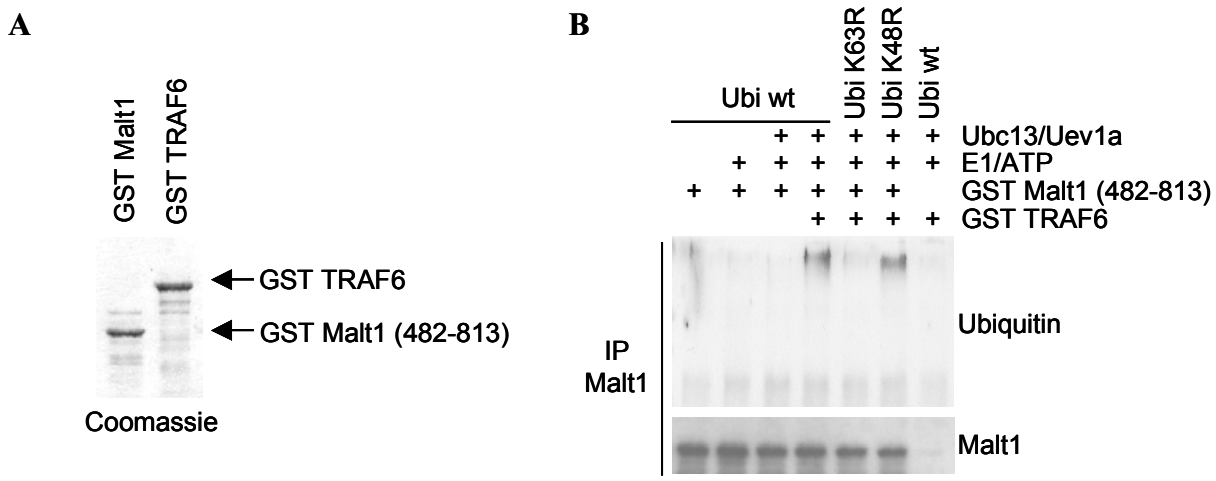


Figure 2.8: TRAF6 mediates the assembly of K63 linked ubiquitin chains to Malt1. (**A**) Coomassie staining showing purified GSTMalt1 482-813 and GSTTRAF6. (**B**) *In vitro* ubiquitin ligation reactions were performed using GSTMalt1 (aa 482-813) as substrate, GSTTRAF6, E1 enzyme, E2 enzyme (Ubc13/Uev1a), Energy regeneration solution (ATP) and ubiquitin wt, K63R, or K48R proteins. Ubiquitination reactions were boiled in 1% SDS containing ubiquitination buffer before ubiquitination was analyzed by Western blotting after Malt1 immunoprecipitation.

2.1.4 TRAF6 mediates Malt1 ubiquitination upon T cell activation.

As a next step, TRAF6 function for stimulus-dependent Malt1 ubiquitination in Jurkat T cells was addressed. First, Jurkat T cells were stimulated with PMA/ionomycin and Malt1 or TRAF6 were immunoprecipitated. Malt1 IPs revealed an inducible binding of TRAF6 to Malt1, which coincides with Malt1 ubiquitination and IκBα degradation (Fig. 2.9 A). *Vice versa*, Malt1 could be co-precipitated with TRAF6 upon T cell stimulation. In side-by-side analysis of IP and lysate samples, it could be seen that most of the TRAF6-associated Malt1 was modified by ubiquitination upon T cell activation (Fig. 2.9 B).

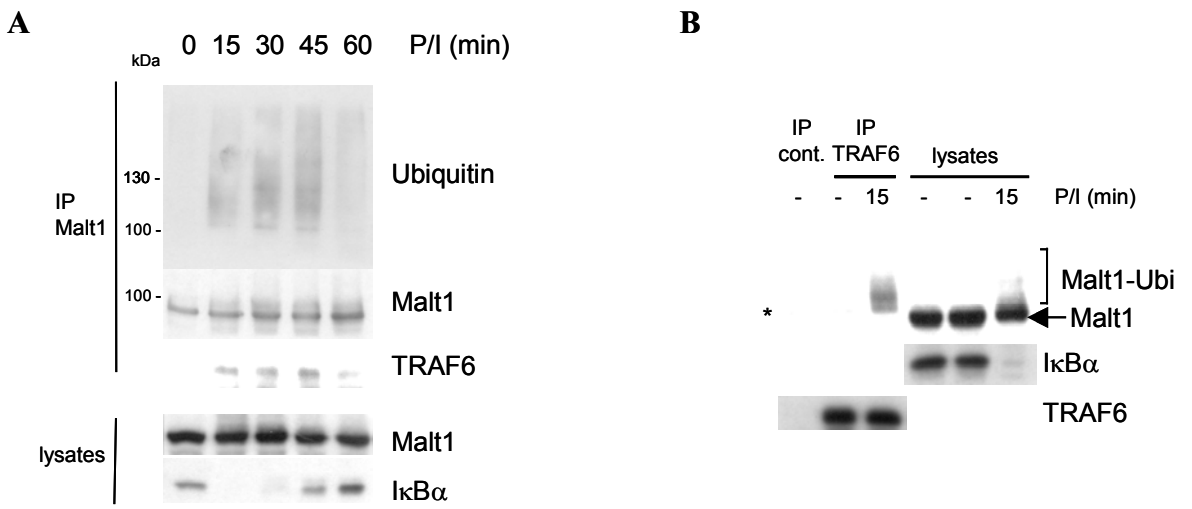


Figure 2.9: Inducible interaction of TRAF6 and Malt1 in Jurkat T cells. Jurkat T cells were stimulated with PMA/ionomycin (P/I) for the indicated time periods and lysed in CoIP buffer. (**A**) Malt1 IP was performed for simultaneous detection of TRAF6 interaction and Malt1 ubiquitination. (**B**) IPs were carried out using TRAF6 or control antibodies. IP and lysate samples were analyzed side by side to demonstrate that the majority of TRAF6 associated Malt1 was modified by ubiquitination. Asterisk marks an unspecific band.

After proof of the stimulus-dependent interaction of endogenous Malt1 and TRAF6 in Jurkat T cells, RNA interference experiments were performed to examine, if TRAF6 could also serve as E3 ubiquitin ligase for Malt1 ubiquitination upon T cell activation. Indeed, the reduction of TRAF6 protein levels in Jurkat T cells led to a significant reduction of Malt1 ubiquitination after PMA/ionomycin stimulation (Fig. 2.10 A). Three different siRNAs against TRAF6 were used to exclude off-target effects.

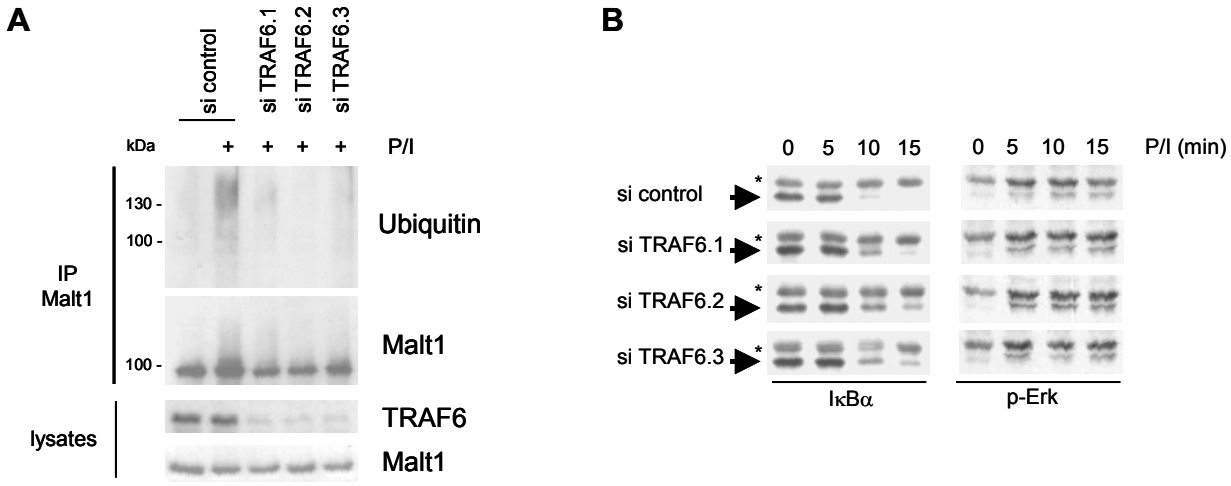


Figure 2.10: siRNA mediated knock-down of TRAF6 impairs inducible Malt1 ubiquitination and NF-κB signaling. (**A**) Jurkat T cells were transfected with three independent TRAF6 siRNAs and control siRNA. 72 hours after transfection, Jurkat T cells were stimulated with P/I for 20 min. Cells were lysed in ubiquitination buffer and Malt1 precipitates were analyzed with an anti-ubiquitin antibody after Western blotting. (**B**) Jurkat T cells were transfected as in (A) and then stimulated with P/I for different time periods. Extracts were analyzed by Western blotting for IκBα degradation and phosphorylation of the MAPKinase Erk.

In addition, IκBα degradation upon PMA/ionomycin stimulation was examined to investigate whether TRAF6 is required for NF-κB activation upon TCR ligation. Congruent with previous results [183], the reduction of TRAF6 protein level resulted in impaired NF-κB activation, monitored by significantly delayed IκBα degradation (Fig. 2.10 B). Phosphorylation of the MAPKinase Erk served as control for equivalent stimulation of the cells.

Thus, these results demonstrate that Malt1 and TRAF6 associate upon T cell activation and that stimulus-dependent Malt1 ubiquitination is mediated by TRAF6. Furthermore, TRAF6 seems to be required for NF-κB activation upon TCR signaling.

2.1.5 TRAF6 binding sites in Malt1 are critical for NF-κB activation in T cells

A consensus sequence for TRAF6 binding sites had been published [186], based on which two putative interaction motifs in the Malt1 protein were proposed [183]: PE**E**TGSY (aa 640-646) and PV**E**TTD (aa 793-798). Disturbance of these binding motifs by mutation of the two glutamic acid residues to alanines has been shown to impair interaction of TRAF6 and the C-terminus of Malt1 [183]. Therefore, it was now first tested in co-transfection experiments whether also interaction of full-length Malt1 with TRAF6 was prevented, when both binding sites were altered. For that purpose, the two glutamates (E642 and E795) were mutated to alanines in FlagMalt1 (Malt1 2EA). FlagMalt1 wildtype and 2EA mutant were then co-expressed with XTRAF6 or MycTRAF6 in HEK293 cells and Flag IPs were analyzed for TRAF6 co-immunoprecipitation and ubiquitination of Malt1 (Fig. 2.11).

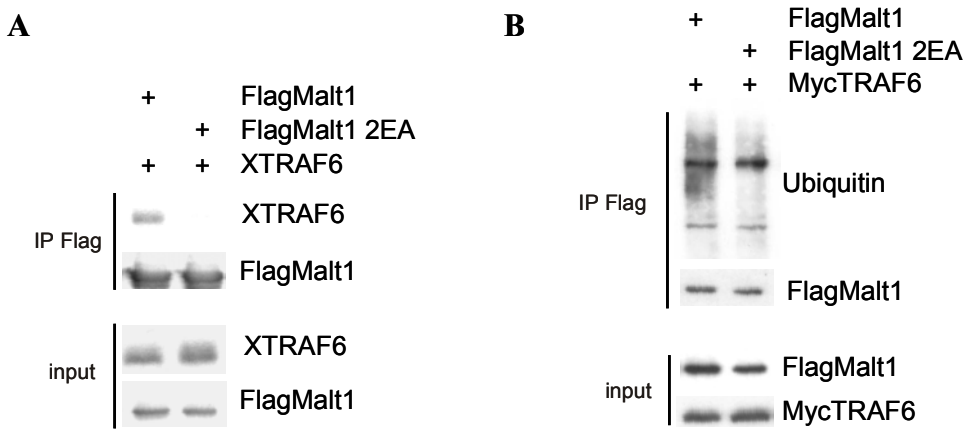


Figure 2.11: Mutation of TRAF6 binding motifs in Malt1 impairs Malt1-TRAF6 interaction and TRAF6-dependent Malt1 ubiquitination. FlagMalt1, FlagMalt1 2EA, XTRAF6, or MycTRAF6 were expressed in HEK293 cells. Cells were lysed in CoIP (**A**) or ubiquitination buffer (**B**) and FlagMalt1 wt or 2EA precipitates were analyzed by Western blotting for co-immunoprecipitated TRAF6 (A) or Malt1 ubiquitination (B).

Full length Malt1 2EA indeed showed impaired binding to TRAF6. In addition, TRAF6-dependent ubiquitination of Malt1 in HEK293 cells was strongly decreased upon introduction of the 2EA mutations.

Although the binding sites for TRAF6 in Malt1 were known before [183], functional importance of these motifs for NF-κB activation upon T cell stimulation had not been proven. Thus, the physiological significance of TRAF6/Malt1 interaction was now investigated using these binding sites. This was addressed by reconstitution experiments with Malt1 deficient T cells, which are defective in NF-κB activation and interleukin-2 (IL-2) production upon CD3/28 co-ligation or P/I stimulation [157, 158]. The ability of Malt1 mutant constructs to restore NF-κB activation and IL-2 production in comparison to wt Malt1 in these T cells thus provides information about the functional importance of the altered protein regions.

Primary CD4 positive (CD4+) T cells were isolated from spleen and lymph nodes of Malt1 deficient mice and retrovirally infected with FlagMalt1 wildtype or 2EA. The retroviral vectors contained an IRES sequence for the simultaneous expression of the surface marker Thy1.1. Malt1 deficient mice only express the Thy1.2 isoform. Consequently, infected cells could be identified by staining with an APC (allophycocyanin)-labeled Thy1.1 antibody and flow cytometry analysis using FACS (Fluorescence Activated Cell Sorting) (see scheme in Fig. 2.12 A).

Viable cells were gated according to forward scatter (FSC) and side scatter (SSC) (Fig. 2.12 B, left panel). While the FSC correlates with cell volume, the SSC reflects the inner complexity (nucleus shape, etc.) and membrane roughness of the cell. Viable cells were then analyzed for expression of Thy1.1 compared to uninfected cells (R2, Fig. 2.12 B, right panels), showing that transfection efficiencies of 30-50% were achieved (45.5 % in the example given in Fig 2.12 B). However, Western blot analysis of lysates from infected Malt1 deficient T cells and T cells from control mice revealed that ectopic expression of Malt1 was low compared to the endogenous Malt1 protein level in CD4+ T cells from wildtype mice (Fig. 2.12 C). In addition, co-staining of Thy1.1 and Flag demonstrated that only cells that showed high levels of Thy1.1 (Thy1.1 high) significantly expressed FlagMalt1 (Fig. 2.12 D). For this analysis, cells were gated for high Thy1.1 expression (R2 in Fig 2.12 D, left panel) and the FlagMalt1 wt or 2EA staining of these gated cells was depicted in a histogram (Fig. 2.12 D, right panel). The similar peak shift for Malt1 wt and 2EA proteins in this Thy1.1/Flag co-staining proves that both proteins were expressed at equivalent levels in Thy1.1 high cells. Since only Thy1.1 high cells showed significant expression of Malt1 proteins and were therefore analyzed in the following experiments, this represents an important control

experiment, proving that any differences in the rescue abilities of the two Malt1 constructs would not be due to different protein expression levels.

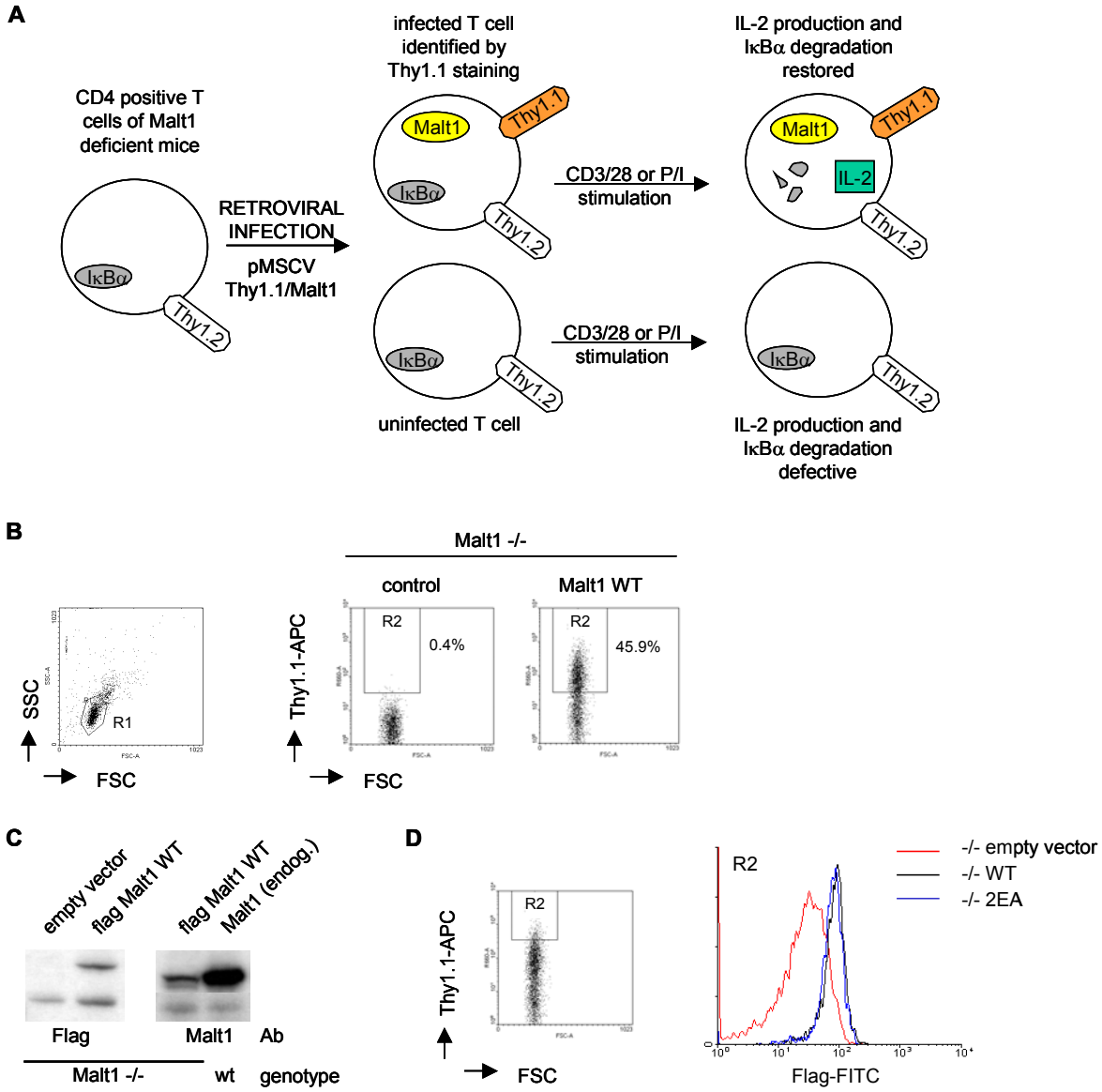


Figure 2.12: Retroviral infection of Malt1 -/- CD4+ T cells (**A**) Schematic representation of retroviral infection of Malt1 deficient CD4 positive T cells. Simultaneous expression of Thy1.1 with Malt1 enabled identification of infected cells, because these T cells normally only express the Thy1.2 isoform. Upon stimulation with P/I or CD3/28 ligation, the ability of ectopically expressed Malt1 constructs to restore defective IκBα degradation and IL-2 production was examined. (**B**) Malt1 -/- T cells were infected with a retroviral Thy1.1-IRES-FlagMalt1 construct. Viable (R1, left panel) cells were analyzed for Thy1.1 expression. Comparison of infected with uninfected cells revealed a transfection efficiency of 45,9 % (R2, right panel). (**C**) Western blot analysis of lysates from primary T cells after infection and wt T cells showed that ectopic expression of FlagMalt1 was low compared to endogenous Malt1 protein level. (**D**) Malt1 -/- T cells were infected with retroviral Thy1.1-IRES-FlagMalt1 wt and 2EA constructs. Viable (R1, (A)) and Thy1.1 high (R2, left panel) cells were analyzed by Flag-FITC staining. Similar peak shifts were observed for the two Malt1 constructs compared to the empty vector control, proving equivalent expression levels in the analyzed cell population (Thy1.1 high cells).

IκBα degradation can be used as determinant for NF-κB activation and should now be investigated in reconstituted cells. Since transfection efficiency was just around 50% and importantly only a small population of infected cells (Thy1.1 high) expressed significant levels of FlagMalt1 constructs, analysis of IκBα degradation by Western blot on the background of unreconstituted cells was not possible. For that reason intracellular staining of IκBα in T cells and FACS analysis were performed. IκBα degradation can hereby be observed as an increase in cells stained for low IκBα protein amounts upon stimulation, as shown for stimulated T cells from wt mice in Fig. 2.13 A. Coupled with Thy1.1 co-staining and gating for high Thy1.1 levels, this provides the opportunity to specifically analyze Malt1 expressing single cells for restored IκBα degradation. Hence, cells infected with FlagMalt1 wt, FlagMalt1 2EA and empty vector control were stimulated with PMA/Ionomycin or left untreated and co-stained with Thy1.1 and IκBα antibodies. Viable (R1), Thy1.1 high cells (R2) were then analyzed for their IκBα protein levels (Fig. 2.13 B).

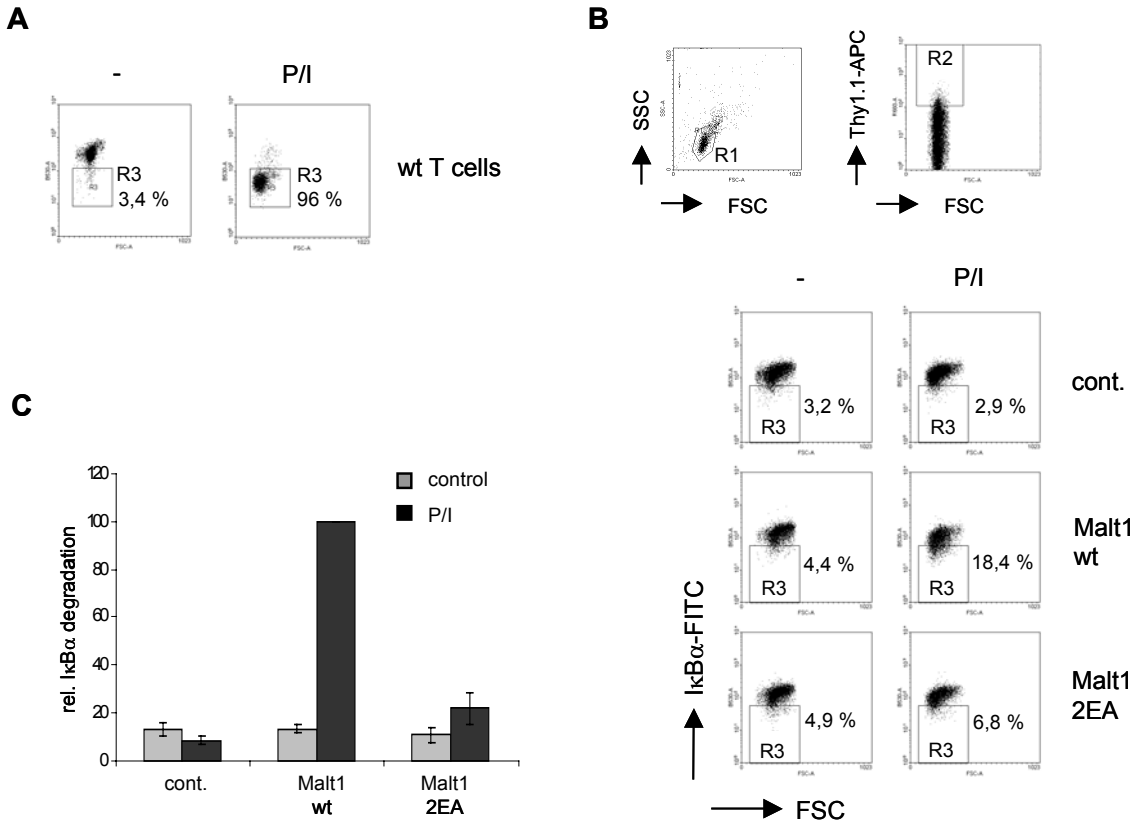


Figure 2.13: TRAF6 binding sites in Malt1 are required for IκBα degradation (**A**) CD4+ T cells from wt mice were stimulated with P/I for 20 min and analyzed by intracellular IκBα staining and FACS. IκBα degradation upon stimulation is monitored by an increase in cells gated for low IκBα protein levels (R3) (**B**) Malt1 -/- T cells were infected with retroviral Thy1.1-IRES-FlagMalt1 wt and 2EA constructs. Viable (R1, upper panel) and Thy1.1 high (R2, upper panel) cells were analyzed by IκBα staining and FACS. Quantification of IκBα degradation was performed by determining the number of cells gated for low IκBα protein amounts (R3). (**C**) Three independent experiments were analyzed as in (B) and numbers for Malt1 wt reconstitution were set to 100 %. Bars and standard deviations are given.

Results showed that ectopic expression of FlagMalt1 wt could partially restore IκBα degradation, monitored by an increase in cells showing low IκBα protein levels after P/I stimulation (18.4 %) compared to empty vector control (2.9 %) (R3 in Fig. 2.13 B). However, FlagMalt1 2EA was significantly impaired in reconstituting IκBα degradation (6.8 %). For quantification, results from three independents were analyzed and numbers for Malt1 wt rescue were set to 100%. Bars and standard deviations for quantification of IκBα degradation are given in Fig. 2.13 C. These results clearly demonstrate that mutation of TRAF6 binding sites in Malt1 impairs PMA/ionomycin induced IκBα degradation in T cells.

To support the hypothesis that Malt1/TRAF6 interaction is important for T cell activation, it was investigated whether the TRAF6 binding sites are also required for CD3/28 ligation induced production of interleukin-2 (IL-2). To enable intracellular staining of IL-2, Brefeldin A was added to the cells to inhibit the budding of vesicles from the ER and therefore secretion. In that way, the amount of produced IL-2 could be measured by intracellular staining with a FITC-labeled IL-2 antibody and FACS analysis. As for IκBα degradation, this experimental setting provides the advantage of a single cell assay system compared to an also conceivable ELISA assay. First, IL-2 production of CD4+ T cells from wt mice upon CD3/28 triggering was determined (Fig 2.14 A). IL-2 production was hereby quantified by gating of viable cells for high IL-2 amounts (R3). Malt1 deficient CD4+ T cells were infected with FlagMalt1 wt and 2EA retroviruses and co-stained for Thy1.1 and IL-2. Viable, Thy1.1 high cells (R1, R2 upper panels Fig 2.14 B) were then analyzed for IL-2 production by determining the number of cells gated for high IL-2 amounts (R3 Fig. 2.14 B). Ectopically expressed Malt1 wt was able to significantly restore CD3/28 ligation-induced IL-2 production, as monitored by an increase of cells showing high IL-2 amounts upon stimulation (21 %, R3, Fig. 2.14 B) compared to empty vector control (1.1%, R3). Similar numbers (30 - 40 %) of IL-2 producing cells were obtained in CD4+ T cells from wt mice (Fig. 2.14 A). In line with the results for reconstituted IκBα degradation, Malt1 2EA could not provoke an increase in IL-2 production comparable to Malt1 wt (10.5 %). Analysis and quantification of three independent experiments is given in Figure 2.14 C, demonstrating that TRAF6 binding sites in Malt1 are required for optimal T cell activation upon TCR triggering.

Taken together, these reconstitution experiments provide evidence that the TRAF6/Malt1 interaction is critical for NF-κB and T cell activation in a close to physiological setting and further support a critical role for TRAF6 in TCR signaling.

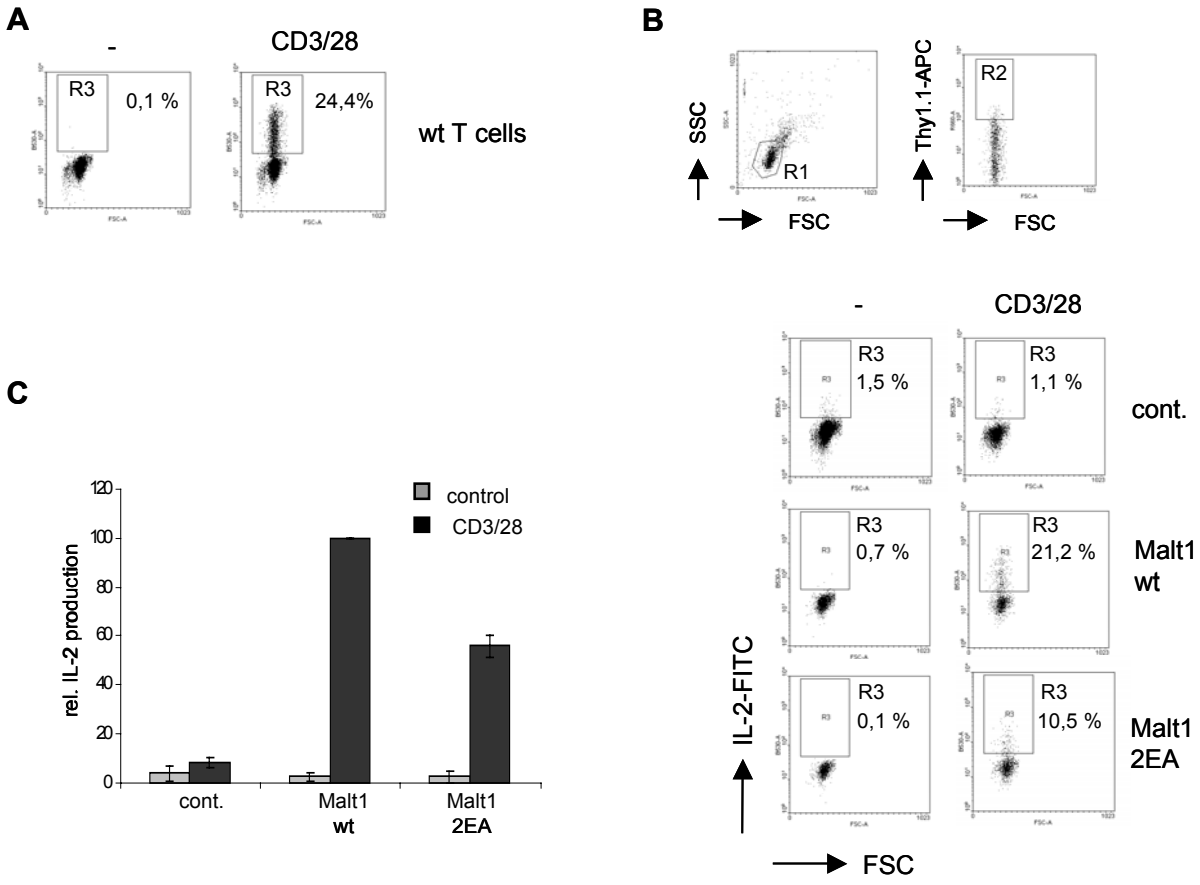


Figure 2.14: TRAF6 binding sites in Malt1 are required for IL-2 production. (**A**) CD4+ T cells from wt mice were stimulated with CD3/28 antibodies for 3 h and analyzed by intracellular IL-2 staining and FACS. IL-2 production upon stimulation is monitored by an increase in cells gated for high IL-2 protein levels (R3) (**B**) Malt1 -/- T cells were infected with retroviral Thy1.1-IRES-FlagMalt1 wt and 2EA constructs. Viable (R1, upper panel) and Thy1.1 positive (R2, upper panel) cells were analyzed for IL-2 staining. Quantification of IL-2 production was performed by determining the number of cells gated for high IL-2 protein amounts (R3). (**C**) Three independent experiments were analyzed as in (B) and numbers for Malt1 wt reconstitution were set to 100 %. Bars and standard deviations are given.

2.1.6 Multiple C-terminal lysines of Malt1 can serve as ubiquitin acceptor sites

As a first step to a functional analysis of Malt1 ubiquitination, mapping of the ubiquitin acceptor lysine(s) in Malt1 was addressed. Consecutive N-terminal deletion analysis of Malt1 revealed that ubiquitination takes place in the C-terminal 200 amino acids of Malt1 (612-813) (see Fig. 2.7 A). The results suggested that the region between aa 612 and 684 contains the acceptor lysine(s), however, a contribution of lysines in the very C-terminal region (684-813) could not be completely excluded (see Fig. 2.7 B). Therefore, the complete C-terminal 200 amino acid sequence of Malt1 was screened for acceptor lysines by consecutive introduction of point mutations, thereby exchanging lysines with arginines (Fig. 2.15).

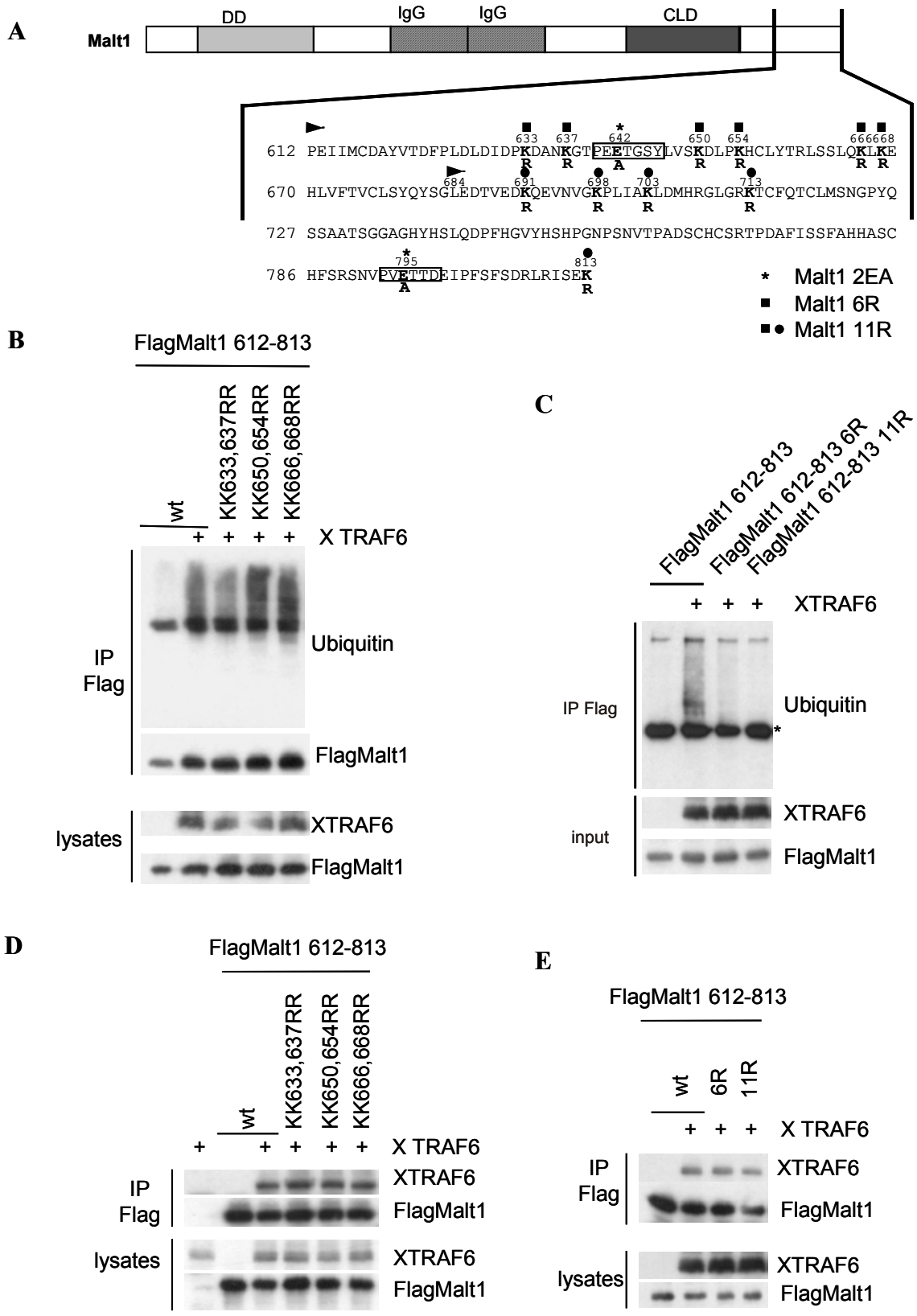


Figure 2.15: TRAF6 mediates ubiquitin conjugation to multiple lysine residues in the Malt1 C-terminus. (**A**) Schematic presentation of the Malt1 domain organization and primary sequence of the Malt1 C-terminus (aa 612-813). Mutated lysine residues are marked by squares (Malt1 6R) or squares/circles (Malt1 11R). TRAF6 binding sites are boxed and mutated glutamates are marked by asterisks (Malt1 2EA) (**B**) and (**C**) HEK293 cells were transfected with FlagMalt1 612-684 lysine to arginine mutants and XTRAF6, and lysed in ubiquitination buffer. Ubiquitination of Malt1 was detected after FlagMalt1 IP. An asterisk marks an unspecific band. (**D**) and (**E**) HEK293 cells were transfected with the indicated Malt1 612-684 constructs, lysed in CoIP buffer and subjected to FlagMalt1 IP to detect interaction with TRAF6.

Six lysine residues are located between aa 623 and 684 (K633, K637, K650, K654, K666, K668) and five additional lysines are present in the very C-terminal part of Malt1 (K691, K698, K703, K713, K813). The primary sequence of the Malt1 C-terminus is depicted in Fig. 2.15 A, showing lysine residues and TRAF6 binding sites.

It is known that mapping of ubiquitination sites can be difficult due to the fact that ubiquitination is often rather promiscuous and not strictly restricted to one site. Therefore, as a first step to determine the acceptor lysines in Malt1, the first six lysines located in the region, which was suggested to contain the acceptor sites by deletion experiments (aa 612 to 684), were pair wise exchanged to arginines (KK633,37RR, KK650,54RR, KK666,68RR). In addition, other mutant constructs were created, in which all six lysines (6R) between aa 612 to 684 or all eleven lysines (11R) of the C-terminal region of Malt1 were mutated.

All lysine mutant proteins were expressed in HEK293 cells and probed for ubiquitination upon TRAF6 co-expression by Flag IP and Western blotting (Fig. 2.15 B and C). It could be shown that none of the double mutants (KK633,37RR, KK650,54RR or KK666,68RR) displayed impaired Malt1 ubiquitination. Only the combined exchange of all six lysines (Malt1 6R) in the suspected ubiquitination region (aa 612-684) significantly reduced TRAF6 dependent Malt1 ubiquitination, which was completely abolished when all eleven lysines in the Malt1 C-terminus were mutated (Malt1 11R). It is important to note that none of the lysine mutations impaired the ability of the respective Malt1 protein to interact with TRAF6 (Fig. 2.15 D and E). This indicates that mutation of the lysines did not alter the TRAF6 binding interface, but removed the respective acceptor sites for TRAF6-dependent Malt1 ubiquitination. Thus, multiple lysines residues in the C-terminus of Malt1 can serve as acceptor sites for TRAF6 dependent ubiquitination.

Equivalent results could also be obtained in an *in vitro* ubiquitin ligation experiment. GSTMalt1 482-813 wt, 6R and 11R were purified from *E.coli* as described in chapter 6.6.1 and used as substrates for a TRAF6 ubiquitination assay. Also in this *in vitro* system, Malt1 ubiquitination was strongly reduced upon 6R and 11R mutations (Fig. 2.16), supporting the conclusion that multiple lysine residues in the Malt1 C-terminus can be ubiquitinated. Furthermore, these results prove specificity for this cell free ubiquitination assay.

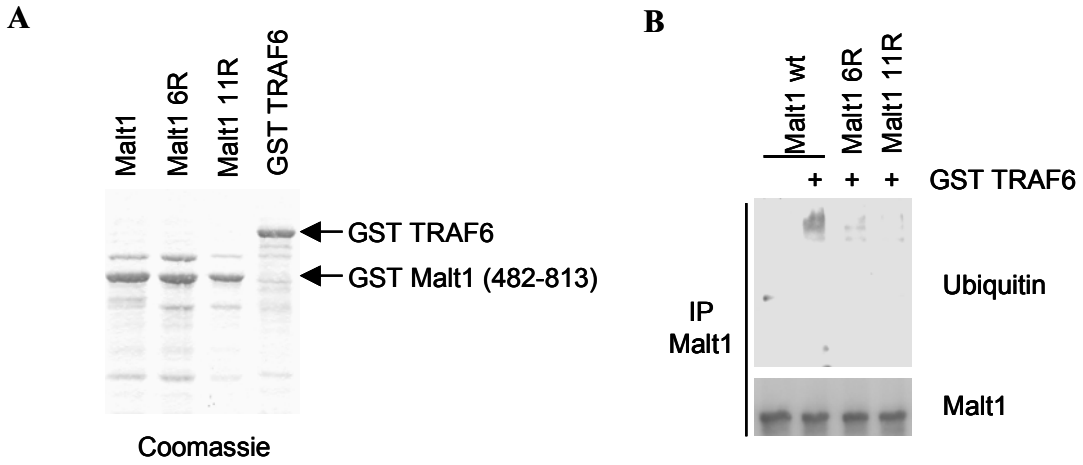


Figure 2.16: TRAF6 mediates *in vitro* assembly of ubiquitin chains to multiple C-terminal lysine residues of Malt1 C. (**A**) Coomassie staining showing purified GSTMalt1 482-813 wt, 6R and 11R, as well as GST TRAF6. (**B**) Malt1 wt, 6R and 11R (aa 482-813), bound to glutathione sepharose, were used as substrates for an *in vitro* ubiquitin ligation reaction using E1, E2 (Ubc13/Uev1a), Energy Regeneration Solution (Mg, ATP and ATP regenerating enzymes), ubiquitin and GST TRAF6. Ubiquitination reactions were boiled in 1 % SDS containing ubiquitination buffer prior to Malt1 immunoprecipitation and subsequent Western blot analysis.

To investigate if Malt1 ubiquitination upon T cell activation is also dependent on these C-terminal lysine residues, full length Malt1 6R and 11R constructs were transfected in Jurkat T cells together with HA-tagged ubiquitin. 72 hours after transfection, Jurkat cells were stimulated with P/I for 20 min and lysed in CoIP buffer. HA IPs from these lysates revealed reduced stimulus-dependent ubiquitination of Malt1 6R and 11R compared to wildtype Malt1 (Fig. 2.17 A). This shows that the C-terminal acceptor lysines mapped in overexpression experiments are also crucial for the observed stimulus-induced TRAF6-dependent ubiquitination of Malt1 in Jurkat T cells.

In line with the results from overexpression in HEK293 cells, ectopically expressed Malt1 6R and 11R did not show an impairment of stimulus-induced association with endogenous TRAF6 in Jurkat cells, whereas Malt1 2EA binding to TRAF6 was, as expected, strongly reduced (Fig. 2.17 B). In addition, lysine (6R, 11R) or glutamate (2EA) exchange in Malt1 did not affect constitutive binding of Malt1 to Bcl10 (Fig. 2.17 B) or stimulus-induced interaction with Carma1 in Jurkat T cells (Fig. 2.17 C). Unaltered interaction of Malt1 6R and 11R with TRAF6 demonstrates that most probably no changes in the structure of the Malt1 C-terminus have been evoked by lysine mutagenesis and that impaired ubiquitination of Malt1 6R and 11R is indeed due to the removal of acceptor sites and not to the disruption of interaction surfaces. Furthermore, these results indicate that Malt1/TRAF6 interaction and C-terminal Malt1 ubiquitination are not involved in CBM complex formation, because stimulus-

dependent interaction of Malt1 with Carma1 was unaffected by the 6R, 11R or 2EA mutations.

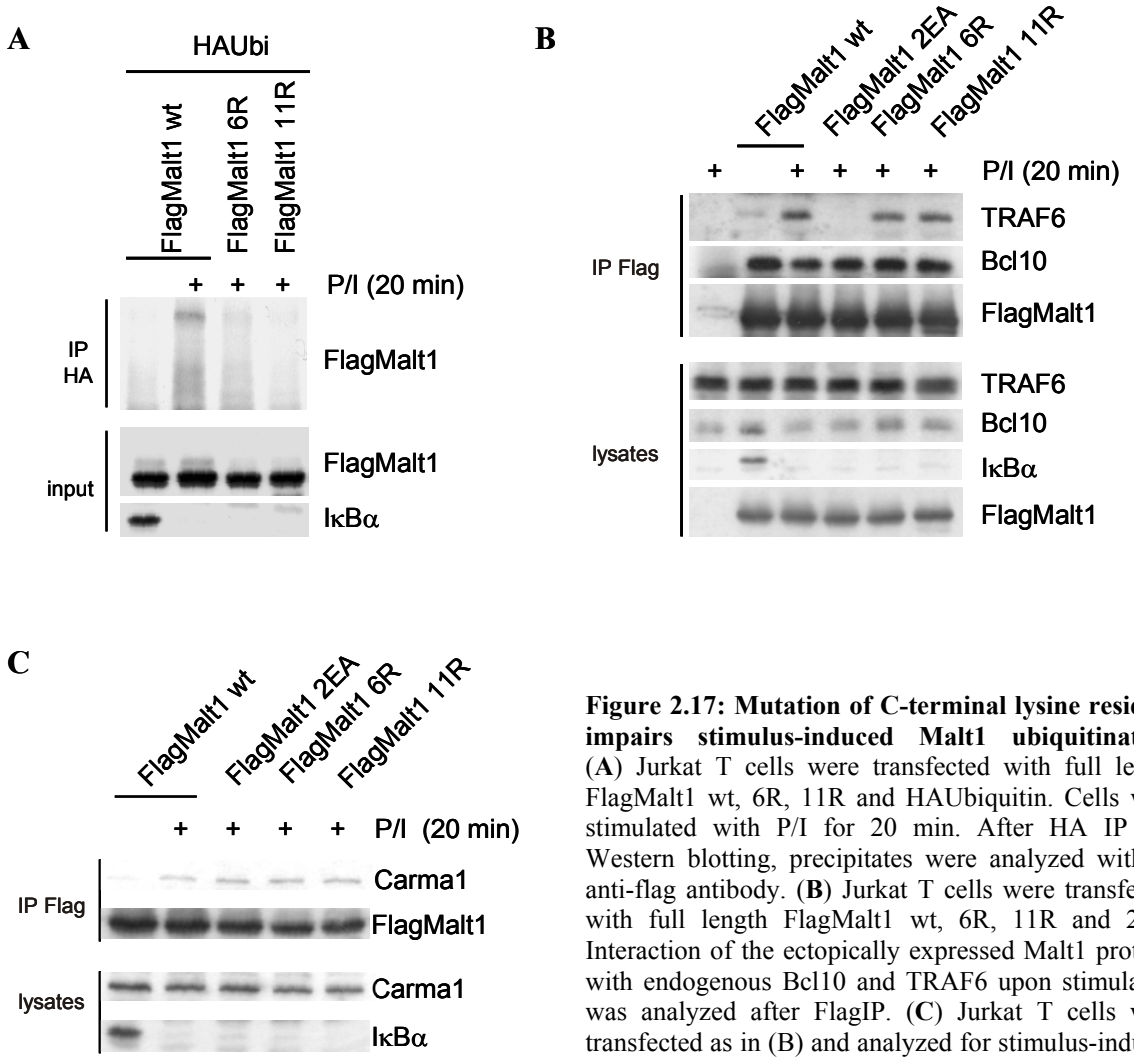


Figure 2.17: Mutation of C-terminal lysine residues impairs stimulus-induced Malt1 ubiquitination. (**A**) Jurkat T cells were transfected with full length FlagMalt1 wt, 6R, 11R and HAUbiquitin. Cells were stimulated with P/I for 20 min. After HA IP and Western blotting, precipitates were analyzed with an anti-flag antibody. (**B**) Jurkat T cells were transfected with full length FlagMalt1 wt, 6R, 11R and 2EA. Interaction of the ectopically expressed Malt1 proteins with endogenous Bcl10 and TRAF6 upon stimulation was analyzed after FlagIP. (**C**) Jurkat T cells were transfected as in (B) and analyzed for stimulus-induced interaction of the transfected constructs with Carma1.

2.1.7 Malt1 ubiquitination is crucial for NF-κB activation and IL-2 production

To examine the physiological relevance of C-terminal Malt1 ubiquitination, CD4 positive T cells from Malt1 deficient mice were reconstituted by retroviral infection with Malt1 lysine mutants as described for Malt1 wt and 2EA in chapter 2.1.5. Malt1 wt, 6R, 11R and an N-terminal deletion construct (Malt1 314-813), that cannot bind to Bcl10 and therefore served as negative control, were used for these rescue experiments. As described before, all constructs contained an IRES sequence for simultaneous expression of the surface marker Thy1.1, and staining with an APC-labeled Thy1.1 antibody was performed to identify infected cells by FACS. As for Malt1 2EA, the infected primary T cells were analyzed for the ability of the

different Malt1 constructs to reconstitute IκBα degradation and IL-2 production as determinants for NF-κB and T cell activation, respectively. As in Malt1 2EA experiments, only cells expressing high amounts of Thy1.1 (Thy1.1 high) and consequently high amounts of Malt1 were analyzed before and after stimulation in the following experiments (R2, Fig 2.18 A, right panel).

To ensure that differences in the rescue ability of the Malt1 constructs would not be due to different expression levels of the respective proteins, a control Flag staining was performed. This Flag/Thy1.1 co-staining showed that Thy1.1 high cells (R2) expressed equivalent amounts of all the transfected FlagMalt1 proteins, as can be seen by similar peak shifts for all constructs compared to cells infected with empty vector (Fig. 2.18 B).

First, IκBα degradation upon PMA/ionomycin stimulation was measured as determinant for NF-κB activation in T cells reconstituted with FlagMalt1 wt, 6R, 11R and 314-813. IκBα degradation could be observed as a partial shift of Thy1.1 high cells into a gate of low IκBα amount (Fig 2.18 C, gate R3) after stimulation. Quantification of the decrease in IκBα protein level was performed by determination of the relative number of cells in this gate. Infection with empty vector or FlagMalt1 314-813 did not lead to rescue of deficient IκBα degradation in Malt1 -/- T cells (1.1% and 1.9%, R3, Fig. 2.18 C). In contrast, rescue of IκBα degradation could be achieved with expression of FlagMalt1 wt: 17.5 % of viable, Thy1.1 high cells were gated for low IκBα protein amounts after 20 min of P/I stimulation (R3). Importantly, FlagMalt1 6R was severely impaired in mediating IκBα degradation (9.8% cells in R3), an effect that was even more pronounced when all eleven lysines were exchanged (FlagMalt1 11R, 4.8 % cells in R3). For quantification of these results, three independent experiments were analyzed. Numbers for Malt1 wt reconstitution were set to 100 %. Bars and standard deviations are given in Fig. 2.18 D.

These results demonstrate that the C-terminal acceptor lysines of Malt1 are required for PMA/ionomycin induced IκBα degradation and therefore NF-κB activation in primary T cells.

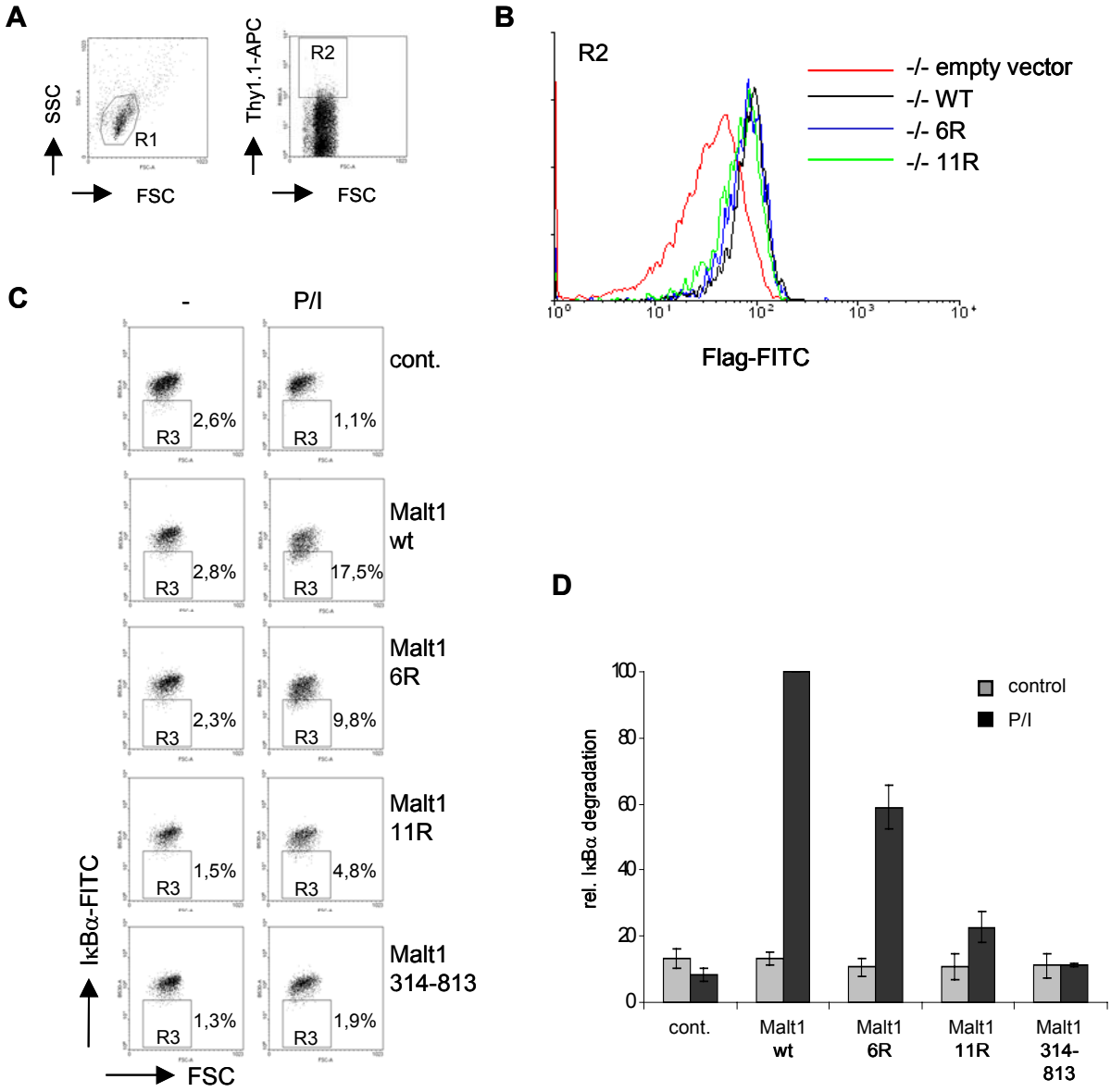


Figure 2.18: C-terminal Malt1 ubiquitination is required for IκBα degradation upon P/I stimulation. (**A**) Malt1 -/- T cells were infected with retroviral Thy1.1-IRES-FlagMalt1 constructs. Viable (R1, left panel) and Thy1.1 positive (R2, right panel) cells were analyzed in all further experiments. (**B**) CD4+ T cells were infected with retroviral Thy1.1-FlagMalt1 wt, 6R and 11R constructs. Viable (R1) and Thy1.1 positive (R2) cells were analyzed by Flag-FITC staining. Similar peak shifts were observed for all Malt1 constructs compared to the empty vector control, proving equivalent expression levels in the analyzed cell population. (**C**) CD4+ T cells were infected with retroviral Thy1.1-FlagMalt1 wt, 6R, 11R, 2EA and 314-813 constructs. IκBα protein amounts of viable, Thy1.1 high cells were determined by intracellular staining and FACS analysis. Cells were gated for low IκBα amounts (R3) to determine reconstituted IκBα degradation. Results for one exemplary experiment are given. (**D**) Quantitative analysis of three independent experiments. Numbers for FlagMalt1 wt reconstitution of IκBα degradation (R3 in (C)) were set to 100%. Standard deviations are given.

To determine whether Malt1 ubiquitination would also affect T cell activation, CD3/CD28 induced production of IL-2 was measured (Fig. 2.19). T cells were infected as described above and stimulated with plate bound CD3/28 antibodies for 3 h. 2 h before the end of stimulation Brefeldin A was added to the cells to inhibit secretion of IL-2. Production of IL-2

by viable, Thy1.1 positive cells (see Fig 2.18 A, R1 and R2) was quantified by the number of cells gated for high IL-2 protein amounts upon stimulation (Fig. 2.19 A, R3). As for IκBα degradation, FlagMalt1 wt expression in Malt1-/- T cells led to a significant increase in IL-2 production upon CD3/28 stimulation (29.1 %), whereas expression of empty vector or Malt1 314-813 had no effect. Again, mutation of the C-terminal acceptor lysines for ubiquitination significantly diminished the ability of Malt1 constructs to reconstitute IL-2 production (FlagMalt1 6R 19.2 %, FlagMalt1 11R 10 %). Quantitative analysis of three independent experiments (Fig. 2.19 B) clearly demonstrated that C-terminal acceptor lysines in Malt1 are required for optimal IL-2 production and therefore T cell activation in primary CD4 positive T cells.

Taken together, the data provide physiological evidence that C-terminal Malt1 ubiquitination is essential for NF-κB activation and IL-2 production in response to T cell activation.

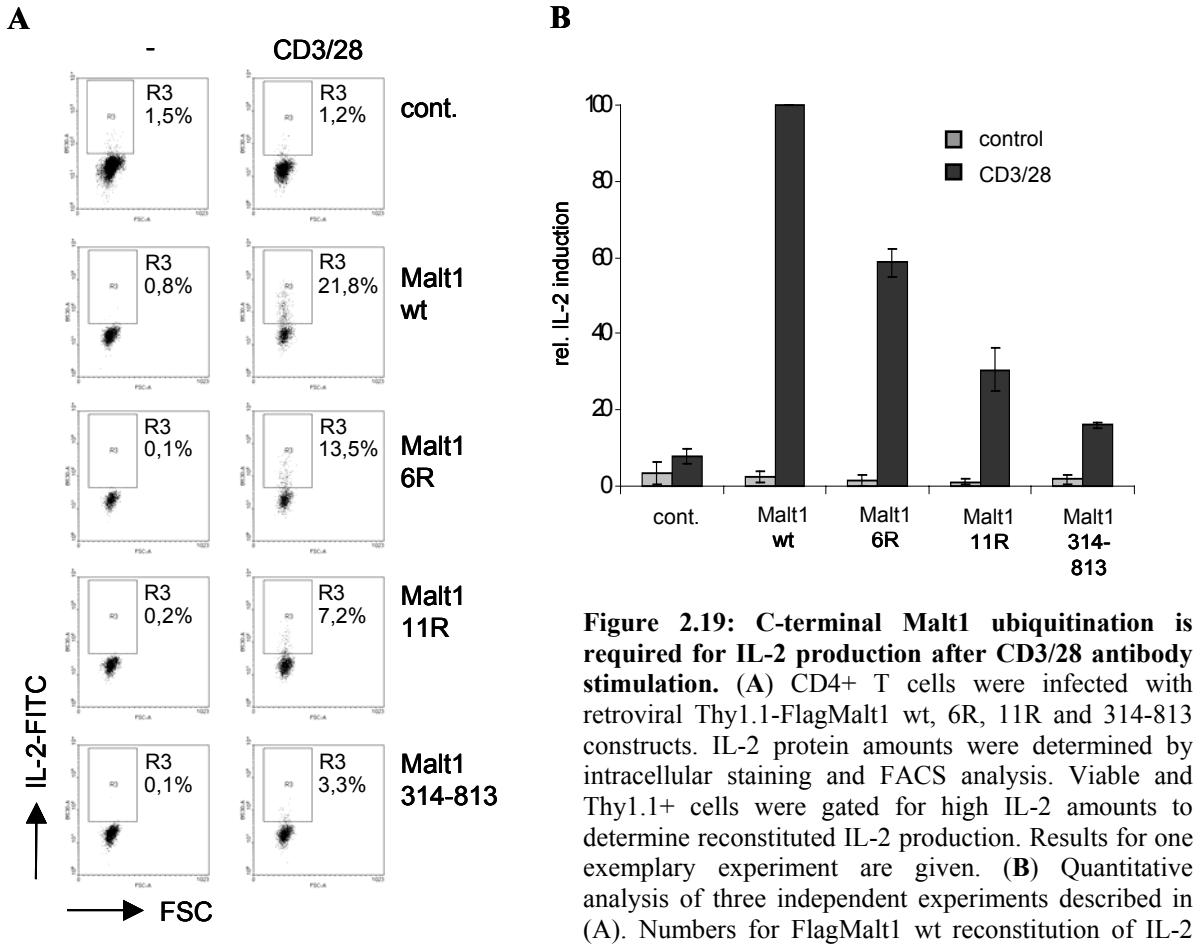


Figure 2.19: C-terminal Malt1 ubiquitination is required for IL-2 production after CD3/28 antibody stimulation. (**A**) CD4+ T cells were infected with retroviral Thy1.1-FlagMalt1 wt, 6R, 11R and 314-813 constructs. IL-2 protein amounts were determined by intracellular staining and FACS analysis. Viable and Thy1.1+ cells were gated for high IL-2 amounts to determine reconstituted IL-2 production. Results for one exemplary experiment are given. (**B**) Quantitative analysis of three independent experiments described in (A). Numbers for FlagMalt1 wt reconstitution of IL-2 production (R3 in (A)) were set to 100%. Standard deviations are given.

2.1.8 IKKγ is recruited to ubiquitinated Malt1.

In recent publications it has been demonstrated that IKKγ can bind to poly-ubiquitin chains. IKKγ preferentially associates with K63-linked ubiquitin chains, using the region between aa 242 and 350 as interaction surface (NEMO ubiquitin-binding domain, NUB), which includes parts of the second coiled-coil region and the leucine zipper. Mutations in this region (L329P, Y308S and D311N) were shown to impair ubiquitin binding [109, 110]. It was further demonstrated that the K63-linked ubiquitin conjugation to RIP1 in TNFα signaling is required for the recruitment of IKKγ and therefore of the IKK complex to the TNF receptor [109, 110].

As a consequence, the question arose, whether IKKγ associates with ubiquitinated Malt1 in response to T cell activation. To answer this question, Jurkat T cells were stimulated with PMA/ionomycin and IKKγ immunoprecipitation was performed. Indeed, ubiquitinated Malt1 could be co-immunoprecipitated with IKKγ after 15 min and up to 45 min of stimulation (Fig. 2.20). This coincides with early NF-κB activation as monitored by IκBα degradation. An unspecific cross reactivity that is detected in Malt1 Western blots after CoIPs is slightly larger than unmodified Malt1 (compare also Fig. 2.9. B). Importantly, IPs from stimulated Jurkat T cells using control IgGs proved that there is no unspecific binding of ubiquitinated Malt1 species (Fig. 2.22 A, first lane).

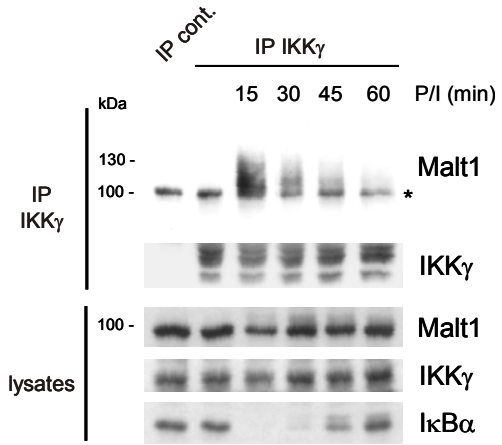


Figure 2.20: IKKγ associates with ubiquitin-conjugated Malt1. Jurkat T cells were stimulated with P/I as indicated, lysed and subjected to IKKγ CoIP or IP with a control antibody. Western blot analysis of the precipitates showed that cellular IKKγ binds to modified Malt1 in a stimulus-dependent manner. Molecular weight markers are given for better comparison of molecular sizes. An unspecific cross-reaction band slightly above 100 kDa in IP samples is marked by an asterisk.

Point mutations in IKKγ that prevent binding to K63-linked ubiquitin chains were then used to investigate, if the stimulus-dependent IKKγ-Malt1 interaction depends on the conjugation of poly-ubiquitin to Malt1. Two of these mutant IKKγ proteins (L329P and Y308S) were tested for their ability to associate with Malt1 in pull-down assays. Strep-tagged IKKγ wt and

mutant proteins were expressed and purified from *E. coli* and used for pull-down experiments from lysates of unstimulated or P/I stimulated Jurkat T cells (Fig. 2.21 A). Western blot analysis of the pull-down samples showed that StrepIKKγ wt preferentially associated with ubiquitinated Malt1 after P/I stimulation, although some affinity to unmodified Malt1 could also be detected. This is in contrast to the results obtained with IKKγ CoIPs from Jurkat T cells, where no interaction between IKKγ and Malt1 in unstimulated cells and IKKγ and unmodified Malt1 in stimulated cells could be detected. This discrepancy is probably due to the experimental conditions of the pull-down assay, where large amounts of recombinant IKKγ are presented as binding partner for cellular Malt1. Hence, a low affinity of IKKγ to unmodified Malt1 might be detected in this setting, but not under physiological conditions inside cells. Importantly however, IKKγ mutations L329P and Y308S reduced binding to ubiquitin-conjugated Malt1 in pull-downs from stimulated Jurkat cell lysates, while association with unmodified Malt1 was unaltered (Fig. 2.21 A).

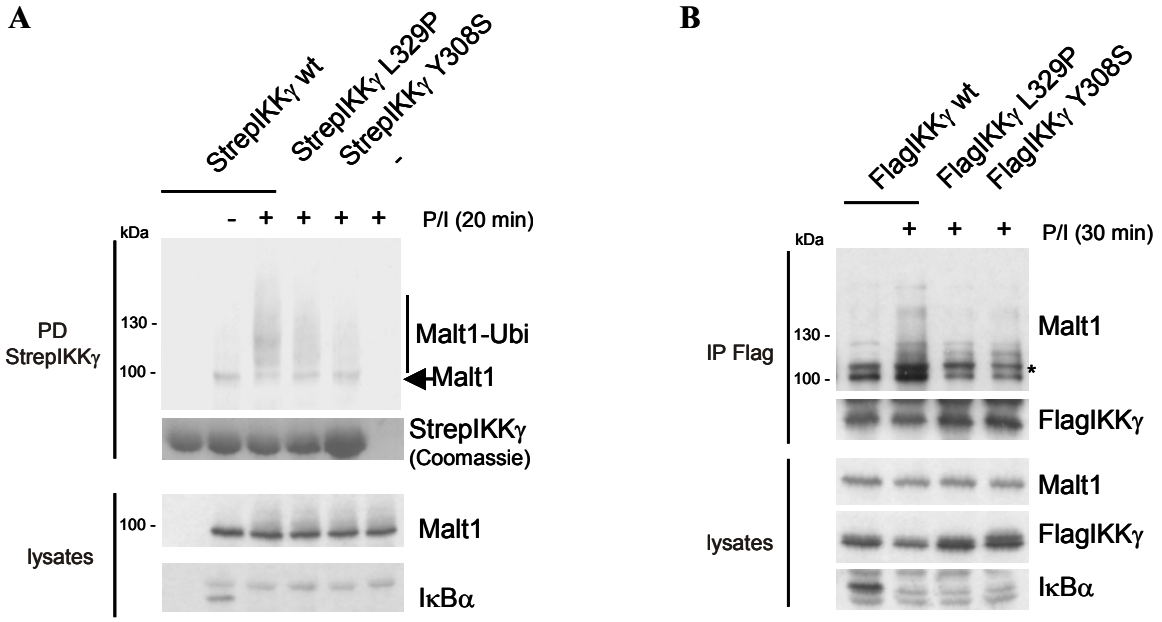


Figure 2.21: IKKγ mutants defective in poly-ubiquitin binding show impaired association with Malt1. (**A**) Recombinant StrepIKKγ wt, L329P and Y308S were used to pull-down Malt1 from lysates of unstimulated and P/I stimulated Jurkat T cells. Pull-down samples were analyzed by Western blotting. (**B**) Jurkat T cells were transfected with FlagIKKγ wt, L329P and Y308S, stimulated with P/I and lysed in CoIP buffer. Proteins were precipitated with Flag antibodies and analyzed for binding of endogenous Malt1. Asterisk marks an unspecific band detected after Flag IP.

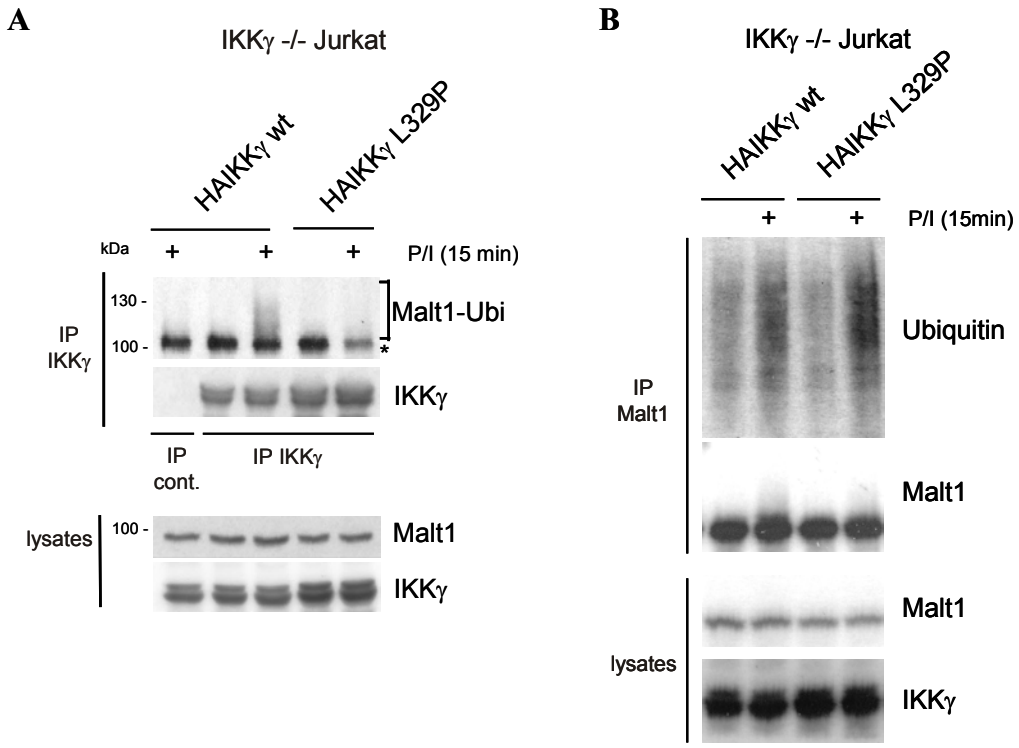
Next, FlagIKKγ wt, L329P and Y308S constructs were transfected in Jurkat T cells. Transfected cells were stimulated with P/I and lysed in CoIP buffer. Ectopically expressed IKKγ proteins were precipitated with Flag antibodies and precipitates were analyzed by Western blotting for bound endogenous Malt1. In line with the results from the StrepIKKγ pull-down experiments, modified Malt1 was co-immunoprecipitated with FlagIKKγ wt from lysates of stimulated cells, while the point mutations L329P and Y308S significantly reduced this interaction (Fig. 2.21 B).

Taken together, this demonstrates that Malt1-IKKγ interaction depends on an intact ubiquitin-binding motif in IKKγ. Thus, IKKγ associates with Malt1 ubiquitin chains upon T cell activation.

IKKγ deficient and reconstituted (IKKγ wt and L329P) Jurkat T cells, which were a kind gift of J.D. Ashwell (National Institutes of Health, Bethesda, USA), were then used to investigate the physiological consequences of IKKγ interaction with ubiquitinated Malt1 for NF-κB activation. First, cells were analyzed for IKKγ-Malt1 interaction. In IKKγ deficient cells, reconstituted IKKγ wt, but not IKKγ L329P, associated with ubiquitin-conjugated Malt1 (Fig. 2.22 A), even though the level of inducible Malt1 ubiquitination was equivalent in both cell lines (Fig. 2.22 B). This strongly supports the results obtained in pull-down experiments and upon overexpression of IKKγ mutant proteins, showing that sensing of ubiquitin chains by IKKγ is crucial for stimulus-dependent IKKγ/Malt1 interaction. In addition, Malt1 ubiquitination was shown to be independent of IKKγ mutation.

Furthermore, the ability of IKKγ wt and L329P to restore NF-κB activation in IKKγ deficient cells was examined by monitoring IκBα degradation upon PMA/ionomycin stimulation (Fig. 2.22 C). Jurkat T cells served as positive control for IκBα degradation. As expected, IKKγ deficient Jurkat T cells were defective in IκBα degradation upon stimulation. Importantly, only IKKγ wt, but not IKKγ L329P, was able to significantly rescue P/I stimulated NF-κB activation in IKKγ deficient Jurkat T cells, as determined by restored IκBα degradation. This demonstrates that the ubiquitin binding of IKKγ is crucial for TCR dependent NF-κB activation.

Thus, an essential function for Malt1 ubiquitination in TCR signaling could be assigned: ubiquitination of Malt1 by TRAF6 functionally links CBM and IKK complexes by the recruitment of IKKγ to Malt1 ubiquitin chains, thereby directing TCR derived signals to NF-κB activation.



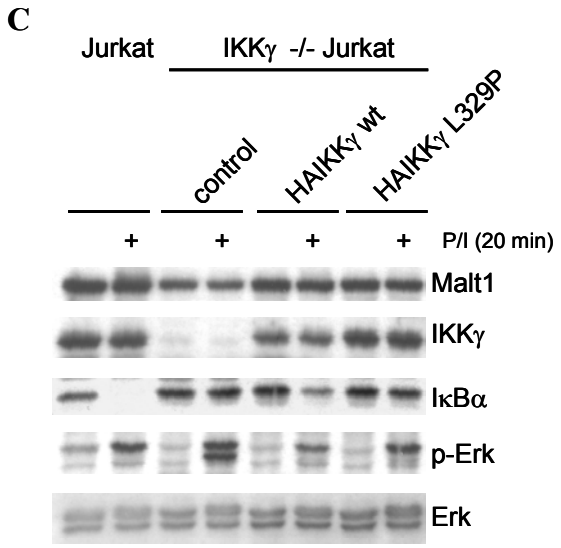


Figure 2.22: IKKγ mutant L329P does not bind to ubiquitinated Malt1 and fails to rescue NF-κB activation in reconstituted IKKγ -/- Jurkat T cells. (**A**) IKKγ -/- Jurkat cells reconstituted with HAIKKγ wt or L329P were stimulated with P/I and lysed in CoIP buffer. Binding of Malt1 upon P/I stimulation was investigated in IKKγ CoIPs by Western blot analysis (**B**) IKKγ -/- Jurkat cells reconstituted with HAIKKγ wt or L329P were stimulated with P/I, lysed in ubiquitination buffer and subjected to Malt1 immunoprecipitation. Malt1 ubiquitination was detected by Western blotting. (**C**) Normal Jurkat T cells, as well as IKKγ deficient and rescued cells were stimulated with P/I for 20min. Lysates were analyzed by Western blotting for degradation of IκBα as indicator for NF-κB activation. Phosphorylation of Erk is shown as stimulation control (p-Erk).

2.2 Nuclear Bcl10 is modified by sumoylation

2.2.1 Sumoylation of Bcl10

To identify unknown interaction partners of Bcl10, a yeast two-hybrid assay had been performed in the lab by Elmar Wegener using Bcl10 (aa 1-116) as bait. The screen revealed interaction of Bcl10 with the E2 enzyme Ubc9 and SUMO1 (not shown). This finding led to the question whether Bcl10 can be modified by SUMO attachment through Ubc9.

To investigate if Bcl10 can be sumoylated, FlagBcl10 was co-expressed with MycSUMO1 and MycUbc9 in HEK293 cells. Cells were lysed in CoIP buffer containing N-ethyl-maleimid (NEM, 20 mM), a potent inhibitor of cysteine proteases, to prevent cleavage of SUMO from substrates by desumoylating enzymes. Western blot analysis of Flag IPs demonstrated that Bcl10 is indeed modified by sumoylation upon co-expression of Ubc9 and SUMO1 (Fig. 2.23 A).

To examine whether endogenous Bcl10 is also sumoylated in Jurkat T cells, MycSUMO1 was ectopically expressed in these cells and Myc precipitates from unstimulated and PMA/ionomycin stimulated cells were analyzed for modified Bcl10 (Fig. 2.23 B). Western blot results showed that upon overexpression of SUMO1 a small amount of endogenous Bcl10 was modified by SUMO conjugation. However, Bcl10 sumoylation was not influenced by PMA/ionomycin or TNFα stimulation, suggesting that SUMO1 attachment to Bcl10 is not involved in TCR triggered signal propagation.

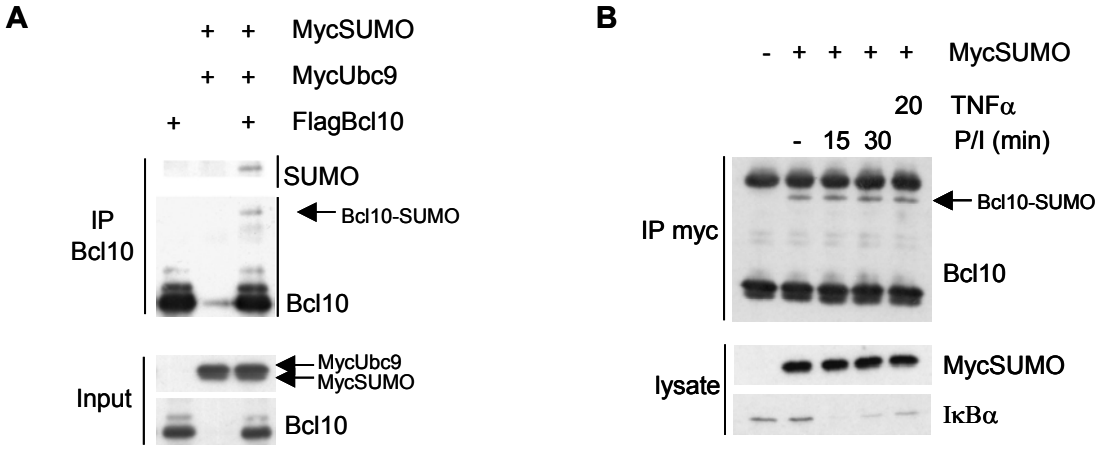


Figure 2.23: Bcl10 is modified by SUMO attachment. (**A**) FlagBcl10, MycUbc9 and MycSUMO1 were transfected in HEK293 cells. Cells were lysed in CoIP buffer containing 20 mM N-ethyl-maleimide (NEM). Western blot analysis of Flag precipitates demonstrated sumoylation of Bcl10 upon Ubc9/SUMO1 co-expression. (**B**) Jurkat T cells were transfected with MycSUMO1. Cells were stimulated with P/I for 15 and 30 min and lysed in CoIP buffer. Stimulation with TNFα for 20 min served as control. Myc Precipitates were analyzed for modified Bcl10 by Western blotting using a Bcl10 antibody.

2.2.2 Nuclear localization enhances Bcl10 sumoylation

A Bcl10 mutant protein, in which a leucine in the CARD domain is altered (L41Q) is deficient in Carma1 interaction and therefore defective in TCR signaling [180]. When this Bcl10 mutant L41Q was included in HEK293 overexpression experiments as a signaling deficient control protein, it was realized that its sumoylation was enhanced compared to wildtype Bcl10 (Fig. 2.24 A). Immunofluorescence studies using GFP tagged Bcl10 constructs revealed that, while wt Bcl10 resides in the cytoplasm, Bcl10 L41Q is to a large extent localized in the nucleus (Fig 2.24 B). Others had shown before that in contrast to Bcl10, Bcl10 L41Q cannot associate with cytoplasmic filaments upon over-expression, suggesting a general difference in the localization properties of the two proteins [212]. It remains however unclear, if association with cytoplasmic filaments possesses any functional relevance under physiological conditions.

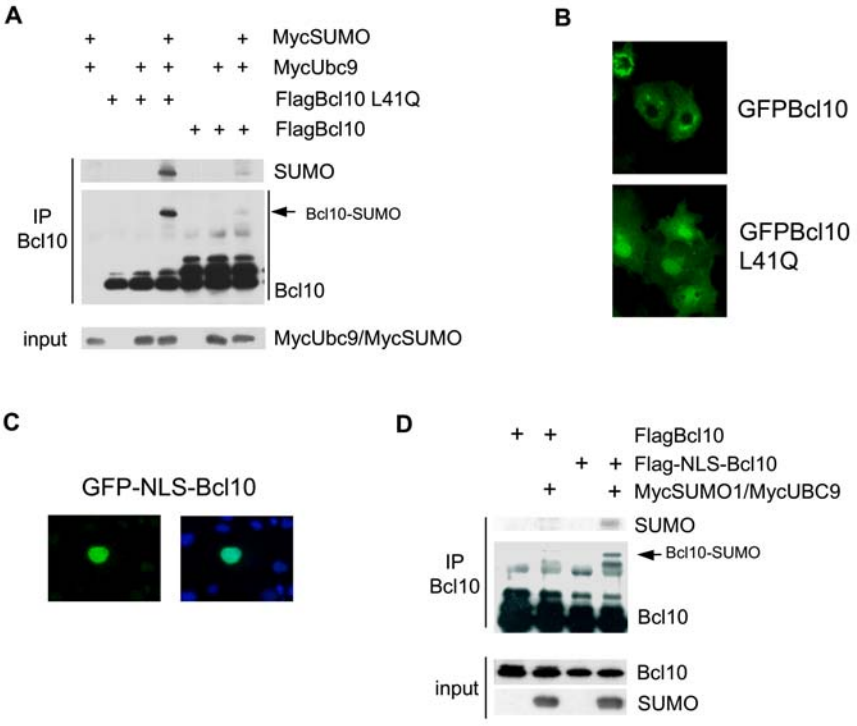


Figure 2.24: Nuclear localization of Bcl10 enhances sumoylation (**A**) Flag Bcl10 wt and L41Q were co-expressed with MycUbc9 and MycSUMO1 in HEK293 cells as indicated. Bcl10 immunoprecipitations were analyzed for sumoylated Bcl10 after Western blotting with Bcl10 and SUMO antibodies. (**B**) Fluorescence microscopy of GFPBcl10 and GFPBcl10 L41Q (green) expressed in COS-7 cells showed cytosolic staining for wt Bcl10, but mainly nuclear localization of the L41Q mutant. (**C**) Fluorescence microscopy of GFP-NLS-Bcl10 expressed in COS-7 cells (green). DAPI staining was used to visualize cell nuclei (blue). An overlay is given in the right panel, showing nuclear localization of GFP-NLS-Bcl10. (**D**) HEK293 cells were transfected with FlagBcl10 wt, Flag-NLS-Bcl10 and MycUb9/MycSUMO as indicated and analyzed as in (A).

Since it is known that sumoylation often represents a nuclear event, the question arose whether enhanced Bcl10 L41Q sumoylation was due to its partially nuclear localization. To answer this question a nuclear localization signal (NLS, amino acids PKKKRKV, from SV40 large T antigen) was introduced in GFPBcl10 to artificially induce its nuclear import. Immunofluorescence analysis of COS-7 cells expressing GFP-NLS-Bcl10 showed that the introduced NLS was functional (Fig. 2.24 C). Co-expression of NLS-Bcl10 with Ubc9 and SUMO1 in HEK293 cells demonstrated that also sumoylation of NLS-Bcl10 was enhanced compared to wt Bcl10 (Fig. 2.24 D). Thus, nuclear localization indeed seems to promote sumoylation of Bcl10.

A cytosolic steady-state localization can be mediated by predominant nuclear export despite actual nucleocytoplasmic shuttling of the respective protein. Thus, the question arose whether Bcl10 shuttles between nucleus and cytosol, so that the nuclear portion of Bcl10 could become sumoylated. Many proteins require a receptor for nuclear export, which mediates their interaction with the nuclear pore complex (NPC) during translocation. Chromosomal region maintenance 1 (CRM1) represents the best characterized nuclear export receptor [213]. CRM1 recognizes nuclear export sequences (NES), which contain 4 hydrophobic amino acid residues, usually leucines (L), in variable distances. The export consensus sequence is normally given as L-X(1-3)-L-X(2-3)-L-X-L with X being any residue, but variations of this motif are found [214, 215, 216]. The *Streptomyces* metabolite leptomycin B (LMB) inhibits CRM1-dependent nuclear export and consequently leads to nuclear accumulation of shuttling, NES-containing proteins [217]. Thus, LMB is widely used to examine the nucleocytoplasmic shuttling of proteins. However, active nuclear export of some proteins has been shown to be driven in a CRM1-independent manner, probably by direct contact of these proteins with the NPC [218 and references therein]. To examine if Bcl10 shuttles between cytoplasm and nucleus, COS-7 cells expressing GFPBcl10 or FlagMalt1 were treated with LMB for 18h and analyzed by fluorescence microscopy (Fig. 2.25 A). LMB treatment did not influence the cytosolic localization of Bcl10, suggesting that Bcl10 does not shuttle between nucleus and cytosol. In contrast, strong nuclear accumulation of Malt1 due to inhibition of nuclear export by LMB demonstrated a shuttling of Malt1. These findings are in line with results published by others during the course of this thesis [219] (see Discussion 3.2.3). However, when both proteins were co-expressed in COS-7 cells, binding of Bcl10 to Malt1 inhibited nuclear accumulation of Malt1 upon LMB treatment (Fig. 2.25 B), indicating that Bcl10 predominantly influences localization of the Bcl10/Malt1 complex.

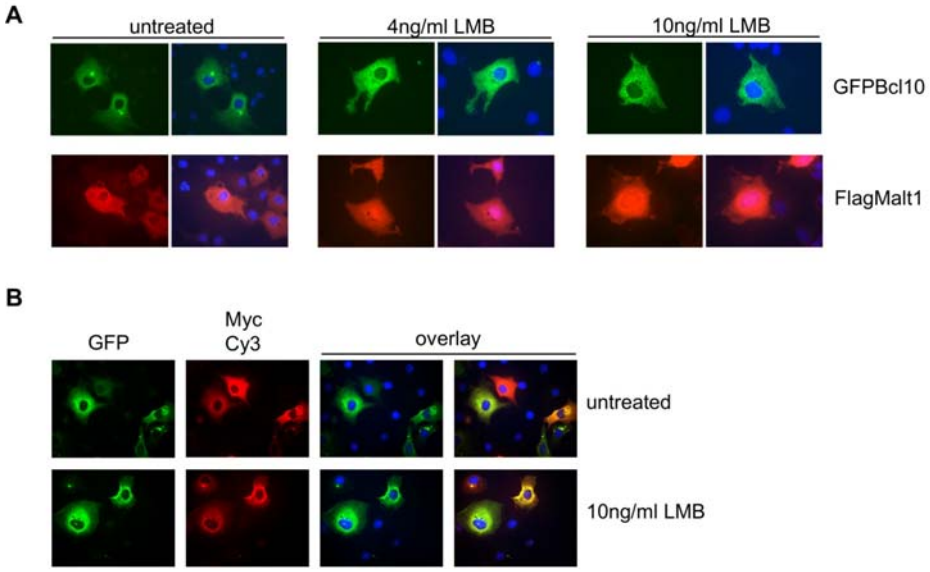


Figure 2.25: Nucleocytoplasmic shuttling of Malt1 but not Bcl10 (**A**) COS-7 cells were transfected with GFPBcl10 or FlagMalt1. 10h after transfection, leptomycin B (LMB) was added to the cells in the indicated concentrations for 18h. FlagMalt1 was stained using anti-FlagM5 primary and Cy3-labeled secondary antibodies (red). Nuclei were visualized by DAPI staining (blue). Subcellular localization of the transfected proteins was analyzed by fluorscence microscopy. (**B**) COS-7 cells were co-transfected with GFPBcl10 and MycMalt1 and treated and analyzed as in (A). Right panels show overlays of the different signals for GFP, Cy3 and DAPI.

2.2.3 Lysine 110 of Bcl10 serves as acceptor lysine for sumoylation.

In contrast to ubiquitination, where an E3 ubiquitin ligase is required, modification of a substrate with SUMO can be directly mediated by the E2 enzyme. Ubc9 represents the only known E2 for sumoylation and was shown to be able to directly attach SUMO to lysine residues of substrate proteins. Since these acceptor lysines are usually found in the sumoylation consensus sequence ψKxE, where ψ represents a bulky hydrophobic amino acid, the Bcl10 amino acid sequence was screened for this motif. Primary sequence analysis revealed two putative SUMO modification consensus sites in Bcl10 with K17 or K110 as possible acceptor lysines (Fig. 2.26 A). Since amino acid 18 of Bcl10 is also a lysine, constructs were generated by PCR based mutagenesis, in which K110 or K17 and K18 together were exchanged with arginines (Bcl10 K110R and Bcl10 K17/18R). Co-expression of the respective NLS Bcl10 constructs with Ubc9 and SUMO1 in HEK293 cells demonstrated that Bcl10 sumoylation is unaffected by mutation of K17 or K18, but completely abolished when K110 was exchanged (Fig. 2.26 B). Similar results were obtained for Bcl10 wt and Bcl10 L41Q mutants (not shown). Thus, this finding clearly reveals lysine 110 as specific acceptor site for SUMO modification of Bcl10.

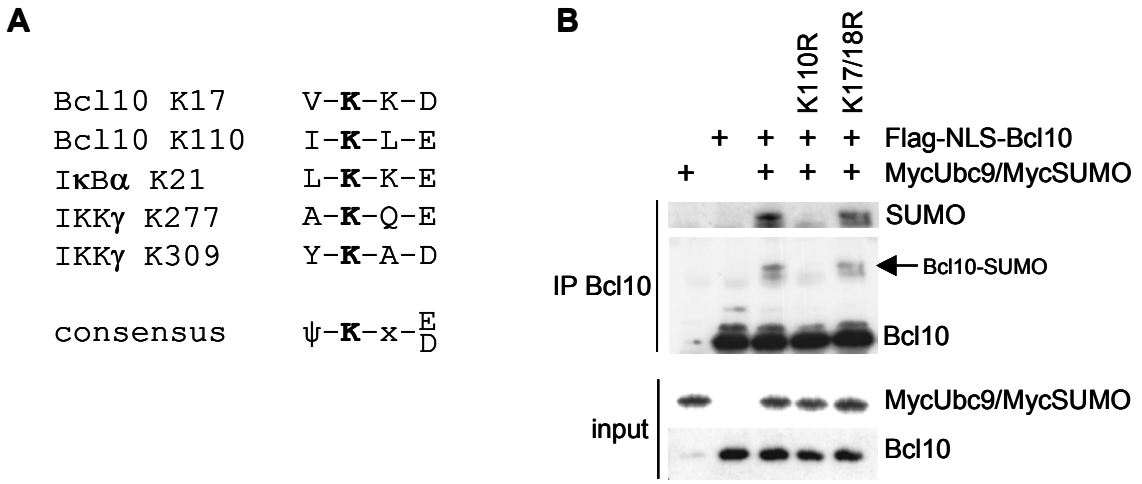


Figure 2.26: Mapping of K110 as acceptor lysine for sumoylation in Bcl10. (**A**) Sumoylation often occurs at lysine residues located in the consensus motif ψ-K-x-E/D, as known for K21 in IκBα, or K277 and K309 in IKKγ. Two sumoylation motifs can be found in Bcl10, suggesting K17 or K110 as putative SUMO acceptor residues. (**B**) Flag-NLS-Bcl10 wt, K17/18R and K110R were co-expressed with MycUbc9/MycSUMO1 in HEK293 cells. Western blot analysis of Bcl10 immunopreciptates using Bcl10 and SUMO antibodies showed that Bcl10 sumoylation was abolished upon mutation of K110, but not K17 or K18.

2.2.4 Bcl10-SUMO fusion promotes nuclear localization.

Since Bcl10 does not shuttle between cytoplasm and nucleus *per se*, it was asked whether sumoylation of Bcl10 might influence its localization, as has been shown for various other substrates. As only small amounts of Bcl10 were modified by sumoylation upon Ubc9/SUMO1 overexpression, immunofluoresence analysis of Ubc9/SUMO1 co-expressed Bcl10 did not show any alteration in cytosolic Bcl10 localization (data not shown). Therefore, to examine localization of sumoylated Bcl10, Bcl10-SUMO fusion constructs were generated. Obviously this does not exactly reflect sumoylation of Bcl10 at K110R, but should be informative to which extent Bcl10 sumoylation may affect Bcl10 localization.

Indeed, expression of GFPBcl10-SUMO in COS-7 cells revealed that fusion of SUMO to Bcl10 leads to a nearly exclusive nuclear localization of Bcl10 (Fig. 2.27 A). Furthermore, when Malt1, which is under normal conditions found in the cytosol, was co-expressed, Bcl10-SUMO mediated nuclear localization of both proteins (Fig. 2.27 B). Nuclear uptake of Malt1 depends on the interaction with Bcl10, as a Bcl10-binding-deficient Malt1 protein (Malt1 aa 314-824) showed cytosolic localization even when co-expressed with Bcl10-SUMO. These data demonstrate that fusion of SUMO to Bcl10 leads to nuclear localization of Bcl10 and Malt1, suggesting that sumoylation of Bcl10 could promote nuclear accumulation not only of Bcl10, but also of Bcl10/Malt1 complexes.

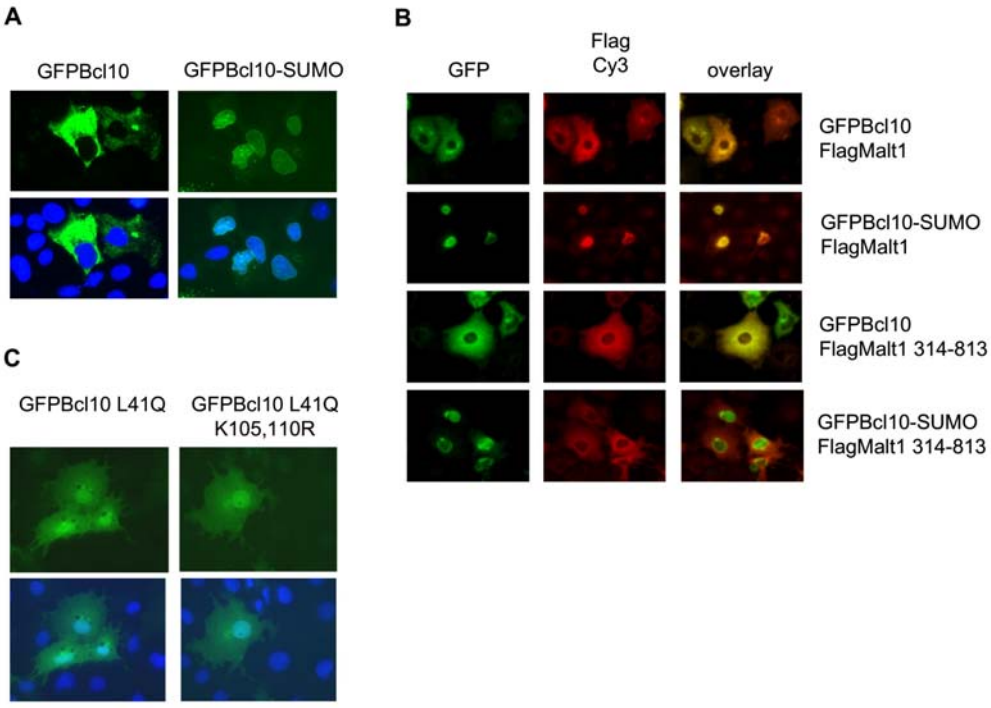


Figure 2.27: SUMO fusion mediates nuclear localization of Bcl10 and Bcl10/Malt1 complexes. (**A**) COS-7 cells were transfected with GFPBcl10 and GFPBcl10-SUMO (green). Nuclei were visualized by DAPI staining (blue). Fluorescence microscoscopy showed that SUMO fusion leads to nuclear localization of Bcl10. (**B**) COS-7 cells were transfected with GFPBcl10, GFPBcl10-SUMO, FlagMalt1 and FlagMalt1 314-813 as indicated. FlagMalt1 was stained using a primary Flag antibody and a secondary Cy3-labeled anti-mouse antibody (red). Dependent on its interaction with Bcl10, Malt1 revealed nuclear localization when co-expressed with Bcl10-SUMO. An overlay (yellow, right panels) demonstrates co-localization of Bcl10 and Malt1. (**C**) COS-7 cells were transfected with GFP Bcl10 L41Q and GFP Bcl10 L41Q K105,110R constucts (green) and analyzed by fluorescence microscopy. DAPI staining was used to visualize cell nuclei (blue).

Taken together, two putative scenarios are conceivable: cytosolic sumoylation of Bcl10 could target Bcl10 to the nucleus or nuclear sumoylation could mediate its nuclear retention. However, a Bcl10 L41Q construct, in which the SUMO acceptor lysine 110 was mutated, still showed partial nuclear localization comparable to Bcl10 L41Q, demonstrating that nuclear targeting of Bcl10 L41Q is not due to sumoylation (Fig. 2.27 C). Thus, other mechanisms besides sumoylation must exist that can mediate nuclear localization of Bcl10 L41Q.

2.2.5 Bcl10-SUMO fusion enhances NF-κB activation by Bcl10.

Aberrant nuclear localization of Bcl10 is observed in several Malt1 lymphomas and seems to be associated with advanced and aggressive lymphoma forms (see introduction 1.5.2.6.5). The mechanism underlying this unusual nuclear localization of Bcl10 is not understood, but the data hint that Bcl10 exerts its oncogenic role in the nucleus [200, 201]. MALT lymphomas

critically depend on constitutive NF-κB activity. Therefore, the ability of Bcl10 wt, NLS-Bcl10, and Bcl10-SUMO to mediate NF-κB activation was investigated in luciferase reporter assays (Fig. 2.28). For that purpose HEK293 cells were transfected with GFP tagged constructs and two reporter plasmids. One reporter plasmid contained six copies of NF-κB binding sites for inducible expression of Firefly luciferase (6x NF-κB), the other one carried a gene for *Renilla* luciferase under control of a thymidine kinase promotor (TkLuc). Constitutive expression of *Renilla* luciferase served as an internal control.

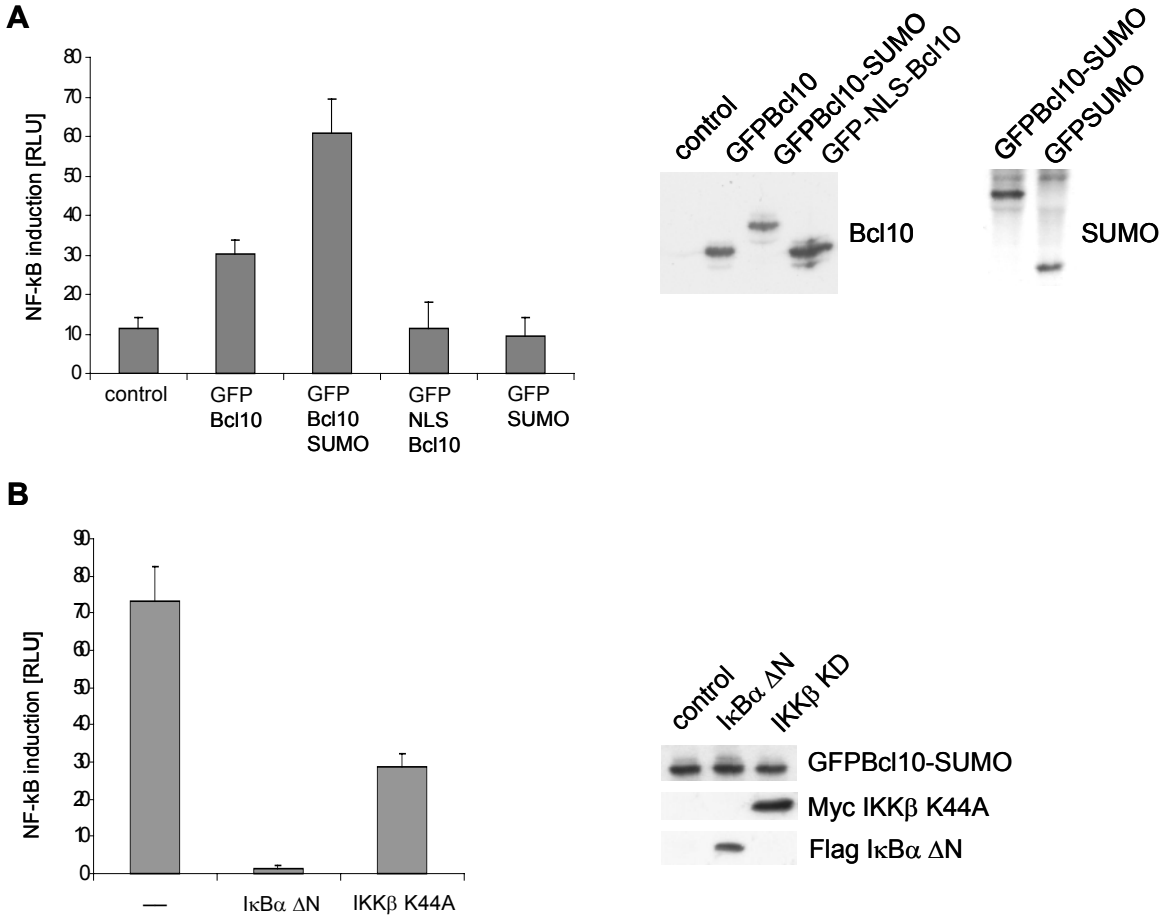


Figure 2.28: SUMO fusion enhances NF-κB activating potential of Bcl10. (**A**) HEK293 cells were transfected with GFPBcl10, GFPBcl10-SUMO, GFP-NLS-Bcl10, GFPSUMO, empty vector control and 6xNF-κB and TkLuc reporter plasmids. 24h after transfection, cells were lysed in Passive lysis buffer. Extracts were analyzed for equivalent expression of the transfected Bcl10 constructs by Western blotting (right panels). Reporter assays were performed according to manufacturer's instructions. Every extract was measured in duplications and ratios of luminescence signals of Firely and *Renilla* luciferases were calculated. Three independent experiments were analyzed for quantification. Bars and standard deviations are given. (**B**) HEK293 cells were transfected as indicated and analyzed as in (A). Bars and standard deviations are given for three independent experiments. Western blot results show similar expression level of GFLBcl10-SUMO in all samples (right panel).

As expected, wt Bcl10 activates an NF-κB reporter about 2.5 to 3 fold in comparison to empty vector control. However, Bcl10-SUMO was even more potent in inducing NF-κB activtity (Fig. 2.28 A). This is not only due to the nuclear localization of the protein since NLS-Bcl10 did not trigger NF-κB activation. In addition, SUMO alone could not provoke NF-κB activity in the reporter assay. Western Blot analysis of the lysates was performed to ensure, that the observed effects are not due to different expression levels of the proteins. Thus, SUMO fusion to Bcl10 enhances the NF-κB activation potential of Bcl10. A characteristic feature of Bcl10-SUMO fusion rather than nuclear localization of Bcl10 or SUMO serves this function. Co-expression of a dominant negative IKKβ construct (IKKβ K44A) or an N-terminally deleted IκBα construct (IκBα ΔN, aa 71-317), in which the serines required for phosphorylation dependent degradation are missing, significantly reduced Bcl10-SUMO induced NF-κB activation. This demonstrates that NF-κB activation by Bcl10-SUMO is mediated by IKKβ dependent degradation of IκBα, thus canonical signaling to NF-κB (Fig. 2.28 B).

Taken together, it could be shown that Bcl10 can be modified by sumoylation at lysine 110. Bcl10 sumoylation is enhanced by its nuclear localization, and fusion of SUMO to Bcl10 leads to nuclear retention of Bcl10 and an enhanced NF-κB activation potential compared to wt Bcl10. Since nuclear localization of Bcl10 is observed under pathological conditions (MALT lymphoma), the presented results hint at a novel putative mechanism, which might influence this aberrant nuclear localization and NF-κB activity.