

Malt1 ubiquitination triggers NF- κ B signaling upon T-cell activation

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Triggering of antigen receptors on lymphocytes is critical for initiating adaptive immune response against pathogens. T-cell receptor (TCR) engagement induces the formation of the Carma1–Bcl10–Malt1 (CBM) complex that is essential for activation of the I κ B kinase (IKK)/NF- κ B pathway. However, the molecular mechanisms that link CBM complex formation to IKK activation remain unclear. Here we report that Malt1 is polyubiquitinated upon T-cell activation. Ubiquitin chains on Malt1 provide a docking surface for the recruitment of the IKK regulatory subunit NEMO/IKK γ . TRAF6 associates with Malt1 in response to T-cell activation and can function as an E3 ligase for Malt1 *in vitro* and *in vivo*, mediating lysine 63-linked ubiquitination of Malt1. Multiple lysine residues in the C-terminus of Malt1 serve as acceptor sites for the assembly of polyubiquitin chains. Malt1 mutants that lack C-terminal ubiquitin acceptor lysines are impaired in rescuing NF- κ B signaling and IL-2 production in Malt1^{–/–} T cells. Thus, our data demonstrate that induced Malt1 ubiquitination is critical for the engagement of CBM and IKK complexes, thereby directing TCR signals to the canonical NF- κ B pathway.

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Introduction

The adaptive immune response is initiated upon specific recognition of antigens presented on the surface of antigen-presenting cells (APC) by T lymphocytes. Productive activation and clonal expansion of T cells requires the concerted action of the T-cell receptor (TCR) and the CD28 co-stimulatory receptor. TCR/CD28 co-engagement induces the forma-

tion of the immunological synapse at the contact site of T cell and APC through clustering of lipid rafts, receptor molecules and cytosolic signaling mediators (van der Merwe, 2002). TCR/CD28-initiated signaling networks ultimately promote the activation of several transcription factors including NFAT, AP-1 and NF- κ B (Okamura and Rao, 2001).

NF- κ B plays a key role for the regulation of T-cell activation by mediating the induction of various genes that control T-cell proliferation, activation and survival (Ghosh *et al*, 1998). The NF- κ B transcription factor family comprises the Rel proteins p65 (RelA), RelB, c-Rel, NF- κ B1 (p105/p50) and NF- κ B2 (p100/p52), which can form various combinations of homo- and heterodimers. In unstimulated cells, NF- κ B proteins are retained in the cytoplasm by their tight association with inhibitory (I κ B) proteins (Hayden and Ghosh, 2004). TCR/CD28 co-ligation induces canonical NF- κ B signaling, which involves phosphorylation and degradation of small cytosolic I κ B inhibitors (I κ B α , I κ B β and I κ B ϵ), and subsequent nuclear translocation and DNA binding of NF- κ B. I κ B phosphorylation is catalyzed by the I κ B kinase (IKK) complex, which consists of the two catalytic subunits IKK α and IKK β and the essential regulatory subunit IKK γ /NEMO. Hence, an elucidation of signaling mechanisms that control IKK activation is critical for understanding the regulatory events involved in T-cell activation (Rawlings *et al*, 2006; Schulze-Luehrmann and Ghosh, 2006; Weil and Israel, 2006).

TCR/CD28 stimulation induces receptor proximal tyrosine phosphorylation, followed by an association of phospholipase C- γ , small G proteins (e.g., Rac and Ras) and guanine nucleotide exchange factors (e.g., SOS and Vav) to the immunological synapse (Weil and Israel, 2006). The protein kinase C (PKC) isoform PKC θ is recruited to the immunological synapse upon T-cell activation (Monks *et al*, 1997), and was found to be indispensable for TCR triggered NF- κ B activation (Sun *et al*, 2000; Bi *et al*, 2001). Genetic ablations in mice have revealed key molecules that couple PKC θ activation to IKK/NF- κ B signaling. These include Carma1 (CARD11), Bcl10, Malt1 and Caspase8 (Ruland *et al*, 2001, 2003; Egawa *et al*, 2003; Hara *et al*, 2003; Jun *et al*, 2003; Ruefli-Brasse *et al*, 2003; Su *et al*, 2005). The CARD-containing Carma1 protein is a member of the MAGUK (membrane-associated guanylate kinase) family of proteins (McAllister-Lucas *et al*, 2001). Carma1 is associated with the plasma membrane and recruited to the immunological synapse upon TCR/APC contact (Gaide *et al*, 2002). Bcl10 and Malt1, which were originally cloned from translocation breakpoints associated with malignant MALT (mucosa-associated lymphoid tissue) lymphomas (Akagi *et al*, 1999; Morgan *et al*, 1999; Willis *et al*, 1999; Zhang *et al*, 1999), are constitutively associated and recruited to Carma1 in a PKC-dependent manner (Matsumoto *et al*, 2005; Sommer *et al*, 2005). In T cells, PKC θ phosphorylates Carma1 in a central linker region, which triggers a conformational change of Carma1 and thereby promotes homotypic interaction between the N-terminal CARDS of Carma1 and Bcl10 and thus Carma1–Bcl10–Malt1

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(CBM) complex formation (Matsumoto *et al*, 2005). PDK1 associates with PKC θ and Carma1 and might function as a molecular bridge to facilitate Carma1 phosphorylation (Lee *et al*, 2005).

The molecular mechanisms that trigger IKK activation downstream of the CBM complex on the route to NF- κ B are not fully understood. Carma1, Bcl10 and Malt1 were shown to promote NF- κ B activation by inducing IKK γ polyubiquitination (Zhou *et al*, 2004; Shambharkar *et al*, 2007). TAK1 (transforming growth factor- β -activated kinase 1) has been proposed to function as an IKK kinase in TCR signaling (Wang *et al*, 2001; Sun *et al*, 2004; Wan *et al*, 2006), and TAB2/3 contain ubiquitin-binding domains (UBDs) that can mediate recruitment of TAK1 to ubiquitinated IKK γ (Kanayama *et al*, 2004). Malt1 was suggested to possess an intrinsic E3 ligase activity that catalyzes IKK γ ubiquitination (Zhou *et al*, 2004). In addition, the RING ubiquitin ligase TRAF6 can either directly associate with the C-terminus of Malt1 via two conserved binding motifs (Sun *et al*, 2004), or is indirectly recruited to Malt1 through association with Caspase8 (Bidere *et al*, 2006). A role for TRAF6 in TCR-induced IKK activation is further supported by RNAi experiments (Sun *et al*, 2004; Bidere *et al*, 2006). However, T-cell-specific ablation of TRAF6 indicates that one or several other E3 ligases, for example, TRAF2, can compensate for the loss of TRAF6 (King *et al*, 2006). TRAF6 was shown to cooperate with the C-terminus of Malt1 to promote IKK γ ubiquitination (Sun *et al*, 2004), and might thus contribute ubiquitin ligase activity. However, abrogation of IKK γ ubiquitination caused only a partial reduction of PKC-dependent NF- κ B activation in T cells (Zhou *et al*, 2004), hinting that other targets for regulatory ubiquitination must be involved in directing CBM complex formation to IKK activation upon TCR/CD28 co-engagement.

We have identified Malt1 as a novel substrate for induced regulatory ubiquitination in response to TCR/CD28 co-ligation. Malt1 ubiquitin chains provide docking surfaces for the recruitment of IKK γ to the CBM complex. Congruently, NF- κ B signaling in T cells critically depends on an intact ubiquitin-binding motif in IKK γ . We further demonstrate that TRAF6 is an E3 ligase for Malt1 and induces the attachment of lysine 63-linked ubiquitin chains to the C-terminus of Malt1. Importantly, the replacement of ubiquitin acceptor sites on Malt1 impairs TCR induced NF- κ B activation, demonstrating a crucial role for regulatory Malt1 ubiquitination. Thus, our data provide evidence that Malt1 ubiquitination is directing CBM complex formation to IKK activation in response to TCR/CD28 engagement.

Results

Carma1-associated Malt1 is ubiquitinated upon T-cell activation

To identify further interactions or modifications critical for T-cell activation, we investigated the formation of the cellular CBM complex by analyzing Bcl10 immunoprecipitates after gel-filtration chromatography of Jurkat T-cell lysates (Figure 1A). In unstimulated Jurkat T cells, a peak of pre-formed Bcl10–Malt1 eluted in the low-molecular-weight fractions. Activation of Jurkat cells by PMA/ionomycin (P/I) induced the association of Bcl10–Malt1 with Carma1. The cellular CBM complex migrated with an apparent molecular

mass of more than 1500 kDa, a size that by far exceeds the expected molecular weight of a heterotrimer (~260 kDa). We noticed the appearance of higher molecular weight forms of Carma1–Bcl10-associated Malt1, indicative of a modification. To determine whether Malt1 is modified by ubiquitin, we analyzed Malt1 precipitates after gel-filtration chromatography under denaturing conditions to prevent non-covalent protein interactions (1% SDS, see below). Indeed, modified Malt1 in the high-molecular-weight fraction was also detected by an anti-ubiquitin antibody (Figure 1B). To see whether Malt1 ubiquitination is a primary event in response to T-cell activation, we determined the onset of Malt1 ubiquitination, IKK phosphorylation and I κ B α degradation after P/I treatment (Figure 1C). After direct immunoprecipitation (IP) of Malt1 from Jurkat lysates, we found that the appearance of ubiquitin-conjugated Malt1 slightly preceded IKK phosphorylation and subsequent I κ B α degradation, suggesting that Malt1 ubiquitination could be involved in delivering the signal to IKK/NF- κ B after T-cell activation. Further, Malt1 ubiquitination was transient and the disengagement of ubiquitin conjugates correlated with a decrease of IKK/NF- κ B signaling as monitored by reappearance of I κ B α 45–60 min after stimulation (Supplementary Figure 1). In line with P/I activation, CD3/CD28 receptor co-ligation promoted polyubiquitin conjugation of Malt1 in Jurkat T cells and primary CD4+ T cells (Figure 1D). Since we could not detect Malt1 degradation during the course of stimulation, the data suggest that Malt1 is modified by non-degradative regulatory ubiquitination that could be involved in signal propagation.

IKK activity is recruited to Malt1 through the ubiquitin-binding domain of IKK γ

IKK γ was recently shown to contain a UBD (Ea *et al*, 2006; Li *et al*, 2006; Wu *et al*, 2006) and we asked whether IKK γ can associate with ubiquitinated Malt1 in response to T-cell activation. Indeed, we detect ubiquitin-conjugated Malt1 in IKK γ co-IPs from the extracts of activated Jurkat T cells (Figure 2A). An unspecific cross-reactivity that is detected in Malt1 western blots after co-IP is slightly larger than unmodified Malt1 (compare Figure 4B). Importantly, control IKK γ IPs from stimulated Jurkat T cells prove that there is no unspecific binding of ubiquitinated Malt1 species (Supplementary Figure 2A). To determine if TAB2 and TAK1 associate with ubiquitin chains attached to Malt1, we performed co-IPs and found that both proteins are recruited to ubiquitinated Malt1 upon P/I stimulation (Figure 2B). Thus, different UBD-containing signaling mediators can be recruited to ubiquitin-conjugated Malt1 upon T-cell activation.

The functional relevance of the IKK γ UBD to mediate interaction with ubiquitin-conjugated RIP1 was recently shown for TNF α signaling (Ea *et al*, 2006; Li *et al*, 2006; Wu *et al*, 2006). IKK γ L329P and Y308S are two point mutants in the coiled-coil 2/leucine zipper of IKK γ (242–350) that impair binding of IKK γ to polyubiquitin chains and therefore its recruitment to ubiquitinated RIP1 (Li *et al*, 2006; Wu *et al*, 2006). To test whether the same mutants have a reduced affinity to ubiquitin-conjugated Malt1, we performed streptactin pull-down experiments from extracts of activated Jurkat cells using recombinant StrepIKK γ wt, L329P and Y308S (Figure 2C). StrepIKK γ wt preferentially associated with ubiquitin-conjugated Malt1 from stimulated Jurkat T-cell extracts, whereas the mutations L329P and Y308S

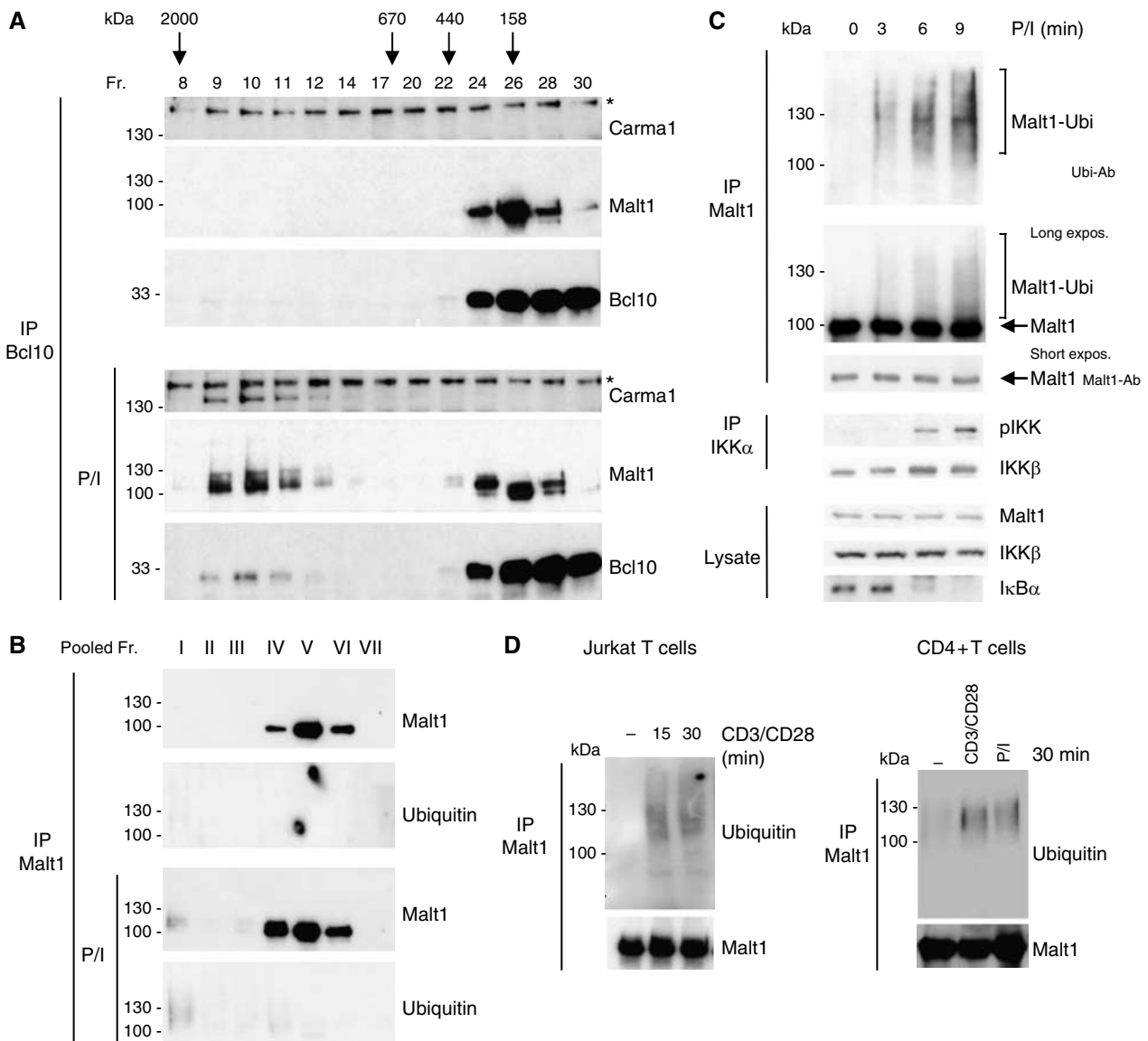


Figure 1 Malt1 ubiquitination in response to T-cell activation. **(A)** Carma1-associated Malt1 is modified in Jurkat T cells. Extracts of untreated and P/I-stimulated (20 min) Jurkat T cells were fractionated by gel-filtration chromatography, followed by Bcl10 IP of collected fractions. Elution profiles of Carma1, Bcl10 and Malt1 were analyzed by western blotting. Molecular-weight standards depict the peak elution of marker proteins. A nonspecific band recognized with the anti-Carma1 antibody is marked by an asterisk. **(B)** Carma1-associated Malt1 is ubiquitinated. Gel filtration was carried out as in panel A. Fractions were pooled (I: 9–11; II: 12–14; III: 16–18; IV: 20–22; V: 24–26; VI: 27–29; VII: 30–32) and denatured, followed by Malt1 IP and western blotting with ubiquitin antibody. **(C)** Onset of Malt1 ubiquitination precedes IKK activation. Jurkat T cells were stimulated for indicated times with P/I and lysed in co-IP buffer containing 1% SDS. Extracts were diluted 10-fold and immunoprecipitated with anti-Malt1 antibody. In parallel, IKK α was precipitated from co-IP buffer lysates for detection of IKK phosphorylation. **(D)** Malt1 ubiquitination in response to CD3/CD28 co-ligation. Jurkat T cells or murine CD4+ T cells were stimulated with anti-CD3/CD28 antibodies or P/I and analyzed as in panel C.

reduce this interaction. Similar, transfected FlagIKK γ L329P and Y308S have a reduced affinity to ubiquitinated Malt1 from P/I stimulated Jurkat T cells (Supplementary Figure 2B). Next, we analyzed the ability of IKK γ L329P to associate with Malt1 and to rescue P/I-induced signaling in IKK γ deficient Jurkat T cells (Figure 2D–F). In reconstitution experiments, the L329P mutation severely impaired the association of IKK γ to ubiquitin-conjugated Malt1, even though the level of inducible Malt1 ubiquitination was equivalent in both cell lines (Supplementary Figure 2C). To verify the association of IKKs to Malt1, we also performed an *in vitro* kinase assay after Malt1 IP, using GSTI κ B α 1–53 as substrate (Figure 2E).

Anti-Malt1 IP coprecipitated inducible IKK activity that specifically phosphorylated serines 32/36 of I κ B α (Supplementary Figure 2D). Moreover, IKK kinase activity was coprecipitated by an anti-Malt1 IP from extracts of Jurkat T cells reconstituted with IKK γ wt, but not IKK γ L329P. Mutation of IKK γ at position L329 did not affect association to IKK α and IKK β (Wu *et al*, 2006, and data not shown). In addition, only IKK γ wt was able to significantly rescue P/I-induced NF- κ B activation, as determined by I κ B α degradation in IKK γ -deficient Jurkat T cells (Figure 2F). This shows that sensing of ubiquitin chains by IKK γ is crucial for TCR-dependent NF- κ B activation, and that Malt1 ubiquitin chains

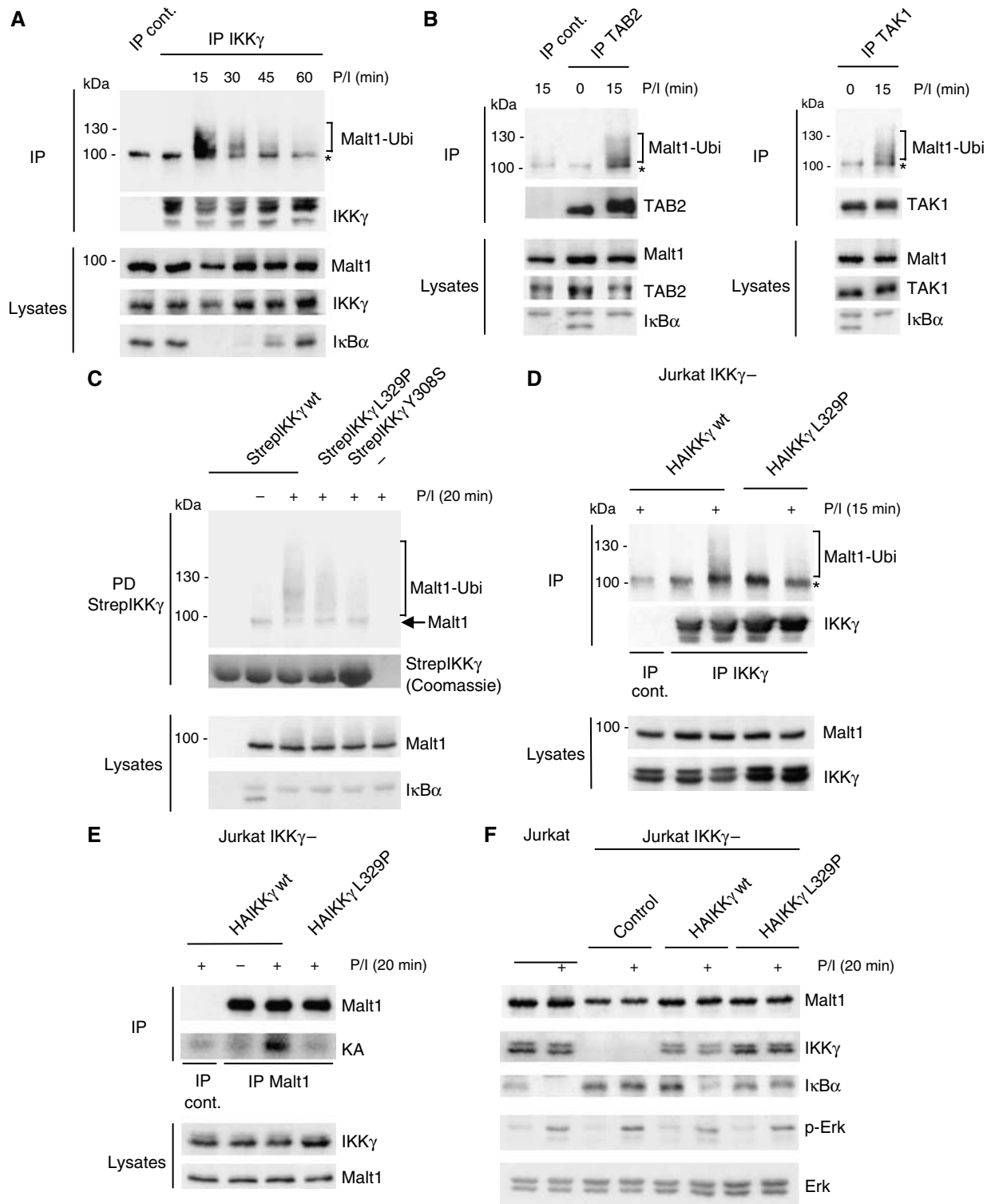


Figure 2 IKK γ associates with ubiquitin-conjugated Malt1. (A) Cellular IKK γ associates predominately with ubiquitinated Malt1. Extracts from P/I-stimulated Jurkat T cells were immunoprecipitated with anti-IKK γ antibody and ubiquitin-modified Malt1 was detected by western blotting. Migration of an unspecific cross-reaction band slightly above 100 kDa in control and IKK γ IPs is marked by an asterisk. Note that no unspecific binding of ubiquitinated Malt1 was observed (Supplementary Figure 2A). (B) TAB2/TAK1 associate with ubiquitin-conjugated Malt1. Experiments were carried out as in panel A, and IP was performed with either anti-TAB2 (left) or anti-TAK1 antibodies (right). An unspecific cross-reaction band also present in control IPs is marked by an asterisk. (C, D) IKK γ mutants defective in polyubiquitin binding show impaired association with ubiquitin-conjugated Malt1. (C) Extracts of Jurkat T cells were incubated with recombinant Strep-IKK γ wt, L329P and Y308S bound to streptactin beads. Malt1 association was analyzed by western blotting. (D) IKK γ -negative Jurkat T cells reconstituted with HAIKK γ wt or L329P were subjected to IKK γ IP as in panel A. (E, F) IKK γ -mutant L329P fails to rescue NF- κ B signaling. (E) IKK kinase assay using GST-I κ B α 1–53 as substrate was performed after Malt1 IP from extracts of IKK γ wt and IKK γ L329P-reconstituted IKK γ -/- Jurkat T cells. (F) Jurkat T cells, IKK γ -negative and rescued Jurkat cells were stimulated with P/I for 20 min and analyzed for I κ B α degradation as an indicator for NF- κ B activation. Detection of Erk phosphorylation served as stimulation control.

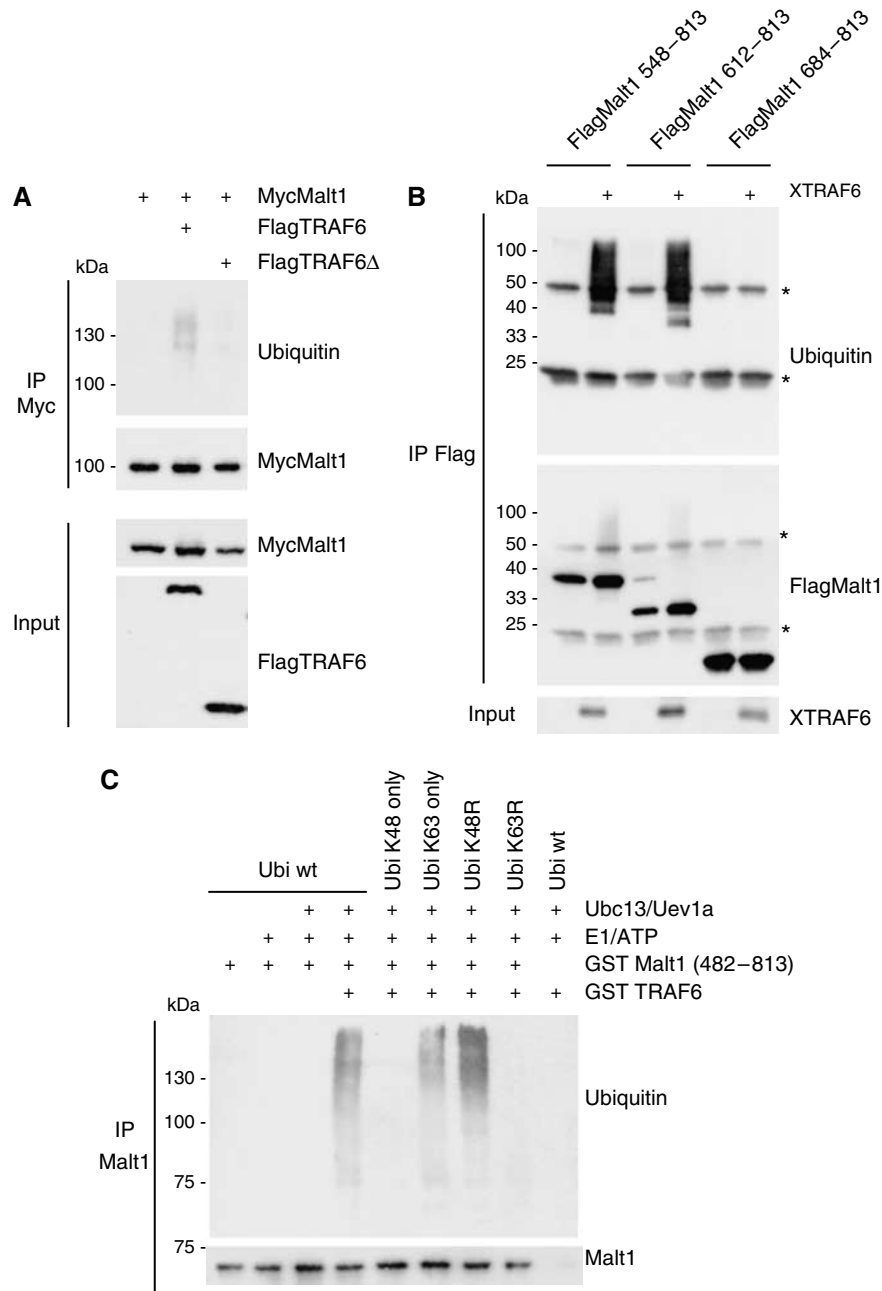


Figure 3 TRAF6 can act as E3 ligase for Malt1. (A) TRAF6 induces Malt1 ubiquitination. HEK293 cells were cotransfected with MycMalt1 and FlagTRAF6 or TRAF6Δ (289–522), and lysed in co-IP buffer containing 1% SDS. Extracts were diluted 10-fold and ubiquitination was detected following MycMalt1 IP. (B) TRAF6 induces ubiquitin conjugation to the C-terminus of Malt1. 293 cells were cotransfected with Malt1 deletion constructs and XTRAF6 and analyzed as in panel A. Cross-reactions with heavy and light antibody chains after IP are marked by asterisks. (C) TRAF6 mediates the assembly of K63-linked ubiquitin chains to Malt1. *In vitro* ubiquitin conjugation to GST Malt1 (aa 482–813) was carried out by adding E1, energy-regenerating solution (ATP), E2 (Ubc13/Uev1a) and GST TRAF6, using either ubiquitin wt or lysine mutants K48-only, K63-only, K48R or K63R. Reactions were boiled in 1% SDS containing co-IP buffer and diluted 10-fold for Malt1 IP. Malt1 precipitates were analyzed for ubiquitination by western blotting.

act as molecular bridges that connect CBM and IKK complexes.

TRAF6 functions as an E3 ligase for Malt1

TRAF6 was shown to interact with the C-terminus of Malt1 upon overexpression and *in vitro* (Sun *et al*, 2004), and we asked whether TRAF6 could act as an E3 ligase for Malt1. Indeed, coexpression of TRAF6 induced Malt1 ubiquitination in 293 cells (Figure 3A). Malt1 ubiquitination was dependent on the N-terminal RING domain of TRAF6 that confers ligase

activity, although TRAF6Δ was still able to associate with Malt1 (Supplementary Figure 3A). To exclude background detection of TRAF6 autoubiquitination or other ubiquitin modifications, ubiquitination experiments were performed after cellular lysis under denaturing conditions (1% SDS). This lysis completely abolished the interactions of transfected or endogenous Malt1/TRAF6 or Malt1/Bcl10 (Supplementary Figure 3B, and data not shown), providing evidence that specifically Malt1 ubiquitination was detected. Since TRAF6 was shown to associate with the C-terminus of Malt1 (Sun

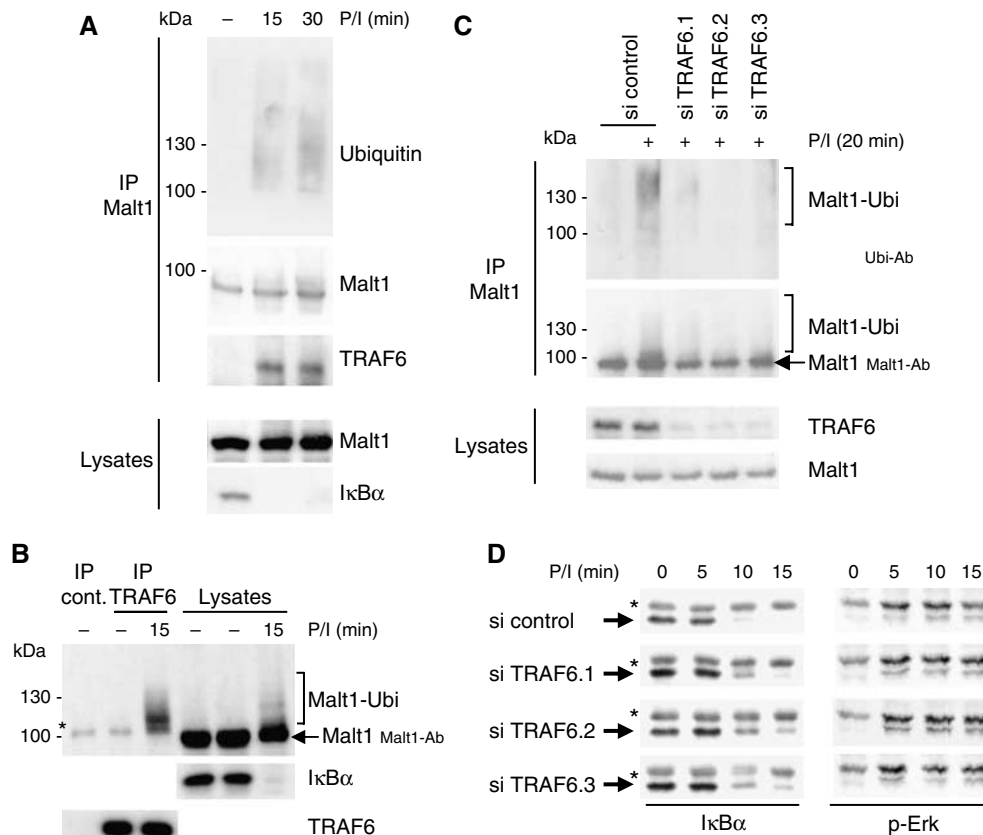


Figure 4 TRAF6 function for Malt1 ubiquitination upon activation of Jurkat T cells. **(A)** Inducible binding of Malt1 to TRAF6 coincides with Malt1 ubiquitination. Jurkat T cells were stimulated with P/I and Malt1 IP was performed for simultaneous detection of TRAF6 association and Malt1 ubiquitination. **(B)** TRAF6-associated Malt1 is ubiquitin conjugated. Jurkat T cells were lysed in co-IP buffer followed by anti-TRAF6 or control IP. Malt1 in lysates and TRAF6-associated Malt1 were analyzed in parallel to demonstrate the modification of TRAF6-associated Malt1. Asterisk indicates nonspecific cross-reaction band after IP. **(C)** siRNA-mediated reduction of TRAF6 impairs inducible Malt1 ubiquitination. Jurkat T cells were transfected with control or TRAF6 siRNAs. Seventy-two hours after transfection, the cells were stimulated for 20 min and lysed. Malt1 ubiquitination was analyzed as in Figure 1C. **(D)** siRNA-mediated reduction of TRAF6 impairs NF-κB signaling. Jurkat T cells were transfected with control or TRAF6 siRNAs and stimulated with P/I. Western blot analysis of lysates demonstrates delayed IκBα degradation upon TRAF6 knockdown. Induction of Erk phosphorylation was not altered. An unspecific band detected with the IκBα antibody is marked by an asterisk.

et al, 2004), we asked whether it is also the C-terminus that is targeted by TRAF6 induced ubiquitination (Figure 3B). Indeed, TRAF6 induced strong ubiquitination of the C-terminal 200 amino acids (aa) (Malt1 612–813). In contrast, the very C-terminus of Malt1 (aa 684–813) was not ubiquitinated by TRAF6, suggesting the existence of potential acceptor lysines in the region between aa 612 and 683. However, reduced ubiquitination could partially result from decreased affinity due to the absence of the second TRAF6-binding site in Malt1 684–813 (data not shown and scheme Figure 5A).

To demonstrate that TRAF6 can function as an E3 ligase for Malt1 *in vitro* and to characterize the linkage of TRAF6 induced C-terminal Malt1 ubiquitin chains, we established a cell-free ubiquitin conjugation assay for Malt1. Detection was performed by anti-ubiquitin western blotting after boiling of ubiquitination assays in 1% SDS buffer, to abolish any non-covalent protein interaction, followed by Malt1 IP (Figure 3C). Depending on E2 (Ubc13/Uev1a) and ATP, TRAF6 mediated the assembly of K63-linked ubiquitin chains to the C-terminus of Malt1.

Next, we addressed TRAF6 function for Malt1 ubiquitination upon activation of Jurkat T cells. Co-IPs revealed inducible binding of endogenous Malt1 and TRAF6 (Figure 4A),

correlating with enhanced Malt1 ubiquitination and degradation of IκBα. Vice versa, the majority of Malt1 associated with TRAF6 was ubiquitin-conjugated after T-cell activation (Figure 4B). Moreover, a reduction of TRAF6 protein amounts using three independent siRNAs diminished stimulus-dependent Malt1 ubiquitination (Figure 4C). Congruent with previous results, siRNA-mediated inactivation of TRAF6 impaired NF-κB activation, as monitored by delayed degradation of IκBα (Figure 4D). These data demonstrate that TRAF6 can function as an E3 ligase mediating the attachment of K63-linked ubiquitin chains to the C-terminus of Malt1.

Multiple lysines in the C-terminus of Malt1 can serve as ubiquitin acceptor sites

For a functional analysis of Malt1 ubiquitination, we mutated C-terminal lysine residue(s) to map potential acceptor site(s) for the attachment of regulatory ubiquitin chains. We concentrated on the entire C-terminus of Malt1 (aa 612–813; 11 lysines), because we could not exclude that the reduced ubiquitination of Malt1 684–813 was at least partially due to decreased affinity to TRAF6 (scheme in Figure 5A). To further narrow the ubiquitination site, we exchanged pairs of lysines and all six lysines in the region 612–683 to arginines

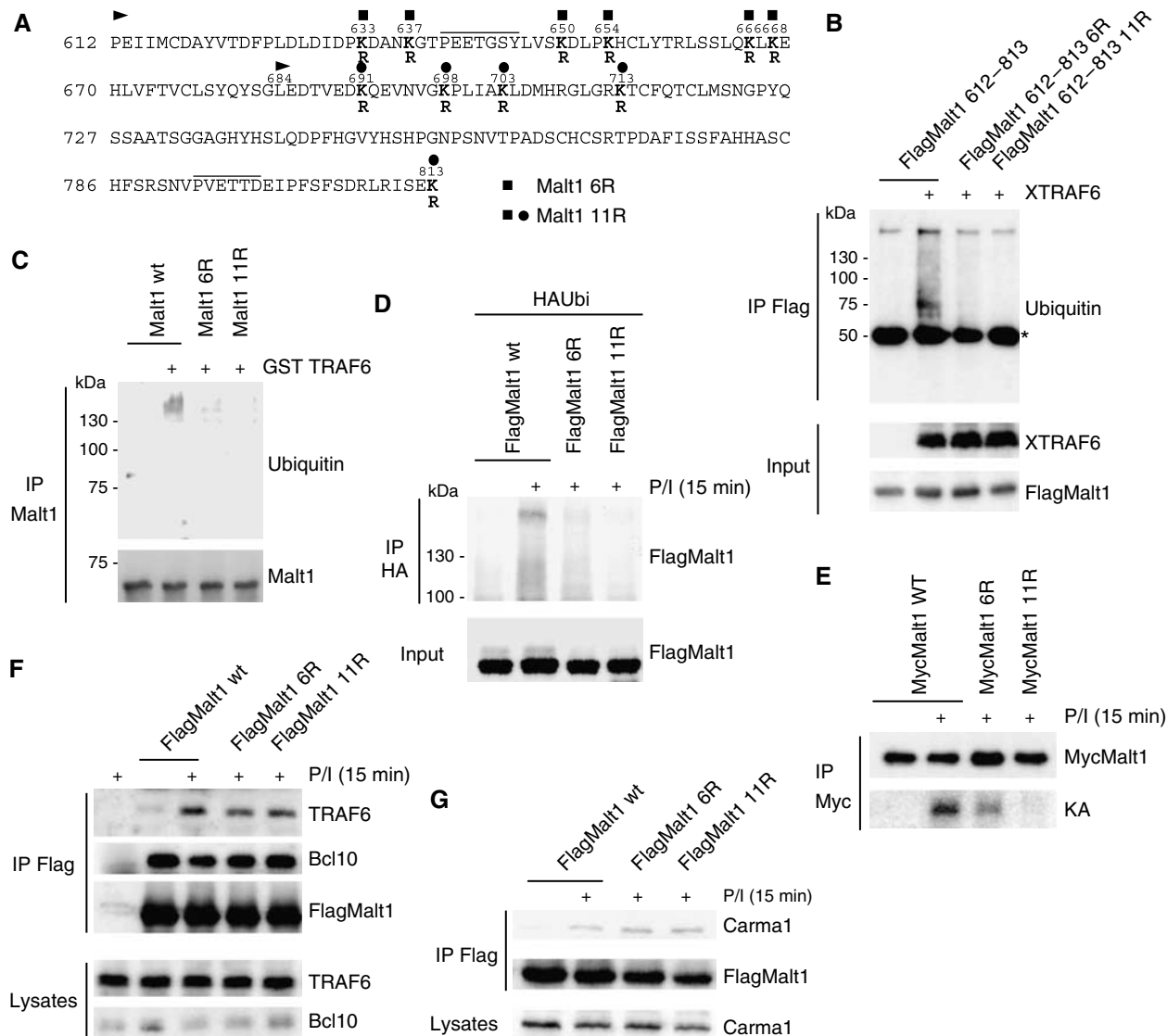


Figure 5 TRAF6 and T-cell activation mediate the assembly of ubiquitin chains to multiple lysine residues in the C-terminus of Malt1. (A) Schematic presentation of lysines (K) in the C-terminus of Malt1. Mutated lysine residues are depicted by squares (Malt1 6R) or squares/circles (Malt1 11R). TRAF6 binding sites are marked by a line. (B) Multiple C-terminal lysine residues of Malt1 serve as acceptor sites for ubiquitin chain attachment. HEK293 cells were cotransfected with Malt1 wt, 6R and 11R constructs (aa 612–813) and XTRAF6, lysed in co-IP buffer containing 1% SDS and diluted 10-fold before FlagMalt1 IP. (C) TRAF6 mediates *in vitro* assembly of ubiquitin chains to multiple C-terminal lysine residues of Malt1. *In vitro* ubiquitin conjugation using GSTMalt1 wt, 6R or 11R (aa 482–813) was carried out as described in Figure 3C. (D) Mutation of C-terminal ubiquitin acceptor lysines impairs inducible Malt1 ubiquitination. Jurkat T cells were transfected with FlagMalt1 constructs and HAUbi as indicated. Cells were stimulated (P/I 20 min) after 72 h and extracts were immunoprecipitated with HA beads followed by Flag western blotting. (E) C-terminal lysine mutations prevent the pull down of IKK activity by anti-Malt1 IP. Transfected Jurkat T cells were stimulated (P/I 15 min) and after lysis and Malt1 IP IKK kinase assay (KA) was carried out in the presence of GST κ B α (1–53). (F) Lysine-to-arginine exchange in the C-terminus of Malt1 does not impair constitutive Bcl10 association and inducible TRAF6 interaction. Jurkat T cells were transfected with FlagMalt1 constructs. The association of endogenous Bcl10 and TRAF6 was determined after Flag IP. (G) C-terminal lysine mutations in Malt1 do not interfere with inducible CBM complex formation. Jurkat T cells were transfected and treated as in panel D. Inducible Carma1 association was determined by western blotting.

and also replaced all 11 lysines (11R) in the Malt1 C-terminus (612–813) (Figure 5B; Supplementary Figure 4A). None of the double mutants displayed reduced TRAF6-dependent Malt1 ubiquitination. Only combined exchange of all six lysine residues in the region 612–683 (Malt1 612–813 6R) significantly reduced Malt1 ubiquitination. As expected, ubiquitination was completely abolished when all eleven C-terminal lysines (Malt1 612–813 11R) were mutated to arginines. The affinity between Malt1 and TRAF6 was not altered by

C-terminal lysine exchange (Supplementary Figure 4B and C). Malt1 ubiquitination was also reduced in an *in vitro* ubiquitin conjugation assay, if Malt1 6R or Malt1 11R instead of Malt1 wt was used as a substrate (Figure 5C). To determine if the presence of C-terminal lysine residues contributes to TCR-dependent Malt1 ubiquitination, we analyzed FlagMalt1 wt, 6R and 11R ubiquitination in Jurkat T cells (Figure 5D). Malt1 6R displayed a marked reduction and replacement of all C-terminal lysines in Malt1 11R nearly abolished inducible

ubiquitin ligation, demonstrating the importance of the mapped acceptor lysines for stimulus-dependent Malt1 ubiquitination. Further, an *in vitro* kinase assay using GSTI κ B α (1–53) as a substrate after IP of MycMalt1 wt, 6R and 11R revealed that C-terminal lysine to arginine exchange severely impaired the ability of Malt1 to pull down IKK kinase activity from the extracts (Figure 5E). Importantly, mutation of C-terminal lysines in Malt1 did not prevent constitutive association with endogenous Bcl10 or inducible interaction with TRAF6 or Carma1 (Figure 5F and G), demonstrating that lysine exchange in the C-terminus did not interfere with CBM complex formation and TRAF6 binding. Thus, TRAF6 and T-cell activation promote the assembly of ubiquitin chains to multiple lysine residues in the C-terminus of Malt1.

C-terminal Malt1 ubiquitination sites are required for NF- κ B activation and IL-2 production

T cells from Malt1-deficient mice are defective in NF- κ B activation and interleukin-2 (IL-2) production upon CD3/CD28 co-ligation or P/I stimulation (Ruefli-Brasse *et al*, 2003; Ruland *et al*, 2003). To investigate the functional consequences of C-terminal Malt1 ubiquitination for NF- κ B signaling and T-cell activation, we rescued naive CD4⁺ T cells from Malt1-deficient mice by retroviral infection using FlagMalt1 wt, 6R, 11R and 314–813 constructs (Figure 6). Retroviral vectors contained an IRES sequence for simultaneous expression of the surface protein Thy1.1 to identify infected cells by FACS. As determined by Flag/Thy1.1 costaining, Thy1.1-positive cells (+) expressed equivalent

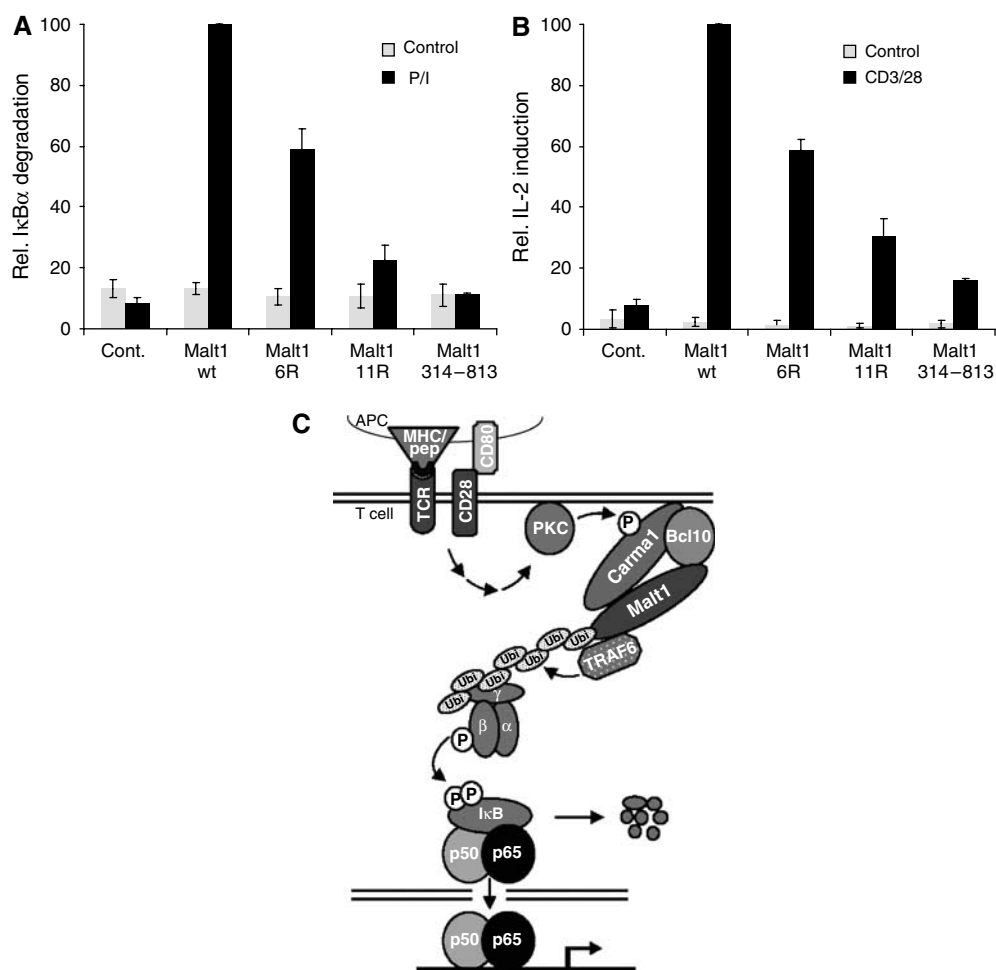


Figure 6 Mutation of ubiquitin acceptor sites in Malt1 abrogates NF- κ B activation and IL-2 production in primary T cells. Malt1^{-/-} CD4-positive T cells were infected with retroviral constructs coupling Malt1 wt, Malt1 6R, Malt1 11R or Malt1 314–813 with Thy1.1 expression. Only Thy1.1-positive cells were analyzed before or after stimulation (see Supplementary Figure 5A). Uninfected Thy1.1 cells behaved like empty vector control (data not shown). (A) C-terminal ubiquitin acceptor lysines of Malt1 are required for P/I-induced I κ B α degradation. I κ B α protein amounts of Thy1.1-positive cells were measured by intracellular staining and FACS analysis, after stimulation with P/I for 20 min. Quantification of I κ B α decrease was performed by determining the number of cells gated for low I κ B α amounts (R3 in Supplementary Figure 5B). Numbers for Malt1 wt reconstitution were set to 100%. Bars and standard deviations are given for three independent experiments. (B) C-terminal ubiquitin acceptor lysines of Malt1 are required for CD3/CD28 co-ligation-induced IL-2 production. IL-2 amounts of Thy1.1-positive cells were measured by intracellular staining and FACS analysis, after stimulation, through CD3/CD28 antibody ligation for 3 h. Quantification of IL-2 increase was performed by determining the number of cells gated for high IL-2 amounts (R3 in Supplementary Figure 5C). Numbers for Malt1 wt reconstitution were set to 100%. Bars and standard deviations are given for three independent experiments. (C) Schematic model of the function of TRAF6-mediated Malt1 ubiquitination in TCR/CD28-induced NF- κ B activation. T-cell activation provokes PKC θ -dependent formation of the CBM complex, and recruitment of TRAF6 to Malt1. TRAF6 mediates ubiquitination of lysine residues in the C-terminal part of Malt1, a process that facilitates the association of IKK γ and thereby the recruitment of the IKK complex to initiate the canonical NF- κ B pathway.

amounts of FlagMalt1 wt and mutant proteins (Supplementary Figure 5A). We measured I κ B α protein levels to monitor NF- κ B activation (Figure 6A; Supplementary Figure 5B) and intracellular IL-2 production as a marker for T-cell activation (Figure 6B; Supplementary Figure 5C). Infection with empty vector or Malt1 314–813, a deletion mutant that cannot bind to Bcl10, did not rescue defective I κ B α degradation in Malt1 $^{-/-}$ T cells in response to P/I. In contrast, P/I treatment of Thy1.1 + Malt1 wt cells induced I κ B α degradation, whereas Malt1 6R and 11R mutants were severely impaired in mediating I κ B α degradation. Further, Malt1 wt, but not empty vector or Malt1 314–813, provoked a robust increase in IL-2 producing Thy1.1 + cells after CD3/CD28 costimulation. Again, mutation of C-terminal lysines (6R, 11R) significantly impaired the ability of Malt1 to rescue IL-2 production in Malt1 $^{-/-}$ T cells. Thus, the data provide evidence that C-terminal ubiquitination of Malt1 is essential for NF- κ B activation and IL-2 induction in response to T-cell activation.

Discussion

In T cells recruitment of Bcl10/Malt1 to Carma1 is essential for IKK activation in response to TCR/CD28 co-ligation, but the molecular mechanisms of IKK activation downstream of the CBM have not been fully elucidated. In our study, we present several lines of evidence that ubiquitination of Malt1 functionally links CBM and IKK complexes. First, Malt1 ubiquitination coincides with the activation of the IKK/NF- κ B-signaling pathway. Second, IKK γ associates with Malt1 ubiquitin chains and the ubiquitin-binding motif of IKK γ is critical for NF- κ B signaling in response to T-cell activation. Third, TRAF6 can catalyze the assembly of K63-linked ubiquitin chains *in vitro* and functions as a potential ubiquitin ligase for Malt1 *in vivo*. Fourth, T-cell activation induces the assembly of ubiquitin chains to multiple C-terminal lysines of Malt1 and conservative replacement (lysine to arginine exchange) of ubiquitin attachment sites strongly decreases the ability of Malt1 to mediate T-cell stimulation-dependent NF- κ B signaling and IL-2 production. Collectively, the data demonstrate that polyubiquitination of Malt1 is essential for directing TCR signaling to the canonical NF- κ B pathway.

We suggest the following model of IKK activation in T cells (Figure 6C). TCR/CD28 co-engagement initiates a series of receptor proximal signaling events that lead to the activation of PKC θ (Sun *et al*, 2000). PKC θ phosphorylation of Carma1 promotes recruitment of Bcl10–Malt1 to Carma1 and thus CBM complex formation (Matsumoto *et al*, 2005; Sommer *et al*, 2005). The ubiquitin ligase TRAF6 is recruited to the C-terminus of Malt1 and mediates the assembly of K63-linked ubiquitin chains to lysine residues in the vicinity. IKK γ binds through its ubiquitin-binding moiety to ubiquitin chains on Malt1. Further, TAB2/TAK1 associate with ubiquitinated Malt1 upon T-cell stimulation, but it remains to be seen whether this is due to a direct interaction of the TAB2 UBD and Malt1-attached ubiquitin chains. TAK1 was shown to function as activating kinase for IKK β kinase (Wang *et al*, 2001; Sun *et al*, 2004; Wan *et al*, 2006), suggesting that recruitment of TAK1 to ubiquitinated Malt1 upon TCR engagement could lead to subsequent IKK activation. However, the necessity for TAK1 at this stage has not been completely resolved and alternatively, binding of IKK γ to

Malt1 ubiquitin chains could induce proximity and autoactivation of IKK complexes (Hayden and Ghosh, 2004). Future studies must determine whether recruitment of several UBD containing proteins is crucial for efficient signal propagation.

The function of Malt1 in TCR/CD28-induced IKK activation seems to be analogous to the role of RIP1 in TNF α -triggered NF- κ B activation. It was shown that TNF α stimulation-induced RIP1 polyubiquitination, potentially catalyzed by the E3 ligase TRAF2, provides a platform for the recruitment of IKKs to the TNF receptor complex (Ea *et al*, 2006; Li *et al*, 2006; Wu *et al*, 2006). Nevertheless, there is a discrepancy between Malt1 and RIP1 regarding the mode of ubiquitination. A single lysine residue (K377) was shown to serve as the attachment site for ubiquitin chains to RIP1 (Ea *et al*, 2006; Li *et al*, 2006). However, mutation of RIP1 at position K377 diminished its inducible interaction with TNF receptor complexes (Ea *et al*, 2006), indicating that lack of ubiquitination could be caused by disturbed recruitment rather than mutation of the substrate attachment site. Based on *in vivo* and *in vitro* evidence, we find that for the assembly of ubiquitin chains to Malt1, any lysine within an acceptable distance seems to be sufficient, which is in agreement with observations that RING E3 ligases often do not precisely position the ubiquitin chain to specific acceptor lysines (Passmore and Barford, 2004). Since the C-terminal lysine mutants of Malt1 associate with Bcl10 and TRAF6 and integrate into the CBM complex, we can exclude that gross structural alterations have been evoked and that upstream signaling is defective.

A previous study has suggested that Bcl10/Malt1-induced ubiquitination of IKK γ is crucial for NF- κ B signaling in T cells, and that Malt1 contains intrinsic ubiquitin ligase activity (Zhou *et al*, 2004). Although we cannot completely exclude that Malt1 is a TRAF6-dependent E3 ligase, in our experiments we did not observe that Malt1 is significantly auto-ubiquitinated after overexpression in cells or *in vitro* (see Figure 3A and C, and data not shown). Thus, Malt1 does not seem to confer sufficient E3 ligase activity. In line with these observations, a separate study suggested that TRAF6 might be the E3 ligase that mediates Malt1-dependent IKK γ ubiquitination (Sun *et al*, 2004). Carma1 and Bcl10–Malt1 can induce ubiquitination of IKK γ on K399; however, K399R mutation has only very little effect on inducible NF- κ B activation in T cells (Zhou *et al*, 2004; Shambharkar *et al*, 2007). Thus, the functional link between IKK γ ubiquitination and NF- κ B activation for T-cell activation is rather vague. It is tempting to speculate that different regulatory mechanisms are required for sustained productive T-cell activation. However, the kinetic of Malt1 ubiquitination and the mutagenesis of C-terminal Malt1 acceptor lysines suggest that attachment of ubiquitin chains to Malt1 is a key event to initialize IKK/NF- κ B signaling in response to TCR/CD28 co-engagement.

Recently, it was suggested that T-cell activation can trigger phosphorylation and ubiquitination of the IKK complex by two distinct mechanisms (Shambharkar *et al*, 2007). Although Carma1 and Bcl10 are involved in TRAF6-dependent ubiquitination of IKK γ , both proteins are dispensable for TAK1-dependent IKK α / β phosphorylation. Mechanistically, it is unclear how these separate pathways are integrated. We find that the critical components, including Carma1, Bcl10, Malt1, TRAF6, TAB2/TAK1 and IKK γ , are directly or indirectly associating, suggesting that they should act in concert.

However, it might be that some components (e.g., TAK1) can perform certain tasks independent of the other mediators.

Previous studies have reported impaired IKK/NF- κ B activation after siRNA-mediated downregulation of TRAF6 (Sun *et al*, 2004; Bidere *et al*, 2006). In line with this, we found that downregulation of TRAF6 resulted in a partial inhibition of Malt1 ubiquitination and NF- κ B signaling. Unexpectedly, mice that lack expression of TRAF6 in T cells have no apparent abnormalities in NF- κ B activation upon TCR engagement (King *et al*, 2006). However, the importance of regulatory K63-linked ubiquitination in TCR signaling is supported by the conditional excision of the UBC13 locus in T cells, as thymocytes from these mice are defective in IKK/NF- κ B activation (Yamamoto *et al*, 2006). Altogether, the data indicate that one or several unknown E3 ligases compensate for the loss of TRAF6 in T cells. Based on siRNA experiments, Sun *et al* (2004) have suggested that TRAF2 could be involved in TCR-dependent NF- κ B activation. Future studies must therefore determine whether TRAF2 or other E3 ligases might have a redundant function with TRAF6 in mediating TCR-induced NF- κ B signaling.

Recent results demonstrated a conserved function of Bcl10-Malt1 in directing antifungal responses and G-protein-coupled receptors (GPCR) to NF- κ B activation (Gross *et al*, 2006; Klemm *et al*, 2007; McAllister-Lucas *et al*, 2007; Wang *et al*, 2007). The Carma1 homologue Carma3 (CARD10) functions as a scaffold for GPCR-initiated NF- κ B activation, which is abrogated by TRAF6 deficiency (Grabner *et al*, 2007). Further, the CBM complex is critical for the survival of a subset of malignant lymphomas (Ngo *et al*, 2006). Chromosomal translocations leading to the generation of API2-Malt1 fusion proteins are associated with aggressive MALT lymphoma, and API2-Malt1 requires the C-terminus of Malt1 for triggering NF- κ B activation (Zhou *et al*, 2005). Thus, C-terminal Malt1 ubiquitination may be relevant for activation of NF- κ B in various physiological and pathological settings.

Materials and methods

Reagents and antibodies

The following antibodies were used: human CD3, human CD28, mouse IgG1, mouse IgG2a, mouse IgG1a-FITC, IKK α and IKK γ (all from BD Biosciences); I κ B α (C21), Myc (9E10), TRAF6 (H274), Malt1 (H300, B12), Bcl10 (331.1), TAB2 (H300), TAK1 (M579) and IKK γ (FL419) (all from Santa Cruz Biotechnology); Carma1 (Abcam); flag M2 and flag M2-FITC (both from Sigma); ubiquitin (FK2; Biomol); I κ B α , phospho-IKK α / β and IKK β (all from Cell Signaling); Thy1.1-APC, IL-2-FITC (both eBioscience) and ICN anti-hamster (MP Biomedicals). The following reagents and siRNAs (100 nM) were used: PMA (200 ng/ml) and ionomycin (300 ng/ml; both from Calbiochem); IL-2 (20 U/ml; Roche), Brefeldin A (10 ng/ml; Sigma); Dynabeads CD4 and DetachaBead mouse CD4 (Dyna Invitrogen) and Streptactin Superflow resin (IBA); si TRAF6.1: GCA CAGCAGUGCAAUGGAAUUUAUA (Invitrogen); si TRAF6.2: CCAGC UCCUGUAGCCGUGAACAAA (Invitrogen); si TRAF6.3: CCACGAA GAGAUAAUGGAU (Eurogentec) and si control: CCAUCCUGAUG UCCGAAUGCCGAAA (Invitrogen).

Plasmids

All Malt1 constructs were cloned with N-terminal Flag (pEF vector; Invitrogen) or Myc (pRK5 vector) epitopes. Mutagenesis was performed by standard PCR. Flag/Myc or Express (X) TRAF6 constructs were expressed from pRK5 or pcDNA4-His-Express vectors (Invitrogen), respectively. FlagIKK γ wt and mutants were cloned in pcDNA3 (Invitrogen). GST-Malt1 (493–824) and GST-TRAF6 were expressed from pGEX6p-1 (GE Healthcare). Retroviral

FlagMalt1 constructs were cloned using the Gateway system (Invitrogen) into pMSCV-Thy1.1 that couples Thy1.1 and Malt1 expression via an IRES (Internal ribosome entry site) sequence.

Cell culture

HEK293 and Phoenix packaging cells were transfected using standard calcium phosphate precipitation protocols. Cell culture, transfection and stimulation of Jurkat T cells (P/I or CD3/CD28 antibody co-ligation) were performed as described (Scharschmidt *et al*, 2004). For RNA interference, Jurkat T cells were transfected with Atufect transfection reagent (Atugen, Berlin) and 100 nM siTRAF6 or control siRNA and analyzed after 72 h. Primary T cells were cultured in RPMI supplemented with 1% pen/strep, 1% glutamine, 10% FCS and 0.1% mercaptoethanol. Positive selection for CD4+ T cells was carried out with Dynabeads.

Retroviral infection of CD4-positive T cells and FACS analysis

Purified CD4+ T cells from spleen and lymph nodes of Malt1^{-/-} mice (Ruland *et al*, 2003) were stimulated with plate-bound CD3/CD28 antibodies for 48 h essentially as described (Wegener *et al*, 2006). For retroviral infection, virus was harvested from Phoenix packaging cells 2 days after transfection and supplemented with Polybrene (4 μ g/ml). CD4+ T cells were incubated with retroviral supernatant for 6 h and then resuspended and cultured in medium with IL-2 (20 U/ml) for 3 days before analysis. Infection efficiencies between 25–50% were achieved. Infected T cells were then stimulated with P/I or plate-bound CD3/28 antibodies for the indicated times. For determination of IL-2 production, Brefeldin A was added 1 h after CD3/28 stimulation. After Thy1.1-APC staining, activated cells were fixed, permeabilized and stained with primary anti-I κ B α and secondary anti-mouse IgG1a-FITC antibodies or anti-IL-2-FITC antibody for FACS analysis. Quantifications of I κ B α degradation and IL-2 production represent statistical analysis of three independent experiments.

Co-IP, cellular ubiquitination and gel filtration

For binding studies, cells were lysed in co-IP buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM DTT, 10 mM sodium fluoride, 8 mM β -glycerophosphate, 20 μ M sodium vanadate and protease inhibitor cocktail). IP was carried out overnight at 4°C, and after washing precipitates were boiled and analyzed by western blotting. For detection of Malt1 ubiquitination, the lysis buffer was supplemented with 1% SDS. Before IPs, extracts were diluted 10-fold with co-IP buffer. For gel-filtration analysis, extracts from Jurkat T cells lysed in co-IP buffer without glycerol were fractionated on a Superose 6 column (GE Healthcare), followed by anti-Bcl10 IP. For detection of Malt1 ubiquitination, fractions were supplemented with 1% SDS and diluted to 0.1% SDS final concentration before anti-Malt1 IP.

Streptactin pull down

StrepIKK γ wt, L329P and Y308S were expressed in *Escherichia coli* BL21(DE3), bound to streptactin beads and incubated overnight with extracts from Jurkat T cells (lysis in co-IP buffer with 1% Triton X-100 instead of NP-40; final concentration of Triton-X100 for pull down was 0.1%). Analysis of material bound to beads was by western blotting.

In vitro ubiquitination and kinase assays

Recombinant GST-Malt1 (482–813) and GST-TRAF6 were expressed in *E. coli* BL21(DE3)RIL and purified using glutathione sepharose. *In vitro* ubiquitination reactions (30 μ l total volume) were performed in ubiquitination buffer (20 mM HEPES pH 7.2, 10 mM MgCl₂, 1 mM DTT, protease inhibitor cocktail) with 50 nM E1, 875 nM E2 (Ubc13/Uev1a), 150 μ M ubiquitin (wt, K63-only, K48-only, K63R or K48R) and energy-regenerating solution (all from Boston Biochemicals). The reactions were incubated for 2 h at 30°C, boiled in co-IP buffer containing 1% SDS and diluted 10-fold before Malt1 IP. For IKK kinase assays, untreated and P/I-stimulated Jurkat cells were lysed in co-IP buffer and Malt1 was precipitated using Malt1 (H300) or Myc antibodies. Kinase assays using GST I κ B α (aa 1–53) as substrate were performed essentially as described previously (Scharschmidt *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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