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ORIGINAL ARTICLE

The API2–MALT1 fusion exploits TNFR pathway-associated RIP1 ubiquitination to promote oncogenic NF-κB signaling

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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 nuclear factor kappa B (NF-κB). API2–MALT1 promotes ubiquitination of RIP1 at lysine (K) 377, which is necessary for full NF-κB activation. Furthermore, we found that TNF receptor-associated factor 2 (TRAF2) recruitment is required for API2–MALT1 to induce RIP1 ubiquitination, NF-κB 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-κB, indicating that API2–MALT1-dependent RIP1 ubiquitination 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.

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INTRODUCTION

Mucosa-associated lymphoid tissue (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)$^+$ 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 heavy-chain (IGH) locus leading to deregulated overexpression.[7–9]

Although 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: nuclear factor kappa B (NF-κB) signaling. NF-κB comprises a family of latent dimeric transcription factors required for proper immune responses. Inactive NF-κB dimers are bound by the inhibitor of NF-κB (IκB) 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, lymphotoxin-β receptor and the receptor for B-cell-activating factor belonging to the TNF family (BAFF). Two signaling mechanisms that activate NF-κB are the canonical and noncanonical pathways. These pathways differ, in part, by the specific IκB kinase (IKK) complex subunits used to transmit the NF-κB signal. Stimulation of the canonical pathway activates IKKβ, which phosphorylates IκBα and targets it for proteasomal degradation, freeing canonical p65(RelA)/p50 NF-κB dimers to translocate to the nucleus and regulate transcription of specific genes. In contrast, noncanonical NF-κB activation, which is stimulated by only a few cell-surface receptors (for example,

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lymphotoxin-β receptor and receptor for BAFF), requires stabilization and activation of NF-κB-inducing kinase (NIK). Activated NIK phosphorylates and activates IKKα, which in turn phosphorylates the NF-κB2 precursor p100 and targets it for partial proteasomal processing to p52, thus freeing noncanonical RelB/p52 heterodimers to transactivate appropriate genes.[10]

We and others determined that Bcl10 and MALT1 interact and synergize to activate canonical NF-κB.[11,12] In the lymphocyte, this interaction is facilitated by the CARMA1 scaffolding protein during B-cell antigen or T-cell antigen stimulation, which is reviewed in Thome *et al.*[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-κB essential modulator (NEMO) to promote IKKβ activation.[14–16]

API2–MALT1 utilizes multiple mechanisms to activate NF-κB by harnessing both the canonical and noncanonical pathways. First, API2–MALT1 binds and proteolytically cleaves NIK, creating a stable, constitutively-active NIK fragment that stimulates IKKα and the noncanonical NF-κB pathway.[17] Second, API2–MALT1 activates canonical NF-κB 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 IKKβ 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 receptor interacting protein-1 (RIP1), a key regulator of TNF-dependent effects on NF-κB 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-κB activation.[19,20] TRAF2, which was originally identified as a TNFR-interacting protein,[21] is required for maximal TNF-induced NF-κB 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 polyubiquitination of RIP1 at K377.[25–28] The ubiquitin chains attached to RIP1 act as a scaffold to which the transforming growth factor β-activated kinase (TAK)-binding protein (TAB)/TAK and NEMO/IKK complexes bind,[28–30] resulting in IKKβ activation and stimulation of canonical NF-κB. 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-κB signaling.

RESULTS

API2–MALT1 interacts with TNFR-associated protein RIP1

We previously showed that TRAF2 is required for API2–MALT1-dependent canonical NF-κB 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-κB activation, and discovered a novel interaction between API2–MALT1 and RIP1 (Figure 1a). Interestingly, we did not detect the association between API2–MALT1 and TRADD, an adapter protein required for the recruitment of TRAF2 and RIP1 to the TNFR. Our findings indicate that API2–MALT1 binds key mediators of the TNFR-dependent NF-κB 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 the interaction with RIP1. We found that, like TRAF2, RIP1 associates with the API2 moiety (Supplementary Figure S1A), but not the MALT1 moiety of

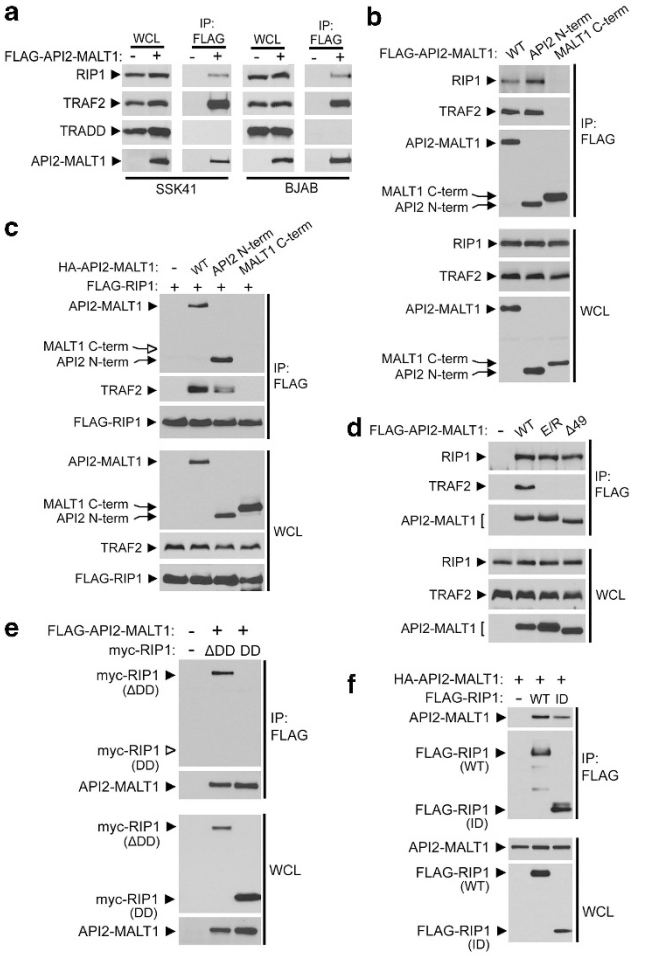

Figure 1. RIP1 is a novel API2–MALT1-associated protein. (**a**) Lysates from SSK41 (left) and BJAB (right) B-cell lines were subjected to anti-FLAG IP, and analyzed for the presence of endogenous RIP1, TRAF2 and TNFR-associated death domain (TRADD) by western blot. (**b**) FLAG–API2–MALT1, API2 moiety alone or MALT1 moiety alone were expressed in HEK-293T cells, and tested for their ability to co-immunoprecipitate (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 (ΔDD) 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 coexpressed with either FLAG–RIP1 or only the 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.

API2–MALT1 (Figures 1b and c, 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 (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-κB activation

To evaluate the contribution of RIP1 to API2–MALT1-induced NF-κB signaling, we next knocked down RIP1 using small interfering RNA (siRNA). Loss of RIP1 reduced NF-κB-dependent luciferase activity by 50% in response to API2–MALT1 expression, which also correlated with the loss of IKK complex activation and IκBα phosphorylation (Figure 2a). Because NF-κB-dependent luciferase activity was not completely lost, and expression of API2–MALT1 is known to activate both canonical and noncanonical NF-κB,[17,31] we hypothesized that API2–MALT1-induced noncanonical NF-κB 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-κB-dependent luciferase activity could be due to unimpaired non-canonical NF-κB signaling, which results in the activation of p52/RelB dimers that may bind to the same DNA consensus as canonical dimers of p50/p65.[10] RIP1 knockdown 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-κB-dependent luciferase activity in response to impaired RIP1 expression (Supplementary Figure S2).

As RIP1 kinase activity is dispensable for TNF-induced NF-κB activation,[32] we investigated whether this was also true for NF-κB activation induced by API2–MALT1. Treatment with the RIP1-specific kinase inhibitor, Necrostatin-1,[33] had no effect on API2–MALT1-induced NF-κB 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-κB activation, and that RIP1 is dispensable for API2–MALT1-induced noncanonical NF-κB activation.

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

We have previously determined, via RNAi-mediated knockdown of either IKKα or IKKβ, that both canonical and noncanonical NF-κB activity are required for API2–MALT1 to optimally promote B-cell adhesion and resistance to dexamethasone-induced cell death.[17] To determine whether 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 vascular cell adhesion molecule 1-coated plates (Figures 3a and b) 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-κB activation.

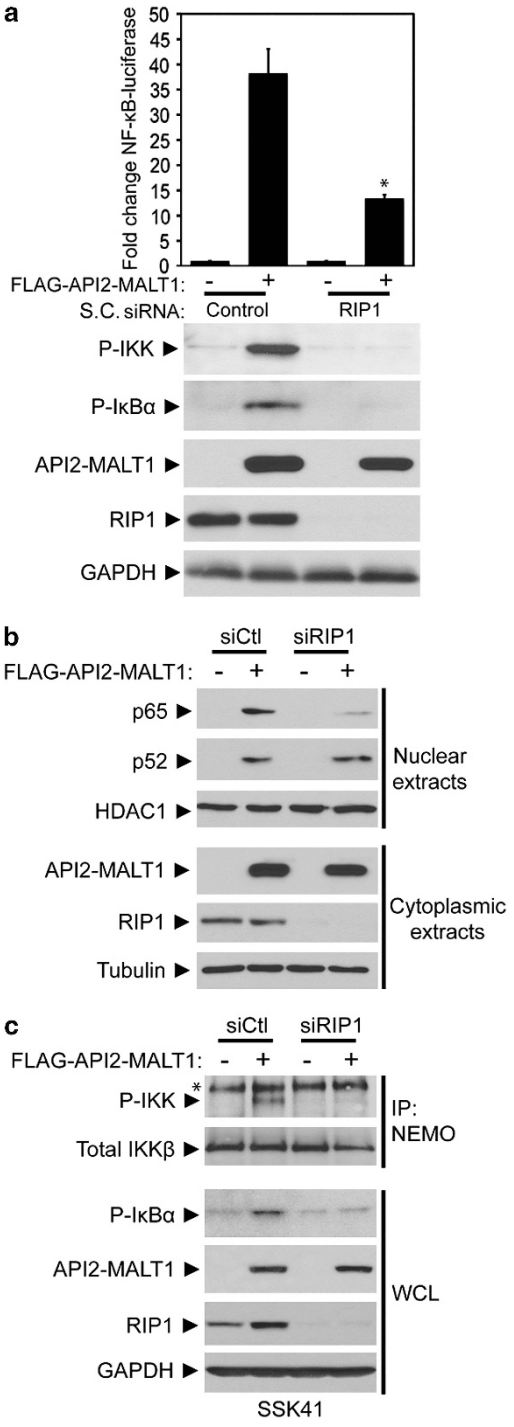

Figure 2. RIP1 is required for API2–MALT1-dependent activation of canonical NF-κB. (**a**) HEK-293T were first transfected with either control or RIP1-specific siRNA on two consecutive days. Then, on the third day, either empty vector or FLAG–API2–MALT1 was co-transfected with both NF-κB-dependent luciferase 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 ± s.e.m. 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.

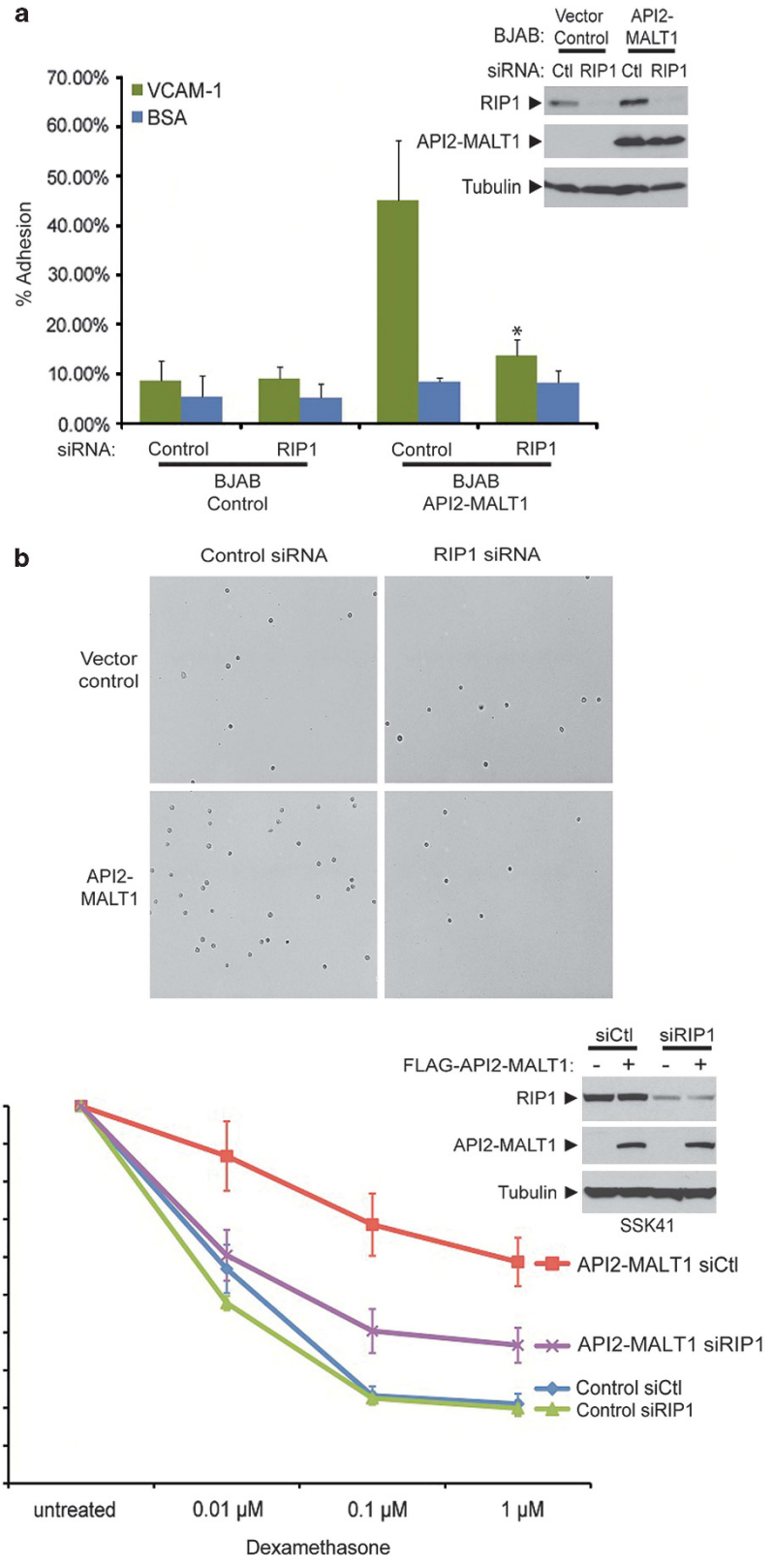

Figure 3. RIP1 is necessary for API2–MALT1-dependent changes in B-cell phenotype. (**a**) BJAB B cell lines were electroporated with either control or RIP1-specific siRNA. After 48 h, cells were treated with doxycycline and adhesion to vascular cell adhesion molecule 1 (VCAM-1) coated plates was assessed. The graph depicts the average ± s.e.m. 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 ± s.e.m. of four separate experiments. (inset) Western blot analysis of a representative experiment shows effective RIP1 knockdown.

API2–MALT1-induced ubiquitination of RIP1 at K377 is required for full NF-κB 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-κB, 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.

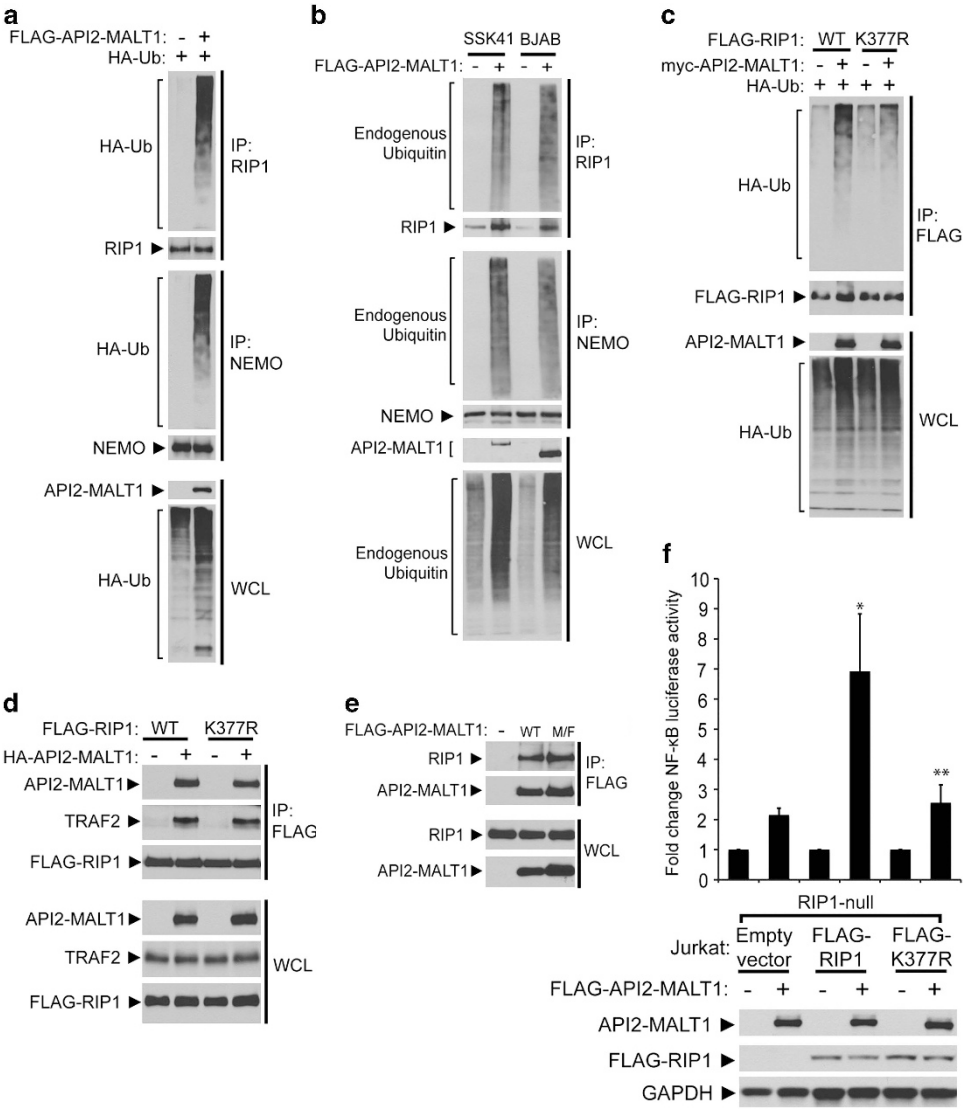

Figure 4. API2–MALT1 expression is associated with RIP1 ubiquitination. (**a**) HEK-293T cells were co-transfected with FLAG–API2–MALT1 and HA-ubiquitin (HA-Ub). Denatured lysates were subjected to IP with either anti-RIP1 or anti-NEMO, and probed with anti-HA to detect the incorporation of HA-Ub. (**b**) Denatured lysates from either SSK41 or BJAB B-cell lines were processed for IP as above and probed with anti-ubiquitin to detect the incorporation of endogenous ubiquitin. The SSK41 cells express the 'A7M3' variant of API2–MALT1 that contains two immunoglobulin (Ig)-like domains 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-κB-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 ± s.e.m. 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.

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 the association with API2–MALT1, indicating that API2–MALT1 can associate with unmodified RIP1 and induce its ubiquitination.

We then asked whether RIP1 ubiquitination is required for API2–MALT1-dependent NF-κB 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-κB. Whereas API2–MALT1 expression in control RIP1-null cells only modestly activated an NF-κB-dependent reporter, API2–MALT1 expression in wild-type RIP1-complemented cells robustly activated NF-κB (Figure 4f). The non-ubiquitinatable K377R RIP1 mutant, however, was unable to rescue API2–MALT1-induced NF-κB activation. These data suggest that, as in TNF-induced NF-κB 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 the activation of canonical NF-κB.[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. As 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 IκBα phosphorylation in response to API2–MALT1 expression (Figure 5b). Furthermore, coexpression of a TRAF2 dominant-negative mutant lacking the N-terminal RING domain, which has been shown to impair API2–MALT1-induced NF-κB activation,[19,20] completely blocked API2–MALT1-induced RIP1 and NEMO ubiquitination (Figure 5c) as well as phosphorylation of IκBα (Supplementary Figure S5).

A recent study demonstrated that TNF-induced TRAF2-dependent RIP1 ubiquitination could occur upon the binding of sphingosine-1-phosphate 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 activity, and therefore the production of sphingosine-1-phosphate, on API2–MALT1-dependent NF-κB activation. We found that treatment with SK1-I, a specific sphingosine kinase 1 inhibitor, impaired API2–MALT1-induced RIP1 and NEMO ubiquitination, as well as IκBα phosphorylation, in a dose-dependent manner (Figure 5d).

To determine whether the 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-κB 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 Δ49) or both TRAF2 binding and oligomerization (Δ98 and Δ166), two characteristics of API2–MALT1 that are required for full NF-κB 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, the 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-κB 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-κB.[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-κB-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 the 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-κB (Figure 7b). To specifically determine whether 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-κB activation (Figure 7c). We then sought to determine whether 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-κB 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

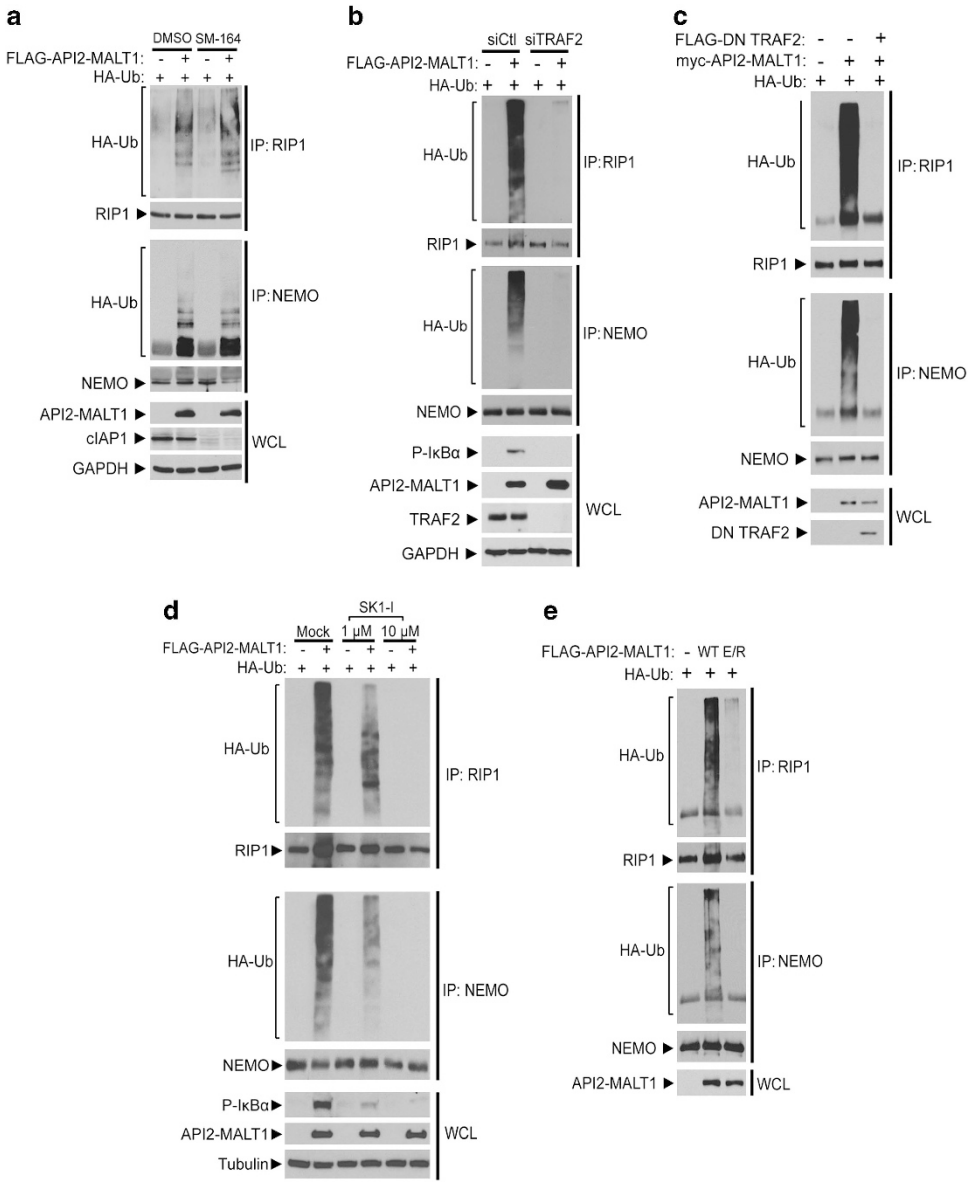

Figure 5. TRAF2 is required for API2–MALT1-induced RIP1 ubiquitination. (**a**) HEK-293T cells were pretreated with either dimethyl sulfoxide (DMSO) or smac mimetic (SM-164) for 16 h before co-transfection with either empty vector or FLAG–API2–MALT1 and HA-Ub plasmids. Denatured lysates were subjected 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-IκBα. (**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-κB. (**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.

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-κB activation.

DISCUSSION

We previously demonstrated that TRAF2 binding to API2–MALT1 is required for API2–MALT1-dependent NF-κB 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-κB 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 ID of RIP1. Through a series of elegant structure/function studies, the ID of RIP1 was recently shown to be essential for the antiapoptotic function of RIP1.[40] The ID 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

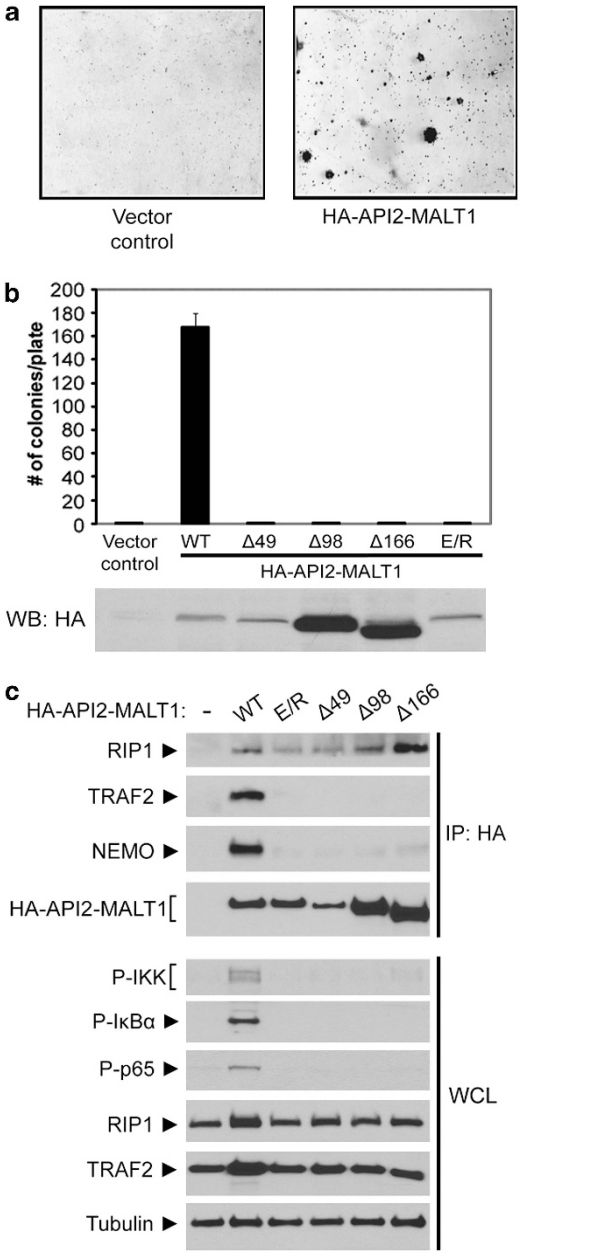

Figure 6. TRAF2 binding is required for API2–MALT1-induced NF-κB activation and cellular transformation. (**a**) Representative brightfield images of NIH-3T3 cells with either empty vector control or stable API2–MALT1 expression after plating in soft agar. (**b**) Quantification of the number of colonies formed per plate of the indicated NIH-3T3 stable cell lines subjected to growth in soft agar. Bar graphs represent the average ± s.e.m. 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-κB activation by probing for phospho-IKK, phospho-p65 and phospho-IκBα.

the absence or presence of RIP1 polyubiquitination at K377. When RIP1 is ubiquitinated, both TAB2 and NEMO bind to the polyubiquitin chains, and these interactions are essential for TNF-induced IKK complex activation, NF-κB 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 the 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-κB 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-κB. Second, we demonstrate that either deletion of the N-terminal RING domain of TRAF2 or impairing production of sphingosine-1-phosphate, which binds the TRAF2 RING domain, suppresses API2–MALT1-induced RIP1 and NEMO ubiquitination and phosphorylation of IκBα. 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-κB. 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.*[27] 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*. Intriguingly, Zhou *et al.*[18] have reported that Ubc13 is required for API2–MALT1 to stimulate NF-κB. 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, which is critical for cellular transformation and colorectal tumor growth *in vivo*.[47] In line with this observation that demonstrates a protumorigenic role for TRAF2, we find that mutants of API2–MALT1 that fail to recruit TRAF2 and cannot activate NF-κB 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-κB. 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] which are 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.

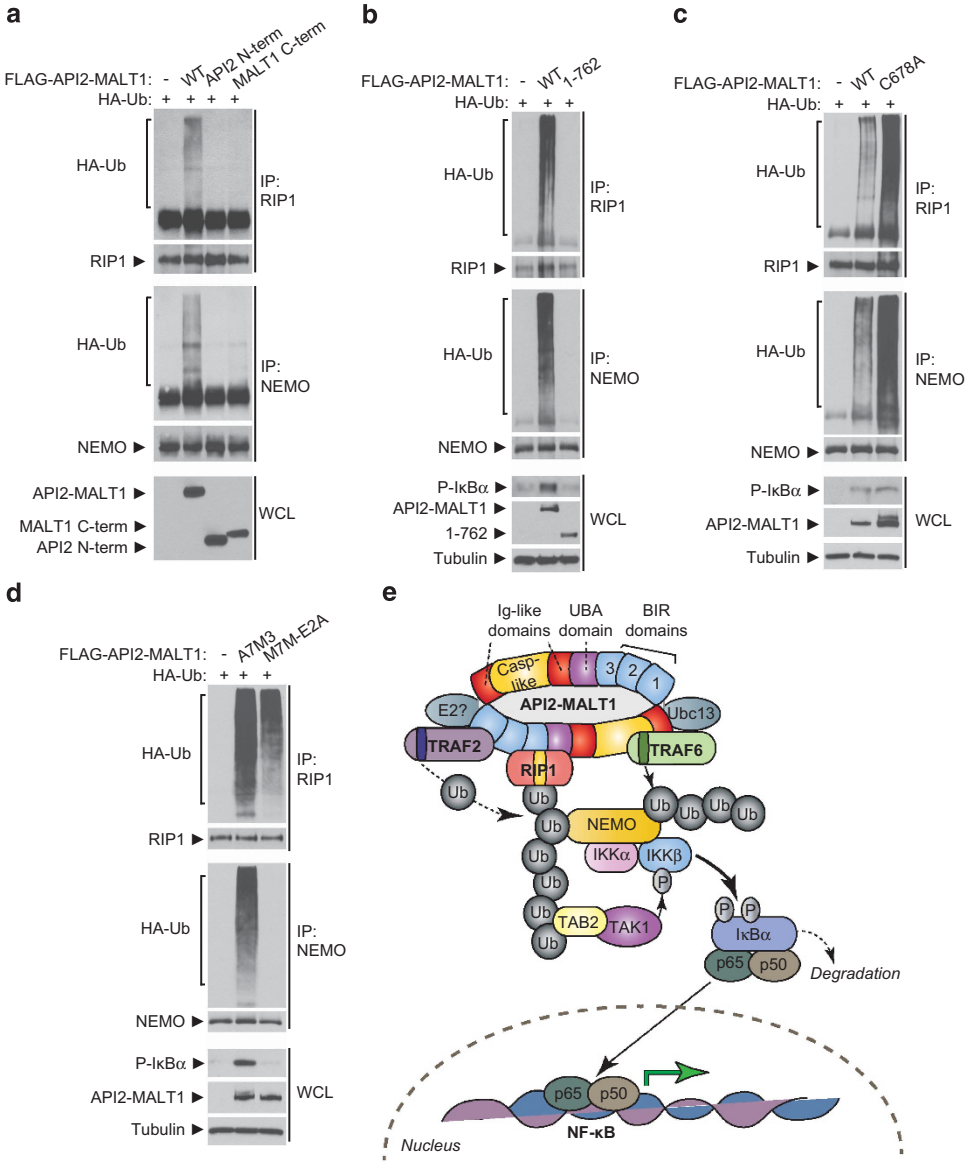

Figure 7. The role of the MALT1 moiety in API2–MALT1-induced RIP1 ubiquitination. (**a**) HEK-293T cells were co-transfected with plasmids encoding FLAG–API2–MALT1, the API2 moiety or the MALT1 moiety and HA-Ub. RIP1 and NEMO ubiquitination were assessed. (**b**, **c**) Either WT API2–MALT1 or a C-terminal 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-κB activation. (**d**) Either WT API2–MALT1 (A7M3) or a TRAF6 binding-deficient mutant (M7M-E2A) were coexpressed with HA-Ub to compare RIP1 and NEMO ubiquitination and NF-κB activation. (**e**) Proposed model of API2–MALT1-induced canonical NF-κB activation.

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-κB activation were totally impaired. This suggested that API2–MALT1 recruitment of TRAF6 is required for canonical NF-κB activation, similar to the antigen receptor-stimulated CARMA1/Bcl10/MALT1-dependent NF-κB activation pathway, wherein oligomerized MALT1 recruits TRAF6 to promote TRAF6-dependent ubiquitination of NEMO and IKK complex activation.[15,16] As 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, as 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.

On the basis of 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) Polyubiquitinated 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-κB 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-κB 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-κB activation by facilitating RIP1 ubiquitination, allowing the recruitment and activation of NF-κB-activating kinase complexes. Possible interventions that disrupt either API2 moiety-mediated TRAF2 or RIP1 interaction could inhibit canonical NF-κB activation. Although MALT1 moiety-associated protease activity is dispensable for stimulating canonical NF-κB, we have previously shown that MALT1 protease activity is critical for API2–MALT1-induced NIK cleavage and activation of noncanonical NF-κB, 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)$^+$ 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, ID, death domain-deleted (ΔDD) and death domain only, were previously described.[34,51] HA-ubiquitin and mutants K48R and K63R were previously described.[52] Wild-type and mutant (Δ49, Δ98, Δ166, E47/R48A) API2–MALT1 complementary DNAs 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-κB luciferase reporter plasmid pGL2-3x-κB-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 the 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 (Thermo Scientific, Rockford, IL, USA) and *N*-ethyl-maleimide (Sigma-Aldrich, St Louis, MO, USA) at 10 mg/ml). Cleared lysates (500 μg for HEK-293T cells transfected with HA-ubiquitin 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 10-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–polyacrylamide gel electrophoresis. 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)