# Finally, MALT1 is a protease!

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**Two groups demonstrate proteolytic activity for MALT1, a component of the signaling pathway mediating antigen receptor–dependent stimulation of the transcription factor NF-κB, and identify its first substrates.**



The gene *MALT1* (mucosa-associated lymphoid tissue lymphoma translocation 1) was first identified by virtue of its involvement in the recurrent t(11;18)(q21;q21) translocation[1]. This chromosomal translocation, associated with a subset of mucosa-associated lymphomas, creates a fusion oncoprotein consisting of the MALT1 carboxyl terminus linked to the amino terminus of the apoptosis inhibitor API2 (also called c-IAP2). Structural analysis showed that the MALT1 carboxyl terminus has a putative proteolytic active site that bears similarity to the active site of caspases, except for the presence of a neutral leucine residue and an acidic glutamate residue in place of two basic amino acids in the substrate pocket[2]. This suggested that as a protease, MALT1 should show specificity for a substrate with a basic or uncharged amino acid in the P1 position (immediately carboxy-terminal to the cleavage site). Thus, MALT1 was classified as the first human 'paracaspase', to distinguish it from caspases, which show specificity for substrates with an acidic P1 residue[2]. Simultaneously, MALT1 was found to bind to the adaptor protein Bcl-10, an interaction that induces the formation of MALT1 oligomers, which suggested that like traditional caspases, MALT1 may be activated through the enforced formation of oligomers[2,3]. Now, 7 years later, this issue of *Nature Immunology* presents the work of two groups who provide the first demonstration that MALT1 has proteolytic activity; their work also identifies two substrates whose proteolysis affects distinct aspects of lymphocyte activation[4,5]. The papers beautifully complement each other, with each providing insights that support and extend the observations of the other.

Since the original identification of MALT1, years of work by many laboratories has culminated in an understanding of the physiological functions of Bcl-10 and MALT1 in lymphocytes[6] (**Fig. 1a**). Stimulation of either the T cell antigen receptor (TCR) or B cell antigen receptor leads to stimulation of protein kinase C isoforms that phosphorylate the scaffolding protein CARMA1, which subsequently recruits both Bcl-10 and MALT1 to form what is now referred to as the CARMA1–Bcl-10–MALT1 (CBM) 'signalosome'. Once the CBM signalosome is assembled, MALT1 functions as the 'effector' protein and mediates activation of the IKK complex, a multisubunit kinase that phosphorylates the IκB proteins, which bind to and sequester the transcription factor NF-κB in the cytoplasm. Phosphorylation and subsequent degradation of IκB leads to the release of NF-κB, which then translocates to the nucleus and regulates the transcription of 'target' genes involved in the immune response to foreign antigens. In contrast to native MALT1, which requires 'upstream' receptor engagement for its recruitment and formation of oligomers in the context of the CBM signalosome, the API2-MALT1 fusion protein was found to form oligomers of itself, thus providing a mechanism for unregulated, autoactivation of the MALT1 carboxy-terminal moiety[7,8] (**Fig. 1b**).

Even 7 years ago, however, it was recognized that any caspase-like activity of MALT1 could not fully explain the ability of MALT1 in oligomers to activate the IKK complex, as substitution of a critical cysteine residue in the putative catalytic domain produced only a modest reduction in NF-κB activation[2,3]. As a result, subsequent work focused on identifying other activities of MALT1, and it is now known that MALT1 induces lysine 63 (K63)–linked ubiquitination of IKKγ, the regulatory subunit of the IKK complex, although it is unclear whether this is a direct action of MALT1 or whether it requires the participation of other proteins, including the known E3 ubiquitin ligase TRAF6 (refs. 8–10; **Fig. 1a**, pathway 1). Ubiquitination of IKKγ is now thought to be perhaps the main mechanism by which MALT1 stimulates 'downstream' NF-κB activity. Given that function for MALT1, and as no demonstrable proteolytic activity had been reported for MALT1 so far, the idea of MALT1 as a 'caspase-like' protease faded from the limelight.

That has all changed with the present issue of *Nature Immunology*. In searching for previously unknown post-translational modifications of Bcl-10, Rebeaud *et al.* astutely noticed the appearance of a Bcl-10 breakdown product in stimulated T cells or B cells[5] and demonstrate that this phenomenon requires activation of the CBM signalosome. With a series of clever mapping studies, the authors definitively show that Bcl-10 cleavage occurs at a single site, just five amino acids from the carboxy-terminal end of the protein, between Arg228 and Thr229 (**Fig. 1c**). They note that this cleavage site, which follows a basic P1 residue, is consistent with the proposed specificity of the MALT1 paracaspase. In further studies, the authors demonstrate MALT1 *in vitro* protease activity by using purified recombinant glutathione *S*-transferase–MALT1 and, as a substrate, either purified Bcl-10 or a fluorogenic peptide consisting of the four amino acids preceding the 'mapped' Bcl-10 cleavage site (Leu-Arg-Ser-Arg). Furthermore, substitution of the critical cysteine residue in the putative MALT1 active site, Cys464, completely abolishes *in vitro* protease activity. The authors next harness what is known about the substrate specificity of the related arginine-lysine–specific family of plant proteases, called 'metacaspases', to design a tetrapeptide inhibitor of MALT1, z-VRPR-fmk (Val-Arg-Pro-Arg conjugated to fluoromethyl ketone (fmk); derived from the well known general caspase inhibitor z-VAD-fmk), and show that this compound inhibits MALT1 protease activity both *in vitro* and in cultured cells.

Notably, z-VRPR-fmk causes only a partial blockade of TCR-dependent NF-κB activation. That finding is in complete agreement with what had been found 7 years earlier[2,3] and supports the idea that MALT1 protease activity only partially accounts for the amount of NF-κB activation achieved by stimulation of the CBM signalosome. Even more notably, the authors find that the initial burst of IκB phosphorylation induced after T cell stimulation is completely unaffected by

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the z-VRPR-fmk inhibitor. This collectively suggests that MALT1 caspase-like activity might not contribute to the direct activation of IKK but instead may influence some other modulatory pathway that controls the maximum achievable NF-κB activation.

Remarkably, the complementary yet completely independent work of Coornaert *et al.*[4] goes a long way toward solving this conundrum. These investigators similarly noticed a proteolytic breakdown product in extracts of stimulated T cells, but instead of Bcl-10, this was an apparent cleavage product of A20, a known inhibitor of the NF-κB pathway[11]. As with Bcl-10, they find that the cleavage of A20 is also dependent on the presence of the CBM components. Moreover, cleavage can be reproduced after expression of an API2-MALT1 fusion protein, which supports the idea that the caspase-like domain of MALT1, which is preserved in the fusion protein, could be responsible. As with the study by Rebeaud *et al.*, the study by Coornaert *et al.* uses the same Cys464 substitution of MALT1 to establish the essential function of MALT1 proteolytic activity in the cleavage reaction. The authors next use an *in vitro* reconstitution assay to definitively prove that MALT1 has catalytic activity toward A20. By careful analysis of the two fragments of A20 produced by MALT1-dependent proteolysis, the authors are able to map the cleavage site to a location after Arg439, which lies between the first two zinc fingers in a stretch of seven zinc fingers in the carboxy-terminal half of the protein (**Fig. 1c**). The result is a 50-kilodalton amino-terminal fragment and a 37-kilodalton carboxy-terminal fragment that seems to undergo rapid proteasome-mediated degradation.

But how do these findings solve the conundrum remaining in the work of Rebeaud *et al.*? A20 has been known for some time to downregulate NF-κB after stimulation of tumor necrosis factor (TNF), interleukin 1 and Toll-like receptors[11]. The work by Coornaert *et al.* now shows that A20 is able to inhibit TCR-dependent activation as well, a notable finding in itself. Published work has shown that the inhibitory effect of A20 on NF-κB signaling can occur through the ability of A20 to use its amino-terminal deubiquitinating OTU domain to remove K63-linked polyubiquitin chains from its substrates, which include TNF receptor–associated factors 2 and 6 (TRAF2 and TRAF6), TNF receptor–interacting protein 1 (RIP1) and IKKγ[11]. In TNF signaling, A20 has dual ubiquitin-editing functions: after K63-linked deubiquitination of RIP1, A20 can then mediate K48-linked polyubiquitination through its carboxy-terminal zinc finger domain, thus targeting RIP1 for proteosome-mediated degradation[11]. Coornaert *et al.*[4] provide the hypothesis that because the A20 carboxyl terminus is also known to bind IKKγ, MALT1-mediated cleavage may at least prevent IKKγ deubiquitination by separating the amino-terminal deubiquitinating OTU domain from the rest of the protein (**Fig. 1a**, pathway 1).

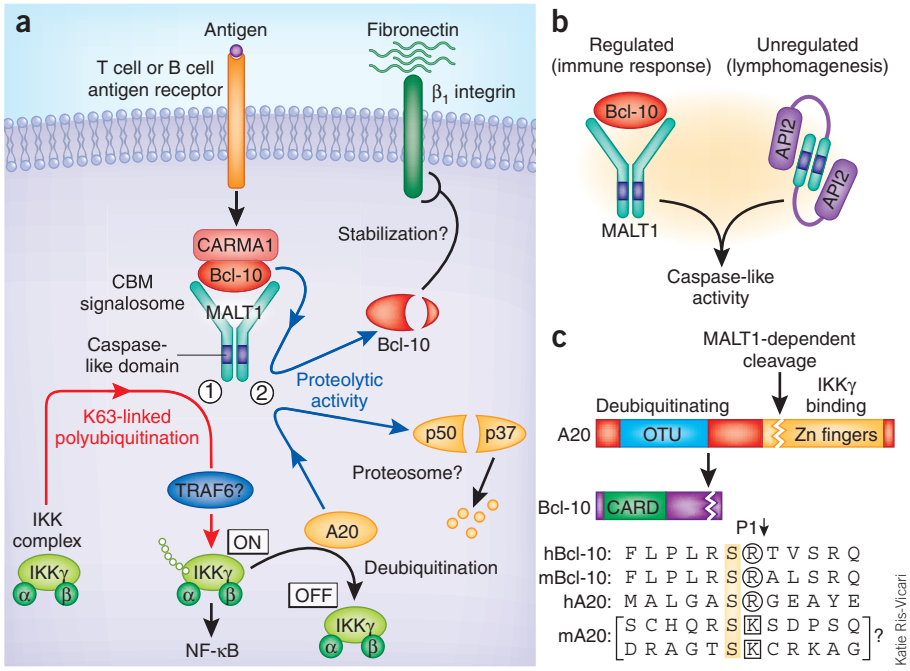


**Figure 1** The MALT1 caspase-like domain has proteolytic activity. (**a**) Proposed model of a dual function for MALT1 in antigen receptor–mediated signaling. Antigen receptor ligation triggers a series of biochemical events culminating in the activation of protein kinase C. Protein kinase C phosphorylates and activates the CARMA1 scaffolding protein, which then recruits Bcl-10 and MALT1 to an active signaling complex. The formation of MALT1 oligomers then leads to activation of the IKK complex and subsequently to stimulation of NF-κB. In pathway 1, MALT1 induces K63-linked ubiquitination of IKKγ either directly or through activation of TRAF6. In pathway 2, the MALT1 caspase-like domain cleaves A20 and Bcl-10. Cleavage of the deubiquitinating enzyme A20 into its p50 and p37 subunits inhibits A20 deubiquitination of IKKγ and thereby promotes maximum antigen-dependent NF-κB activation. MALT1-mediated cleavage of Bcl-10 promotes integrin-mediated adhesion of activated T cells by an as-yet-unknown mechanism. (**b**) Bcl-10-mediated formation of MALT1 oligomers or autoformation of oligomers of the API2-MALT1 fusion oncoprotein can lead to activation of MALT1 caspase-like proteolytic activity. (**c**) Sites of MALT1-mediated cleavage of human A20 and Bcl-10 (above), and alignment of the amino acid sequences corresponding to putative MALT1 cleavage sites (below) of human Bcl-10 (hBcl-10), mouse Bcl-10 (mBcl-10), human A20 (h A20) and mouse A20 (mA20). CARD, caspase recruitment domain; OTU, ovarian tumor domain.

In summary, then, the MALT1 caspase-like activity does not provide a direct means for activating the IKK complex, but instead it cleaves and inactivates a protein (A20) that serves to ultimately dampen the amount of IKK activation. Thus, the MALT1 caspase-like protease activity is not the 'on-off switch' for antigen-dependent NF-κB activation. Instead, MALT1 'fine tunes' the degree of NF-κB activation by protecting the IKK complex from downregulation.

So what then is the function of MALT1-dependent Bcl-10 cleavage? By comparing the capacity of wild-type Bcl-10 and an uncleavable R228A Bcl-10 mutant to reconstitute NF-κB activation in T cells in which expression of endogenous Bcl-10 is silenced by RNA interference, Rebeaud *et al.* demonstrate that cleavage of Bcl-10 is not required for antigen-dependent NF-κB activation. They next explore the possibility that Bcl-10 cleavage may be involved in stimulation-induced, integrin-mediated T cell adhesion. First, they show that 'knockdown' of MALT1 or Bcl-10 by small interfering RNA decreases activation-induced adhesion to the $\beta_1$ integrin ligand fibronectin; they also find that treatment of T cells with the MALT1 protease inhibitor z-VRPR-fmk decreases antigen receptor–mediated adherence to fibronectin. They then demonstrate that T cells expressing an uncleavable R228A Bcl-10 mutant show less adhesion to fibronectin than do cells with wild-type Bcl-10. These studies provide evidence that MALT1-mediated cleavage of

Bcl-10 may be involved in TCR-induced cellular adhesion. Thus, MALT1 protease activity may have a dichotomous role in T cell activation: for Bcl-10, MALT1-dependent cleavage seems to activate or 'unmask' a function, whereas for A20, cleavage brings about a loss of function (**Fig. 1a**).

Alignment of the specific cleavage sites in human Bcl-10 and A20 shows that in both cases, a serine residue precedes the P1 arginine residue (**Fig. 1c**). This pattern is present in several substrate cleavage sites of metacaspases, the related family of plant proteases that specifically cleave after either lysine or arginine. Unexpectedly, the MALT1 cleavage site in human A20 is not conserved in the mouse[4]. Instead, experimental data indicate that MALT1 cleaves mouse A20 at a site in the linker region between zinc fingers 3 and 4. Analysis of this region shows the presence of two lysine residues, each preceded by serine residues (**Fig. 1c**), as well as several arginine residues, any of which might represent the actual cleavage site. Obviously, further studies are needed to define the substrate site specificity of the MALT1 protease. It seems likely that the publication of these two landmark manuscripts will lead to a search for additional physiological substrates of MALT1 and much more will be learned about the nature of this proteolytic activity.

Identification of MALT1 proteolytic activity represents a major step in understanding the molecular events that regulate the normal immune response to antigen and the molecular pathogenesis of lymphoma. The work of Coornaert *et al.* indicates that this proteolytic activity serves to inactivate an inhibitor of NF-κB-dependent gene expression, and the studies by Rebeaud *et al.* suggest involvement of MALT1-mediated proteolysis in promoting integrin-mediated cellular adhesion. Both NF-κB signaling and integrin action are critical to normal immune function, and dysregulation of these activities is associated with immunological and inflammatory diseases as well as malignancy. Thus, both groups wisely point out that the proteolytic activity of MALT1 could represent a promising new target for the development of immunomodulatory and antineoplastic agents. These exciting new discoveries were clearly worth waiting for.


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# Innocents abroad: regulating where naive T cells go

**Barrett J Rollins**

**Kruppel-like factor 2 is now shown to regulate chemokine receptor expression in lymphocytes, which leads to their homing to nonlymphoid organs after they leave the thymus.**

In one sense, adaptive immunity is all about cell migration. Although specific antigen-recognition structures and the signal-transduction cascades they activate are essential for adaptive immunity, the cells that have those structures and signaling pathways must be at 'the right place at the right time' for immunity to occur. For example, after T lymphocytes mature, they leave the thymus and, as naive cells, circulate through blood, secondary lymphoid organs, lymph and back to blood with regulated 'dwell times' in each environment. When activated, T lymphocytes either travel to peripheral organs as effector cells or become memory cells with 'central' or 'peripheral' patterns of migration. Elegant experimentation has identified many of the molecular controls that govern these movements. In particular, several papers have provided an apparently complete picture of T cell egress from the thymus and secondary lymphoid organs. However, a report in this issue of *Nature Immunology* by Kahn and colleagues puts an unexpected twist on the model and provides an important insight into how naive T cells are kept out of peripheral organs until they are needed[1].

The story begins 10 years ago with work on how transcription factors determine T cell phenotypes. One set of studies focused on Kruppel-like factor 2 (KLF2), a member of a family with homology to the drosophila Kruppel transcription factor. KLF2 was originally known as 'Lklf' because of its expression in lung, but it is also present in lymphoid tissue, erythroid cells and endothelial cells. Its influence on T cell activity was inferred from its higher expression in thymocytes after positive selection and lower expression in T cells after activation[2]. Study of KLF2 *in vivo* is complicated by the fact that *Klf2*$^{-/-}$ embryos die *in utero*. Therefore, chimeric mice were developed by injection of *Klf2*$^{-/-}$ cells into blastocysts deficient in recombination-activating gene 2, so that mature B cells and T cells in surviving mice were KLF2 deficient[2]. Notably, these mice had almost no detectable circulating T cells and many fewer T cells in their spleens and lymph nodes, and because the few surviving peripheral T cells had a 'partially activated' phenotype (CD44$^{hi}$CD62L$^{lo}$CD69$^{hi}$ but CD25$^{-}$), their near absence was thought to be a consequence of upregulation of the cytokine FasL, leading to apoptosis. Thus KLF2 was identified as a 'master regulator' of mature T lymphocyte quiescence and, perhaps, survival[3]. One curious property of these initial mice with cell-specific KLF2 deficiency[2] was that despite lacking normal numbers of peripheral T cells, they had more single-positive (SP) cells in the thymus; to explain these phenotypes, the authors mentioned in passing that they might be due to a defect in the export of SP cells[2]. However, it would be several more years before a detailed molecular explanation was forthcoming.

Thymic egress had been shown to be dependent on G proteins, which suggested the involvement of a chemoattractant receptor[4]. Its eventual identification was facilitated by elucidation of the mechanism of action of FTY720, an immune suppressant with an interesting mechanism of action: FTY720 'depletes' animals of circulating T cells by causing the cells to accumulate in secondary

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