



Published in final edited form as:

*Oncogene*. 2014 May 8; 33(19): 2520–2530. doi:10.1038/onc.2013.195.

## The API2-MALT1 Fusion Exploits TNFR Pathway-associated RIP1 Ubiquitination to Promote Oncogenic NF- $\kappa$ B Signaling

Shaun Rosebeck<sup>1,£</sup>, Aasia O. Rehman<sup>1</sup>, Ingrid J. Apel<sup>2</sup>, Dawn Kohrt<sup>1,+</sup>, Alex Appert<sup>3</sup>, Marie Anne O'Donnell<sup>4</sup>, Adrian T. Ting<sup>4</sup>, Ming-Qing Du<sup>3</sup>, Mathijs Baens<sup>5,6</sup>, Peter C. Lucas<sup>2,\*</sup>,<sup>7</sup>, and Linda M. McAllister-Lucas<sup>1,\*</sup>,<sup>8</sup>,<sup>¥</sup>

<sup>1</sup>Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI 48109, USA

<sup>2</sup>Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

<sup>3</sup>Division of Molecular Histopathology, Department of Pathology, University of Cambridge, Laboratory Block, Addenbrooke's Hospital, Cambridge, UK

<sup>4</sup>Immunology Institute, Mount Sinai School of Medicine, New York, NY 10029, USA

<sup>5</sup>Human Genome Laboratory, Molecular Genetics, Center for Human Genetics, Catholic University Leuven, Belgium

<sup>6</sup>Human Genome Laboratory, Department of Molecular and Developmental Genetics, Flanders Institute for Biotechnology (VIB), B-3000 Leuven, Belgium

### Abstract

The API2-MALT1 fusion oncoprotein is created by the recurrent t(11;18)(q21;q21) chromosomal translocation in mucosa-associated lymphoid tissue (MALT) lymphoma. We identified receptor interacting protein-1 (RIP1) as a novel API2-MALT1-associated protein and demonstrate that RIP1 is required for API2-MALT1 to stimulate canonical NF- $\kappa$ B. API2-MALT1 promotes ubiquitination of RIP1 at lysine (K) 377, which is necessary for full NF- $\kappa$ B activation.

Furthermore, we found that TRAF2 recruitment is required for API2-MALT1 to induce RIP1 ubiquitination, NF- $\kappa$ B activation, and cellular transformation. Although both TRAF2 and RIP1 interact with the API2 moiety of API2-MALT1, this moiety alone is insufficient to induce RIP1 ubiquitination or activate NF- $\kappa$ B, indicating that API2-MALT1-dependent RIP1 ubiquitination

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: [http://www.nature.com/authors/editorial\\_policies/license.html#terms](http://www.nature.com/authors/editorial_policies/license.html#terms)

<sup>¥</sup>Correspondence: [linda.mcallister@chp.edu](mailto:linda.mcallister@chp.edu) (L.M.M.-L.), [lucaspc@upmc.edu](mailto:lucaspc@upmc.edu) (P.C.L.), Contact information: Linda M. McAllister-Lucas, Department of Pediatrics, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh, 5120 Rangos Research Building, 4401 Penn Avenue, Pittsburgh, PA 15224.

<sup>\*</sup>These authors contributed equally to this work

<sup>7</sup>Present address: Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15224, USA

<sup>8</sup>Present address: Department of Pediatrics, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh, Pittsburgh, PA 15224, USA

<sup>£</sup>Present address: Multiple Myeloma Program, University of Chicago, Chicago, IL 60637, USA

<sup>+</sup>Present address: Department of Biology, Connecticut College, New London, CT 06320, USA

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### SUPPLEMENTARY INFORMATION

Supplementary information accompanies this paper and is available at the *Oncogene* website (<http://www.nature.com/onc>).

represents a gain of function requiring the concerted actions of both the API2 and MALT1 moieties of the fusion. Intriguingly, constitutive RIP1 ubiquitination was recently demonstrated in several solid tumors, and now our study implicates RIP1 ubiquitination as a critical component of API2-MALT1-dependent lymphomagenesis.

### Keywords

MALT1; TRAF2; RIP1; lymphoma; ubiquitination

---

## INTRODUCTION

MALT lymphoma accounts for 8% of non-Hodgkin's lymphomas and typically presents at sites that acquire lymphoid tissue as a result of chronic inflammation, such as the gastric mucosa. In many cases MALT lymphomas are cured by eliminating the source of inflammation. For example, *Helicobacter pylori* infection is associated with the majority of gastric MALT lymphomas, and antibiotic eradication of *H. pylori* leads to regression in 70% of Stage I patients (1). Specific acquired chromosomal abnormalities also contribute to MALT lymphomagenesis. The first-described and most commonly occurring translocation is t(11;18)(q21;q21), which fuses the *cellular inhibitor of apoptosis 2* (*ciAP2*; also known as *API2*) and *MALT translocation protein 1* (*MALT1*) genes (2–4). Interestingly, t(11;18)<sup>+</sup> gastric MALT lymphomas are typically resistant to antibiotic eradication of *H. pylori* and are associated with increased rates of dissemination (5, 6). Several additional translocations have been identified in MALT lymphoma, including t(1;14)(p22;q32) and t(14;18)(q32;q21), which place the *B cell CLL/lymphoma 10* (*BCL10*) and *MALT1* genes, respectively, under the control of the immunoglobulin (Ig) heavy-chain (*IGH*) locus leading to deregulated over-expression (7–9).

While the MALT lymphoma-specific *API2-MALT1*, *IGH-BCL10*, and *IGH-MALT1* translocations occur in a mutually exclusive manner, all three deregulated proteins expressed as a result of these translocations affect a common cell survival pathway: NF- $\kappa$ B signaling. NF- $\kappa$ B comprises a family of latent dimeric transcription factors required for proper immune responses. Inactive NF- $\kappa$ B dimers are bound by inhibitor of NF- $\kappa$ B (*I $\kappa$ B*) proteins in the cytoplasm and are then activated in response to stimulation by cell-surface receptors, including tumor necrosis factor receptor (TNFR), B- and T-cell antigen receptors (BCR and TCR), lymphotoxin- $\beta$  receptor (LT- $\beta$ R), and the receptor for B-cell activating factor belonging to the TNF family (BAFFR). Two signaling mechanisms that activate NF- $\kappa$ B are the canonical and non-canonical pathways. These pathways differ, in part, by the specific *I $\kappa$ B* kinase (IKK) complex subunits used to transmit the NF- $\kappa$ B signal. Stimulation of the canonical pathway activates IKK $\beta$ , which phosphorylates *I $\kappa$ B $\alpha$*  and targets it for proteasomal degradation, freeing canonical p65(RelA)/p50 NF- $\kappa$ B dimers to translocate to the nucleus and regulate transcription of specific genes. In contrast, non-canonical NF- $\kappa$ B activation, which is stimulated by only a few cell-surface receptors (*e.g.* LT- $\beta$ R and BAFFR), requires stabilization and activation of NF- $\kappa$ B-inducing kinase (NIK). Activated NIK phosphorylates and activates IKK $\alpha$ , which in turn phosphorylates the NF- $\kappa$ B2

precursor p100 and targets it for partial proteasomal processing to p52, thus freeing non-canonical RelB/p52 heterodimers to transactivate appropriate genes (10).

We and others determined that Bcl10 and MALT1 interact and synergize to activate canonical NF- $\kappa$ B (11, 12). In the lymphocyte, this interaction is facilitated by the CARMA1 scaffolding protein during BCR or TCR stimulation (reviewed in 13). Assembly of this CARMA1/Bcl10/MALT1 complex results in oligomerization of MALT1, a necessary step for subsequent activation of the IKK complex. Although the precise mechanisms are still being investigated, it is likely that oligomerized MALT1 recruits TNF receptor associated factor 6 (TRAF6), which then catalyzes K63-linked ubiquitination of both MALT1 and NF- $\kappa$ B essential modulator (NEMO) to promote IKK $\beta$  activation (14–16).

API2-MALT1 utilizes multiple mechanisms to activate NF- $\kappa$ B by harnessing both the canonical and non-canonical pathways. First, API2-MALT1 binds and proteolytically cleaves NIK, creating a stable, constitutively-active NIK fragment that stimulates IKK $\alpha$  and the non-canonical NF- $\kappa$ B pathway (17). Second, API2-MALT1 activates canonical NF- $\kappa$ B by virtue of its ability to constitutively auto-oligomerize via the baculovirus IAP repeat (BIR)-1 domain of the API2 moiety (18, 19). This auto-oligomerization recapitulates the oligomerization of wild-type MALT1 that occurs in the context of antigen-receptor stimulation and assembly of the CARMA1/Bcl10/MALT1 complex. Yet the ability of API2-MALT1 to auto-oligomerize and recruit TRAF6 for the purpose of IKK $\beta$  activation has, alone, never fully explained the exceptionally robust level of canonical pathway activation achieved by expression of this fusion oncoprotein.

Work presented in this manuscript now reveals that API2-MALT1 induces the ubiquitination of RIP1, a key regulator of TNF-dependent effects on NF- $\kappa$ B activity and cellular survival. This discovery stems from the previous demonstration, by our group and others, that TRAF2 binding to API2-MALT1 is required for optimal NF- $\kappa$ B activation (19, 20). TRAF2, which was originally identified as a TNFR-interacting protein (21), is required for maximal TNF-induced NF- $\kappa$ B activation. In this capacity, stimulated TNFR recruits TNFR-associated death domain (TRADD), which can bind RIP1 and TRAF2 (22, 23). TRAF2 then associates with cIAP1/2 (24), and these proteins mediate poly-ubiquitination of RIP1 at K377 (25–28). The ubiquitin chains attached to RIP1 act as a scaffold to which the transforming growth factor  $\beta$ -activated kinase (TAK)-binding protein (TAB)/TAK and NEMO/IKK complexes bind (28–30), resulting in IKK $\beta$  activation and stimulation of canonical NF- $\kappa$ B. Here, we demonstrate that API2-MALT1 also recruits RIP1, making it an available target for ubiquitination. In this respect, API2-MALT1 can function in much the same way as the activated TNFR to promote a RIP1-dependent pathway of canonical NF- $\kappa$ B signaling.

## RESULTS

### API2-MALT1 interacts with TNFR-associated protein RIP1

We previously showed that TRAF2 is required for API2-MALT1-dependent canonical NF- $\kappa$ B activation, however, the mechanism by which TRAF2 contributes to this aspect of API2-MALT1 signaling has remained unclear (19). To investigate the role of TRAF2, we affinity

purified FLAG-API2-MALT1 from B cell lines engineered to either stably express (SSK41) or to inducibly express (BJAB) API2-MALT1 and screened for interacting proteins known to functionally associate with TRAF2. Because TRAF2 has been extensively characterized as a mediator of TNFR signaling, we focused on proteins involved in TNF-dependent NF- $\kappa$ B activation and discovered a novel interaction between API2-MALT1 and RIP1 (Figure 1A). Interestingly, we did not detect association between API2-MALT1 and TRADD, an adaptor protein required for recruitment of TRAF2 and RIP1 to the TNFR. Our findings indicate that API2-MALT1 binds key mediators of the TNFR-dependent NF- $\kappa$ B signaling, but does so in a manner that is not dependent on TRADD.

We next sought to determine which domains of the API2-MALT1 fusion are required for interaction with RIP1. We found that, like TRAF2, RIP1 associates with the API2 moiety (Supplementary Figure S1A), but not the MALT1 moiety of API2-MALT1 (Figure 1B and 1C, and Supplementary Figure S1B), and that neither the TRAF2 binding site (Figure 1D) nor the oligomerization region of the API2 BIR1 domain within API2-MALT1 are necessary for this interaction (Supplementary Figure S1B). The RIP1 death domain (DD; Supplementary Figure S1A), which is responsible for its recruitment to the TNFR complex, is dispensable for the interaction with API2-MALT1 (Figure 1E), but the RIP1 intermediate domain (ID) is sufficient to mediate the interaction with API2-MALT1 (Figure 1F).

### **RIP1 is required for API2-MALT1-dependent canonical NF- $\kappa$ B activation**

To evaluate the contribution of RIP1 to API2-MALT1-induced NF- $\kappa$ B signaling, we next knocked down RIP1 using siRNA. Loss of RIP1 reduced NF- $\kappa$ B-dependent luciferase activity by 50% in response to API2-MALT1 expression, which also correlated with loss of IKK complex activation and I $\kappa$ B $\alpha$  phosphorylation (Figure 2A). Because NF- $\kappa$ B-dependent luciferase activity was not completely lost, and expression of API2-MALT1 is known to activate both canonical and non-canonical NF- $\kappa$ B (17, 31), we hypothesized that API2-MALT1-induced non-canonical NF- $\kappa$ B activation remains intact in the absence of RIP1. Indeed, loss of RIP1 suppressed only API2-MALT1-induced p65 nuclear translocation and had no effect on p52 nuclear accumulation (Figure 2B). This suggested that the residual NF- $\kappa$ B-dependent luciferase activity could be due to unimpaired non-canonical NF- $\kappa$ B signaling, which results in activation of p52/RelB dimers that may bind to the same DNA consensus as canonical dimers of p50/p65 (10). RIP1 knock down in SSK41 B cells stably expressing API2-MALT1 likewise impaired IKK activation (Figure 2C). Using another set of RIP1-specific siRNA, we confirmed the loss of API2-MALT1-induced NF- $\kappa$ B-dependent luciferase activity in response to impaired RIP1 expression (Supplementary Figure S2).

Since RIP1 kinase activity is dispensable for TNF-induced NF- $\kappa$ B activation (32), we investigated if this was also true for NF- $\kappa$ B activation induced by API2-MALT1. Treatment with the RIP1-specific kinase inhibitor, Necrostatin-1 (33), had no effect on API2-MALT1-induced NF- $\kappa$ B activation, but did disrupt necrosome formation in response to TNF-induced necrosis (Supplementary Figure S3). Altogether, these data suggest that RIP1, but not its kinase activity, is required for API2-MALT1-induced canonical NF- $\kappa$ B activation, and that RIP1 is dispensable for API2-MALT1-induced non-canonical NF- $\kappa$ B activation.

### **RIP1 is required for API2-MALT1-dependent enhanced B cell adhesion and survival**

We have previously determined, via RNAi-mediated knockdown of either IKK $\alpha$  or IKK $\beta$ , that both canonical and non-canonical NF- $\kappa$ B activity are required for API2-MALT1 to optimally promote B cell adhesion and resistance to dexamethasone-induced cell death (17). To determine if RIP1 is required for these effects, we knocked down RIP1 expression using siRNA. Loss of RIP1 impaired API2-MALT1-induced adhesion of BJAB B cells to VCAM-1-coated plates (Figure 3A and 3B) and reduced API2-MALT1-dependent protection of SSK41 B cells from dexamethasone-induced cell death (Figure 3C). These results suggest that RIP1 may be a critical mediator of API2-MALT1-dependent tumor progression and/or chemotherapy resistance via canonical NF- $\kappa$ B activation.

### **API2-MALT1-induced ubiquitination of RIP1 at K377 is required for full NF- $\kappa$ B activation**

The ubiquitination status of RIP1 determines whether RIP1 promotes either cell survival, via association of ubiquitinated RIP1 with TAK1/TAB2/NEMO and activation of NF- $\kappa$ B, or cell death, via association of de-ubiquitinated RIP1 with caspase 8, in response to TNFR stimulation (34). We detected ubiquitination of endogenous RIP1 in response to API2-MALT1 expression, either in HEK-293T cells (Figure 4A and Supplementary Figure S4A) or in B cells expressing API2-MALT1 (Figure 4B), consistent with the ability of API2-MALT1 to promote cell survival. As expected, we also detected NEMO ubiquitination in cells expressing API2-MALT1 (18, 35). In contrast to wild-type RIP1, the ubiquitination of a mutant RIP1 lacking lysine 377 (K377R) is not significantly enhanced in the presence of API2-MALT1 (Figure 4C). We also determined that ubiquitination of RIP1 in response to API2-MALT1 does not occur exclusively as K48- or K63-linked ubiquitin chains (Supplementary Figure S4B). This suggests that, as in TNF signaling, API2-MALT1-induced RIP1 ubiquitination is likely quite complex (36) and further investigation will be required to fully characterize the precise biochemical nature of this modification.

Next, we wanted to further characterize the nature of the association between RIP1, TRAF2, and API2-MALT1 and whether this depends on RIP1 ubiquitination. We found that both wild-type and K377R mutant RIP1 were equally able to bind API2-MALT1 and only associated with endogenous TRAF2 when API2-MALT1 was present (Figure 4D). In addition, we determined that the recently identified ubiquitin binding domain within API2-MALT1 was dispensable for the API2-MALT1/RIP1 association (Figure 4E; M/F mutant is depicted in Supplementary Figure S1A). Altogether, these data suggest that ubiquitination of RIP1 at K377 is not needed for association with API2-MALT1, indicating that API2-MALT1 can associate with unmodified RIP1 and induce its ubiquitination.

We then asked if RIP1 ubiquitination is required for API2-MALT1-dependent NF- $\kappa$ B activation. To this end, we expressed API2-MALT1 in RIP1-null Jurkat T cells complemented with either control empty vector, wild-type RIP1, or mutant K377R RIP1 (34) and assessed the ability of API2-MALT1 to activate NF- $\kappa$ B. Whereas API2-MALT1 expression in control RIP1-null cells only modestly activated an NF- $\kappa$ B-dependent reporter, API2-MALT1 expression in wild-type RIP1-complemented cells robustly activated NF- $\kappa$ B (Figure 4F). The non-ubiquitinatable K377R RIP1 mutant, however, was unable to rescue API2-MALT1-induced NF- $\kappa$ B activation. These data suggest that, as in TNF-induced NF-

$\kappa$ B activation, API2-MALT1-induced K377-dependent ubiquitination of RIP1 may serve as a scaffold to link API2-MALT1 to the TAB2/TAK1 and NEMO/IKK complexes and promote activation of canonical NF- $\kappa$ B (28).

### **TRAF2 is required for API2-MALT1-induced RIP1 ubiquitination**

Because all translocation variants of API2-MALT1 lack the RING domain of wild-type cIAP2, which possesses E3 ubiquitin ligase activity (25), we reasoned that API2-MALT1-induced RIP1 ubiquitination was not the result of intrinsic enzymatic activity of API2-MALT1 itself. Since TRAF2 and cIAP1/2 are both necessary for TNF-induced RIP1 ubiquitination (26, 27), we speculated that these proteins might somehow facilitate API2-MALT1-induced RIP1 ubiquitination, and therefore investigated their potential roles in this process. First, we found that treatment with a smac mimetic (SM-164), which causes degradation of cIAP1 (37) without affecting API2-MALT1 levels, did not affect API2-MALT1-induced ubiquitination of either RIP1 or NEMO (Figure 5A). In contrast, siRNA-mediated silencing of TRAF2 abrogated both RIP1 and NEMO ubiquitination and impaired I $\kappa$ B $\alpha$  phosphorylation in response to API2-MALT1 expression (Figure 5B). Furthermore, co-expression of a TRAF2 dominant negative mutant lacking the N-terminal RING domain, which has been shown to impair API2-MALT1-induced NF- $\kappa$ B activation (19, 20), completely blocked API2-MALT1-induced RIP1 and NEMO ubiquitination (Figure 5C) as well as phosphorylation of I $\kappa$ B $\alpha$  (Supplementary Figure S5).

A recent study demonstrated that TNF-induced TRAF2-dependent RIP1 ubiquitination could occur upon binding of sphingosine-1-phosphate (S1P) to the TRAF2 RING domain (27). To further investigate the similarities between API2-MALT1- and TNFR-induced RIP1 ubiquitination, we tested the effects of impairing sphingosine kinase 1 (Sphk1) activity, and therefore production of S1P, on API2-MALT1-dependent NF- $\kappa$ B activation. We found that treatment with SK1-I, a specific Sphk1 inhibitor, impaired API2-MALT1-induced RIP1 and NEMO ubiquitination, as well as I $\kappa$ B $\alpha$  phosphorylation, in a dose-dependent manner (Figure 5D).

To determine if recruitment of TRAF2 to API2-MALT1 was necessary for RIP1 ubiquitination, we expressed a TRAF2 binding-deficient point mutant of API2-MALT1 (E47/R48A; E/R) (Supplementary Figure S1A), which is still able to associate with RIP1 (Figure 1D). We found that, in contrast to wild-type API2-MALT1, expression of this API2-MALT1 mutant was unable to promote either RIP1 or NEMO ubiquitination (Figure 5E). Altogether, these data indicate that TRAF2 is required to facilitate API2-MALT1-dependent RIP1 ubiquitination and subsequent NF- $\kappa$ B activation.

### **TRAF2 binding is required for API2-MALT1-dependent cellular transformation**

To further confirm an important role for TRAF2 in API2-MALT1-dependent oncogenic signaling, we generated NIH-3T3 cell lines stably expressing wild-type API2-MALT1 and a series of N-terminal mutants that disrupt either TRAF2 binding only (E47/R48A and 49) or both TRAF2 binding and oligomerization (98 and 166), two characteristics of API2-MALT1 that are required for full NF- $\kappa$ B activation (18–20). In agreement with previous work (38), we found that wild-type API2-MALT1 imparted anchorage-independent growth

to NIH-3T3 fibroblasts suspended in soft agar (Figure 6A), indicating a transformed phenotype. However, expression of TRAF2 binding-defective API2-MALT1 mutants failed to promote transformation (Figure 6B), which also correlated with a loss of NEMO binding (Figure 6C; top) and canonical NF- $\kappa$ B pathway activity (Figure 6C; bottom). These data indicate that TRAF2 binding, which is required for API2-MALT1 to induce RIP1 ubiquitination, is also required for API2-MALT1 to induce cellular transformation.

### **Both the API2 and the MALT1 moieties are required for API2-MALT1-induced RIP1 ubiquitination**

Thus far, our data suggested that the API2 moiety of API2-MALT1 recruits TRAF2 and RIP1 in BIR1 domain-dependent and independent manners, respectively. We next found, however, that expression of the API2 moiety alone is insufficient to induce either RIP1 or NEMO ubiquitination (Figure 7A) or to activate NF- $\kappa$ B (20). The MALT1 moiety is similarly insufficient on its own. This suggests that fusion of the API2 and MALT1 moieties is essential for providing a gain of function to the oncoprotein *per se*, which culminates in RIP1 ubiquitination and recruitment of the NF- $\kappa$ B-activating kinase complexes. The MALT1 moiety of API2-MALT1 possesses multiple binding sites for the E3 ubiquitin ligase, TRAF6, as well as a proteolytically active caspase-like domain (see Supplementary Figure S1A) (17, 39). We found that expression of a C-terminal deletion mutant of API2-MALT1 (1–762; depicted in Supplementary Figure S1A), which lacks the binding sites for the TRAF6 E3 ubiquitin ligase and the E2 conjugating enzyme, UBC13, and also lacks MALT1 proteolytic activity, failed to induce RIP1 and NEMO ubiquitination and to activate NF- $\kappa$ B (Figure 7B). To specifically determine if the proteolytic activity of the API2-MALT1 caspase-like domain is necessary for RIP1 ubiquitination, we compared wild-type API2-MALT1 to the C678A point mutant, which lacks proteolytic activity but retains TRAF6 binding, and found that loss of MALT1 protease activity does not impair API2-MALT1-induced RIP1 or NEMO ubiquitination, nor does it impair canonical NF- $\kappa$ B activation (Figure 7C). We then sought to determine if MALT1 moiety-dependent recruitment of TRAF6 is required for API2-MALT1-induced RIP1 ubiquitination by comparing wild-type API2-MALT1 with a point mutant of API2-MALT1 that cannot interact with TRAF6 (M7M-E2A), but retains TRAF2 binding (39). Interestingly, expression of the TRAF6 binding-deficient API2-MALT1 mutant still promoted RIP1 ubiquitination, albeit to a lesser degree, but NEMO ubiquitination and subsequent NF- $\kappa$ B activation were completely lost (Figure 7D). We were unable to specifically investigate the impact of TRAF6 deficiency on API2-MALT1-dependent RIP1 ubiquitination because siRNA-mediated knockdown of TRAF6 resulted in dramatically decreased levels of API2-MALT1 (data not shown). Our analysis thus far indicates that two of the known functions attributed to the MALT1 moiety of API2-MALT1, MALT1 proteolytic activity and TRAF6 binding, are not required for API2-MALT1 to induce RIP1 ubiquitination, although TRAF6 binding is required for API2-MALT1 to induce NEMO ubiquitination. Further investigations will be required to not only determine how the MALT1 moiety contributes to API2-MALT1-induced RIP1 ubiquitination but also the nature of the critical interplay between the API2 and MALT1 moieties to promote RIP1 ubiquitination and NF- $\kappa$ B activation.

## DISCUSSION

We previously demonstrated that TRAF2 binding to API2-MALT1 is required for API2-MALT1-dependent NF- $\kappa$ B activation (19), but the mechanism by which TRAF2 contributes to API2-MALT1-induced signaling has remained unclear. In the present study, we investigated the contribution of proteins that are functionally associated with TRAF2 and TNF-induced NF- $\kappa$ B activation to API2-MALT1-dependent signaling. We identified a novel interaction between RIP1 and API2-MALT1, which requires the API2 moiety of API2-MALT1 and the intermediate domain of RIP1. Through a series of elegant structure/function studies, the intermediate domain of RIP1 was recently shown to be essential for the anti-apoptotic function of RIP1 (40). The intermediate domain contains K377, a crucial ubiquitin acceptor site on RIP1. Perhaps the best-understood function of RIP1 relates to its ability to direct the outcome of TNF-induced signaling by differentially recruiting specific proteins to the TNFR complex. This function is dictated by the absence or presence of RIP1 poly-ubiquitination at K377. When RIP1 is ubiquitinated, both TAB2 and NEMO bind to the poly-ubiquitin chains, and these interactions are essential for TNF-induced IKK complex activation, NF- $\kappa$ B stimulation and cell survival (28, 34, 41, 42). In contrast, when de-ubiquitinated at K377, RIP1 interacts with caspase 8 and Fas-associated via death domain (FADD) and promotes apoptosis (28, 29, 34).

The importance of RIP1 ubiquitination in promoting cell survival is highlighted by the increasing number of cancer types that are reported to exhibit constitutive RIP1 ubiquitination, including breast and ovarian carcinoma (25) and glioblastoma (43), as well as other tumor types that acquire chemoresistance as a result of chemotherapy-induced RIP1 ubiquitination (44). Our current findings are the first to demonstrate that expression of a fusion oncoprotein can induce the ubiquitination of RIP1 and to reveal a novel role for deregulated RIP1 ubiquitination in B cell malignancy. We show that API2-MALT1-induced RIP1 ubiquitination occurs on K377 and that this event is required for NF- $\kappa$ B activation. These data suggest that API2-MALT1, a MALT lymphoma-specific oncoprotein, perturbs TNFR-associated signaling proteins to promote sustained survival signals while counteracting cell death signals.

We present three lines of evidence that support a role for TRAF2 in API2-MALT1-induced RIP1 ubiquitination. First, we demonstrate that RNAi-mediated knockdown of TRAF2 abrogates both RIP1 and NEMO ubiquitination as well as activation of canonical NF- $\kappa$ B. Second, we demonstrate that either deletion of the N-terminal RING domain of TRAF2 or impairing production of S1P, which binds the TRAF2 RING domain, suppresses API2-MALT1-induced RIP1 and NEMO ubiquitination and phosphorylation of I $\kappa$ B $\alpha$ . Third, we show that a TRAF2 binding-deficient point mutant of API2-MALT1, which still binds RIP1, fails to induce RIP1 and NEMO ubiquitination and activate NF- $\kappa$ B. Although recent studies suggest that structural features of TRAF2 may prevent it from interacting with E2 ubiquitin-conjugating enzymes such as Ubc13 (45), our data, taken together with recent work of other laboratories, point toward a possible role for TRAF2 as an E3 ubiquitin ligase. For example, Alvarez *et al.* demonstrate that, in the presence of sphingosine-1-phosphate, TRAF2 is able to use the E2s Ubc13 and UbcH5 to facilitate RIP1 ubiquitination *in vitro* (27). Intriguingly, Zhou *et al.* have reported that Ubc13 is required for API2-MALT1 to stimulate NF- $\kappa$ B (18).



In addition, it was demonstrated that purified recombinant TRAF2 possesses E3 ubiquitin ligase activity *in vitro* in the presence of Ubc13 (46). A new report demonstrated that TRAF2 also functions downstream of the epidermal growth factor receptor and promotes ubiquitination of ribosomal S6 kinase 2 (RSK2), which is critical for cellular transformation and colorectal tumor growth *in vivo* (47). In line with this observation that demonstrates a pro-tumorigenic role for TRAF2, we find that mutants of API2-MALT1 that fail to recruit TRAF2 and cannot activate NF- $\kappa$ B are incapable of transforming NIH-3T3 cells.

Although the API2 moiety of API2-MALT1 facilitates the recruitment of RIP1 and TRAF2, expression of the API2 portion alone is insufficient to promote either RIP1 or NEMO ubiquitination or to activate NF- $\kappa$ B. This suggests that fusion of the MALT1 moiety to the API2 domains imparts a gain of function to the API2-MALT1 oncoprotein. We found that a C-terminal deletion mutant of API2-MALT1 (1–762), which lacks a significant portion of the MALT1 moiety, failed to promote ubiquitination of both RIP1 and NEMO. This deletion has several structural consequences in that it removes the binding sites for both the TRAF6 E3 ubiquitin ligase and the E2 ubiquitin-conjugating enzyme Ubc13, and it inactivates the MALT1 protease domain (18, 39). By analyzing a catalytically-dead API2-MALT1 point mutant (C678A), we determined that loss of MALT1 protease activity does not account for the inability of the 1–762 C-terminal mutant to induce RIP1 ubiquitination. To the contrary, MALT1 protease activity is dispensable for API2-MALT1-dependent RIP1 ubiquitination. This is despite the fact that two substrates of the MALT1 protease domain, A20 and CYLD, are known to possess deubiquitinase activity and can remove K63-linked ubiquitin chains from RIP1 (48–50), observations that had led us to speculate that MALT1 protease-dependent cleavage of A20 and/or CYLD might inhibit the activity of these enzymes and thereby protect or promote RIP1 ubiquitination.

To determine what other attributes of the MALT1 moiety might be contributing to API2-MALT1-dependent RIP1 ubiquitination, we analyzed an API2-MALT1 point mutant that fails to bind TRAF6. Interestingly, RIP1 ubiquitination remained intact, but NEMO ubiquitination and NF- $\kappa$ B activation were totally impaired. This suggested that API2-MALT1 recruitment of TRAF6 is required for canonical NF- $\kappa$ B activation, similar to the antigen receptor-stimulated CARMA1/Bcl10/MALT1-dependent NF- $\kappa$ B activation pathway, wherein oligomerized MALT1 recruits TRAF6 to promote TRAF6-dependent ubiquitination of NEMO and IKK complex activation (15, 16). Since we found that neither MALT1 proteolytic activity nor MALT1-dependent TRAF6 association are required for API2-MALT1 to induce RIP1 ubiquitination, the precise mechanism by which the MALT1 moiety contributes to API2-MALT1-dependent RIP1 ubiquitination remains unknown. MALT1 moiety-dependent interaction with Ubc13 could be critical to this process since this is a third known function of the MALT1 domain that is abolished by the 1–762 C-terminal deletion, and this possibility has not yet been evaluated.

Based on our data, we propose the following model: 1) API2-MALT1 recruits both TRAF2 and RIP1. 2) Via an incompletely understood mechanism that depends on the TRAF2 RING domain, API2-MALT1 promotes RIP1 ubiquitination at K377. 3) Poly-ubiquitinated RIP1 may then act as a scaffold to bind TAB2/TAK1/NEMO. 4) This allows MALT1 moiety-bound TRAF6 to ubiquitinate NEMO and 5) promote IKK complex activation, 6) stimulate

canonical NF- $\kappa$ B, and 7) ultimately promote cell survival (Figure 7E). Thus, our data posit that the API2-MALT1 fusion oncoprotein can harness components of both TNFR-dependent and antigen receptor-dependent NF- $\kappa$ B signaling to promote cell survival and transformation.

API2-MALT1-positive MALT lymphomas tend to be more aggressive than t(11;18)-negative tumors and elucidating the molecular mechanisms by which API2-MALT1 promotes cell survival may identify putative targets for therapeutic intervention. Our current study suggests that TRAF2 binding is key to API2-MALT1-induced NF- $\kappa$ B activation by facilitating RIP1 ubiquitination, allowing recruitment and activation of NF- $\kappa$ B-activating kinase complexes. Possible interventions that disrupt either API2 moiety-mediated TRAF2 or RIP1 interaction could inhibit canonical NF- $\kappa$ B activation. While MALT1 moiety-associated protease activity is dispensable for stimulating canonical NF- $\kappa$ B, we have previously shown that MALT1 protease activity is critical for API2-MALT1-induced NIK cleavage and activation of non-canonical NF- $\kappa$ B, which represents another pathway through which API2-MALT1 can influence cell survival signaling (17). Developing compounds that disrupt the essential protein interactions and/or enzymatic activities associated with API2-MALT1 may provide a means to treat t(11;18)<sup>+</sup> MALT lymphoma. Furthermore, evaluation of such compounds could inform new treatment options for other tumor types that harbor analogous deregulated signaling pathways, such as uncontrolled ubiquitination of RIP1.

## MATERIALS AND METHODS

### Plasmids and reagents

Plasmids for FLAG-, HA-, myc-API2-MALT1 and dominant negative TRAF2 were described previously (17, 19). RIP1 constructs, including mutants K377R, intermediate domain (ID), death domain-deleted (DD), and DD-only, were previously described (34, 51). HA-ubiquitin and mutants K48R and K63R were previously described (52). Wild-type and mutant (49, 98, 166, E47/R48A) API2-MALT1 cDNAs described previously (19) were inserted into the bicistronic RET10 retroviral backbone. Resultant vectors were transfected into Phoenix-E packaging cells to generate virus capable of infecting NIH-3T3 cells.

The NF- $\kappa$ B luciferase reporter plasmid pGL2-3 $\times$ - $\kappa$ B-luc was co-transfected with the control renilla plasmid phRL-TK (Promega, Madison, WI, USA) and luciferase activity was measured as previously described (19).

### Ubiquitination analyses

RIP1 and NEMO ubiquitination were detected as described previously (53) with minor alterations. HEK-293T cells were transfected with the appropriate plasmids to assess RIP1 and NEMO ubiquitination by detecting incorporation of either endogenous ubiquitin or co-transfected HA-tagged ubiquitin where indicated. SSK41 and BJAB B cell lines were cultured appropriately and harvested upon reaching  $\sim 2 \times 10^6$  cells/mL to assess incorporation of endogenous ubiquitin into RIP1 and NEMO. Lysates were prepared with Bit/Ash buffer (20 mM Tris HCl pH7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2

mM EDTA, including freshly added protease inhibitor cocktail (Pierce) and *N*-ethylmaleimide [NEM; Sigma-Aldrich] at 10 mg/mL). Cleared lysates (500 µg for HEK-293T cells transfected with HA-Ub or 5 mg for detecting endogenous ubiquitin in HEK-293T cells and SSK41 and BJAB B cells) were mixed with SDS (1% final) and heated for 10 min at 95°C. Denatured lysates were diluted ten-fold with Bit/Ash buffer and incubated with mixing overnight at 4°C with 2 µg of either anti-RIP1 (sc-7881) or anti-NEMO (sc-8330). The following day, 20 µL of Protein A/G Agarose (Thermo Scientific) was added and samples were processed for SDS-PAGE. Western blot analysis used either anti-HA to detect incorporation of transfected HA-ubiquitin or anti-ubiquitin to detect endogenous protein. Efficient capture of RIP1 and NEMO was verified by Western blot with mouse anti-RIP1 and anti-NEMO monoclonal antibodies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

We would like to thank Gabriel Nuñez for contributing plasmids, Shaomeng Wang for providing smac mimetic compounds, and Linda Klei for laboratory management. This work was supported by the Shirley K. Schlafer Foundation, the Elizabeth Caroline Crosby Fund, and grants from the University of Michigan Comprehensive Cancer Center (G007839) and National Cancer Institute, NIH (R01CA124540). S.R. was supported by National Heart, Lung, and Blood Institute, NIH (T32-HL007622-21A2), the Nancy Newton Loeb Pediatric Cancer Research Award, and the AACI Fellowship for Translational Cancer Research.

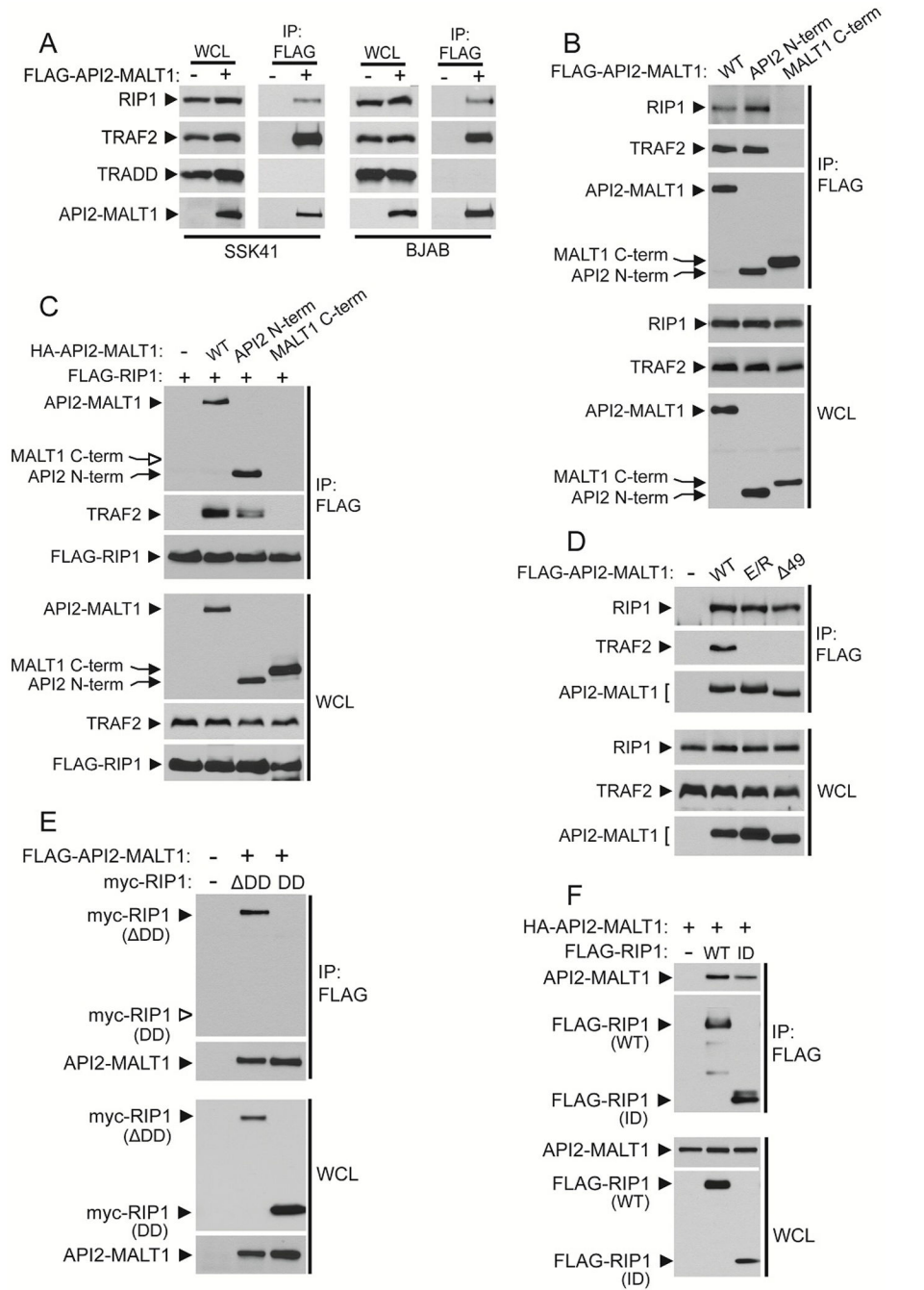
## REFERENCES

1. Isaacson PG, Du MQ. MALT lymphoma: from morphology to molecules. *Nat Rev Cancer*. 2004 Aug; 4(8):644–653. [PubMed: 15286744]
2. Dierlamm J, Baens M, Wlodarska I, Stefanova-Ouzounova M, Hernandez JM, Hossfeld DK, et al. The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. *Blood*. 1999 Jun 1; 93(11):3601–3609. [PubMed: 10339464]
3. Akagi T, Motegi M, Tamura A, Suzuki R, Hosokawa Y, Suzuki H, et al. A novel gene, MALT1 at 18q21, is involved in t(11;18)(q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. *Oncogene*. 1999 Oct 14; 18(42):5785–5794. [PubMed: 10523859]
4. Morgan JA, Yin Y, Borowsky AD, Kuo F, Nourmand N, Koontz JI, et al. Breakpoints of the t(11;18)(q21;q21) in mucosa-associated lymphoid tissue (MALT) lymphoma lie within or near the previously undescribed gene MALT1 in chromosome 18. *Cancer Res*. 1999 Dec 15; 59(24):6205–6213. [PubMed: 10626814]
5. Liu H, Ye H, Ruskone-Fourmesttraux A, De Jong D, Pileri S, Thiede C, et al. T(11;18) is a marker for all stage gastric MALT lymphomas that will not respond to *H. pylori* eradication. *Gastroenterology*. 2002 May; 122(5):1286–1294. [PubMed: 11984515]
6. Liu H, Ye H, Dogan A, Ranaldi R, Hamoudi RA, Bearzi I, et al. T(11;18)(q21;q21) is associated with advanced mucosa-associated lymphoid tissue lymphoma that expresses nuclear BCL10. *Blood*. 2001 Aug 15; 98(4):1182–1187. [PubMed: 11493468]
7. Willis TG, Jadayel DM, Du MQ, Peng H, Perry AR, Abdul-Rauf M, et al. Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. *Cell*. 1999 Jan 8; 96(1):35–45. [PubMed: 9989495]
8. Zhang Q, Siebert R, Yan M, Hinzmann B, Cui X, Xue L, et al. Inactivating mutations and overexpression of BCL10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32). *Nat Genet*. 1999 May; 22(1):63–68. [PubMed: 10319863]

9. Streubel B, Lamprecht A, Dierlamm J, Cerroni L, Stolte M, Ott G, et al. T(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma. *Blood*. 2003 Mar 15; 101(6):2335–2339. [PubMed: 12406890]
10. Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. *Immunol Rev*. 2006 Apr; 210:171–186. [PubMed: 16623771]
11. Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, et al. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol Cell*. 2000 Oct; 6(4):961–967. [PubMed: 11090634]
12. Lucas PC, Yonezumi M, Inohara N, McAllister-Lucas LM, Abazeed ME, Chen FF, et al. Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway. *J Biol Chem*. 2001 Jun 1; 276(22):19012–19019. [PubMed: 11262391]
13. Thome M, Charton JE, Pelzer C, Hailfinger S. Antigen receptor signaling to NF-kappaB via CARMA1, BCL10, and MALT1. *Cold Spring Harb Perspect Biol*. 2010 Sep.2(9):a003004. [PubMed: 20685844]
14. Zhou H, Wertz I, O'Rourke K, Ultsch M, Seshagiri S, Eby M, et al. Bcl10 activates the NF-kappaB pathway through ubiquitination of NEMO. *Nature*. 2004 Jan 8; 427(6970):167–171. [PubMed: 14695475]
15. Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ. The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Mol Cell*. 2004 May 7; 14(3):289–301. [PubMed: 15125833]
16. Oeckinghaus A, Wegener E, Welteke V, Ferch U, Arslan SC, Ruland J, et al. Malt1 ubiquitination triggers NF-kappaB signaling upon T-cell activation. *EMBO J*. 2007 Nov 14; 26(22):4634–4645. [PubMed: 17948050]
17. Rosebeck S, Madden L, Jin X, Gu S, Apel IJ, Appert A, et al. Cleavage of NIK by the API2-MALT1 fusion oncoprotein leads to noncanonical NF-kappaB activation. *Science*. 2011 Jan 28; 331(6016):468–472. [PubMed: 21273489]
18. Zhou H, Du MQ, Dixit VM. Constitutive NF-kappaB activation by the t(11;18)(q21;q21) product in MALT lymphoma is linked to deregulated ubiquitin ligase activity. *Cancer Cell*. 2005 May; 7(5):425–431. [PubMed: 15894263]
19. Lucas PC, Kuffa P, Gu S, Kohrt D, Kim DS, Siu K, et al. A dual role for the API2 moiety in API2-MALT1-dependent NF-kappaB activation: heterotypic oligomerization and TRAF2 recruitment. *Oncogene*. 2007 Aug 16; 26(38):5643–5654. [PubMed: 17334391]
20. Garrison JB, Samuel T, Reed JC. TRAF2-binding BIR1 domain of c-IAP2/MALT1 fusion protein is essential for activation of NF-kappaB. *Oncogene*. 2009 Apr 2; 28(13):1584–1593. [PubMed: 19234489]
21. Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell*. 1994 Aug 26; 78(4):681–692. [PubMed: 8069916]
22. Hsu H, Shu HB, Pan MG, Goeddel DV. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*. 1996 Jan 26; 84(2):299–308. [PubMed: 8565075]
23. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity*. 1996 Apr; 4(4):387–396. [PubMed: 8612133]
24. Vince JE, Pantaki D, Feltham R, Mace PD, Cordier SM, Schmukle AC, et al. TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf- $\kappa$ b and to prevent tnf-induced apoptosis. *J Biol Chem*. 2009 Dec 18; 284(51):35906–35915. [PubMed: 19815541]
25. Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, et al. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol Cell*. 2008 Jun 20; 30(6):689–700. [PubMed: 18570872]

26. Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K, et al. c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. *J Biol Chem.* 2008 Sep 5; 283(36):24295–24299. [PubMed: 18621737]
27. Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, et al. Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature.* 2010 Jun 24; 465(7301):1084–1088. [PubMed: 20577214]
28. Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol Cell.* 2006 Apr 21; 22(2):245–257. [PubMed: 16603398]
29. Li H, Kobayashi M, Blonska M, You Y, Lin X. Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. *J Biol Chem.* 2006 May 12; 281(19):13636–13643. [PubMed: 16543241]
30. Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. *Nat Cell Biol.* 2006 Apr; 8(4):398–406. [PubMed: 16547522]
31. Conze DB, Zhao Y, Ashwell JD. Non-canonical NF-kappaB activation and abnormal B cell accumulation in mice expressing ubiquitin protein ligase-inactive c-IAP2. *PLoS Biol.* 2010; 8(10):e1000518. [PubMed: 21048983]
32. Lee TH, Shank J, Cusson N, Kelliher MA. The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. *J Biol Chem.* 2004 Aug 6; 279(32):33185–33191. [PubMed: 15175328]
33. Degterev A, Hitomi J, Gemscheid M, Ch'en IL, Korkina O, Teng X, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol.* 2008 May; 4(5):313–321. [PubMed: 18408713]
34. O'Donnell MA, Legarda-Addison D, Skountzos P, Yeh WC, Ting AT. Ubiquitination of RIP1 regulates an NF-kappaB-independent cell-death switch in TNF signaling. *Curr Biol.* 2007 Mar 6; 17(5):418–424. [PubMed: 17306544]
35. Baens M, Fevery S, Sagaert X, Noels H, Hagens S, Broeckx V, et al. Selective expansion of marginal zone B cells in Emicro-API2-MALT1 mice is linked to enhanced IkappaB kinase gamma polyubiquitination. *Cancer Res.* 2006 May 15; 66(10):5270–5277. [PubMed: 16707452]
36. Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, et al. Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature.* 2011 Mar 31; 471(7340):591–596. [PubMed: 21455173]
37. Lu J, Bai L, Sun H, Nikolovska-Coleska Z, McEachern D, Qiu S, et al. SM-164: a novel, bivalent Smac mimetic that induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP. *Cancer Res.* 2008 Nov 15; 68(22):9384–9393. [PubMed: 19010913]
38. Stoffel A, Chaurushiya M, Singh B, Levine AJ. Activation of NF-kappaB and inhibition of p53-mediated apoptosis by API2/mucosa-associated lymphoid tissue 1 fusions promote oncogenesis. *Proc Natl Acad Sci U S A.* 2004 Jun 15; 101(24):9079–9084. [PubMed: 15184680]
39. Noels H, van Loo G, Hagens S, Broeckx V, Beyaert R, Marynen P, et al. A Novel TRAF6 binding site in MALT1 defines distinct mechanisms of NF-kappaB activation by API2middle dotMALT1 fusions. *J Biol Chem.* 2007 Apr 6; 282(14):10180–10189. [PubMed: 17287209]
40. Duprez L, Bertrand MJ, Vanden Berghe T, Dondelinger Y, Festjens N, Vandenabeele P. Intermediate domain of receptor-interacting protein kinase 1 (RIPK1) determines switch between necroptosis and RIPK1 kinase-dependent apoptosis. *J Biol Chem.* 2012 Apr 27; 287(18):14863–14872. [PubMed: 22362767]
41. Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB. TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. *J Mol Biol.* 2003 Feb 7; 326(1):105–115. [PubMed: 12547194]
42. Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, et al. TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Mol Cell.* 2004 Aug 27; 15(4):535–548. [PubMed: 15327770]

43. Bellail AC, Olson JJ, Yang X, Chen ZJ, Hao C. A20 Ubiquitin Ligase-Mediated Polyubiquitination of RIP1 Inhibits Caspase-8 Cleavage and TRAIL-Induced Apoptosis in Glioblastoma. *Cancer Discov.* 2012 Feb; 2(2):140–155. [PubMed: 22585859]
44. Yang Y, Xia F, Hermance N, Mabb A, Simonson S, Morrissey S, et al. A cytosolic ATM/NEMO/RIP1 complex recruits TAK1 to mediate the NF-kappaB and p38 mitogen-activated protein kinase (MAPK)/MAPK-activated protein 2 responses to DNA damage. *Mol Cell Biol.* 2011 Jul; 31(14): 2774–2786. [PubMed: 21606198]
45. Yin Q, Lamothe B, Darnay BG, Wu H. Structural basis for the lack of E2 interaction in the RING domain of TRAF2. *Biochemistry.* 2009 Nov 10; 48(44):10558–10567. [PubMed: 19810754]
46. Cardamone MD, Kronen A, Tanasa B, Taylor H, Ricci L, Ohgi KA, et al. A protective strategy against hyperinflammatory responses requiring the nontranscriptional actions of GPS2. *Mol Cell.* 2012 Apr 13; 46(1):91–104. [PubMed: 22424771]
47. Peng C, Zhu F, Wen W, Yao K, Li S, Zykova T, et al. TRAF2 is a key mediator of the EGF-induced RSK2/CREB/Fos signaling pathway. *J Biol Chem.* 2012 Jun 8.
48. Shembade N, Ma A, Harhaj EW. Inhibition of NF-kappaB signaling by A20 through disruption of ubiquitin enzyme complexes. *Science.* 2010 Feb 26; 327(5969):1135–1139. [PubMed: 20185725]
49. Coornaert B, Baens M, Heyninck K, Bekaert T, Haegman M, Staal J, et al. T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. *Nat Immunol.* 2008 Mar; 9(3):263–271. [PubMed: 18223652]
50. Staal J, Driege Y, Bekaert T, Demeyer A, Muylaert D, Van Damme P, et al. T-cell receptor-induced JNK activation requires proteolytic inactivation of CYLD by MALT1. *EMBO J.* 2011 May 4; 30(9):1742–1752. [PubMed: 21448133]
51. Ting AT, Pimentel-Muinos FX, Seed B. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J.* 1996 Nov 15; 15(22):6189–6196. [PubMed: 8947041]
52. Hasegawa M, Fujimoto Y, Lucas PC, Nakano H, Fukase K, Nunez G, et al. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. *EMBO J.* 2008 Jan 23; 27(2):373–383. [PubMed: 18079694]
53. Biton S, Ashkenazi A. NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling. *Cell.* 2011 Apr 1; 145(1):92–103. [PubMed: 21458669]



**Figure 1. RIP1 is a novel API2-MALT1-associated protein**

(A) Lysates from SSK41 (*left*) and BJAB (*right*) B cell lines were subjected to anti-FLAG IP and analyzed for the presence of endogenous RIP1, TRAF2, and TRADD by western blot. (B) FLAG-API2-MALT1, API2 moiety alone, or MALT1 moiety alone were expressed in HEK-293T cells and tested for their ability to co-IP endogenous RIP1 and TRAF2. (C) HEK-293T were co-transfected with plasmids encoding FLAG-RIP1 and either HA-tagged API2-MALT1, the API2 moiety, or the MALT1 moiety and lysates were subjected to anti-FLAG IP and western blot with appropriate antibodies. (D) FLAG-tagged WT or mutants of

API2-MALT1 incapable of binding TRAF2 were expressed in HEK-293T cells and assessed for their ability to co-IP endogenous RIP1 and TRAF2. (E) Plasmids encoding FLAG-API2-MALT1 and death domain-deleted myc-RIP1 ( DD) or only the death domain (DD) of RIP1 were co-transfected into HEK-293T cells and lysates were subjected to anti-FLAG IP and western blot. (F) HA-API2-MALT1 co-expressed with either FLAG-RIP1 or only the intermediate domain (ID) of RIP1 in HEK-293T cells were tested for their ability to co-IP by anti-FLAG IP. WCL – whole cell lysate. Open arrowheads indicate the position of proteins that fail to co-IP.

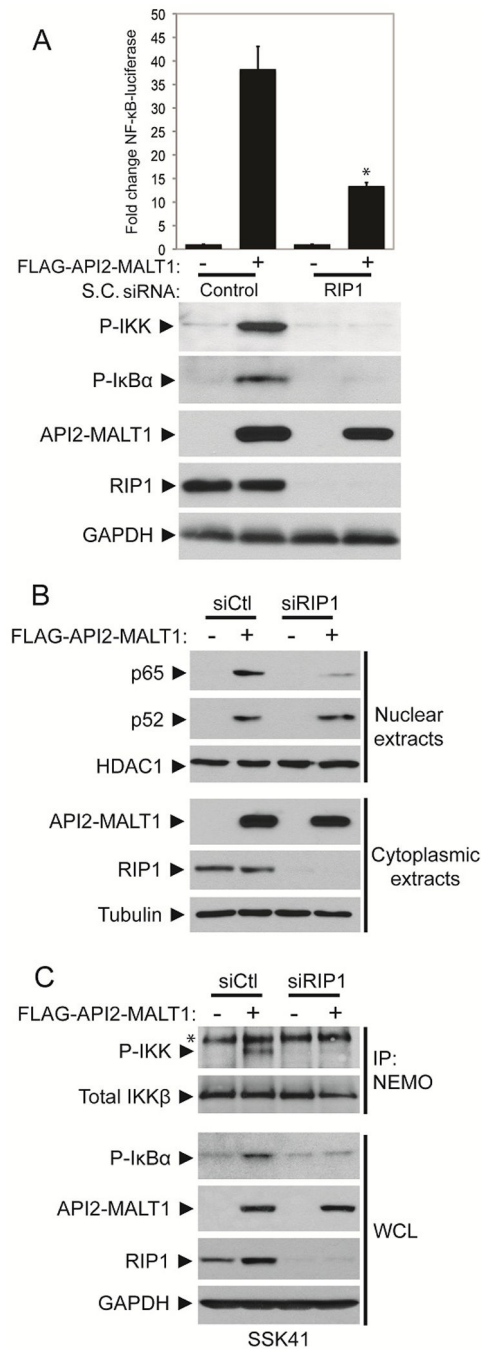
Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript





**Figure 2. RIP1 is required for API2-MALT1-dependent activation of canonical NF-κB**  
 (A) HEK-293T were first transfected with either control or RIP1-specific siRNA on two consecutive days. Then, on the third day, either empty vector or FLAG-API2-MALT1 was co-transfected with both NF-κB-dependent luciferase and control renilla reporter plasmids. Lysates were processed 24 h later to measure the amount of luciferase activity (*top*) and analyzed for the indicated proteins (*bottom*). The graph depicts the average  $\pm$  SEM of triplicate determinations and is representative of three separate experiments. (\* $p = 0.01232$ ; S.C. = Santa Cruz) (B) HEK-293T were transfected with siRNA and either empty vector or

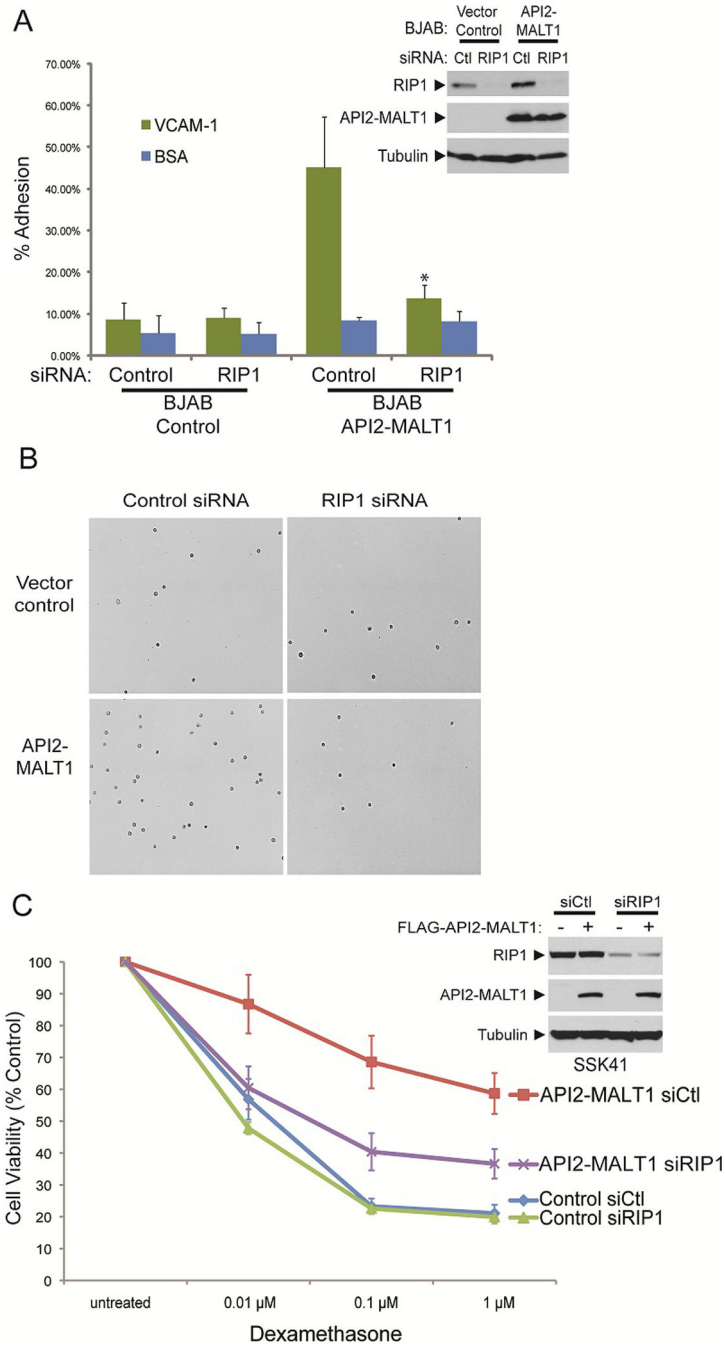
FLAG-API2-MALT1 plasmids as in (A). Nuclear and cytoplasmic extracts were generated and nuclear translocation of both p65 and p52 was determined by western blot. Probing for HDAC1 serves as a loading control. (C) SSK41 B cell lines were electroporated with either control or RIP1-specific siRNA. After 48 h, lysates were prepared and either subjected to IP with anti-NEMO antibody or analyzed directly by western blot for the indicated proteins. (\*) represents a nonspecific band.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 3. RIP1 is necessary for API2-MALT1-dependent changes in B cell phenotype**  
 (A) BJAB B cell lines were electroporated with either control or RIP1-specific siRNA. After 48 h, cells were treated with doxycycline and adhesion to VCAM-1 coated plates was assessed. The graph depicts the average ± SEM of triplicate determinations and is representative of three separate experiments. (\**p* = 0.01305) (*inset*) Western blot analysis of a representative experiment showing effective RIP1 knockdown. (B) Representative brightfield images used for quantification in (A). (C) SSK41 B cell lines were electroporated as stated above, subjected to increasing doses of dexamethasone, and the percent cell

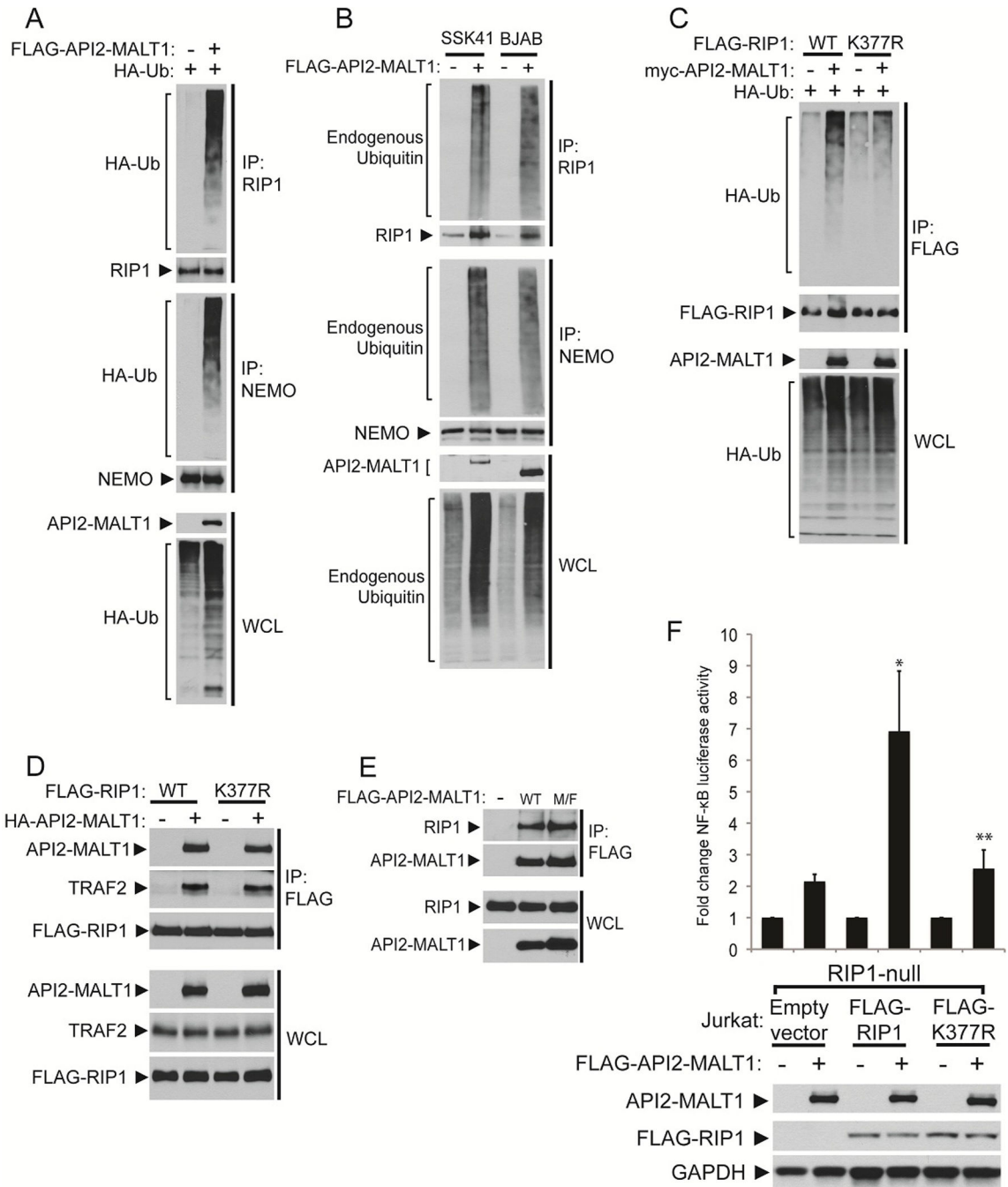
viability was compared. Data are expressed as the average  $\pm$  SEM of four separate experiments. (*inset*) Western blot analysis of a representative experiment shows effective RIP1 knockdown.

Author Manuscript

Author Manuscript

Author Manuscript

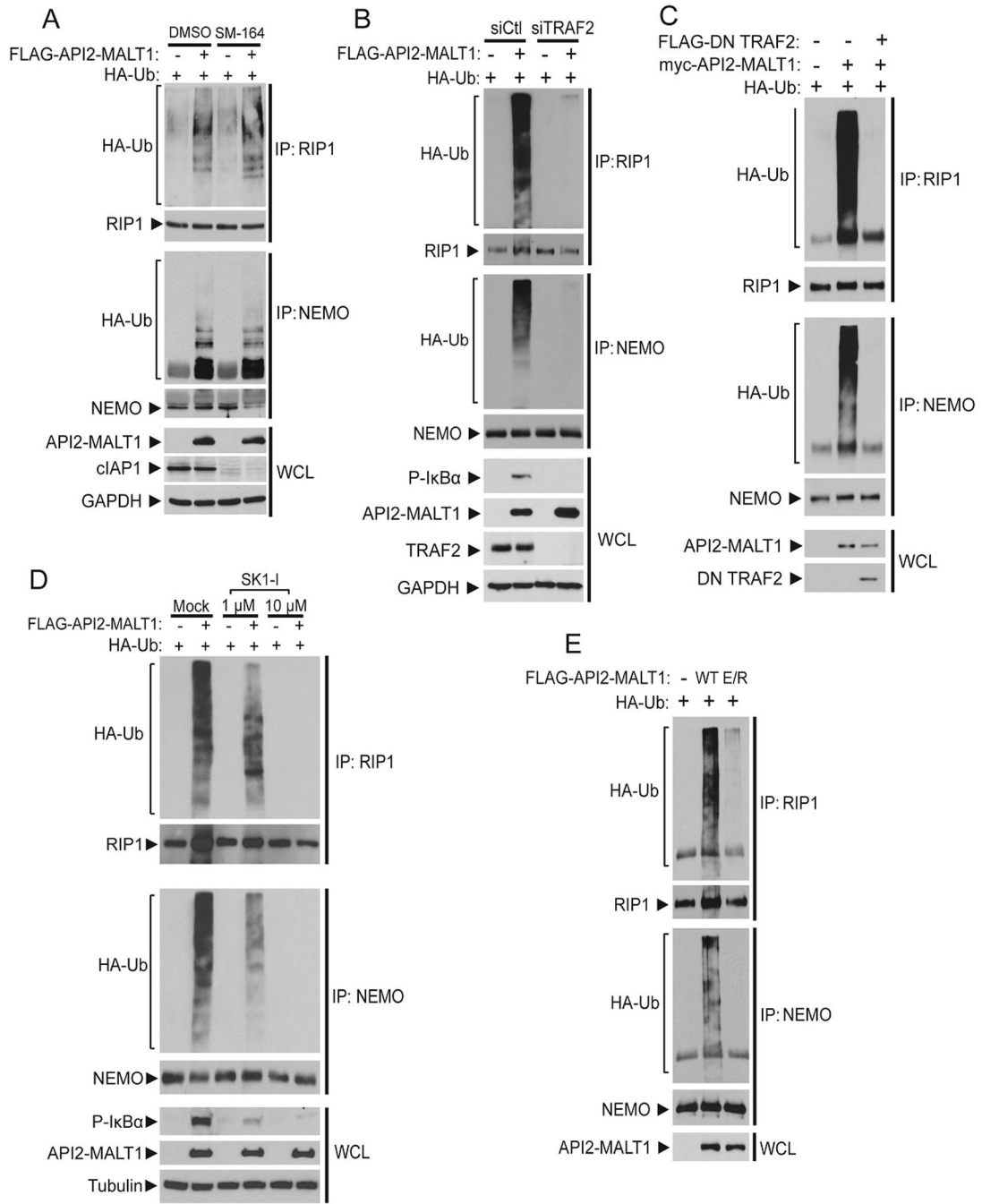
Author Manuscript



**Figure 4. API2-MALT1 expression is associated with RIP1 ubiquitination**

(A) HEK-293T cells were co-transfected with FLAG-API2-MALT1 and HA-ubiquitin (HA-Ub). Denatured lysates were subjected to IP with either anti-RIP1 or anti-NEMO and probed with anti-HA to detect the incorporation of HA-Ub. (B) Denatured lysates from either SSK41 or BJAB B cell lines were processed for IP as above and probed with anti-ubiquitin to detect incorporation of endogenous ubiquitin. The SSK41 cells express the "A7M3" variant of API2-MALT1 that contains two Ig-like domain near the N-terminus of the MALT1 moiety, whereas the BJAB cells express the "A7M5" variant that contains only one

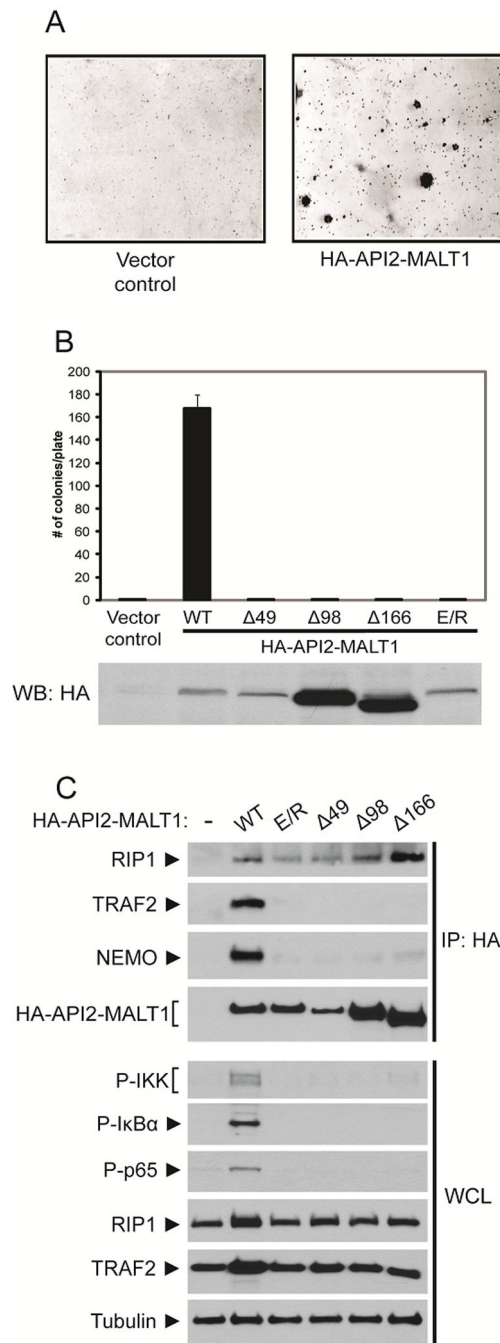
Ig-like domain, which accounts for the differences in apparent molecular weight of the API2-MALT1 proteins expressed in these cell lines (see Supplementary Figure S1A). (C) HEK-293T cells were co-transfected with plasmids encoding HA-Ub, myc-API2-MALT1, and either FLAG-tagged WT or K377R mutant RIP1. Denatured lysates were analyzed to detect ubiquitination of transfected RIP1. (D) HA-API2-MALT1 was co-transfected with either FLAG-tagged WT or K377R mutant RIP1, and the ability of the indicated proteins to co-IP was compared. (E) Either FLAG-tagged WT or UBA domain mutant API2-MALT1 (M/F) were transiently expressed in HEK-293T cells. Lysates were subjected to anti-FLAG IP to compare RIP1 association. (F) RIP1-null Jurkat T cells stably reconstituted with either empty vector, FLAG-RIP1, or FLAG-K377R RIP1 were co-transfected with HA-API2-MALT1 and both NF- $\kappa$ B-dependent luciferase and control renilla reporter plasmids. Lysates were processed 24 h later to measure luciferase activity (*top*) and subjected to western blotting for the indicated proteins (*bottom*). The graph depicts the average  $\pm$  SEM of triplicate determinations and is representative of three separate experiments. (\* $p = 0.00721$ ; \*\* $p = 0.01383$ ) Western blot analysis of a representative experiment shows expression of transfected API2-MALT1 and reconstituted FLAG-RIP1 proteins.



**Figure 5. TRAF2 is required for API2-MALT1-induced RIP1 ubiquitination**  
 (A) HEK-293T cells were pre-treated with either DMSO or smac mimetic (SM-164) for 16 h prior to co-transfection with either empty vector or FLAG-API2-MALT1 and HA-Ub plasmids. Denatured lysates were subjected to IP and western blot as stated previously. Probing WCL for cIAP1 confirms smac mimetic treatment was effective. (B) HEK-293T cells were transfected with either control or TRAF2-specific siRNA on two consecutive days, followed by co-transfection with HA-Ub and either empty vector or FLAG-API2-MALT1 plasmids. RIP1 and NEMO ubiquitination was assessed, and NF-κB activation was

determined by probing for phospho-I $\kappa$ B $\alpha$ . (C) myc-API2-MALT1 was expressed alone or with dominant negative (DN)-TRAF2 and RIP1 and NEMO ubiquitination was assessed. (D) HEK-293T cells transiently transfected with either empty vector or FLAG-API2-MALT1 and HA-Ub were treated after 3 h of transfection with either vehicle alone or the indicated doses of SK1-I for 24 h total. Lysates were analyzed for RIP1 and NEMO ubiquitination and activation of NF- $\kappa$ B. (E) HEK-293T cells were co-transfected with either FLAG-API2-MALT1 or a point mutant that fails to bind TRAF2 (E/R) and HA-Ub to compare RIP1 and NEMO ubiquitination.





**Figure 6. TRAF2 binding is required for API2-MALT1-induced NF- $\kappa$ B activation and cellular transformation**

(A) Representative brightfield images of NIH-3T3 cells with either empty vector control or stable API2-MALT1 expression after plating in soft agar. (B) Quantification of the number of colonies formed per plate of the indicated NIH-3T3 stable cell lines subjected to growth in soft agar. Bar graphs represent the average  $\pm$  SEM of four separate experiments. Western blotting confirms expression of the indicated proteins. (C) (*top*) Lysates from the NIH-3T3 stable cell lines were subjected to IP with anti-HA antibody to compare the ability of either

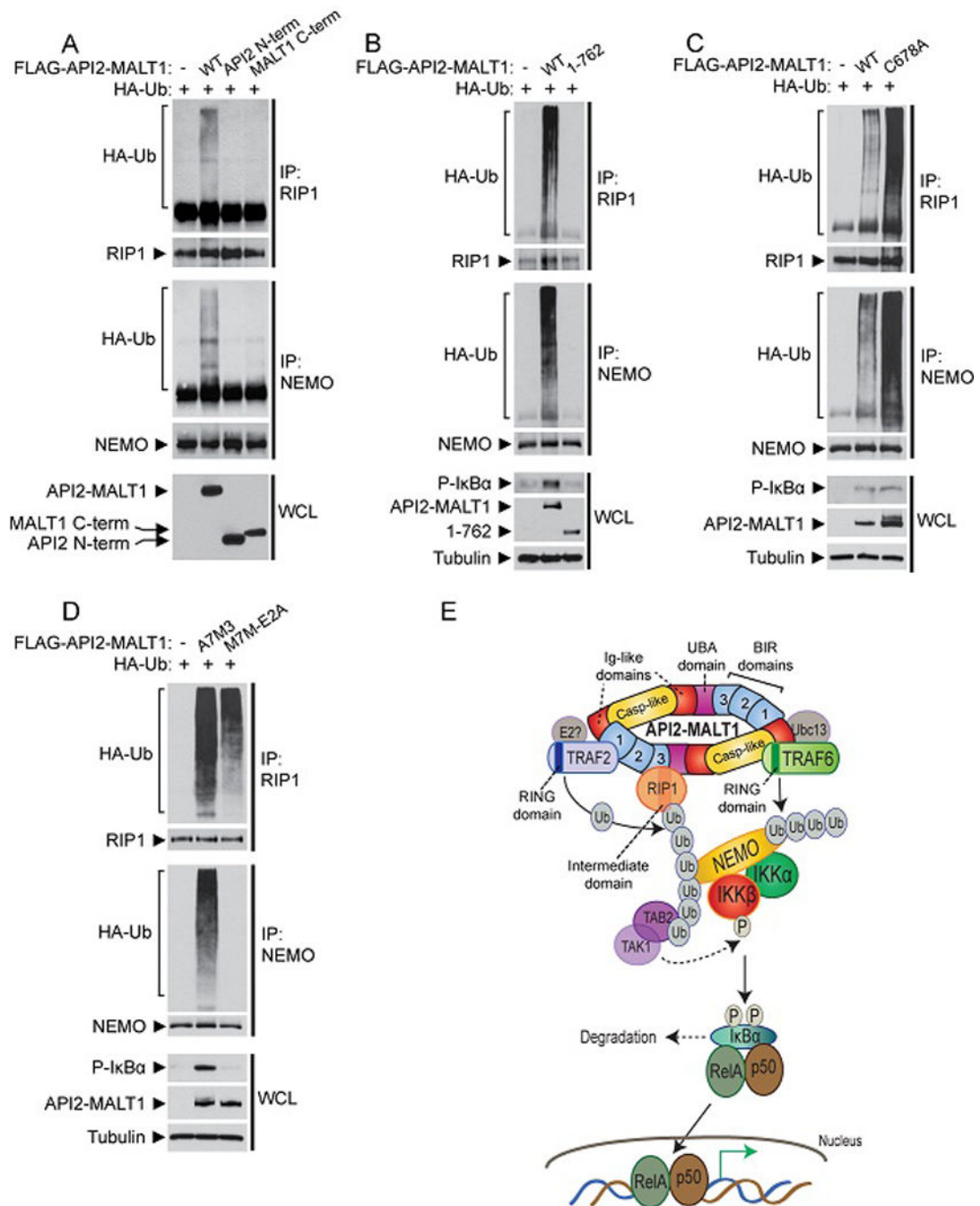
WT or mutant API2-MALT1 proteins to co-IP endogenous RIP1, TRAF2, and NEMO. (*bottom*) WCL were also analyzed for NF- $\kappa$ B activation by probing for phospho-IKK, phospho-p65, and phospho-I $\kappa$ B $\alpha$ .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 7. The role of the MALT1 moiety in API2-MALT1-induced RIP1 ubiquitination**  
 (A) HEK-293T cells were co-transfected with plasmids encoding FLAG-API2-MALT1, the API2 moiety, or the MALT1 moiety and HA-Ub. RIP1 and NEMO ubiquitination were assessed. (B and C) Either WT API2-MALT1 or a C-terminal deletion (1-762) (B) or the proteolytically inactive mutant (C678A) (C) were co-transfected into HEK-293T cells with HA-Ub to compare RIP1 and NEMO ubiquitination and NF- $\kappa$ B activation. (D) Either WT API2-MALT1 (A7M3) or a TRAF6 binding-deficient mutant (M7M-E2A) were co-

expressed with HA-Ub to compare RIP1 and NEMO ubiquitination and NF- $\kappa$ B activation.  
(E) Proposed model of API2-MALT1-induced canonical NF- $\kappa$ B activation.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript