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Atypical Mechanism of NF-κB activation by TRE17/Ubiquitin-Specific Protease 6 (USP6) oncogene and its requirement in tumorigenesis

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Abstract

The NF-κB transcription factor plays a central role in diverse processes, including inflammation, proliferation and cell survival, and its activity is dysregulated in diseases such as auto-immunity and cancer. We recently identified the TRE17/ubiquitin-specific protease 6 (USP6) oncogene as the first de-ubiquitinating enzyme to activate NF-κB. *TRE17*/*USP6* is translocated and overexpressed in aneurysmal bone cyst (ABC), a pediatric tumor characterized by extensive bone degradation and inflammatory recruitment. In the current study, we explore the mechanism by which TRE17 induces activation of NF-κB, and find that it activates the classical NF-κB pathway through an atypical mechanism that does not involve IκB degradation. TRE17 co-precipitates with IκB kinase (IKK), and IKK activity is augmented in stable cell lines overexpressing TRE17, in a USP-dependent manner. Optimal activation of NF-κB by TRE17 requires both catalytic subunits of IKK, distinguishing its mechanism from the classical and non-canonical pathways, which require either IKKβ or IKKα, respectively. TRE17 stimulates phosphorylation of p65 at serine 536, a modification that has been associated with enhanced transcriptional activity and nuclear retention. Induction of S536 phosphorylation by TRE17 requires both IKKα and IKKβ, as well as the IKKγ/NEMO regulatory subunit of IKK. We further demonstrate that TRE17(long) is highly tumorigenic when overexpressed in NIH3T3 fibroblasts, and that inhibition of NF-κB significantly attenuates tumor formation. In summary, these studies uncover an unexpected signaling mechanism for activation of classical NF-κB by TRE17. They further reveal a critical

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

role for NF-κB in TRE17-mediated tumorigenesis, and suggest that NF-κB inhibitors may function as effective therapeutic agents in the treatment of ABC.

Keywords

TRE17; USP6; aneurysmal bone cyst; NF-κB; IκB

Introduction

NF-κB is a ubiquitous transcription factor that plays a central role in innate immunity, inflammation, cellular proliferation, survival, and differentiation (Gilmore, 2006; Karin, 2006). This transcription factor comprises five family members, Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p105/p50), and NF-κB2 (p100/p52), which form various homo- and heterodimeric complexes to regulate the transcription of numerous target genes, including cytokines, components of the cell cycle machinery, and genes that control proliferation (Hacker and Karin, 2006; Rollins, 2006). In addition to its role in these physiological processes, dysregulation of NF-κB has been documented under pathological conditions, such as autoimmune disease and cancer.

NF-κB is typically divided into classical and non-canonical pathways, which are distinguished by the NF-κB subunits activated, the upstream regulatory proteins involved, and their biological functions. The classical pathway is associated with innate immunity and the inflammatory response, and is activated by agents such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1). Classical NF-κB consists of p65/p50 heterodimers or p65 homodimers, which are kept inactive in the cytoplasm through their association with Inhibitor of NF-κB (IκB). Agonists induce phosphorylation of IκB by IκB kinase (IKK), triggering its ubiquitination and proteasomal degradation, with subsequent translocation of p65/p50 to the nucleus. IKK consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO. Both IKKβ and NEMO, but not IKKα, are required for phosphorylation and degradation of IκB.

The non-canonical pathway is associated with B cell differentiation and a distinct array of agonists, such as B-cell activating factor (BAFF) and lymphotoxin β. The NF-κB subunits in this pathway are RelB and p100/p52. Agonists of the non-canonical pathway induce phosphorylation of p100 by IKK, leading to its processing to the p52 form, and nuclear translocation with RelB. Notably, IKKα, but not IKKβ or NEMO, is essential for activation of this pathway.

While classical vs. non-canonical NF-κB are commonly depicted as described above, increasing evidence suggests a higher degree of complexity and crosstalk between these pathways. For example, the p100 gene is a direct target of classical NF-κB. In addition, recent work indicates that in mouse embryo fibroblasts with targeted deletion of IKKβ, IKKα can mediate IL-1-induced activation of classical NF-κB in a NEMO-dependent manner (Solt *et al*., 2007). Another example of atypical IKK subunit involvement is exemplified by a study showing that reovirus requires IKKα and NEMO, but not IKKβ, for activation of classical NF-κB (Hansberger *et al*., 2007).

Numerous components of the NF-κB pathway are regulated by poly-ubiquitination (Harhaj and Dixit, 2011). While some modifications occur through lysine 48 (K48) ubiquitin linkages, others arise through lysine 63 (K63)-linked chains, which promote the formation of signaling complexes. Multiple de-ubiquitinating enzymes (DUBs), including A20, CYLD, Cezanne, USP21, and USP15, have been shown to function as negative regulators of NF-κB (Brummelkamp *et al.*, 2003; Kovalenko *et al.*, 2003; Trompouki *et al.*, 2003; Wertz *et al.*, 2004; Harhaj and Dixit, 2011). However, only one DUB, TRE17/ubiquitin-specific protease 6 (USP6), induces NF-κB activation (Ye *et al.*, 2010).

The *TRE17* locus is translocated in aneurysmal bone cyst (ABC), an aggressive bone tumor characterized by inflammation and destruction of the surrounding bone (Mankin *et al.*, 2005; Mendenhall *et al.*, 2006; Saccomanni, 2008). The majority of ABC cases harbor rearrangement of *TRE17*, resulting in high level expression of wild type TRE17 (Oliveira *et al.*, 2004a; Oliveira *et al.*, 2004b; Oliveira *et al.*, 2005). We recently undertook studies to determine the mechanism by which TRE17 contributes to ABC pathogenesis. The cells harboring *TRE17* translocation/overexpression are believed to be pre-osteoblasts or fibroblasts (Oliveira *et al.*, 2004a; Oliveira *et al.*, 2004b; Oliveira *et al.*, 2005), leading us to examine the effects of TRE17 overexpression in this cell type (Lau and Oliveira, 2010; Ye *et al.*, 2010). We recently reported that TRE17 induces the expression of matrix metalloproteinase 9 (MMP9) through activation of NF-κB (Ye *et al.*, 2010). In the current study we examine the mechanism by which it functions, demonstrating that TRE17 activates classical NF-κB through an atypical mechanism that is independent of IκB degradation. We further demonstrate that NF-κB is a critical effector of TRE17 in tumorigenesis.

RESULTS

TRE17 activates canonical but not non-canonical NF-κB

We recently reported that TRE17 activates NF-κB, in a manner dependent on its USP activity (Ye *et al.*, 2010). However, the mechanism by which TRE17 activates NF-κB and the selective involvement of the classical vs. non-canonical pathways were not examined. To address these issues, we initiated studies in two distinct cell culture models, HeLa cells and stable MC3T3-E1 cell lines. HeLa are human cervical carcinoma cells that endogenously express low levels of *TRE17* (Martinu *et al.*, 2004). MC3T3-E1 (referred to hereafter as MC3T3) are murine pre-osteoblasts, the cell type in which *TRE17* is translocated/overexpressed in at least a subset of ABCs. MC3T3 cell lines stably expressing TRE17 in a doxycyclin (dox)-inducible manner were previously generated, and shown to recapitulate multiple features of ABC when xenografted into nude mice (Ye *et al.*, 2010), validating it as a useful model for dissecting TRE17's functions in ABC pathogenesis.

To determine whether TRE17 activates classical or non-canonical NF-κB, electrophoretic mobility shift assays (EMSAs) were performed. As shown in Figure 1A (left panel), expression of the full length TRE17 isoform, TRE17(long), in HeLa cells induced activation of NF-κB. This NF-κB complex was supershifted by antibodies against classical NF-κB subunits p65 and p50, but not non-canonical subunits RelB and p52. To ensure that antibody against non-canonical NF-κB was functional, we confirmed its ability to perturb NF-κB

activation induced by overexpression of NIK, an activator of the non-canonical pathway (Figure 1A, right panel).

We next examined the composition of NF-κB complexes in the MC3T3 stable cell lines. Addition of dox induced expression of HA-TRE17(long) (Figure 1B, left panel), with concomitant activation of NF-κB (Figure 1B). Interestingly, only antibody against p65 but not p50 caused a supershift, indicating activation of a p65 homodimer. We also tested a point mutant of TRE17 that ablates its de-ubiquitinating activity (TRE17(long)/USP-) (Figure 1B). This mutant failed to activate NF-κB above levels seen in control samples (Figure 1B). Furthermore, as with HeLa cells, we confirmed that TNFα activated classical NF-κB in control vector-expressing MC3T3 cells (Figure 1B, right panel). The major TNFα-induced complex in these cells was composed of p65 homodimers, rather than the p65/p50 heterodimers commonly seen. This led us to consider whether the p65 homodimers induced by TRE17(long) in MC3T3 might reflect an atypical classical NF-κB response in this cell line.

To explore this issue, we examined NF-κB complexes in NIH3T3 cells, where TNFα has been shown to activate p65/p50 heterodimers. Stable NIH3T3 cell lines expressing TRE17(long) or TRE17(long)/USP- in a dox-inducible manner were generated (Figure 1). EMSA confirmed USP-dependent activation of NFκB by TRE17 (Figure 1C). Supershift analysis revealed that the TRE17-induced complexes were composed of p65 and p50 (Figure 1C).

As an independent means of confirming selective activation of classical NF-κB, we examined the subcellular localization of NF-κB by immunofluorescence confocal microscopy. In both transiently transfected HeLa (Figure 1D) and stable MC3T3 cell lines (Supplementary Figure 1), TRE17(long) induced nuclear accumulation of p65, but not of non-canonical NF-κB, as monitored by p100/p52 staining. In contrast, a constitutively active mutant of IKKα stimulated translocation of p100/p52 to the nucleus (Figure 1D). In sum, these data indicate that TRE17(long) selectively activates classical NF-κB, consisting of either homo- or heterodimeric p65 complexes, in three different cell types.

TRE17 activates canonical NF-κB independently of IκB phosphorylation and degradation

In resting cells, classical NF-κB is sequestered in the cytoplasm by Inhibitor of NF-κB (IκB). Upon agonist stimulation IκB is phosphorylated by IKK triggering its proteasomal degradation. Since TRE17(long) induced activation of classical NF-κB, we anticipated that it would induce IκB degradation. However, immunoblotting revealed that steady state levels of IκBα and IκBβ, the two major IκB isoforms, were comparable in TRE17(long) and control cells (Figure 2A). Given this discrepancy, we analyzed IκB processing in greater detail. Following initial agonist-induced degradation of IκB, activation of NF-κB leads to re-synthesis of IκB within several hours. Thus, transient downregulation of IκB might have been missed due to the chronic nature of TRE17(long) expression in our system (i.e. over a 24–48 hour time period). To address this issue we exploited our dox-inducible expression system to monitor IκB phosphorylation and degradation upon acute TRE17(long) expression. As seen in Figure 2B, a time-dependent increase in TRE17(long) expression was observed from 8 to 24 hours of dox treatment. However, no consistent phosphorylation or

reduction in total levels of IκBα or IκBβ was observed in TRE17(long)/MC3T3 (Figure 2B). In contrast, TNFα induced phosphorylation and degradation of IκBβ, but not IκBα, in MC3T3 cells (Figure 2B). Probing of TNFα-treated HeLa extracts confirmed that the phospho-IκBα antibody was functional (Figure 2B).

As an alternative approach to unmasking TRE17's potential effects on IκB degradation we tested the effects of cycloheximide (CHX) to monitor the stability of the pre-existing pool of IκB in the absence of *de novo* synthesis. Both IκB isoforms were highly stable in control and TRE17(long)/MC3T3, with comparable levels present throughout the 2 hour time course of CHX treatment (Figure 2C). As a positive control, we confirmed that TNFα caused a significant reduction in the half-life of IκBβ but not IκBα (Figure 2C). In aggregate these data indicate that TRE17(long) activates classical NF-κB through a mechanism that does not appear to involve phosphorylation and degradation of IκB.

This uncoupling of p65 activation from IκB degradation prompted us to further characterize how p65/IκB complexes are regulated by TRE17. Immunoprecipitation of p65 followed by IκB immunoblotting revealed that TRE17 did not induce dissociation of p65 from either IκB isoform (Figure 2A, right panels). Nevertheless, TRE17 induced the accumulation of nuclear p65 which was free of IκB (Figure 2D). We speculated that the only means by which this could occur is if there was an IκB-free pool of p65 in resting cells. Supporting this notion, we detected a population of p65 that was resistant to immunodepletion using anti-IκB antibodies (Figure 2E). Notably, TRE17 did not increase levels of IκB-free p65, suggesting that it does not function by stimulating dissociation of p65 from IκB.

TRE17 co-immunoprecipitates with IκB kinase

The findings above suggest that TRE17(long) may activate NF-κB by regulating nuclear translocation of an IκB-free population of p65. To explore how this might occur we examined whether TRE17(long) associates with the IKK complex, which consists of the catalytic subunits IKKα and IKKβ, and the regulatory subunit NEMO. TRE17(long) co-immunoprecipitated with endogenous IKKα but not control IgG in MC3T3 cells (Figure 3A). Association did not require USP activity, since TRE17(long)/USP- bound at levels comparable to the WT protein (Figure 3A). Similarly, TRE17(long) tagged with either HA or GST co-immunoprecipitated with endogenous IKKα in transiently transfected HeLa cells (Figure 3B). To confirm this association, the reciprocal immunoprecipitation/blot was performed. As seen in Figure 3C, IKKα and IKKβ were present in anti-HA immunoprecipitates from TRE17(long) but not vector-expressing control cells.

IKKα and IKKβ are required for TRE17-induced NFκB activation

We next sought to determine which IKK subunits were required for activation of NF-κB by TRE17. In the classical pathway, IKKβ and NEMO, but not IKKα, typically mediate activation of p65 complexes by inducing IκB phosphorylation and degradation. However, since IκB degradation was not elicited by TRE17, it was unclear which IKK subunit(s) might be required. To address this question, siRNA-mediated depletion of IKK was performed in HeLa cells. Specific knockdown of IKKα, IKKβ, and NEMO by their respective siRNAs was confirmed (Figure 4A). Depletion of each subunit significantly

inhibited TRE17(long)'s ability to induce NF-κB activation and p65 nuclear translocation (Figure 4B).

The contribution of IKKα in particular was surprising, since it is largely viewed as dispensable for activation of classical NFκB. We therefore confirmed that this result was not due to non-specific effects of IKKα depletion on IKKβ or NEMO function. Indeed, in the absence of IKKα, TNFα was able to elicit normal degradation of IκB and phosphorylation of p65 on serine 536 (see below for further discussion of this regulatory site) (Figure 4C). These results indicate that IKKβ, NEMO and, unexpectedly, IKKα all contribute to activation of NF-κB by TRE17(long). Thus, TRE17(long) functions through a novel mechanism distinct from classical and non-canonical NFκB activators, which typically require IKKβ or IKKα, respectively.

IKK activity is elevated in TRE17-expressing cells, in a USP-dependent manner

The requirement for all IKK subunits raised the possibility that TRE17 induces activation of the complex. To test this, *in vitro* kinase assays were performed. MC3T3 extracts were immunoprecipitated with anti-IKKα, then subjected to *in vitro* kinase assays using recombinant I B or a fragment of p65 as substrates. As seen in Figure 5A, IKK activity was elevated in TRE17(long)/MC3T3 relative to vector control cells. This required TRE17's de-ubiquitinating activity, since TRE17(long)/USP- failed to induce IKK activation (Figure 5A).

TRE17 induces phosphorylation of p65 on serine 536 through IKKα and IKKβ

While IκB served as a convenient *in vitro* substrate for the kinase assays, our results above indicated that it is not targeted by TRE17 *in vivo*, leading us to explore p65 as a potential substrate. Serine 536 (S536) of p65 is a major regulatory site within the carboxy-terminal transactivation domain (Viatour *et al.*, 2005). Phosphorylation of S536 has been associated with enhanced nuclear localization of p65, decreased association of p65 with co-repressors, enhanced association with co-activators, and elevated transcriptional activity (Yang *et al.*, 2003; Hu *et al.*, 2004; Mattioli *et al.*, 2004; Viatour *et al.*, 2005; Hoberg *et al.*, 2006). TRE17(long) induced phosphorylation of S536-p65 in both the MC3T3 stable cell lines and transiently transfected HeLa cells (Figure 5B,C).

We next assessed the contributions of the IKK subunits to S536 phosphorylation induced by TRE17. Previous studies have indicated that a number of kinases, including IKKα and IKKβ, can mediate phosphorylation of this site *in vivo* (Sakurai *et al.*, 1999; Sizemore *et al.*, 2002; Sakurai *et al.*, 2003; Buss *et al.*, 2004; Viatour *et al.*, 2005). As seen in Figure 5C, TRE17-induced phosphorylation of p65 was significantly attenuated in cells depleted of each of the IKK subunits. To confirm the functional specificity of the siRNAs, we determined that NEMO but not IKKα was required for p65 S536 phosphorylation in response to TNFα (Figures 4C and 5C). Together, these data suggest that TRE17 activates NF-κB by promoting S536 phosphorylation of p65, in manner that optimally requires IKKα, IKKβ, and NEMO.

Our results showing that TRE17 overexpression induces NF-κB activation and p65 phosphorylation is relevant for understanding its pathological functions in ABC, which is a disease arising from TRE17 overexpression. However, we also sought to determine whether TRE17 modulates NF-κB activation in response to physiological agonists. Knockdown of TRE17 in HeLa cells using SmartPool siRNA partially attenuated p65 phosphorylation in response to TNFα and LIGHT, an agonist for the lymphotoxin β receptor (Figure 5D). Inhibition of NF-κB was further confirmed by EMSA and blotting for nuclear translocation of p65 (Figure 5E, F). Thus, TRE17 also participates in agonist-mediated NF-κB activation when expressed at native levels.

TRE17 induces tumorigenesis in a manner requiring NF-κB

We recently demonstrated that overexpression of TRE17(long) in MC3T3 osteoblasts induces tumor formation in nude mice, in a USP-dependent manner (Ye *et al.*, 2010). Interestingly, our results contradicted an earlier report suggesting that when overexpressed in NIH3T3 fibroblasts, the truncated, catalytically inactive TRE17(short) isoform is tumorigenic, but TRE17(long) is not (Nakamura *et al.*, 1992). To determine whether this difference arises from the use of different cell types, we examined the tumorigenic potential of our NIH3T3 cell lines. As seen in Figure 6A, TRE17(long)/NIH3T3 were highly tumorigenic, with all injected animals exhibiting palpable tumors visible as early as 5 days post-injection. In contrast, no tumors formed in mice injected with NIH3T3 cells expressing TRE17(long)/USP-, vector control NIH3T3, or TRE17(long)/NIH3T3 in the absence of dox (Figure 6A and data not shown). These results thus refute initial reports suggesting that USP activity inhibits TRE17's tumorigenic potential in NIH3T3 (Nakamura *et al.*, 1992), and instead confirm our recent work in MC3T3 cells demonstrating that USP activity is essential for TRE17's oncogenicity (Ye *et al.*, 2010).

We next examined whether activation of NF-κB by TRE17 was required for tumorigenesis. We performed these studies in NIH3T3 rather than MC3T3 cells, since the former yielded much larger tumors, providing a wider dynamic range to assess the effects of NF-κB inhibition. Our studies above indicated that depletion of any of the IKK subunits might block activation of NF-κB. However, we encountered difficulties with sustained and efficient knockdown in NIH3T3 cells (unpublished observations). Although IκB degradation is not normally elicited by TRE17, we speculated that overexpression of the IκB Super Repressor (IκB-SR) might nevertheless effectively sequester p65 and block NF-κB activation by TRE17(long). IκB-SR is an amino-terminal truncation mutant lacking the IKK phosphorylation sites, and has been used as a potent, agonist-resistant inhibitor of classical NFκB (Brockman *et al.*, 1995). IκB-SR was introduced under the control of a dox-inducible promoter into the TRE17(long) cell line (Figure 6B). IκB-SR completely abrogated activation of NF-κB by TRE17 as determined by EMSA (Figure 6C).

Xenografting of the TRE17(long)/IκB-SR cells in parallel with the parental TRE17(long) cells revealed that inhibition of NF-κB significantly attenuated tumor formation (Figure 6D). The average mass of TRE17(long)/IκB-SR tumors was approximately 10% that of TRE17(long) tumors (0.83 g for TRE17(long); 0.07 for TRE17(long)/IκB-SR). However, equally striking to the reduction in tumor mass was the virtually complete inhibition of

tumor vascularization (Figure 6E). These results demonstrate that NF-κB is a critical effector of TRE17 in tumorigenesis.

DISCUSSION

Here we demonstrate that TRE17 activates canonical p65-containing NF-κB through an atypical mechanism that is independent of IκB phosphorylation/degradation. TRE17 associates with IKK and promotes its activation, leading to phosphorylation of p65 on S536. Our data suggest that TRE17 targets a pool of p65 that is free of IκB. Optimal phosphorylation of S536 requires both IKKα and IKKβ, distinguishing TRE17's mechanism from the proto-typical classical and non-canonical pathways. We further demonstrate that NF-κB is a critical effector of TRE17 in tumor formation. Inhibition of NF-κB dramatically attenuates tumor growth and vascularization, indicating that this may be a highly effective avenue for treatment of ABC patients.

Our finding that TRE17 activates classical NF-κB in the absence of IκB degradation, and in a manner requiring both IKKα and IKKβ, adds to the growing list of stimuli that activate NF-κB through mechanisms distinct from simplified depiction of the classical pathway (Hansberger *et al.*, 2007; Solt LA, 2007). Interestingly, it has been shown that agonists such as glutamate, angiotensin II, lysophosphatidic acid, and thrombin activate p65 NF-κB by inducing S536 phosphorylation, independently of IκB downregulation (Douillette *et al.*, 2006; Choudhary *et al.*, 2007; Sitcheran *et al.*, 2008; Sun *et al.*, 2009). Additional studies have shown that IKKα and IKKβ can phosphorylate a pool of p65 that is not bound by IκB (Sasaki *et al.*, 2005), and we suggest TRE17 functions by such a mechanism. S536 phosphorylation of p65 has been reported to promote its nuclear retention, enhance its association with the transcriptional co-factors, inhibit its association with co-repressors, and potentiate activation of NF-κB target genes.

We recently reported that activation of NF-κB by TRE17 is mediated in part by RhoA and ROCK (Ye *et al.*, 2010). Previous work from others has shown that RhoA and ROCK can promote p65 S536 phosphorylation (Khandaker *et al.*, 2004; Shimada and Rajagopalan, 2010). However, we found that ROCK inhibitors did not significantly affect TRE17's ability to induce S536 phosphorylation (unpublished observations). Thus, our current results suggest that TRE17 functions through multiple parallel pathways to activate NF-κB, one of which involves interaction with IKK, a core regulatory component of the pathway. It remains to be determined if this interaction is direct, and how it contributes to IKK activation. However, TRE17 does not appear to directly activate IKK activity (Supplemental Figure 2). TRE17's USP activity is not required for association with IKK, but is required for NF-κB activation. Thus, one possibility is that TRE17 promotes interaction of IKK with an upstream positive regulator, perhaps by de-ubiquitinating it and rescuing it from proteasomal degradation. It is most likely that TRE17 targets a protein modified by K48-linked rather K63-linked poly-ubiquitin, since all K63-mediated ubiquitination events described thus far in the pathway promote NF-κB activation. Nevertheless, it remains possible that TRE17 acts on an as-yet identified K63-modified protein which functions as a negative regulator of NF-κB. Given this complexity, identifying the substrate of TRE17's

USP domain and determining how it induces the activation of IKK remain goals for the future.

Our xenograft studies establish that USP activity is critical for TRE17's tumorigenic potential. Two prior studies yielded contradictory results: one, performed using MC3T3 cells, demonstrated TRE17(long), but not the catalytically inactive point mutant, induced tumor formation (Ye *et al.*, 2010). The other study utilized NIH3T3, the cell line used in our current work, yet arrived at the opposite conclusion regarding the role of TRE17's USP activity in tumorigenesis. The reason for this discrepancy is unclear. However, it is noted that the earlier studies did not confirm expression of the different TRE17 isoforms (Nakamura *et al.*, 1992). Furthermore, their tumors arose with much greater latency than we observed (4–6 weeks vs. 5–14 days).

Our study establishes NFκB as an essential effector of TRE17 in tumorigenesis. Although TRE17 does not normally elicit IκB degradation to activate NF-κB, overexpression of IκB-SR served as an effective tool to extinguish NF-κB activation in TRE17(long)/NIH3T3 cells, and revealed its requirement in tumor formation. TRE17(long)/IκBSR tumors were not only significantly smaller but also much less vascularized. It remains to be determined how inhibition of NF-κB contributes to these responses. It does not appear to be required for cell survival *in vitro* (Supplemental Figure 3), but may function cell autonomously to promote proliferation of the xenografted cells. Another possibility, which is not mutually exclusive, is that NF-κB is required non-cell autonomously. NF-κB has been shown to regulate production of vascular endothelial growth factor (VEGF), a key physiological regulator of angiogenesis. Whether NF-κB plays a direct role in angiogenesis, or functions indirectly by regulating proliferation of the TRE17(long) cells remains to be determined.

ABC is a tumor for which there are few treatment options, in part because little is known about its pathogenesis (Mendenhall *et al.*, 2006; Cottalorda and Bourelle, 2007). They are typically treated by surgical curettage, however recurrence is relatively common. Furthermore, ABCs can arise in inoperable locations. Thus, alternative modes of treatment would be of great value. Our work strongly suggests that inhibitors of NF-κB might serve as effective therapeutic agents for the treatment of ABC. A variety of NF-κB inhibitors are being avidly explored for the treatment of cancer, autoimmune and inflammatory diseases (Pahl, 1999; Orlowski and Baldwin, 2002; Dobrovolskaia and Kozlov, 2005; Hacker and Karin, 2006; Rollins, 2006; Calzado *et al.*, 2007; Lee and Hung, 2008). Our studies reveal that all three IKK subunits are required for TRE17-mediated activation of NF-κB, suggesting that inhibitors for any of them might be efficacious for management of ABC.

MATERIAL AND METHODS

Tissue Culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin, streptomycin, and fungizone. Transfections were performed with Lipofectamine 2000 (Life Technologies, Inc.) or Fugene (Roche Biochemicals) according to the manufacturer's instructions. MC3T3-E1 (hereafter referred to as MC3T3) pre-osteoblastic stable cell lines that express TRE17 alleles in a doxycyclin

(dox)-inducible manner have been described previously (Ye *et al.*, 2010). MC3T3-derived lines were grown in α-Modified Eagle Media with tetracycline-free FBS, penicillin/streptomycin, sodium pyruvate, and GlutaMax. All cell lines were maintained in 5% CO2. NIH3T3 cell lines expressing TRE17 in a dox-inducible manner were generated, using the methods previously described (Ye *et al.*, 2010). NIH3T3-derived cell lines were grown in DMEM containing 10% calf serum and the supplements described above.

Plasmids and constructs

TRE17 constructs have been described (Masuda-Robens *et al.*, 2003; Martinu *et al.*, 2004; Shen *et al.*, 2005). c-myc-NIK was provided by Dr. Jing Hu (University of Pittsburgh Medical Center). GST-p65 (354–551) was provided by Hiroaki Sakurai (Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd.). Purified recombinant GST-IκB was provided by Dr. Sergei Fuchs (University of Pennsylvania School of Veterinary Medicine). IκB-SR cDNA (provided by Dean NF-KB. Ballard, Vanderbuilt University) was subcloned into pRevTRE (Clontech) prior to introduction into the TRE17(long)/NIH3T3 cell line.

Antibodies and reagents

Antibodies against p65 (sc-372 and sc-8008), p100/52 (sc-7386), IKKα (sc-7218), IKKγ/NEMO (sc-8330), IκBα (sc-847 and sc-371), IκBβ (sc-945), HDAC2 (sc-7899), and actin (sc-8432) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against IKKα (#2682), IKKβ (#2678), IKKγ/NEMO (#2695), phospho-serine 536 p65 (#3031), phospho-IκBα (#9246) and phospho-IκBβ (#9245) were purchased from Cell Signaling Technology. Affinity purified anti-TRE17 antibody was generated against the N-terminus of TRE17. TNFα (210-TA) and LIGHT was purchased from R&D Systems.

Cell fractionation and electrophoretic mobility shift assays

Cells were harvested in ice cold PBS, then resuspended in hypotonic lysis buffer (10 mM HEPES, 0.1 M EDTA, 10 mM KCl, pH 7.9), and supplemented with 1 mM DTT, 5 mM β-glycerophosphate, and protease inhibitors) and incubated for 30 min on ice. Nonidet P-40 was added to a final concentration of 0.1%, and extracts were incubated for 5 min at room temperature. Samples were centrifuged at 375 × g for 10 min at 4°C. The supernatant (cytoplasmic fraction) was collected, and the cell pellet (containing nuclei) was resuspended in ice cold nuclear extraction buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM β-glycerophosphate, and protease inhibitors), incubated for 30 min on ice, then centrifuged at 18,000 × *g* for 20 min at 4°C. The supernatant containing nuclear proteins was used for EMSA or western.

EMSA was performed as described (Ye *et al.*, 2010). Antibodies for supershift assays were purchased from Santa Cruz Biotechnology: p65 (sc-372X), p50 (sc-7178X), RelB (sc-226X), and p52 (sc-7386X). For supershift assays, antibodies were incubated with nuclear extracts for 40 min prior to the addition of labeled oligonucleotide. Complexes were separated by electrophoresison 5% native polyacrylamide gels, and subjected to autoradiography.

Confocal immunofluorescence microscopy

Cells were seeded, transfected, and treated as previously described (Martinu *et al.*, 2004). Samples were viewed on a Zeiss confocal microscope with LSM510 software, using excitation wavelengths of 488 nm (FITC), 546 nm (Cy3), or 358 nm (DAPI).

Immunoprecipitations

Cells were lysed in Lysis Buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 5 mM β-glycerophosphate, 10% glycerol) supplemented with leupeptin, aprotinin, pepstatin, and 10 mM N-ethylmaleimide (NEM) and pelleted at 16,000 × g for 10 min at 4°C. The clarified supernatant was immunoprecipitated with the indicated antibody for 4 hours to overnight. Beads were washed three times in Lysis Buffer then separated by SDS-PAGE and blotted as indicated.

In vitro kinase assays

Cells were treated as indicated, then lysed in Lysis buffer. Extracts were immunoprecipitated with anti-IKKα (Santa Cruz Biotechnologies, Inc; sc-7218) overnight at 4°C, then washed three times in Lysis buffer, and twice in kinase buffer (25 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, pH 7.4). Immunoprecipitates were incubated at 30°C for 30 minutes in kinase buffer containing 10 μCi [γ-^{32}P]-ATP and substrate. GST fusions of IκB or a fragment of p65 encompassing amino acids 354–551 (Sakurai *et al.*, 1999) were purified from *E. coli*; 0.5 μg of each was used as substrate. Reactions were terminated by boiling in sample buffer, and subjected to SDS-PAGE and autoradiography.

Silencing of IKK and TRE17

SMARTpool siRNAs targeting IKKα (#M-003473-02), IKKβ (#M-003503-03), NEMO (#M-003767-02), and TRE17 (M-006096-03) were purchased from Dharmacon RNA Technologies (Lafayette, CO). As a negative control, non-specific siRNA (#D-001210) was used. HeLa cells were subjected to two rounds of transfection with the indicated siRNAs using Oligofectamine (Invitrogen) according to manufacturer instructions. Cells were subsequently transfected and treated as indicated.

Tumorigenesis in nude mice

Mouse procedures were performed in accordance with the institutional animal care and use committee of the Children's Hospital of Philadelphia. Nude mice (Jackson Laboratories, Bar Harbor, ME, USA) were fed water with dox (1 mg/ml containing 5% sucrose) for 1–2 weeks before xenografting. NIH3T3 cells were pre-treated with dox for 1 day before subcutaneous injection (2.5E6 cells per site), using 4–7 mice per cell line, per experiment. Dox was maintained in the drinking water.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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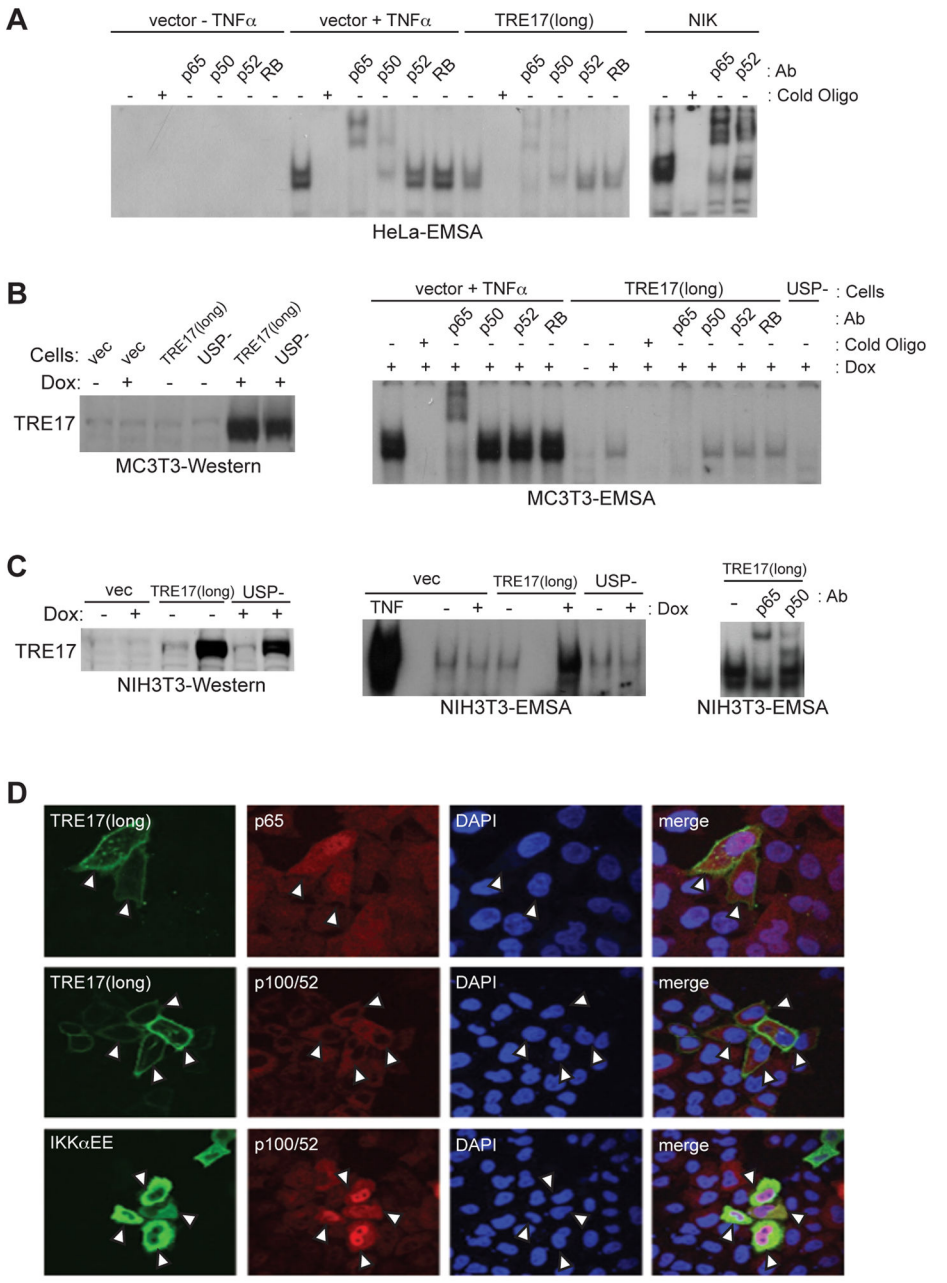

Figure 1. TRE17 activates the canonical NFκB pathway

(A) HeLa cells were transiently transfected with HA-TRE17(long), NIK, or control vector, and treated with TNFα (10 ng/ml for 30 minutes) where indicated. Nuclear extracts were prepared and subjected to EMSA. Unlabelled oligonucleotide (cold oligo) and antibodies (Ab) against the specific NFκB subunits were added where indicated. RB, RelB. (B) Stable MC3T3 cell lines expressing HA-TRE17(long), a USP-inactive point mutant (USP-), or control vector in a doxycyclin (dox)-inducible manner were subjected to immunoblotting with TRE17 antibody (left). Nuclear extracts were prepared from the cell lines treated as specified, then subjected to EMSA/supershift assays (right). (C) Stable NIH3T3 cell lines expressing HA-TRE17(long), TRE17(long)/USP-, or control vector were blotted with

TRE17 antibody (left). EMSA was performed using equal amounts of nuclear extract from the indicated cell line (middle). Supershift assays were performed using the indicated antibodies (right). (D) HeLa cells were transfected with GFP-TRE17(long) or a constitutively active phospho-mimetic mutant of IKKα, then starved. Samples were subjected to immunofluorescence confocal microscopy, using antibodies for p65 and p100/p52. Arrowheads indicate transfected cells.

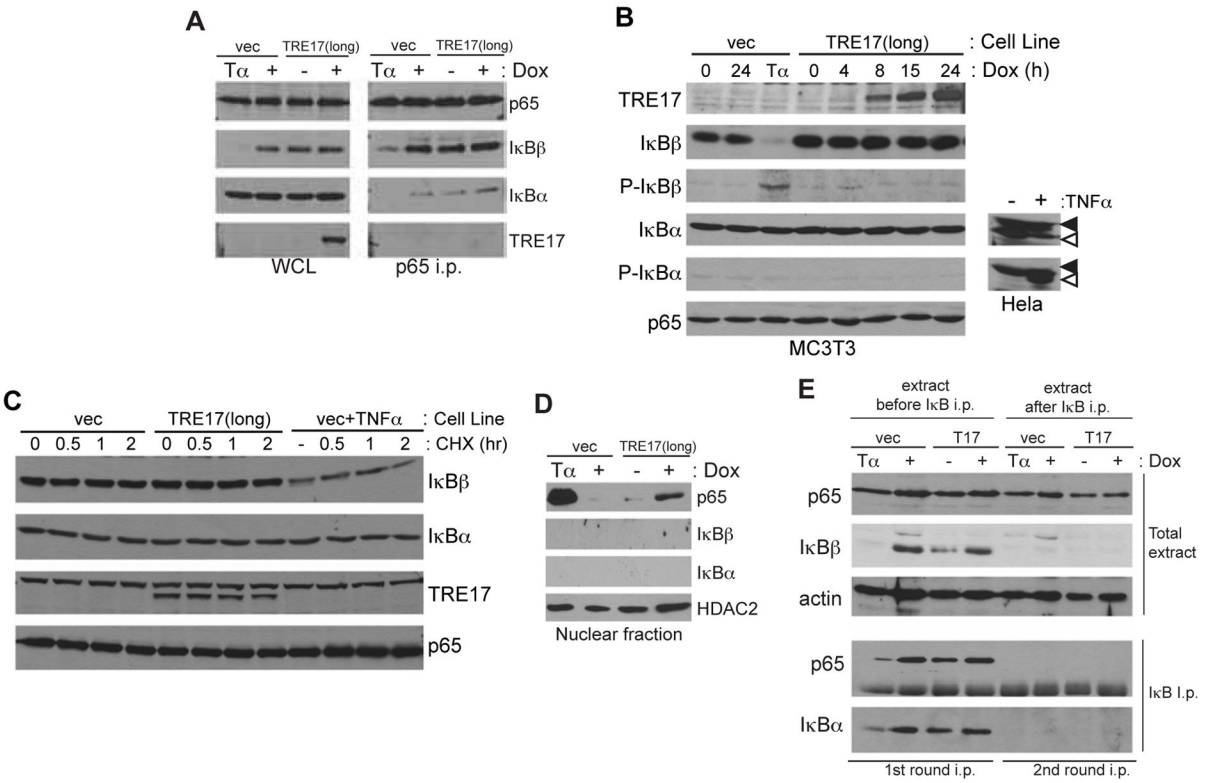

Figure 2. TRE17 activates classical NFκB independently of IκB phosphorylation and degradation

(A) MC3T3 cell lines were treated with dox as indicated. Whole cell extracts (left) or anti-p65 immunoprecipitates were subjected to immunoblotting with the indicated antibodies. (B) TRE17(long)/MC3T3 cells were treated with dox for various times, then subjected to immunoblotting with the indicated antibodies. HeLa extracts were probed with IκBα and phospho-IκBα (right). Open arrowheads indicate migration of IκBα and phospho-IκBα; black arrowheads indicate non-specific crossreactive band. (C) Control or TRE17(long)MC3T3 cell lines were treated with cycloheximide (CHX; 25 μg/ml) for the indicated times, in the absence or presence of TNFα as indicated. Extracts were blotted with the indicated antibody. Note that in cells treated with TNFα for 2 hr in the absence of CHX (lane 9), IκBβ re-synthesis starts to occur. (D) MC3T3 cell lines were treated as indicated, and subjected to cell fractionation. Nuclear extracts were immunoprecipitated with anti-p65, then blotted for IkB proteins. HDAC2 blotting of total nuclear extracts confirmed that equal amounts of protein were used. (E) Control or TRE17(long)-expressing (T17) MC3T3 cells were treated with dox or TNFα (Tα), then immunoprecipitated simultaneously with anti-IκBα and -IκBβ. Supernatants were blotted directly, or subjected to a second round of IκB immunoprecipitation. Both IκB α and IκBβ were efficiently immunodepleted after the first round of immunoprecipitation (note that IκBβ was monitored by blotting extracts after immunodepletion rather than directly in the immunoprecipitates, since it co-migrated with rabbit IgG).

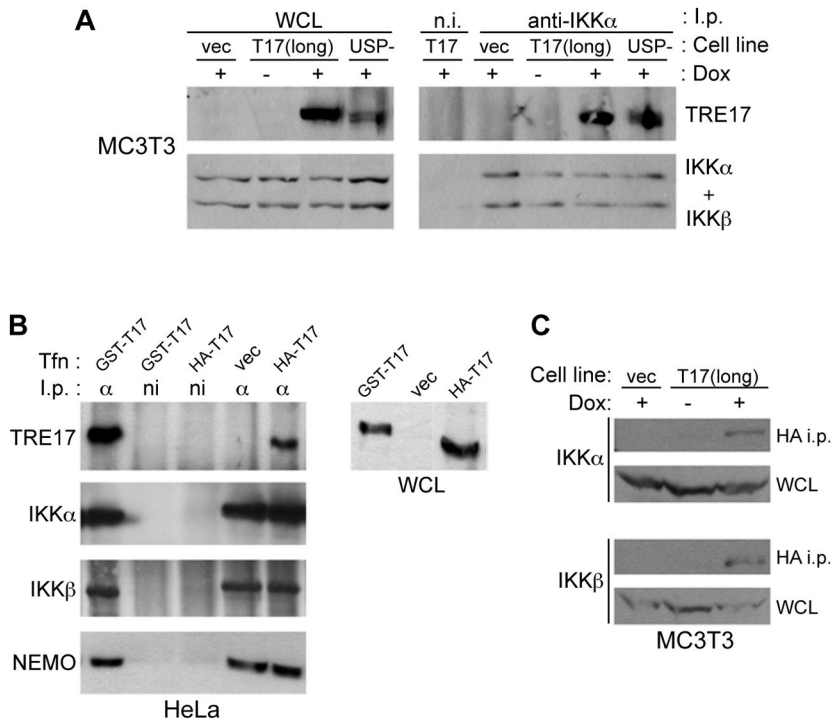

Figure 3. TRE17 co-immunoprecipitates with IKK

(A) MC3T3 cell lines expressing vector, TRE17(long) (denoted T17 or TRE17(long)), or TRE17(long)/USP- (USP-) were treated with dox as indicated. Cell extracts were immunoprecipitated with anti-IKKα or non-immune (n.i.) antibody, then blotted back for TRE17, IKKα, and IKKβ. WCL, whole cell lysate. (B) HeLa cells were transfected with HA- or GST-tagged TRE17(long), then immunoprecipitated with IKKα (α) or non-immune (ni) antibody. Samples were blotted for TRE17, IKKα, IKKβ, and NEMO. (C) Control (vec) or HA-TRE17(long)-expressing MC3T3 cell lines were subjected to immunoprecipitation using anti-HA antibody, then blotted back for IKKα and IKKβ.

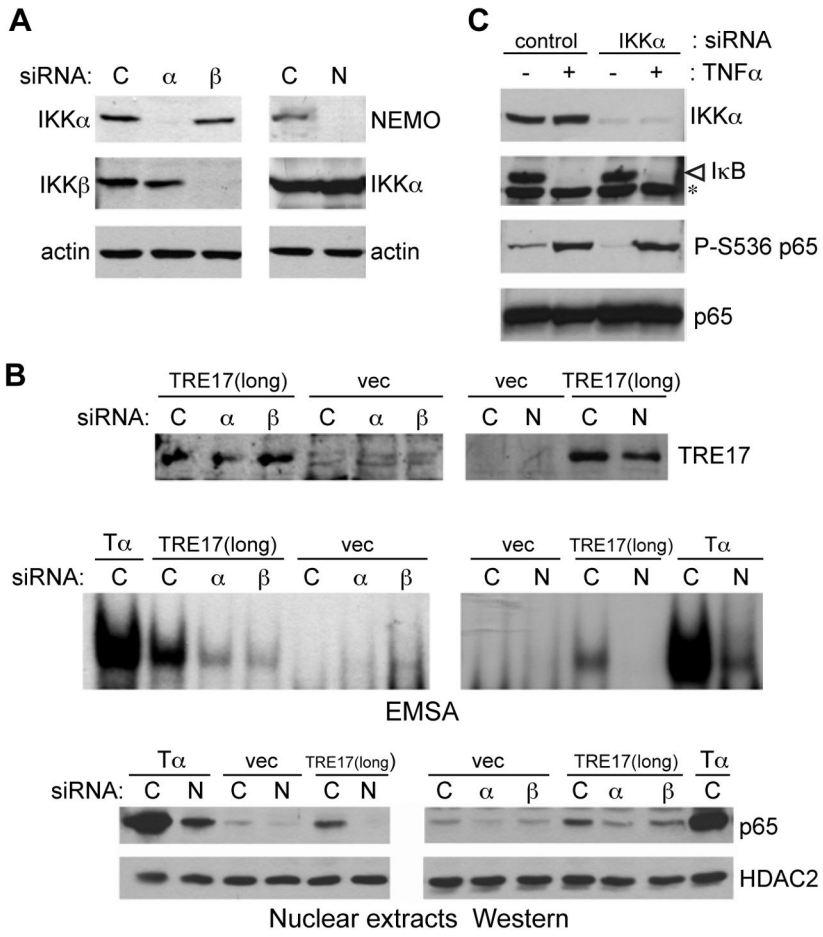

Figure 4. IKKα and IKKβ are both required for optimal activation of NFκB by TRE17

(A) HeLa cells were subjected to two rounds of transfection with SmartPool siRNAs targeting IKKα (α), IKKβ (β), NEMO (N), or a control non-targeting sequence (C). Whole cell lysates were blotted as indicated to confirm specific knockdown of the different IKK subunits. (B) HeLa cells treated with the indicated siRNA were transfected with HA-TRE17(long) or vector, and vector cells were treated with TNFα (Tα) where indicated. Samples were subjected to cell fractionation; cytosolic fractions were blotted for TRE17 to confirm uniform expression (top). Nuclear fractions were subjected to EMSA (middle), or blotted for p65 (bottom). HDAC2 was monitored as a loading control.

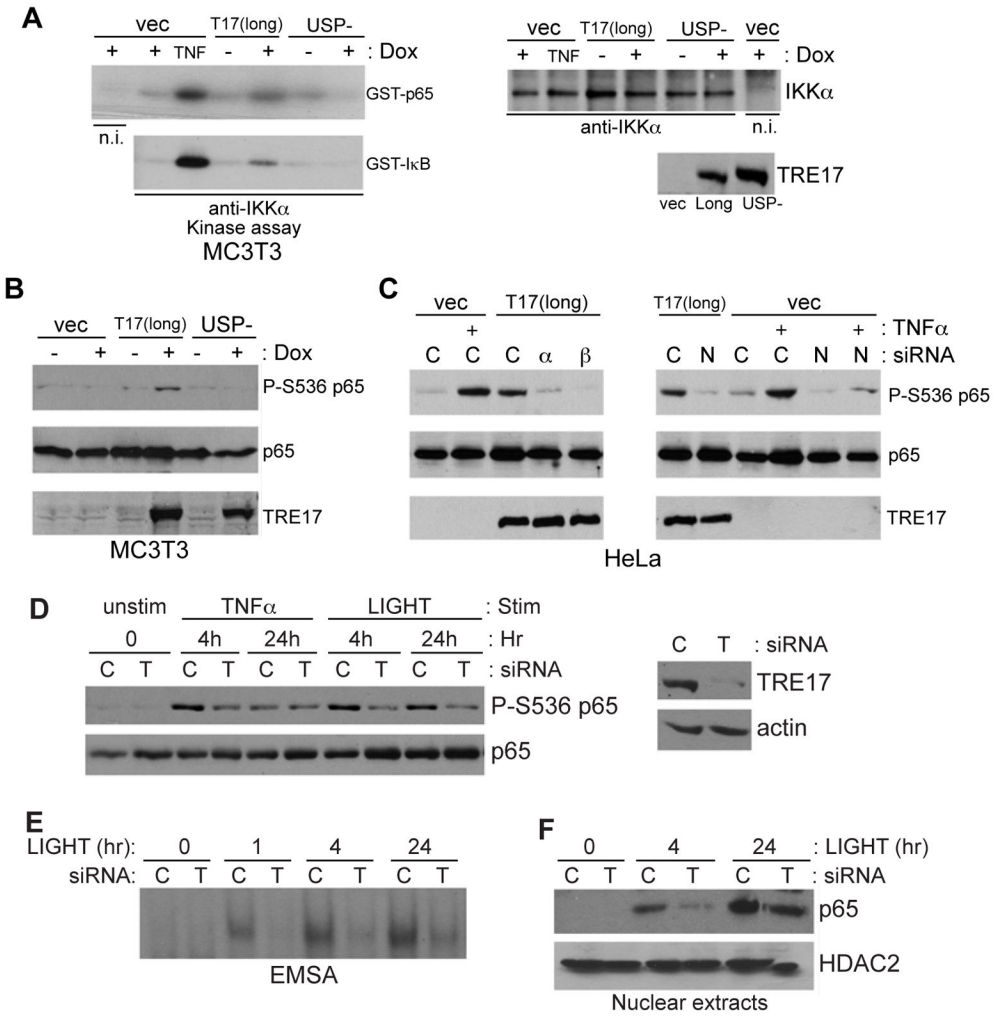

Figure 5. TRE17 stimulates IKK activity and p65 S536 phosphorylation in a manner dependent on both IKKα and IKKβ

(A) MC3T3 cells expressing control vector, TRE17(long) (T17(long)), or TRE17(long)/ USP- (USP-) were treated with dox or TNFα. Extracts were immunoprecipitated with anti-IKKα or non-immune (n.i.) antibody, then subjected to *in vitro* kinase assays using recombinant GST-p65 (top) or GST- I B (bottom) as a substrate. Half of the reaction was subjected to autoradiography (left panels). The other half was blotted for anti-IKKα to confirm uniform immunoprecipitation; whole cell extracts were blotted with TRE17 (right panels). (B) MC3T3 cell lines were treated with or without dox, and extracts were blotted as indicated. (C) HeLa cells were with the indicated siRNA (IKKα, α; IKKβ, β; NEMO, N; and control, C). Samples were then transfected with vector or HA-TRE17(long), and treated with TNFα. Extracts were blotted as indicated. (D) HeLa cells were transfected with siRNA against TRE17 (T) or control siRNA (C), then stimulated with TNFα or LIGHT (100 ng/ ml). Extracts were blotted as shown. (E and F) Nuclear extracts were prepared from HeLa cells transfected with TRE17 (T) or control (C) siRNA, then subjected to EMSA (E) or blotting for p65 and HDAC2 (F).

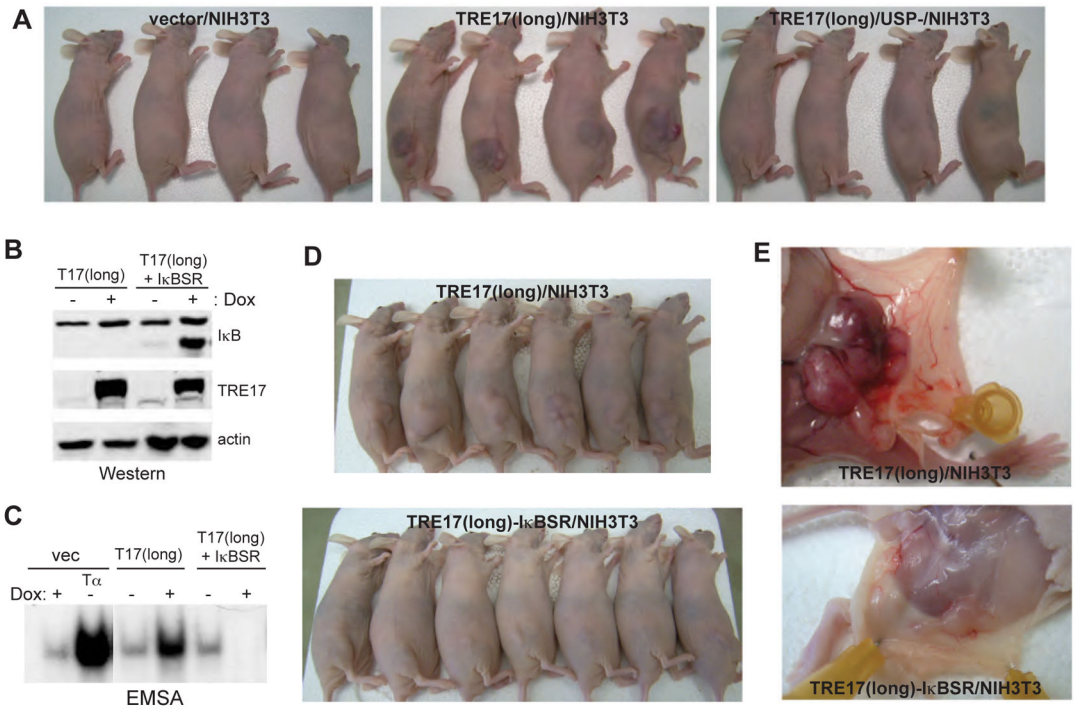

Figure 6. TRE17-induced tumorigenesis is dependent on USP activity and NFκB

(A) Nude mice were injected subcutaneously with stable NIH3T3 cell lines expressing vector, TRE17(long), or TRE17(long)/USP-. Animals are shown 11 days post-injection. (B) Stable NIH3T3 cell lines expressing TRE17(long) alone or together with IκBSR were treated with dox, then subjected to immunoblotting with the indicated antibodies. (C) EMSA was performed using the indicated cell lines, treated with dox or TNFα (Tα) as shown. (D) Nude mice were subcutaneously injected with TRE17(long) (top) or TRE17(long)-IκBSR cells (bottom). (E) Extensive tumor vascularization was observed in mice injected with TRE17(long)/NIH3T3 but not TRE17(long)-IκBSR/NIH3T3.