Molecular Cancer Therapeutics

TRAF2 Cooperates with Focal Adhesion Signaling to Regulate Cancer Cell Susceptibility to Anoikis

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Abstract

TRAF2, a RING finger adaptor protein, plays an important function in tumor necrosis factor (TNF)- and TNF-like weak inducer of apoptosis (TWEAK)-dependent signaling, in particular during inflammatory and immune responses. We identified a functional interaction of TRAF2 with focal adhesion (FA) signaling involving the focal adhesion kinase (FAK) in the regulation of cell susceptibility to anoikis. Comparison of TRAF2-proficient ($TRAF2^{+/+}$) versus TRAF2-deficient ($TRAF2^{-/-}$), and FAK-proficient ($FAK^{+/+}$) versus FAK-deficient ($FAK^{-/-}$) mouse embryonic fibroblasts and their matched reconstituted cells demonstrated that TRAF2 interacts physically with the N-terminal portion of FAK and colocalizes to cell membrane protrusions. This interaction was found to be critical for promoting resistance to cell anoikis. Similar results were confirmed in the human breast cancer cell line MDA-MB-231, where TRAF2 and FAK downregulation promoted cell susceptibility to anoikis. In human breast cancer tissues, genomic analysis of The Cancer Genome Atlas database revealed coamplification of TRAF2 and FAK in breast cancer tissues with a predictive value for shorter survival, further supporting a potential role of TRAF2–FAK cooperative signaling in cancer progression.

Introduction

Tumor necrosis factor (TNF)–associated signaling plays a determinant physiologic function in the regulation of proinflammatory and immune response, with a broad implication in multiple pathologic conditions (1). In particular, TNF exerts its functions through the activation of two distinct receptors, TNFR1 and TNFR2; these can activate canonical nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK; ref. 2). In addition, TNFR2 can activate the noncanonical NF-κB pathway leading to activation of genes that drive inflammation, and cell proliferation and survival, while TNFR1 can activate mechanisms leading to cell death via either apoptosis or necrosis mechanisms, depending on cellular context. A key player for TNFR1- and TNFR2 functions is the RING finger protein named TNF receptor–associated factor 2 (TRAF2), a member of the large TRAF family of adapter proteins that integrates intracellular signaling from plasma membrane receptors such as TNFR and Fn14 receptors to regulate diverse aspects of immune and inflammatory responses (3).

Several studies have shown that TRAF2 plays a role in carcinogenesis (4–6). TRAF2 regulation and activation involves a dynamic interplay of multiple posttranslational events and the detailed mechanisms during tumorigenesis remain partially understood (7, 8). In this study, we report a functional cooperation between TRAF2 and the focal adhesion (FA) network via direct interaction between TRAF2 and focal adhesion kinase (FAK; PTK2). FAK is a key regulator of FA signaling, activated via phosphorylation upon stimulation by integrins and a broad range of growth factors and chemokines (9, 10). Activation of FAK affects the conformational dynamics on C-terminal FAT (focal adhesion-targeting) domain and leads to differential phosphorylation of the tyrosine (Y) residue Tyr397 to create high-affinity binding sites for the SRC homology 2 (SH2) domain of Src kinases. This association triggers further phosphorylations and recruitment of numerous signaling and adapter proteins involved in cell–matrix interaction, cell survival, and cell locomotion. As a multidomain protein that changes conformations upon activation, FAK can act as an assembly platform for protein complexes or as a bridge between proteins (10). Here we provide functional evidence for a cooperation between TRAF2 and FAK in promoting cell resistance to anoikis, a form of apoptosis that occurs in anchorage-dependent cells triggered by cell detachment from the extracellular matrix (ECM). Furthermore, genomic analysis of public breast cancer databases revealed a coamplification of TRAF2 and FAK as predictive of poor survival probability supporting a relevance of TRAF2–FAK cooperative signaling for breast carcinogenesis.

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Materials and Methods

Cell culture

FAK-deficient and FAK-proficient ($FAK^{-/-}$ or $FAK^{+/+}$) mouse embryonic fibroblasts (MEF) were originally provided by Dr. Dusko Ilic (University of California, San Francisco, CA) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Corporation) supplemented with 100 μmol/L 2-mercaptoethanol, and penicillin/streptomycin, 10% fetal bovine serum (FBS), 1 mmol/L sodium pyruvate, 1% nonessential amino acids. MEFs with TRAF2-deficient and proficient ($TRAF2^{-/-}$ or $TRAF2^{+/+}$) were kindly provided by Dr. Tak W. Mak (Campbell Family Institute for Breast Cancer Research at Princess Margaret Cancer Centre, Toronto) and were described earlier (11). These cells were maintained in DMEM supplemented with 10% FBS, 1% nonessential amino acids and penicillin/streptomycin. Cell lines SYF, SYF $Src^{+/+}$, MDA-MB-231, and HEK293T (ATCC) were maintained in RPMI-1640 medium (Mediatech) or DMEM supplemented with 10% FBS, penicillin/streptomycin antibiotics and antimycotic solution. Cells were cultured at 37°C with 5% CO_2. Cell line use was limited to passage nine or lower and periodically authenticated by morphologic inspection and *mycoplasma* testing.

Protein knockdown by gene silencing

For knockdown of FAK (sc-35353) and TRAF2 (sc-36711), transfections were carried out using 100 nmol/L of small interfering RNA (siRNA) oligonucleotides incubated with Dharma-FECT1 (Thermo Fisher Scientific Inc.) in Opti-MEM I reduced serum medium (Invitrogen Corporation) according to the manufacturer's instructions (Santa Cruz Biotechnology).

For short hairpin RNAs (shRNA) experiments, pEBG-TRAF2 (GST) plasmid was provided by Dr. John M. Kyriakis (ref. 12; Addgene #21586). pBabe-GFP FAK wild-type (WT) and mutant FAK-F397 were obtained from Dr. David D. Schlaepfer (13). FAK N-terminal (1–1306nt) and C-terminal (2090–3156nt) were cloned from full-length pBabe-GFP FAK-WT by PCR into pEGFP-N2 vector. His-FAK was cloned from pBabe-GFP FAK-WT into pcDNA3.1/His A plasmid. Transfections were performed using Lipofectamine (Invitrogen Corporation) according to the manufacture's instruction.

Western blot and immunoprecipitation assay

Total cell extracts were used for Western blot and immunoprecipitation assays as previously described (14, 15). Blots were detected using the antibodies for anti-TRAF2 (Cell Signaling Technology, 1:1,000); anti-FAK (Millipore,; 1:500); anti-GST (Santa Cruz Biotechnology, 1:1,000), anti-Fn14 (R&D Systems, 1:2,000), anti-TNFα (R&D Systems, 1:1,000), anti–caspases 3 and 7 (Cell Signaling Technologies, 1:2,000), and anti-GAPDH (Sigma-Aldrich, 1:10,000). Signals were detected with peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection system.

Immunofluorescence

Cells were seeded on coverslips and processed for immunofluorescence as previously described (15). Cells were incubated with primary antibodies: anti-TRAF2 (Cell Signaling Technology, Inc. C192; 1:100) and anti-FAK (clone 4.47; Upstate; 1:200). After labeling, the cells were viewed with a fluorescent microscope (Axiophot; Carl Zeiss MicroImaging, Inc.) equipped with a 63 × plan Apochromat objective and selective filters. Images were acquired from a cooled CCD camera (Retiga 1300; Q Imaging) and displayed on a high-resolution monitor. Images were analyzed by the Northern Eclipse Image analysis system (Carl Zeiss MicroImaging, Inc.). Confocal analyses were performed with an inverted confocal microscope (McGill University; model LSM 510; Carl Zeiss MicroImaging, Inc.).

Luciferase assay for NF-κB activity

The NF-κB activities were analyzed by luciferase assay as described earlier (15). Cells seeded in 12-well plates (10^5 cells/well) were transiently transfected by lipofectamine (Invitrogen) with 500 ng/well of NF-κB-Luc (reporter plasmid) plus 250 ng/well of CMVβ-galactosidase plasmid (control for transfection efficiency) in serum-free medium according to the manufacturer's instructions. After 5 hours of transfection, the cells were incubated with fresh serum-free medium when indicated containing TWEAK (TNF-like weak inducer of apoptosis: 10 ng/mL, R&D Systems). After 24 hours, the cells were rinsed with cold phosphate-buffered saline, and extracts were collected and assayed for luciferase activity following manufacturer's protocol. Luciferase activity was normalized for β-galactosidase activity.

Anoikis assay

For anoikis evaluation, the CytoSelect 96-well anoikis assay kit was used (Cell Biolabs, Inc.). Cells were seeded (5×10^4 cells/mL) and incubated up to 24 hours at 37°C. Cell viability was assessed by MTT proliferation assay (colorimetric) and calcein AM (485 nm/515 nm, fluorimetric) detection. Anoikis-propelled cell death was measured by Ethidium homodimer (EthD-1). Anoikis percentage indicated by relative immunofluorescence of EthD-1 (525 nm/590 nm) on anchorage resistant plate compared with its related calcein AM on control plate. All analyses were performed in at least three independent replicate experiments.

TCGA database analysis

Molecular and clinicopathologic information from patients with invasive breast cancer was retrieved from The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov) using cBioPortal tool (www.cbioportal.org) and consisted of 1,105 samples (data set "Breast Invasive Carcinoma, TCGA, Cell 2015, 1105 samples") with RNA-seq and copy-number data (16, 17). Kaplan–Meier survival analysis was performed to estimate the survival distributions and the log-rank test to assess the statistical significance of the differences between the groups.

Protein–protein interaction (PPI) analyses and functional annotation

FpClass (https://www.ncbi.nlm.nih.gov/pubmed/25402006) was used to predict a high-confidence PPI network of TRAF2 and FAK using the total score (probability of interaction based on all evidence) as parameter. Functional annotation of the network was performed in WEB-based GEne SeT AnaLysis Toolkit (WebGestalt; ref. 18). The whole-genome was used as background, adjusted P value <0.000001 was given by hypergeometric test corrected by Benjamin-Hoe and at least 10 genes were considered in each category. Diseases database (GLAD4U), Gene Ontology analysis (GO), and Kyoto Encyclopedia of Gene and Genome (KEGG) cellular signaling pathways were also explored.

Results

TRAF2 interacts with the N-terminus domain of FAK

We investigated the interaction between TRAF2 and FAK using TRAF2-proficient ($TRAF2^{+/+}$), -deficient ($TRAF2^{-/-}$), and their matched TRAF2-reconstituted with GST-TRAF2 recombinant protein in MEFs. Cells were stimulated with TWEAK at a concentration of 10 ng/mL. We observed that FAK was able to immunoprecipitate endogenous TRAF2 in $TRAF2^{+/+}$ cells and TRAF2-reconstituted $TRAF2^{-/-}$ but not in $TRAF2^{-/-}$ cells (Fig. 1A). This interaction was not observed in FAK-deficient MEF cells; however, when FAK was reconstituted, the interaction between FAK and TRAF2 was restored. To further verify this interaction in cancer cells, similar immunoprecipitation assays were carried out on MDA-MB-231 human breast cancer cell line showing that FAK interacts with TRAF2 (Fig. 1B). Immunofluorescence results also confirmed the intracellular colocalization of FAK and TRAF2 in these cells with predominant costaining in the cytoplasm and cell protrusions (Fig. 1C).

FAK comprises a highly conserved central catalytic domain flanked by N- and C-terminal noncatalytic domains that contain N-terminal FERM region, proline-rich residues with binding motifs for Src homology 3 (SH3) domain-containing proteins, along with a FAT domain located in the C-terminus and which is critical for FAK recruitment and for its association with paxillin and talin. To determine which FAK domain contributes to the interaction with TRAF2, we coexpressed GFP-tagged FAK full-length (WT), N-terminal (FAK-NT), C-terminal (FAK-CT) or phosphosite 397 mutant (F397) with GST-TRAF2 in HEK293T

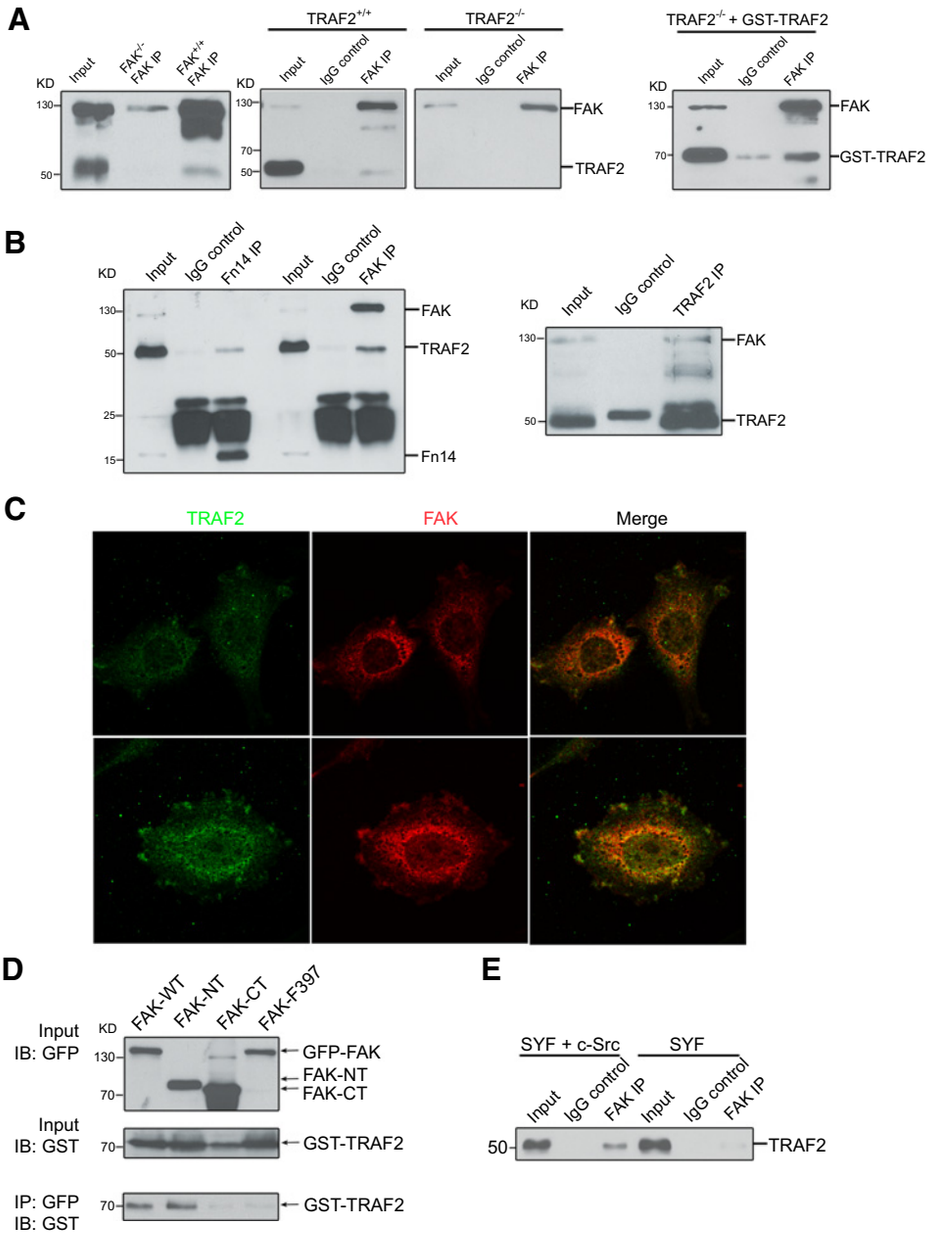

Figure 1.
TRAF2 interacts with the N-terminus domain of FAK. **A,** FAK immunoprecipitation from cell lysates of $FAK^{+/+}$, $FAK^{-/-}$, $TRAF^{+/+}$, $TRAF2^{-/-}$, and GST-TRAF2–reconstituted $TRAF2^{-/-}$ cells reveals that FAK physically interacts with both endogenous and exogenously expressed TRAF2. **B,** FAK immunoprecipitation from cell lysates of the breast cancer MDA-MB-231 cells reveals endogenous FAK to interact with TRAF2 in human cells. Fn-14, which interacts with TRAF2, is shown as a control. **C,** Immunofluorescence microscopy confirmed FAK colocalization with TRAF2. $FAK^{+/+}$ cells grown on cover slides were fixed, incubated with TRAF2 and FAK antibodies, and then immunostained with secondary antibodies conjugated to Cy2 or Texas Red as described in Materials and Methods. **D,** Results showing that TRAF2 interacted with N-terminus domain of FAK. Cells cotransfected with GST-tagged TRAF2 and GFP-tagged full-length FAK, FAK mutants expressing the N-terminal (FAK-NT), the C-terminal (FAK-CT), or full-length 397-phospho-mutant FAK, for 48 hours. Total lysates were immunoprecipitated with GFP antibody and probed for GFP or GST (to determine GST-TRAF2) by immunoblotting. Input shows expression levels of GFP FAK full-length, N-terminus FAK, C-terminus FAK, FAK 397 phosphomutant (F397), and GST-TRAF2. **E,** Cell lysates from SYF (deficient for Src, Yes, and Fyn) and Src-reconstituted (SYF + c-Src) cells were used to immunoprecipitate FAK followed by immunoblotting for TRAF2.

cells, and performed immunoprecipitation using anti-GFP antibody. As shown in Fig. 1D, both FAK full-length and N-terminal, but not C-terminal interacted with TRAF2, which indicates that FAK N-terminal which contains phosphosite 397 is necessary for FAK interaction with TRAF2. FAK activation triggers an autophosphorylation of FAK at Tyr397, which forms a binary complex with Src family kinases, promoting Src catalytic domain activation and subsequent FAK tyrosine phosphorylation. Mutant F397 does not interact with TRAF2 (Fig. 1D). Because FAK Try397 is important for the interaction with TRAF2, we used SYF (deficient for Src, Yes, and Fyn) and Src-reconstituted (SYF reconstituted with c-Src) cells to explore if Src is a part of the FAK–TRAF2 complex. Immunoprecipitation assay shows that FAK can interact with TRAF2 in Src-reconstituted SYF cells but not SYF-deficient cells, which indicates the interaction is Src-dependent (Fig. 1E). However, we were unable to detect the interaction between TRAF2 and Src in FAK-deficient but Src-proficient MEF cells, suggesting that the reduced interaction seen between TRAF2 and Src in SYF cells is the results of indirect interaction or other posttranslational modifications.

TRAF2 and FAK cooperate to regulate NF-κB activity

The activation of NF-κB as a result of stimuli triggers IκB phosphorylation and IκB degradation. In TWEAK-stimulated MDA-MB-231 cells, the inhibition of FAK by siRNA knockdown attenuates NF-κB signaling as revealed by inhibition of p-IκBα and enhanced stability of IκBα (Fig. 2A). Noticeable, we observed that TWEAK receptor (Fn-14) expression was downregulated in FAK-deficient cells (Fig. 2A). Stable knockdown for Fn-14 was constructed and after TWEAK stimulation, the cells did not show increases in p-IκBα compared with their controls. These results indicate that both FAK and Fn-14 are critical components of TWEAK induced NF-κB activation.

Because TRAF2 interacts with FAK (Fig. 1A–E) and FAK regulates NF-κB activation (Fig. 2A), we investigated the impact of TRAF2–FAK interaction on NF-κB signaling by exogenous expression of NF-κB-luciferase reporter plasmid in TRAF2-proficient ($TRAF2^{+/+}$) and -deficient cells ($TRAF2^{-/-}$), and FAK-proficient ($FAK^{+/+}$) and FAK-297T-mutant cells ($FAK^{-/-}$). Luciferase activity was measured after stimulation with TWEAK in comparison with nonstimulated cells (Fig. 2B and C). As shown in Fig. 2B FAK deficiency attenuated NF-κB activity, especially when both TRAF2 and FAK ($FAK^{-/-}$ + siTRAF2) are inhibited. The same results were obtained for TRAF2-deficient cells ($TRAF2^{-/-}$ + siFAK; Fig. 2B). These data further support that TRAF2–FAK cooperation has a great impact on NF-κB function.

TRAF2–FAK cooperates for the regulation of anoikis

Anoikis is an anchorage-dependent form of cell death triggered by detachment from ECM. To evaluate if TRAF2 and FAK cooperate to promote resistance to anoikis, we downregulated TRAF2 in FAK-deficient cells ($FAK^{-/-}$ expressing siTRAF2) and knockdown FAK in

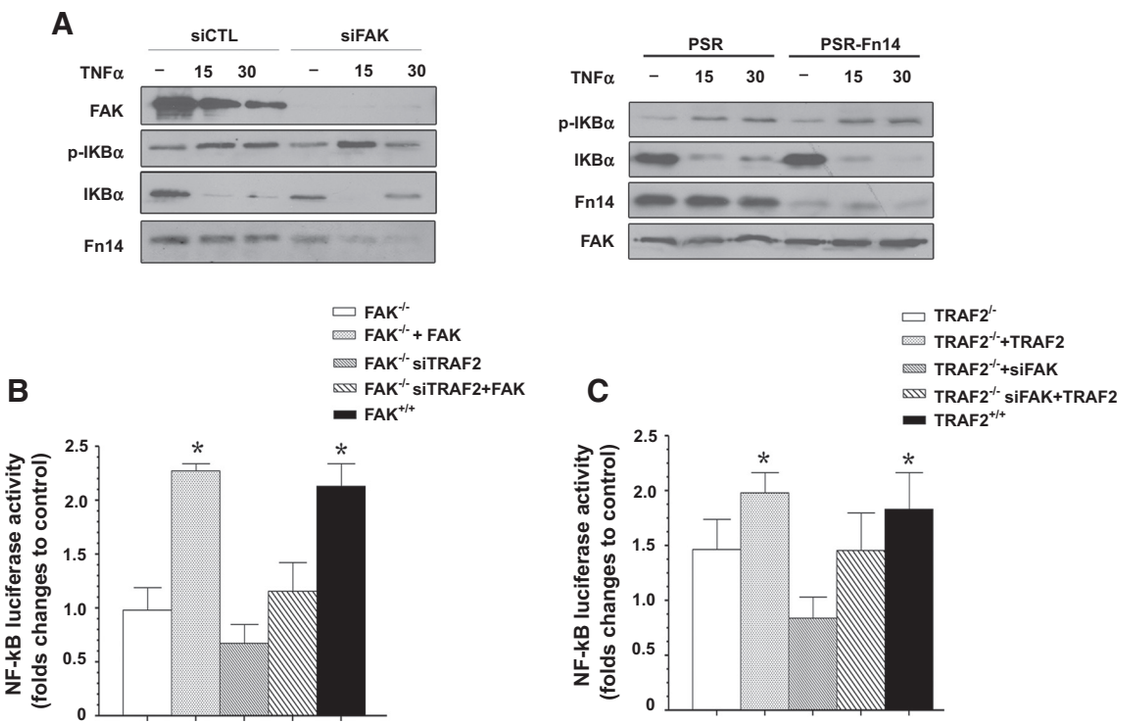

Figure 2.

TRAF2 and FAK cooperate to regulate NF-κB activity and promote cell survival. **A,** FAK and Fn14 were downregulated in MDA-MB-231 cells. Control cells were transfected with matched empty expression vectors alone. Cells were treated with TWEAK (10 ng/mL) at the indicated time intervals, and phospho-IκBα (p-IκBα) and IκBα levels were measured by immunoblotting. **B,** FAK-deficient ($FAK^{-/-}$) and proficient cells ($FAK^{+/+}$) were downregulated for TRAF2 (siTRAF2), and exposed to TWEAK (10 ng/mL). **C,** TRAF2-deficient ($TRAF2^{-/-}$) and -proficient cells ($TRAF2^{+/+}$) were downregulated for FAK (siFAK) and exposed to TWEAK (10 ng/mL). The luciferase reporter assay was performed as described in Materials and Methods (*, $P<0.05$). The NF-κB activities compared with controls represented as folds change (mean ± SD).

TRAF2-deficient cells (TRAF2$^{-/-}$ expressing siFAK). Cells were seeded on control or precoated anchorage-resistant 96-well plates for 24 hours, stimulated with 100 ng/mL TWEAK for 24 hours, and cell viability was determined by MTT and Calcein detection assays as described in Materials and Methods. Noticeably, we observed a significant number of both TRAF2- and FAK-deficient cells become nonviable with rounded shapes and detach rapidly, particularly when cells were cultured on plates coated with fibronectin. The results summarized in Fig. 3A and B demonstrate the percentage of cells undergoing anoikis quantified by the relative immunofluorescence of EthD-1 on anchorage-resistant plates compared with Calcein on control plates. As noted, anoikis is enhanced in both FAK- and TRAF2-deficient cells and this was exacerbated following stimulation with TWEAK ($P < 0.05$). Furthermore, TRAF2- and FAK-deficient cells where FAK or TRAF2 were downregulated, respectively (TRAF2$^{-/-}$-siFAK and FAK$^{-/-}$-siTRAF2, showed significant increase of anoikis. Under similar conditions, increased expression of activated caspases was observed in MDA-MB-231 cells where both FAK and TRAF2 were downregulated (Fig. 3C). Together, these results indicate that TRAF2 and FAK cooperate to regulate cell susceptibility to anoikis.

TRAF2 and FAK are overexpressed and coamplified in breast cancer tissues

Our preclinical data indicates that downregulation of both FAK and TRAF2 renders breast cancer cells more susceptible to anoikis, supporting that co-overexpression of these dual markers in tissues could affect disease progression and patient's survival. To investigate the potential clinical significance of co-overexpression of TRAF2 and FAK in human breast cancer, previously reported expression and genomic database was retrieved from TCGA and visualized using cBioPortal. We focused on a study comprised of 1,105 invasive breast cancer samples (data set "Breast Invasive Carcinoma, TCGA, Cell 2015, 1105 samples"; ref. 17). We observed that TRAF2 and FAK were amplified (Fig. 4A) and overexpressed (Fig. 4B) in breast cancer compared with morphologically normal breast epithelial tissue especially considering basal and triple-negative subtypes. Further analysis revealed significantly lower survival probability in this set of 1,105 patients with breast cancer overexpressing FAK ($P = 0.00891$) and TRAF2 ($P = 0.0263$). The co-overexpression of the both TRAF2 and FAK revealed a predictive value of poor survival probability ($P = 0.0316$; Fig. 4C).

Interestingly, the schematic representation of PPI involving TRAF family members and FAK network using NAViGaTOR, Reactome, BioCarta, and KEGG databases assessing functional interactions predicted a predominance of interacting proteins involved in anoikis genes, inflammatory process, and epithelial–mesenchymal transition (EMT; $P < 0.01$; Supplementary Fig. S1).

Discussion

Cells normally undergo apoptosis after they lose contact with their ECM or their neighboring cells. This cell death process has

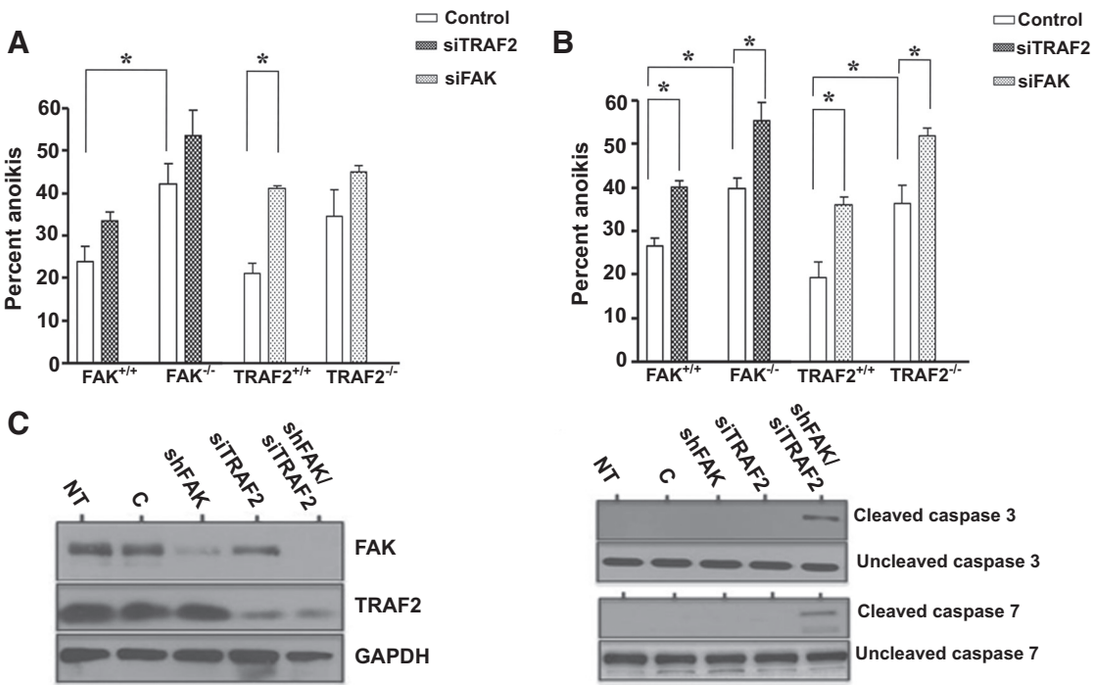

Figure 3.

FAK–TRAF2 cooperation promotes cell survival via resistance to anoikis. FAK and TRAF2 were downregulated in MEF deficient for TRAF2- or FAK-proficient and deficient cells, respectively (FAK$^{+/+}$-siTRAF2, FAK$^{-/-}$-siTRAF2, TRAF2$^{+/+}$-siFAK, TRAF2$^{-/-}$-siFAK) using siRNA. Cells (5×10^4) were seeded onto control or anchorage-resistant 96-well plates for 24 hours. Cells were kept unstimulated in serum-free medium (**A**) or stimulated with TWEAK (100 ng/mL) for 24 hours (**B**). Cell viability was determined by MTT (colorimetric) and calcein AM detection. Anoikis propelled cell death was measured by Ethidium homodimer (EthD-1) and percentages of cells undergoing anoikis quantified by the relative immunofluorescence of EthD-1 on anchorage-resistant plate compared with its related calcein AM on control plates. (*, $P < 0.05$). **C,** The expression of activated caspases was increased in MDA-MB-231 cells, where both FAK and TRAF2 were downregulated.

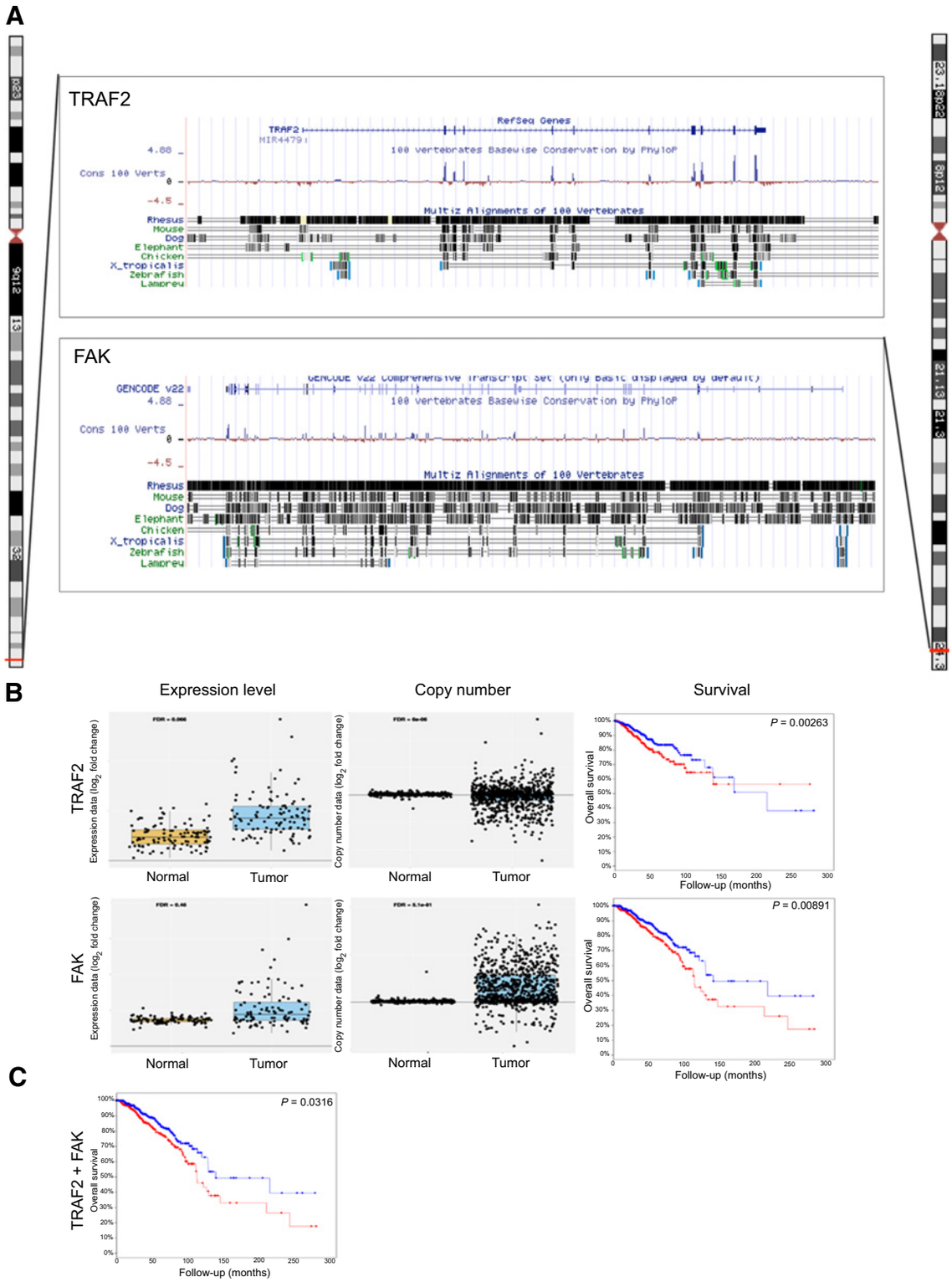

Figure 4.

TRAF2 and FAK are overexpressed and amplified in cancer. **A,** Genomic profiling of 9q34.3 and 8q24.3 amplification in human breast cancer. **B,** Expression data analysis showing TRAF2 and FAK overexpressed (left column) and amplified (middle column). Both are upregulated in tumors in relation to the normal tissues. Survival analysis using TCGA data (17) showed significant lower survival probability in 1,105 patients with breast cancer cases overexpressing TRAF2 (log-rank test, $P = 0.0263$) and FAK (log-rank test, $P = 0.00891$). **C,** Co-overexpression of the both TRAF2 and FAK also revealed a predictive value of poor survival probability in this cohort of breast cancer cases (log-rank test, $P = 0.0316$).

been termed "anoikis." Tumor cells that acquire malignant potential have developed mechanisms to resist anoikis and thereby survive after detachment from their primary site and while traveling through the lymphatic and circulatory systems (19). The interaction between tumor cells and their tissue microenvironment plays a key role in the regulation to anoikis. FAK, a tyrosine kinase that is overexpressed in a variety of human tumors, mediates one of these survival signals. Here we identified that the RING finger protein involved with proinflammatory and immune response, TRAF2, cooperates with FA signaling to regulate cancer cell susceptibility to anoikis.

TRAF2, as an adaptor protein, belongs to the TRAF family constituted by seven members (TRAF1–7). TRAF2 expression is upregulated in multiple cancer types and is a valuable prognostic biomarker in patients (20, 21). In addition to the role of TRAF2 in cancer, our study demonstrated that TRAF2 interacts with FAK. Using MEF proficient and deficient for TRAF2 or FAK, and their matched reconstituted cells, we observed that TRAF2 selectively interacts with the N-terminal portion of FAK and colocalizes with FAK at cell plasma cell membrane protrusions where FA sites are formed. This TRAF2-dependent interaction with FAK requires FAK active conformation induced secondary to Y397 phosphorylation, as expression of nonphosphorylated FAK mutant failed to interact with TRAF2. In addition, this occurs in a TNF-TWEAK-dependent manner, which is required for the subsequent recruitment of IKBα. Lee and colleagues (22) showed that TRAF2 is essential for the activation of JNK (c-Jun N-terminal kinase), suggesting that TRAF2 is at the bifurcation point of two kinase cascades leading to the activation of NF-κB and JNK (23, 24). Our data showed that TRAF2–FAK cooperation has a great impact on NF-κB function. Attention has been paid to activation of the downstream IKK (I kappa B kinase) complex, which is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (23, 25). Activated IKKs thus phosphorylate IκBs at serine residues 32 and 36, leading to their degradation and the subsequent activation of NF-κB (23, 26). We assumed that FAK acts as a bridge with TRAF2 to serve as a platform for the interaction of these molecules as a critical step in the NF-κB signaling cascade.

Our observation about the implication of NF-κB on TRAF2–FAK regulated cell survival is predictable given the established role of NF-κB in the activation of target genes involved in cell survival such as members of the inhibitor of apoptosis (IAP) family, survivin and XIAP, BCL-XL, and BCL-2 (27). Even though we did not directly address here, our study cannot rule out that TRAF2–FAK affects anoikis via its effect on EMT because FAK is known to regulate EMT signaling (28) and NF-κB is implicated in the regulation of switches between MET and EMT (29). Enhanced cancer cell plasticity such as the EMT process associated with proinvasiveness can lead to a switch from anoikis-sensitive to anoikis-resistant phenotype (30, 31).

Anoikis is regulated by diverse signaling mechanisms such as aberrant ECM composition and deregulated chemokine activity. Here, we demonstrated that TRAF2–FAK interaction greatly affects NF-kB activity. The downregulation of either TRAF2 or FAK inhibits NF-kB activity and renders cells more susceptible to anoikis. Previous reports have documented the function of FAK as a survival protein, via multiple mechanisms including through PI3K–AKT signaling, suppression of FAK interaction with the death domain kinase receptor–interacting protein, a component of the death receptor complex that also interacts with Fas and TNFR1 and possibly via EMT-dependent regulation (9, 10, 32). Inhibition of FAK by injection of FAK antibody or a dominant-negative integrin peptide into unattached fibroblasts inhibits assembly of F-actin and focal adhesion complexes (FAC) and promotes apoptosis of anchorage-dependent cells (33, 34). Consistently, overexpression of constitutively active FAK (CD2-FAK) in epithelial cells generates anoikis resistance and transforms epithelial cells into anchorage-independent growth, resulting in tumor formation in nude mice (33, 35). Activation of FAK also protects human ovarian cancer cells from anoikis and promotes cancer progression (33, 36).

We used public genomic platform to investigate the impact of TRAF2–FAK cooperative signals in cancer progression and metastasis. In human breast cancer tissues, genomic analysis of a TCGA database revealed coamplification of TRAF2 and FAK in breast cancer tissues with a predictive value for shorter survival, supporting a potential role of TRAF2–FAK cooperative signals in cancer progression. However, we cannot rule out contribution to outcome of additional TRAF and FAK partners and posttranslational modifications. As noted in Supplementary Fig. S1, PPI analysis of TRAF2 and FAK network (using the NAViGaTOR, Reactome, BioCarta, and KEGG databases assessing functional interactions) indicated an enrichment of proteins involved in anoikis, inflammatory process, and EMT related-genes. Furthermore, both TRAF2 and FAK undergo extensive phosphorylations at multiple sites, many of which have been reported to regulate expression and secretion of several chemokines in cell models, which can contribute to regulate tumor cell–matrix interactions and survival signals (37, 38). Future studies on these alternative events are needed to establish the clinical significance of FAK and TRAF interacting network versus anoikis.

In summary, our data support that pharmacologic approaches targeting TRAF2 and FAK have the potential to overcome resistance to anoikis and improve therapeutic outcome. This is possible given the newly developed selective agents targeting many of the signaling components involved in the regulation of TNF signaling, TRAF2 and FAK.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: B. Xu, M.A. Alaoui-Jamali
Development of methodology: S. Daniela da Silva, B. Xu, M.A. Alaoui-Jamali
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Daniela da Silva, B. Xu, M.I. Alkailani, D. Xiao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Daniela da Silva, B. Xu, M. Maschietto, F.A. Marchi
Writing, review, and/or revision of the manuscript: S. Daniela da Silva, M. Maschietto, K. Bijian, M.A. Alaoui-Jamali
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Daniela da Silva, K. Bijian, M.A. Alaoui-Jamali
Study supervision: K. Bijian, M.A. Alaoui-Jamali

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