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TRAF2 is an NF-κB activating oncogene in epithelial cancers

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Abstract

Aberrant NF-κB activation is frequently observed in human cancers. Genome characterization efforts have identified genetic alterations in multiple components of the NF-κB pathway, some of which have been shown to be essential for cancer initiation and tumor maintenance. Here using patient tumors and cancer cell lines, we identify the NF-κB regulator, TRAF2 as an oncogene that is recurrently amplified and rearranged in 15% of human epithelial cancers. Suppression of TRAF2 in cancer cells harboring *TRAF2* copy number gain inhibits proliferation, NF-κB activation, anchorage-independent growth and tumorigenesis. Cancer cells that are dependent on TRAF2 also require NF-κB for survival. The phosphorylation of TRAF2 at serine 11 is essential for the survival of cancer cells harboring TRAF2 amplification. Together these observations identify TRAF2 as a frequently amplified oncogene.

Keywords

TRAF2; NF-κB; cancer; 9q34 amplification

Introduction

Nuclear Factor kappa B (NF-κB) transcription factors play pivotal roles in immunity, inflammation, cell differentiation, proliferation and survival. In addition to its roles in immunity, constitutive NF-κB activity is frequently detected in both hematopoietic and epithelial cancers (1). In tumor cells, activation of NF-κB occurs in response to inflammatory stimuli within a tumor microenvironment, and cancers associated with chronic inflammation are dependent on NF-κB (2–7).

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Conflict of interest

RB and WCH are consultants for Novartis.

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Cancer genome characterization efforts have identified alterations in many components of the NF-κB pathway. Translocations and mutations of NF-κB regulators have been identified in several different cancer types. In particular, *CARD11* is both mutated and amplified in diffuse large B cell lymphomas, and *CYLD* and *A20* are tumor suppressor genes deleted in familial cylindromatosis and marginal zone B cell lymphomas, respectively (8–12). Other NF-κB components such as *CD40*, *NIK*, *NFKB1*, *NFKB2* are amplified in multiple myeloma (8, 13–15). In solid tumors, amplification, somatic mutations, chromosomal translocations of *IKBKA*, *IKBKE* and *IKBKB* are observed in breast and prostate cancers respectively (16–18). Moreover, NF-κB activity is essential in KRas-driven lung and pancreatic cancer progression that occur in a p53-deficient background (19–22). Similarly, TRAF6 is an amplified oncogene present in non-small cell lung cancers with activated RAS (23), and loss of the tumor suppressor *DAB2IP* contributes to prostate cancer progression in part through activating NF-κB signaling (24). These observations implicate aberrant NF-κB signaling in the initiation or progression of many types of human cancers.

TRAF2 is an adaptor molecule that assembles active NF-κB signaling scaffolds. After TNF receptor engagement, TRAF2 forms multimeric complexes with several intracellular proteins including CIAP1, RIPK, TANK, and TAK1, initiating a kinase cascade that activates NF-κB and JNK (25, 26). One key function of TRAF2 is to facilitate Lys63 ubiquitination of components in these scaffolds (27). TRAF2-mediated Lys63 ubiquitination is essential for the recruitment of the canonical IKK complex, the central mediator of NF-κB activation.

Several studies suggest that TRAF2 plays an important role in cancer. In Ras-transformed cells, TRAF2 promotes resistance to stress-induced apoptosis (28). Similarly, TRAF2 also facilitates resistance to MAPK pathway inhibitors in BRAF V600E mutant melanoma (29). We recently identified TRAF2 as a substrate of the IKKε breast oncogene (30). IKKε phosphorylates TRAF2 at Ser11 to activate NF-κB and promote malignant transformation. Here we report that TRAF2 is amplified in a substantial fraction of human epithelial cancers where it functions independently of IKKε to induce tumorigenicity.

Results

TRAF2 is amplified in a substantial fraction of human epithelial cancers

In prior work, we identified TRAF2 and the tumor suppressor CYLD as key effectors in IKKε driven tumorigenesis in breast cancer (30, 31). We found that expression of TRAF2 could replace IKKε to confer anchorage independent growth in NIH3T3 cells and immortalized human embryonic kidney cells (HA1EM) in a manner that is dependent on TRAF2 Ser11 phosphorylation, an activity that promotes NF-κB activation (Supplementary Figure 1A). To determine whether genetic alterations involving *TRAF2* occur in human cancers, we analyzed genome-wide somatic copy number alterations in 3131 cancer samples including 2520 carcinomas and 611 cancer cell lines (32). We identified a focal region of recurrent amplification (9q34) that encompasses the *TRAF2* locus. We found increased copy number of *TRAF2* in 15.1% of epithelial cancers and 13.1% of all human cancers across multiple tissue types including breast, lung, colorectal, gastric, melanoma, ovarian, and esophageal cancers (Figure 1A). In contrast to broad regions of amplification that include

more than half of the chromosome arm, *9q34* is significantly amplified (q = 0.11) across all lineages and TRAF2 lies within a peak region containing genes most likely to be the targets of these amplifications (32). To validate this finding, we performed FISH on a panel of cancer cell lines using a *TRAF2*-specific fosmid probe and confirmed increased *TRAF2* copy number in six cancer cell lines classified by GISTIC as harboring a 9q34 amplification (RKO, KYSE30, KYSE510, MDA-MB-453, H2009, SUM52) in comparison to two copy neutral cell lines (A2780, AU565) (Table 1). We further observed that *TRAF2* is rearranged to alternative chromosomes in the six cancer cell lines that harbor *TRAF2* amplification, and one additional cell line (MCF7) without *TRAF2* amplification (Supplementary Figure 2A). These observations suggest that *9q34* amplification and rearrangement drives *TRAF2* dysregulation in a subset of human cancers.

To determine whether 9q34 amplification influences TRAF2 expression, we analyzed 9q34 copy number and transcript levels in both the Cancer Cell Line Encyclopedia (CCLE) and The Cancer Genome Atlas (TCGA) datasets (33–35). We determined that median TRAF2 expression levels were increased in breast, ovarian and colon samples with 9q34 amplification in comparison to samples without 9q34 amplification in both the TCGA and CCLE data sets (Figure 1B). In contrast, samples that harbor 9q34 amplification did not exhibit increased IKKε expression in comparison to non-amplified samples, suggesting that TRAF2 amplification is unrelated to IKKε expression or dependency (Supplementary Figure 2B). Comparative marker selection analysis using the CCLE collection of cell lines with matched copy number and expression data likewise revealed that *TRAF2* transcript levels scored as the 5th most highly correlated with 9q34 amplification among 18,988 transcripts (Supplementary Table S1). We then profiled TRAF2 expression in a panel of cancer cell lines and found that TRAF2 protein levels were elevated in all cell lines harboring 9q34 amplification in comparison to cell lines lacking this amplification (Figure 1C). *TRAF2*-amplified cell lines also exhibited higher levels of TRAF2 Ser11 phosphorylation, suggesting that 9q34 gain facilitates both TRAF2 expression and activation (30, 36). IKKε expression levels and dependency, however, did not correlate with TRAF2-amplification in these cell lines, suggesting that IKKε-independent mechanisms also facilitate TRAF2 amplification, overexpression and activation (Figure 1C, Supplementary Figure 1B). Combined, these findings indicate that *TRAF2* is frequently altered and overexpressed in cancers harboring 9q34 amplification and implicate a role for *TRAF2* as a driver oncogene in 9q34 amplified cancers independently of IKKε expression or amplification.

9q34 amplification confers TRAF2 dependency

We next determined whether cell lines that harbor increased copy number of *TRAF2* depend on *TRAF2* for proliferation and/or survival. We suppressed *TRAF2* expression in a panel of eighteen cancer cell lines derived from various lineages that either harbor or lack *TRAF2* copy number gain and measured proliferation, apoptosis, and anchorage-independent growth (Supplementary Figure 3). Twelve cell lines (KYSE510, KYSE30, RKO, EFO21, EFM19, IGROV1, LS513, SUM52, KYSE150, H2009, H1568, and T47D) with increased *TRAF2* copy number exhibited decreased proliferative potential in a 7 day proliferation assay when we suppressed *TRAF2* with two distinct *TRAF2*-specific shRNA (Figure 2A, Supplementary Figure 3). In contrast, suppression of *TRAF2* failed to affect the proliferation of cell lines

(A2780, LOVO, NCI-H661, AU565, OV90, and SW48) that lacked *TRAF2* amplification (Figure 2A). In long term proliferation assays, we found that suppression of *TRAF2* in cells harboring increased *TRAF2* copy number (KYSE30, RKO, SUM52, KYSE150, H2009, led to a mean 41.6% decrease in the doubling time of such cells (Figure 2B). In contrast, we failed to detect evidence of apoptosis after *TRAF2* suppression (Supplementary Figure 4). Depletion of *TRAF2* in three cell lines that exhibited delayed proliferative capacity (KYSE30, RKO, SUM52) also inhibited anchorage-independent growth (Figure 3A). We further found that *TRAF2* suppression in three 9q34 amplified cell lines, KYSE30, RKO and H2009 cells inhibited tumorigenesis in immunodeficient mice (Figure 3B).

To confirm that the proliferative defect induced by our *TRAF2*-directed shRNAs was due to specific *TRAF2* suppression, we transduced *TRAF2*-dependent cells with both an shRNA targeting the 3′UTR of TRAF2 (shTRAF2 #3) and a TRAF2 cDNA lacking the 3′ UTR. In both RKO and MCF7 cells, forced expression of TRAF2 restored TRAF2 protein levels and rescued the proliferative defect (Figure 3C, 3D and 4F). Combined, these findings demonstrate that epithelial cancers with 9q34 amplification depend on aberrant TRAF2 expression.

TRAF2 dependency is conferred through NF-κB activation

TRAF2 promotes NF-κB activation through its ability to facilitate recruitment and activation of the canonical IKK complex (37). To assess the consequences of TRAF2 amplifications on NF-κB activity, we stably expressed a NF-κB reporter in cell lines that do and do not harbor 9q34 amplification. In comparison to copy-neutral cell lines, the mean NF-κB activity was higher in the TRAF2 amplified cells by 5.3 fold (Figure 4A). Suppression of *TRAF2* in RKO and cells also resulted in decreased NF-κB activity as measured by the stable NF-κB reporter (Figure 4B). We further evaluated the expression of several NF-κB target genes including *IL6*, *CIAP1*, *CIAP2*, *CCND1*, and *cFLIP*, and observed that the expression of these genes were decreased in RKO cells. Under conditions where TRAF2 overexpression rescues *TRAF2* dependency, we found that TRAF2 expression restored the expression of these NF-κB target genes (Figure 4C).

To determine whether *TRAF2* dependent cell lines are also dependent on canonical NF-κB activation, we introduced a dominant interfering allele of IκBα (IκBα super repressor) in cells with and without 9q34 copy number gain and assessed cell proliferation. We found that *TRAF2* dependent cell lines were particularly sensitive to inhibition of NF-κB activity, as proliferation was inhibited by up to 7-fold in comparison to control cells (Figure 4). We note that we also found cell lines that lacked the 9q34 amplification, which depended on NF-κB, suggesting that there are other TRAF2-independent mechanisms that activate NF-κB signaling.

In prior work, we found that TRAF2 phosphorylation at Serine 11 promotes Lys63 ubiquitination, recruitment of the IKK complex and downstream NF-κB activation necessary for tumorigenesis (30). Since we observed elevated TRAF2 Ser11 phosphorylation in 9q34 amplified cells, we assessed whether overexpression of a TRAF2 S11A mutant is sufficient to rescue the proliferative defect induced by *TRAF2* suppression. We overexpressed both wildtype TRAF2 and the TRAF2 S11A mutant in RKO cells prior to

TRAF2 depletion with shRNAs (Figure 4E). We found that in contrast to wildtype TRAF2, TRAF2 S11A failed to rescue the proliferative defect after *TRAF2* depletion (Figure 4F). Collectively, these observations provide evidence that *TRAF2* is an oncogene that promotes NF-κB activation that is essential in human cancers that harbor 9q34 amplification.

Discussion

Here we identified TRAF2 as a bona fide oncogene that is essential for the proliferation and transformation of several types of epithelial cancer cell lines. By analyzing more than 3000 primary tumor samples, we found recurrent amplifications at 9q34 involving *TRAF2* that are present in 15% of human epithelial cancers across multiple lineages. Although other candidate oncogenic driver genes may be present at 9q34, we demonstrated that human cancer cell lines harboring *TRAF2* amplifications are dependent on *TRAF2* expression as suppression of *TRAF2* in these cells inhibited proliferative capacity, anchorage independent growth, and tumorigenesis. Moreover, although we previously showed that TRAF2 expression is required for IKKε to transform mammary epithelial cells (30), TRAF2 promotes transformation in NIH3T3 cells and immortalized HA1EM cells independently of IKKε. Taken together, these observations support the notion that *TRAF2* is an oncogene.

Our observations provide evidence that TRAF2 can contribute to cell transformation in two ways. As a substrate of IKKε in cancer cells that harbor amplification or overexpression of IKKε, TRAF2 is required to mediate the activation of NF-κB signaling induced by IKKε amplification (30). Here we demonstrate that in other tumors where 9q34 is amplified, TRAF2 dysregulation occurs as a consequence of increased copy number of TRAF2. We observed increased TRAF2 Ser11 phosphorylation in cells that harbor TRAF2 amplifications. Although such cells do not always exhibit increased IKKε expression, the basal levels of IKKε may suffice to induce this phosphorylation. Alternatively, other kinases may also induce TRAF2 phosphorylation in cells that harbor TRAF2 amplifications (30, 36, 38). Thus, TRAF2 induces cell transformation through both IKKε-dependent and IKKε-independent mechanisms.

Genetic alterations of the NF-κB pathway have recently emerged in different cancer types (39). In addition to *TRAF2* amplifications identified here, TRAF2 mutations are detected in diffuse large cell lymphoma, multiple myeloma and marginal zone lymphomas (8, 11, 14). More recently, focal of amplifications of TRAF6, a structural and functional counterpart of TRAF2, were identified in non-small cell lung cancer (23). Like TRAF2, TRAF6 overexpression induces malignant transformation and TRAF6 depletion inhibits lung cancer cell proliferation and tumorigenesis. We also identified lung cancer cell lines that exhibit TRAF2 dependency, suggesting that TRAF2 may share a similar role as TRAF6 in driving the pathogenesis of non-small cell lung cancers.

TRAF2 regulation and activation involves a dynamic interplay of multiple postranslational events. Phosphorylation of TRAF2 occurs at multiple residues including Thr117, Ser55 and Ser11 and is mediated by PKC and IKKε kinases respectively (30, 36, 38). Both PKC and IKKε-induced phosphorylation of TRAF2 results in Lys63-ubiquitination of TRAF2 required for recruitment and activation of the IKK complex (30, 36, 38). We demonstrated

that TRAF2-dependent cancer cells have increased levels of TRAF2 Ser11 phosphorylation, suggesting that IKKε is important for TRAF2 transformation. Coincidentally, TRAF2 promotes Lys63 ubiquitination of IKKε, and both TRAF2 Ser11 phosphorylation and IKKε Lys63-ubiquitination are required for IKKε oncogenesis in breast cancer (40). This feed-forward regulation suggests a co-dependency between TRAF2 and IKKε in cancer cells with either *TRAF2* or IKKε amplification.

Since TRAF2 plays a key role in the assembly of protein complexes necessary for canonical NF-κB activation, TRAF2 amplification and overexpression would naturally lead to constitutive NF-κB activation. Upon activation, TRAF2 itself undergoes Lys63 ubiquitination and also recruits CIAP1, RIPK, TANK, and TAK1 (25, 26). This activity is a prerequisite for the phosphorylation and activation of the IKK complex, which is essential for the removal of IκBα, the central inhibitor of NF-κB transcription factors. TRAF2-amplified cells are dependent on both TRAF2 and NF-κB activation for proliferation. Thus, dysregulation of TRAF2 by increased copy number facilitates a continuous proliferative signal through the release of NF-κB.

We demonstrate that *TRAF2* is essential for the proliferation of many epithelial cancers that harbor *TRAF2* amplification. *TRAF2*-dependent cancer cells also appear to be dependent on NF-κB activity, suggesting that TRAF2 amplification and overexpression may underscore constitutive NF-κB activity in carcinomas. Identification of TRAF2 dependency may therefore provide a targeted therapy in NF-κB activated or 9q34 amplified cancers.

Materials and Methods

Antibodies and plasmids

The antibodies used include: HSP90, Lamin A/C, p50, p52/p100, TRAF2 (Cell Signaling Technologies, Danvers, MA, USA), p65 (Abcam, Cambridge, MA, USA), and β actin (Sigma-Aldrich, St. Louis, MO, USA). The phospho-TRAF2 (Ser11) was a gift from Dr. Hasem Habelhah.

pWZL-TRAF2, pLEX-V5-TRAF2, pLKO-shLACZ, pLKO-shTRAF2#1 and pLKO-shTRAF2#2 lentiviral constructs are previously described(30). Additional shRNA constructs were obtained from the RNAi Consortium (Broad Institute, Cambridge, MA, USA) and include pLKO-shTRAF2#3 (TRCN0000004572). The plasmid for the NF-κB super-repressor (pWZL-IκBαMUT) was previously described (18).

Cell Culture, Transfection, Subcellular Fractionation

HEK293T, MCF7, MDA-MB-453 and RKO cells were obtained from ATCC (Manassas, VA, USA) and were grown in DMEM containing 10% FBS. A2780, EFM19, EFO21, H1568, H2009, IGROV1, KYSE30, KYSE510, LOVO, LS513, NCIH661, OV90, SW48, and T47D were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI1640 containing 10% FBS. KYSE150 were maintained in RPMI1640:HamsF-12 (1:1) with 2% FBS. SUM52 cells were maintained in HamsF-12:MEGM (1:1) with 10% FBS. Transfection experiments were performed using Fugene (Roche, Indianapolis, IN, USA). Subcellular fractionation experiments were performed as previously described (19).

FISH analysis

Fluorescent in situ hybridization (FISH) analysis is previously described (41) and was performed using a TRAF2 specific fosmid probes (PR11-769N4) and a Chromosome 9 centromeric reference probe on metaphase spreads of indicated cell lines.

NF-κB reporter assays and quantitative RT-PCR

For NF-κB luciferase reporter assays, cell lines were transduced with a lentiviral construct containing a 10xNF_B response element cloned into the PstI and NheI sites of the previously described 7TFP reporter. (42) NF-κB activity was measured using the Dual-Glo Luciferase assay (Promega, Madison, WI, USA) 4 days post-transduction. Luciferase values were normalized to CTG values to yield relative NF-κB activity. Quantitative RT-PCR for NF-κB target genes was conducted as described (18).

Tumorigenicity assay

2×10^6 cells were subcutaneously implanted into immunodeficient mice (Balb/c Nude, Charles River Laboratories, Wilmington, MA USA) anesthetized with isofluorane. Three mice were used per group, and three implantation sites were made per mouse. Tumors were measured at 21 d after implantation.

Viability and proliferation measurements

Relative proliferation was measured 7d post-infection using Cell-Titer Glo (Promega, Madison, WI, USA) in triplicate. Apoptosis was assessed with Annexin V/ PI staining (BD, Franklin Lakes, NJ, USA) and flow cytometric analysis 6d post–infection according to the manufacturer's protocol. Additional proliferation assays were performed my measuring duplicate population doubling (PD) using a Vi-Cell counter every 7d for 21 d. PD were defined as [$\log_2$(cells counted/cells plated)].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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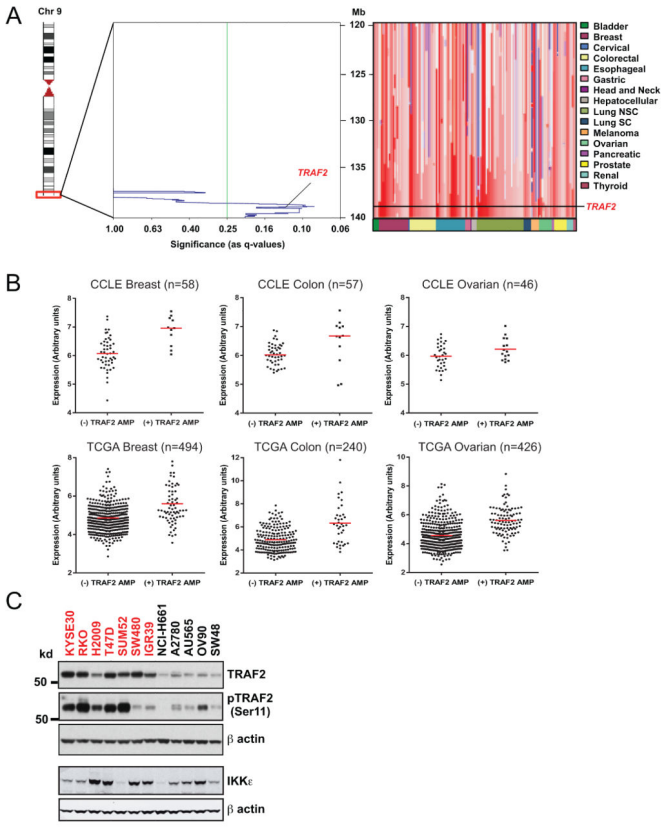

Figure 1. TRAF2 is amplified in human cancers

(A) Copy number profiles at the 9q34 locus. Left panel, Significance of *TRAF2* amplifications across 3131 cancer samples was determined by GISTIC (genomic identification of significant targets in cancer) and shown as q-values (false discovery rate corrected significance of amplification frequency). Right panel, copy number profiles for 50 cancer samples harboring *TRAF2* amplifications. Genomic location and the TRAF2 locus are indicated on the vertical axis and lineages on the horizontal axis are denoted by color. Copy number gain and loss are indicated as red and blue signals respectively. (B) Scatterplots of TRAF2 mRNA expression in TRAF2 amplified or non-amplified primary breast, ovarian, and colon tumors in TCGA (33–35) and cell lines in CCLE (43). These data are log2-transformed signal intensities with the median of each sample set denoted by a red line. (C) Immunoblot of TRAF2 and Ser11 phosphorylated TRAF2, and IKKε in 9q34 amplified (red) and copy neutral (black) cell lines. β-actin is displayed as a loading control.

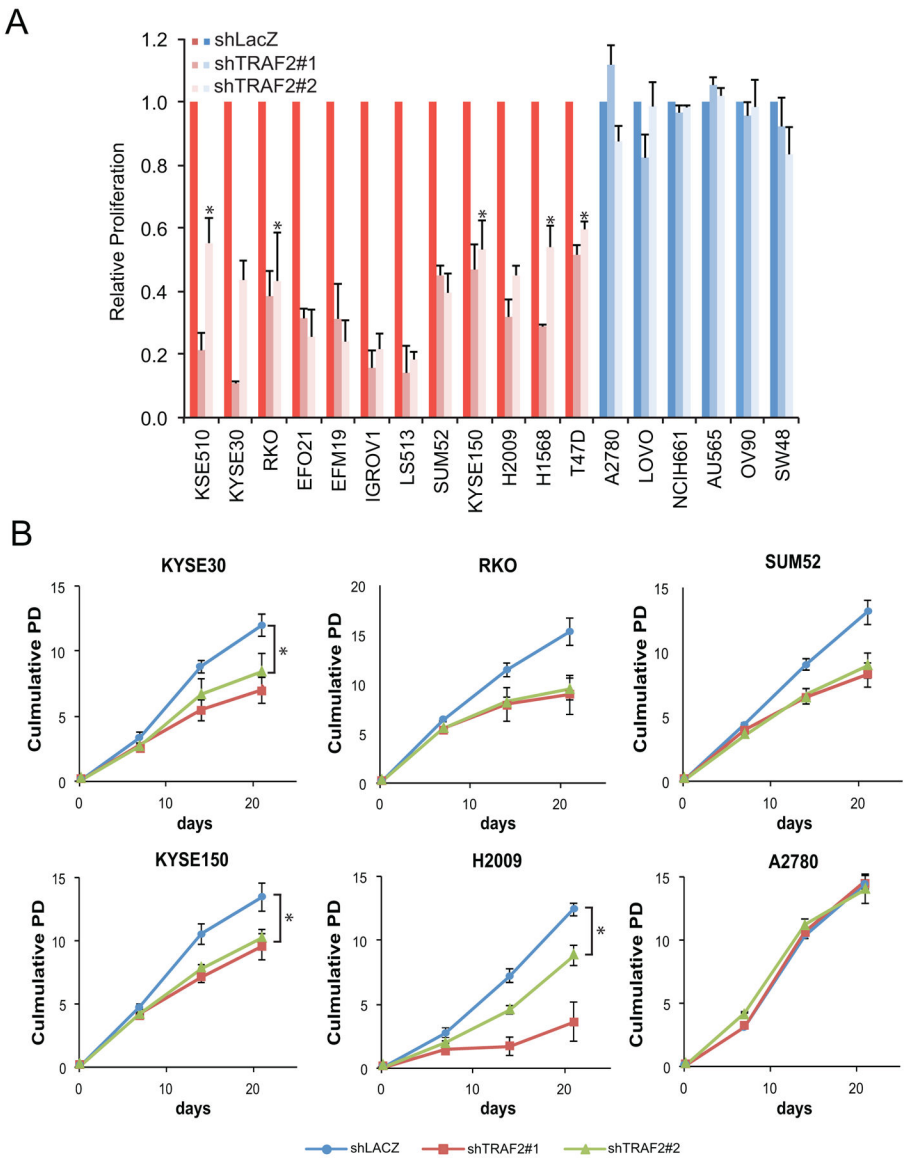

Figure 2. Cancer cells with *TRAF2* amplification depend on TRAF2 for proliferation

(A) Proliferation of cells harboring (red) and lacking (blue) *TRAF2* copy number gain after transduction with two distinct *TRAF2*-specific shRNA (shTRAF2#1 and shTRAF#2). Proliferation was normalized to cells expressing control shRNA (shLacZ). Results reported as mean ± SD of three experiments. * p<= 0.02 as calculated by a standard t test (B) Long-term proliferative capacity of cell lines following TRAF2 suppression. Population doubling (PD) of cells harboring (KYSE30, RKO, SUM52, KYSE50, and H2009) and lacking (A2780) *TRAF2* copy number gain after transduction with control shRNA (shLACZ), shTRAF2#1, or shTRAF2#2. Cells were assayed for 21d. * p<= 0.04 as calculated by a standard t test.

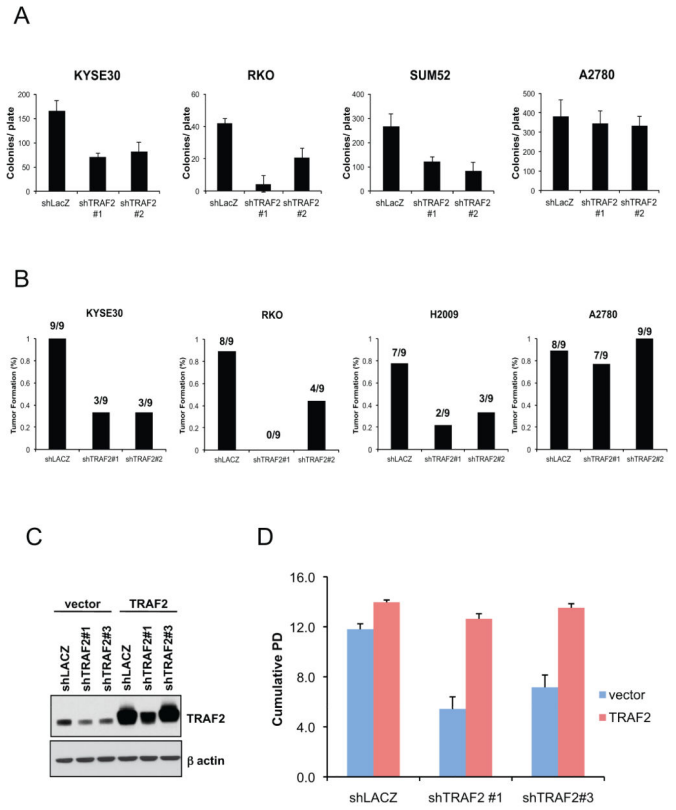

Figure 3. TRAF2 is essential for tumorigenicity in cancer cells with *TRAF2* amplification

(A) Anchorage-independent growth of cell lines harboring (KYSE30, RKO, SUM52) and lacking (A2780) *TRAF2* copy number gain. Cells were transduced with control shRNA (shLACZ) or two distinct *TRAF2*-specific shRNA (shTRAF2#1 and shTRAF2#2). Colony formation was measured after 21 d. p values were calculated by a standard t-test. (B) Tumorigenesis of cancer cells following *TRAF2* suppression. Indicated cells transduced with a control shRNA (shLACZ), shTRAF2 #1 or shTRAF2#2 were subcutaneously introduced into immunodeficient mice. Tumor formation was assessed after 21 d. (C) TRAF2 expression in MCF7 cells transduced to coexpress either control (shLACZ), shTRAF2#1 or a UTR-specific shTRAF2 (shTRAF2 #3) with TRAF2 or control (vector). (D) Long term proliferative capacity of MCF7 cells after TRAF2 suppression and overexpression. Cumulative population doubling (PD) of MCF7 cells from (C) 21d after transduction.

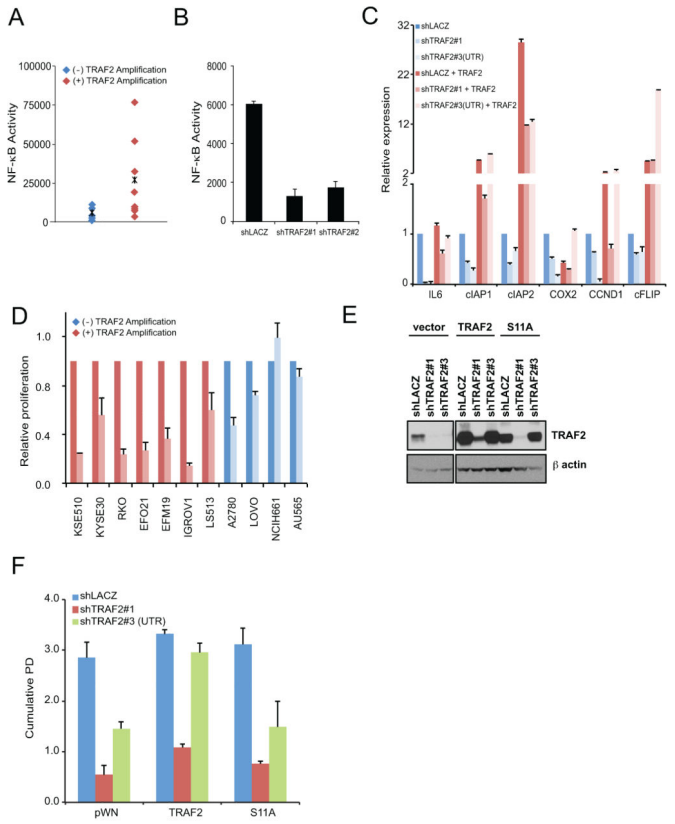

Figure 4. TRAF2 activates NF-κB in cancer cells with *TRAF2* amplification

(A) NF-κB activity in cancer cell lines with (RKO, KYSE30, H2009, SUM52, T47D, LS513, EFO21, EFM19) or without (A2780, NCIH661, OV90, SW48, BT474) *TRAF2* amplification. Cell lines were transduced with a stable NF-κB luciferase reporter and raw light united (RLU) activity was measured and normalized to baseline viability activity (B) NF-κB activity in RKO cells after *TRAF2* suppression. Stable NF-κB reporter RKO cells were transduced as indicated with *TRAF2*-specific shRNA and luciferase raw light unit (RLU) activity was measured and normalized to activity observed with control vector. Results reported as mean ± SD of three experiments. (C) NF-κB target gene expression in RKO cells following expressing TRAF2 specific shRNA (TRAF2#1 or shTRAF2 #3(UTR)) specific shTRAF2 (shTRAF2#3) with TRAF2 or control (vector). Relative expression was quantified by RT-PCR using ΔΔCT and normalized to levels observed with control (V5 and shLACZ) cells. (D) Proliferative capacity of cell lines after NF-κB inhibition. Cells line with (red) and without (blue) *TRAF2* amplification were transduced with the NF-κB super-repressor and proliferation was normalized to cells expressing control vector (Dark colors). Results reported as mean ± SD of three experiments. (E) TRAF2 expression in RKO cells coexpressing either control (shLACZ), shTRAF2#1 or a UTR-specific shTRAF2 (shTRAF2 #3) with TRAF2, TRAF2 S11A, or control (vector). (F) Long term proliferative capacity of RKO cells after TRAF2 suppression and forced expression of TRAF2 or TRAF2 S11A. Cumulative population doubling (PD) of RKO cells from (E) 21d after transduction.

Table 1

FISH analysis of TRAF2 in cancer cell lines

Cell line	TRAF2 Copy Number	9q34 Amplification	9q34 Rearrangement
KYSE30	4.1	+	+
RKO	3.8	+	+
MDA-MB-453	3.7	+	+
H2009	2.7	+	+
SUM52	2.6	+	+
MCF7	2.5	−	+
KYSE510	2.3	+	+
A2780	2.0	−	−
AU565	1.8	−	−