

Growth Inhibition and Radiosensitization of Glioblastoma and Lung Cancer Cells by Small Interfering RNA Silencing of Tumor Necrosis Factor Receptor–Associated Factor 2

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Abstract

Radiotherapy combined with chemotherapy is the treatment of choice for glioblastoma and locally advanced lung cancer, but radioresistance of these two types of cancer remains a significant therapeutic hindrance. To identify molecular target(s) for radiosensitization, we screened a small interfering RNA (siRNA) library targeting all protein kinases and E3 ubiquitin ligases in the human genome and identified tumor necrosis factor receptor–associated factor 2 (TRAF2). Silencing of TRAF2 using siRNA caused a significant growth suppression of glioblastoma U251 cells and moderately sensitized these radioresistant cells to radiation. Overexpression of a really interesting new gene (RING)–deleted dominant-negative TRAF2 mutant also conferred radiosensitivity, whereas overexpression of wild-type (WT) TRAF2 significantly protected cells from radiation-induced killing. Likewise, siRNA silencing of TRAF2 in radioresistant lung cancer H1299 cells caused growth suppression and radiosensitization, whereas overexpression of WT TRAF2 enhanced radioresistance in a RING ligase-dependent manner. Moreover, siRNA silencing of TRAF2 in UM-SCC-1 head and neck cancer cells also conferred radiosensitization. Further support for the role of TRAF2 in cancer comes from the observations that TRAF2 is overexpressed in both lung adenocarcinoma tissues and multiple lung cancer cell lines. Importantly, TRAF2 expression was very low in normal bronchial epithelial NL20 cells, and TRAF2 silencing had a minimal effect on NL20 growth and radiation sensitivity. Mechanistically, TRAF2 silencing blocks the activation of the nuclear factor- κ B signaling pathway and down-regulates several G₂-M cell cycle control proteins, resulting in enhanced G₂-M arrest, growth suppression, and radiosensitization. Our studies suggest that TRAF2 is an attractive drug target for anticancer therapy and radiosensitization. [Cancer Res 2008;68(18):7570–8]

Introduction

Glioblastoma multiforme is the deadliest and most common type of human primary brain tumor with a median survival of less than 1 year (1). Unfortunately, this prognosis has not changed significantly over the past 2 decades despite advances in

neurosurgery, radiation, and chemotherapy (2). Two characteristic features of glioblastoma play a major role in the deadly nature of the disease. First, glioblastoma cells extensively invade the normally functioning brain, which essentially prevents a surgical cure. Second, glioblastoma is resistant to all the current therapeutic modalities, including radiotherapy (1). External beam radiotherapy remains an important local treatment modality in both high- and low-grade gliomas. However, its effectiveness is modest due to the radioresistance of these tumors observed in the clinic (3). Although preclinical and correlative clinical data suggested an involvement of an epidermal growth factor receptor signaling pathway in the radioresistance of glioblastoma (4), the mechanism for such extreme radioresistance was not well understood. Laboratory studies have found that glioblastoma cell lines are very resistant to radiation-induced apoptosis due to failure in p53 and PTEN signaling pathways (5, 6).

Lung cancer is the leading cause of cancer death in the United States and throughout the world, claiming more than 1 million lives each year (7). Although significant progress has been made in our understanding of the molecular mechanisms of lung carcinogenesis, the therapeutic interventions for lung cancer have achieved only modest benefits (8). For non-small cell lung cancer (NSCLC), radiotherapy as a curative modality has been disappointing as evidenced by low tumor response rate and a 5-year survival rate of only 7% to 10% (8). Two major issues that limit the effectiveness of radiotherapy of NSCLC are radioresistance of the tumor and radiation-induced toxicity to normal tissues such as the lung and esophagus. The mechanism of radioresistance of NSCLC remains unclear, although some studies have shown the potential involvement of either p53 mutations (9), overexpression of survival genes such as *XIAP* and *survivin* (10), or activation of the Akt pathway (11). Presently, there are no molecularly targeted therapies that have been effectively combined with radiation for the treatment of lung cancer. Thus, the identification of gene(s) responsible for carrying resistance would be of great importance to discover drugs that enhance sensitivity to radiation.

One approach for identifying targets that may play a role in radioresistance is through the screening of small interfering RNA (siRNA) libraries. Here, we describe a siRNA-based screen to identify genes that confer radioresistance. Using a siRNA library targeting the kinases and E3 ubiquitin ligases, tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2) was identified as a candidate radiosensitizing target. TRAF2 belongs to a family with seven protein members (TRAF1–7) that play a pivotal role in diverse biological processes, including immunity, inflammation, and apoptosis (12, 13). Among TRAF family members, TRAF2 is

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doi:10.1158/0008-5472.CAN-08-0632

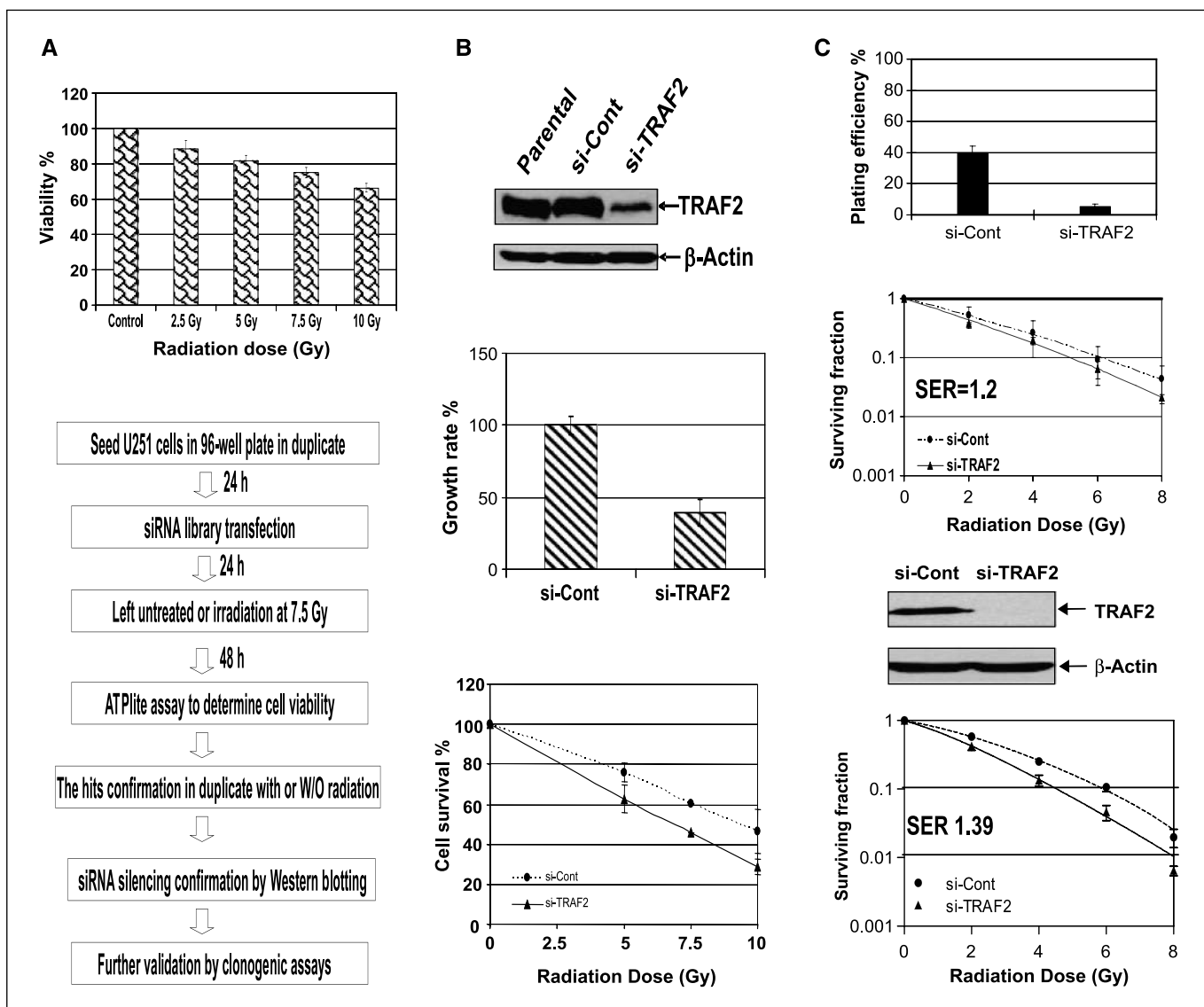


Figure 1. Identification of TRAF2 as a radiosensitizing target: glioblastoma U251 cells are radioresistant. U251 cells were seeded in 96-well plates and irradiated at the indicated doses 24 h later. Cell viability was measured 48 h after radiation by ATPlite assay. *A*, top, results from three independent experiments, each run in quadruplet; bottom, flow chart for siRNA library screening in U251 cells. TRAF2 silencing inhibits cell growth and sensitizes cells to radiation as measured by 96-well ATPlite short-term assay (*B*) or standard clonogenic assay (*C*): U251 cells were infected with lenti-si-Cont (from scrambled sequence) or lenti-si-TRAF2 (targeting TRAF2). *B*, top, cells were harvested 72 h after infection and subjected to Western blot analysis. TRAF2-silenced cells, along with control cells, were assessed for growth rate using 96-well assay up to 5 d after no radiation (*middle*) or radiation with indicated doses (*bottom*). *C*, TRAF2-silenced cells were subjected to standard clonogenic assay for plating efficiency in six-well plate without (*top*) or with (*middle*) radiation. U251 cells were transiently transfected with siRNA oligonucleotide against TRAF2. Bottom, cells were exposed to radiation 48 h after transfection when TRAF2 was silenced and subjected to standard clonogenic assay. Colony number was counted 9 d after radiation, and surviving fraction was calculated and plotted after comparison with 0 Gy corresponding controls. SER was calculated as described (22, 49). Points, mean from three independent experiments, each run in quadruplicate (*B*) or duplicate (*C*); bars, SE.

unique as an adaptor protein that mediates several signaling pathways leading to apoptosis protection (see refs. 14, 15 for reviews). TRAF2 consists of 501 amino acids with three distinct domains: the COOH-terminal domain responsible for homodimerization and heterodimerization of the TRAF proteins, four repeats of zinc finger domain, and a really interesting new gene (RING) finger domain at the NH₂ terminus required for its E3 ubiquitin ligase activity (16). We found that TRAF2 silencing remarkably inhibited growth of glioblastoma U251 and lung cancer H1299 cells and sensitized several cancer lines to radiation. These anticancer effects are mediated at least in part through blocking nuclear factor- κ B (NF- κ B) activation and targeting G₂-M checkpoint control proteins.

TRAF2 may, therefore, serve as an attractive target for anticancer therapy and radiosensitization.

Materials and Methods

Cell culture. Human glioblastoma U251, human non-small cell lung carcinoma H1299, human head and neck cancer UM-SCC-1 cells, and TRAF2^{+/+} or TRAF2^{-/-} mouse embryonic fibroblast (MEF) cells (17) were grown in DMEM with 10% fetal bovine serum (FBS; Atlantic Bioscience). Normal bronchial epithelial cells, NL20, were grown in Ham's F12 medium with 1.5 g/L sodium bicarbonate, 2.7 g/L glucose, 2.0 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 5 μ g/mL insulin, 10 ng/mL EGF, 1 μ g/mL transferrin, 500 ng/mL hydrocortisone, and 4% FBS.

ATPlite cell viability assay and radiation exposure. Cells were seeded in 96-well plates and the following day treated with a range of radiation doses using a 250 kV orthovoltage unit (Philips). Cell viability was measured 48 h after irradiation using an ATPlite kit (Perkin-Elmer; ref. 18).

siRNA library screen. The siRNA library has been described previously and targets each gene with a pool of siRNAs consisting of a combination of four siRNA duplexes directed at different regions (19). For this study, we used a portion of the library that targets the kinases and E3 ubiquitin ligases using four siRNAs per target as a pool at each concentration of 12.5 nmol/L. U251 cells were seeded in duplicate on day 1 at 6,000 per well in 96-well plates (Dot Scientific). Cells were transfected the next day with each siRNA pool using EasyTransgater-si (American Pharma Source). As controls, siRNA to the *polo-like kinase-1* (*PLK-1*) gene (NM_005030) and a universal nontargeting siRNA were used in the screening. The cells were transfected for a total of 4 h, after which the medium was changed. On day 3, one plate was left unirradiated, whereas the other was subjected to 7.5 Gy of radiation. The cells were then allowed to grow for an additional 48 h followed by cell viability determination using the ATPlite assay. A Z factor was calculated (>0.5) to validate the robustness of the assay for high-throughput screening (20). The average of each treatment was normalized to the average of the negative control samples to determine cell viability.

Lentivirus-based short hairpin RNA silencing of TRAF2. The siRNA oligonucleotide or a lentivirus-based short hairpin RNA (shRNA) construct was used to silence TRAF2. The sequence of siRNA oligonucleotides is as follows: *siTRAF2-01*, 5'-GGAGCATTGGCCTCAAGGATTCAAGAGATCCTT-GAGGCCAATGCTCCTTTTTTGT-3'; *siTRAF2-02*, 5'-CTAGACAAAAAAG-GAGCATTGGCCTCAAGGATCTCTTGAATCCTTGAGGCCAATGCTCC-3'. The control siRNA sequences are as follows: *siControl-01*, 5'-ATTGTATGC-GATCGCAGACTTTTCAAGAGAAAGTCTGCGATCGCATAACAATTTTTTGT-3'; *siControl-02*, 5'-CTAGACAAAAAATTGTATGCGATCGCAGACTTCTCTT-GAAAAGTCTGCGATCGCATAACAAT-3'. The lentivirus-based vector was prepared and used to infect U251 or H1299 cells. Cell lysates were prepared 48 to 72 h later for Western blotting analysis (21).

Western blot analysis. The assay was performed as described (18) using antibodies against TRAF2, Wee1, I κ B α , Cdc25C (Santa Cruz Biotechnology), cellular inhibitor of apoptosis protein (cIAP)-1, cIAP-2, receptor interacting protein (RIP)-1, cyclin B1, Cdc2 (Cell Signaling), Cdc25B (BD), Chk2 (Upstate), PLK-1, Aurora-A/B, and β -actin (Sigma).

Clonogenic assay. Cells after lentivirus-based shRNA silencing or siRNA oligonucleotide transfection were seeded in six-well plates at three different cell densities in duplicate. The next day, cells were exposed to different doses of radiation followed by incubation at 37°C for 7 to 9 d. The colonies formed were fixed and the surviving fraction was determined by the proportion of seeded cells following irradiation to form colonies relative to untreated cells as described (22).

Construction of RING mutant and establishment of TRAF2 stable clones. A TRAF2 RING mutant (C49A/H51A/C54A/C57A), previously shown to have a substantial reduction of ligase activity (23), was made by the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing. Plasmids expressing wild-type (WT) FLAG-TRAF2, a dominant-negative mutant, FLAG- Δ N-TRAF2 (24), or a ligase-dead mutant, FLAG-TRAF2-RING-mutant (23), were transfected into U251 or H1299 cells, respectively. Stable clones were selected with G418 and pooled.

Immunohistochemical staining. A lung tissue microarray was constructed from the most representative area using the methodology of Nocito and colleagues (25). Immunohistochemical staining was performed on the DAKO Autostainer (DAKO) using DAKO LSAB+ and 3,3'-diaminobenzidine as the chromogen. Deparaffinized sections of formalin-fixed tissue at 5- μ m thickness were labeled with rabbit anti-TRAF2 (1:400; Santa Cruz Biotechnology) after microwave epitope retrieval in citric acid (pH 6). Appropriate negative (no primary antibody) and positive controls (spleen) were stained in parallel with each set of tumors studied.

Luciferase reporter assay. U251 cells were infected with lentivirus targeting TRAF2, along with the control virus. Forty-eight hours after

infection, cells were seeded into 96-well plate and transfected with a luciferase reporter driven by NF- κ B consensus sequence (pNifty plasmid; Invivogen), along with *Renilla* construct. Twenty-four hours after transfection, cells were either treated with TNF α (10 ng/mL; BD biospheres) for 3 h or exposed to 10 Gy X-ray. Cells were harvested 24 h later for luciferase activity assay (Promega). The results were presented as the fold activation after normalization with *Renilla*.

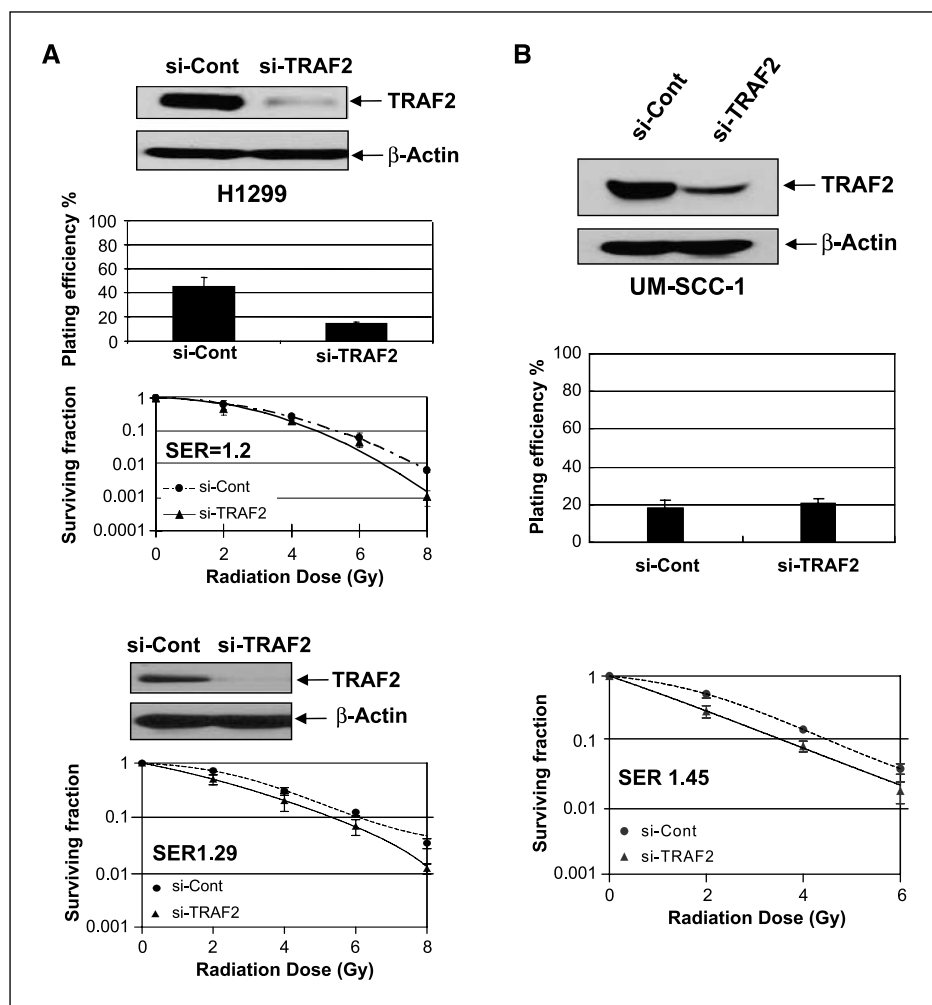
Fluorescence-activated cell sorting analysis. Cells were infected with lentivirus targeting TRAF2, along with the control virus. Forty-eight hours after infections, cells were seeded into 60-mm dish and exposed 24 h later to ionizing radiation (10 Gy). Cells were harvested 24 h after radiation and analyzed by flow cytometry (18).

Statistical analysis. The paired Student's *t* test was used for statistical analysis of luciferase reporter assay and clonogenic assay using Statistical Analysis System software.

Results

Identification of TRAF2 as a target for radiosensitization and cell survival in U251 cells. To identify novel radiosensitizing target(s), we screened a siRNA library directed against the kinases and E3 ubiquitin ligases (19) using the well-characterized radio-resistant U251 glioblastoma cells (2). U251 cell proliferation, as measured by an ATPlite assay in a 96-well plate, is inhibited by only 30% in response to a high dose of ionizing radiation (10 Gy) as shown in Fig. 1A (*top*). Based on these observations, a dose of 7.5 Gy, which caused a 25% growth inhibition, was chosen for the siRNA screen as outlined in Fig. 1A (*bottom*). Hits were defined as those that sensitized cells to radiation by $\geq 10\%$ compared with the control siRNA. These hits were then confirmed in duplicate with or without radiation followed by siRNA silencing confirmation by Western blotting analysis. The confirmed hits were subjected to further validation using classic clonogenic assays on siRNA silencing. Results from our siRNA library screen yielded four hits that reproducibly sensitized cells to radiation and include TRAF2, FLT1, LIMK1, and TRIM5. Among these hits, we decided to validate TRAF2 as a novel radiosensitizing target because literature evidence supports its function in the TNFR signaling pathway, NF- κ B activation, and cell survival (14). To validate TRAF2, four individual siRNA oligonucleotides, each targeting different regions of the TRAF2 gene, were made and tested for radiosensitization. Multiple TRAF2 siRNAs moderately sensitized U251 cells to radiation treatment, with one siRNA oligonucleotide (TRAF2-1) being the most active (data not shown). To confirm that TRAF2 siRNA-induced radiosensitization correlated with TRAF2 knock-down, we continued our validation by cloning the best TRAF2-1 siRNA into a lentivirus-based shRNA system. As shown in Fig. 1B (*top*), infection of the scrambled control siRNA did not cause any change in TRAF2 levels, whereas infection of TRAF2-1 siRNA caused a significant reduction in TRAF2 levels compared with parental cells. Using the ATPlite short-term assay, siRNA knock-down of TRAF2 induced a 2.5-fold reduction of cell growth rate (Fig. 1B, *middle*). In addition, TRAF2 knockdown in combination of different dose of ionizing radiation conferred radiosensitivity at all doses tested (Fig. 1B, *bottom*). Furthermore, in a standard clonogenic assay, the plating efficiency of U251 in the absence of radiation decreased ~ 6 -fold in siTRAF2-infected cells compared with the control (Fig. 1C, *top*). A dose-dependent radiosensitization on TRAF2 silencing was also observed (Fig. 1C, *middle*) with a sensitizing enhancement ratio (SER) of 1.2. This moderate radiosensitizing effect, although statistically significant ($P < 0.05$), might be underestimated due to a high degree of cytotoxicity induced by lentivirus-based constitutive TRAF2 silencing (Fig. 1C,

Figure 2. TRAF2 silencing sensitized lung cancer cells as well as head and neck cancer cells to radiation. *A*, TRAF2 silencing caused growth inhibition and radiosensitization in H1299 cells. H1299 cells were infected with lenti-si-Cont or lenti-si-TRAF2. Cell lysates were prepared for Western blot analysis (*top*) or to standard clonogenic assay in the absence (*panel 2*) or presence (*panel 3*) of radiation. *Bottom*, H1299 cells were also transiently transfected with siRNA oligonucleotide targeting TRAF2 and exposed to radiation 48 h after transfection when TRAF2 was silenced and subjected to standard clonogenic assay. Three independent experiments were conducted. Points, mean; bars, SE. SER was calculated as above. *B*, transient TRAF2 silencing caused radiosensitization in UM-SCC-1 cells. Cells were transiently transfected with siRNA oligonucleotide targeting TRAF2. Cells were harvested 48 h after transfection. One portion was subjected to Western blot analysis (*top*) and the other portion was subjected to clonogenic assay after exposure to indicated dose of radiation. Shown are plating efficiency (*middle*) and surviving fraction (*bottom*) from three independent experiments. Bars, SE. SER was calculated as above.



top). We, therefore, transfected TRAF2 siRNA oligonucleotide to transiently silence TRAF2 at the time of radiation. As shown in Fig. 1C (*bottom*), transient TRAF2 knockdown gave rise to a greater radiosensitization, with SER value increasing from 1.2 to 1.39 (again, $P < 0.05$). Thus, TRAF2 silencing not only remarkably inhibited cancer cell growth and survival but also sensitized cells, to a lesser extent, to radiation in both the monolayer growth assay and the standard clonogenic assay.

TRAF2 siRNA silencing inhibits cell growth and moderately sensitizes H1299 and UM-SCC-1 cells to radiation. We next extended our TRAF2 silencing study to another radioresistant H1299 lung cancer line. H1299 cells were found to be the most radioresistant lung cancer line among four lines tested, including A549, H460, and SKLU-1, with a 25% growth inhibition at dose of 7.5 Gy, similar to U251 (data not shown). H1299 infection of lenti-si-TRAF2 induced up to 80% protein knockdown (Fig. 2A, *top*), which correlated with a significant 3-fold reduction in plating efficiency using a clonogenic assay (Fig. 2A, *panel 2*). Furthermore, reduction of TRAF2 sensitized H1299 cells to radiation, particularly at higher doses with a SER of 1.2 ($P < 0.05$; Fig. 2A, *panel 3*). To avoid overt toxicity associated with constitutive TRAF2 silencing, we also transiently silenced TRAF2 using a siRNA oligonucleotide. As shown in Fig. 2A (*bottom*), transient TRAF2 silencing indeed increased radiation sensitivity with SER value increasing from 1.2 to 1.29 ($P < 0.01$).

Finally, we determined radiosensitizing effect of TRAF2 silencing in UM-SCC-1, a head and neck squamous cell carcinoma line. As shown in Fig. 2B (*top*), transient transfection of si-TRAF2 oligonucleotide caused ~70% TRAF2 knockdown at 48 h after transfection when radiation was delivered. This transient TRAF2 knockdown had no effect on cell survival as reflected by plating efficiency (*middle*) but did sensitize cells to radiation with a SER value of 1.45 ($P < 0.05$). Thus, the results from three human cancer cell lines indicate that TRAF2 knockdown, particularly at the time of radiation in a transient basis, could sensitize cancer cells to radiation.

Radioprotection by WT TRAF2 and radiosensitization by a dominant-negative TRAF2 mutant in U251 cells. We next determined whether overexpression of TRAF2 would protect U251 cells from radiation-induced killing, whereas overexpression of dominant-negative TRAF2 would induce radiosensitization. To avoid clonal heterogeneity, we used a pool of G418 stable clones from individual transfection, along with the empty vector neo control, and confirmed the exogenous expression of TRAF2 and its mutant by anti-FLAG antibody (Fig. 3A, *top*). A standard clonogenic assay showed that, compared with the vector neo control, overexpression of TRAF2 rendered cells radioresistant by significantly increasing the number of radiation-resistant colonies with a protective factor (PF) of 1.24 ($P < 0.01$; Fig. 3A, *bottom*). In contrast, overexpression of a dominant-negative TRAF2 mutant

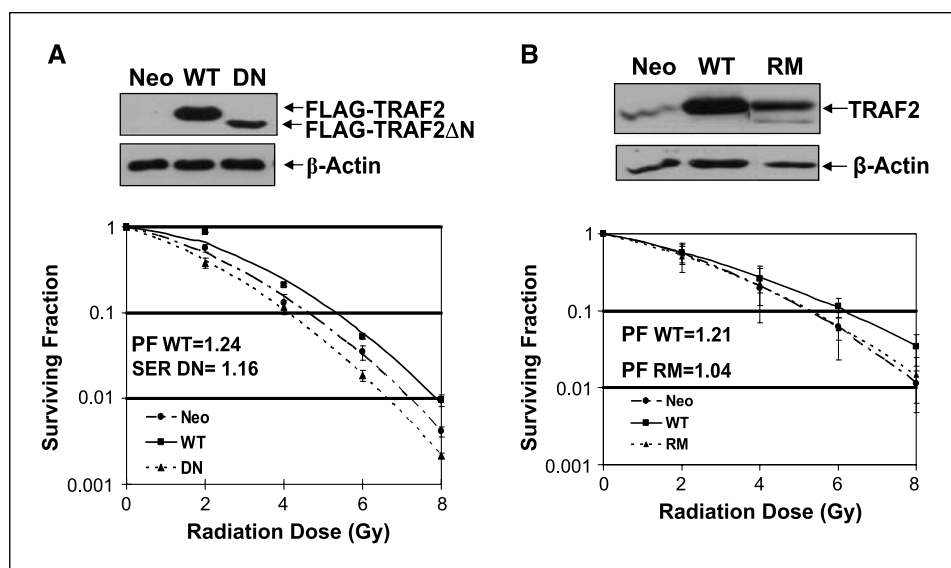


Figure 3. Radioprotection and radiosensitization by TRAF2 or its RING mutants in U251 (A) and H1299 (B) cells. U251 cells were transfected with FLAG-TRAF2 or FLAG-TRAF2 Δ N (a dominant-negative mutant), whereas H1299 cells were transfected with FLAG-TRAF2 or TRAF2 RING mutant (H1299-RM, a ligase-dead mutant), respectively, along with the empty vector neo control. *Top*, the G418-resistant stable clones were pooled after 2 wk of selection, and expression of exogenous TRAF2 was determined by Western blotting using anti-FLAG antibody (A) or anti-TRAF2 antibody (B); *bottom*, pooled stable clones were then subjected to standard clonogenic assay. *Points*, mean from three independent experiments, each run in duplicate; *bars*, SE. PF or SER was calculated as above.

induced a significant reduction of growth rate (data not shown) and moderately enhanced sensitivity to radiation with a SER of 1.16 ($P < 0.05$; Fig. 3A, *bottom*). Thus, overexpression of TRAF2 protects cells from radiation, whereas knockdown of TRAF2 by siRNA or inhibition of TRAF2 by a dominant-negative mutant suppresses cell growth and sensitizes cells to radiation.

TRAF2 radioprotection is RING domain ligase dependent in H1299 cells. We further determined TRAF2 radioprotection and if this protection is ligase activity dependent in H1299 cells using a

TRAF2 RING mutant (C49A/H51A/C54A/C57A) with minimal ligase activity (23). On overexpression (Fig. 3B, *top*), WT TRAF2 conferred radiation protection with a PF of 1.2, whereas the RING mutant abrogated this protection (Fig. 3B, *bottom*). Statistical analysis revealed that the protection by WT TRAF2 is significant ($P < 0.05$). Taken together, the results from U251 (RING deletion) and H1299 (RING mutation) indicate that the RING domain structure and ligase activity is required for TRAF2-mediated protection against radiation.

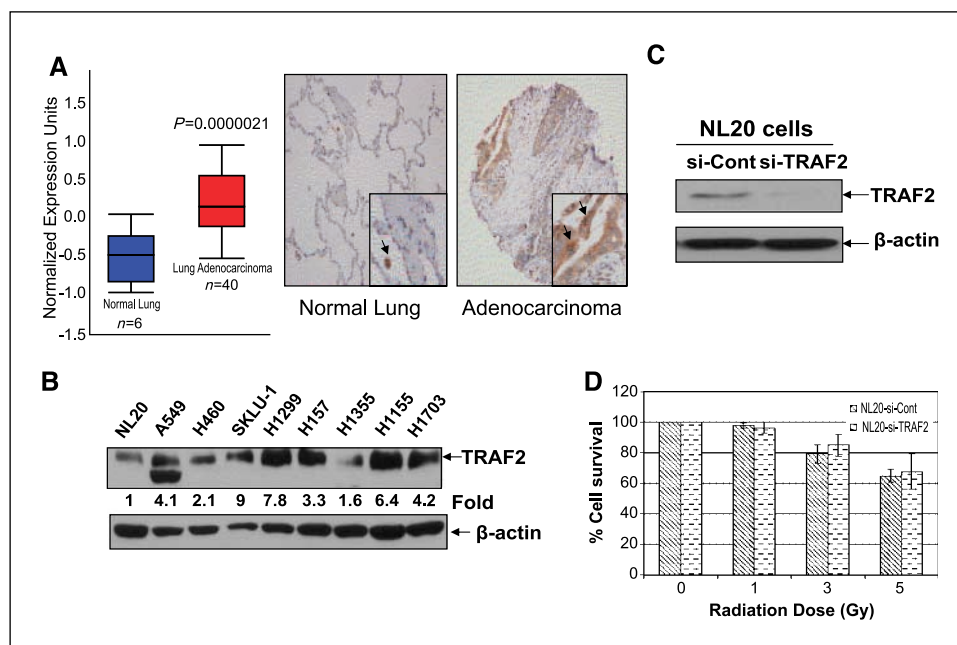


Figure 4. TRAF2 overexpression in lung cancer tissues and cell lines. *A*, overexpression of TRAF2 in lung cancer tissues. *Left*, microarray expression data (26) were compiled by OncoPrint and plotted. Normal lung tissue and lung adenocarcinoma were subjected to immunohistochemical analysis using TRAF2 antibody. *Right*, representative areas from three independent sections with arrows pointing to positively stained adenocarcinoma cells and an alveolar macrophage. Magnification, $\times 400$. *B*, overexpression of TRAF2 in multiple lung cancer cell lines. One immortalized bronchial NL20 cell and eight lung cancer cell lines were harvested for Western blot analysis using antibody against TRAF2. The fold change was calculated after densitometry quantification with β -actin normalization, setting the value in NL20 as 1. *C* and *D*, TRAF2 silencing has no effect on NL20 cell growth and radiosensitivity. NL20 cells were infected with lenti-si-TRAF2, along with lenti-si-Cont. *C*, 72 h after infection, one portion of cells was harvested and subjected to Western blotting. The other portion was exposed to radiation at different doses followed by cell growth evaluation 48 h later using ATPite assay. *D*, columns, mean from three independent experiments; *bars*, SE.

Table 1. TRAF2 silencing enhances radiation-induced G₂-M arrest

Cell lines/treatment	Sub-G ₁	G ₁	S	G ₂ -M
U251-si-Cont (0 Gy)	6.1	77.2	16.3	6.6
U251-si-Cont (10 Gy)	11.5	51.5	2.6	45.9
U251-si-TRAF2 (0 Gy)	12.4	76.7	13.8	9.4
U251-si-TRAF2 (10 Gy)	18.9	40.3	0.3	59.4
H1299-si-Cont (0 Gy)	2.9	37.5	61.1	1.4
H1299-si-Cont (10 Gy)	0.1	45.6	42.9	11.5
H1299-si-TRAF2 (0 Gy)	3.1	46.2	51.1	2.7
H1299-si-TRAF2 (10 Gy)	13.6	47	33.8	19.2
MEF-WT (0 Gy)	0.4	61.9	20	18.1
MEF-WT (10 Gy)	2.5	53.4	17.2	29.4
MEF-TRAF2 ^{-/-} (0 Gy)	1.2	59.1	28.5	12.4
MEF-TRAF2 ^{-/-} (10 Gy)	9.5	44.3	16.1	39.6
MEF-WT-SS (0 Gy)	4.3	30	56	14
MEF-WT-SS (10 Gy)	4.3	29.4	52.3	18.3
MEF-TRAF2 ^{-/-} SS (0 Gy)	1.8	34.5	54.2	11.3
MEF-TRAF2 ^{-/-} SS (10 Gy)	3.9	22.1	38.5	39.4

TRAF2 overexpression in lung cancer tissues and cell lines.

Oncomine database⁴ search for potential TRAF2 overexpression in tumor cells revealed that TRAF2 mRNA is dramatically overexpressed in lung adenocarcinoma compared with normal tissues (26). The data were retrieved and summarized in Fig. 4A (*left*). To further confirm TRAF2 overexpression in human lung adenocarcinoma tissues, we performed immunohistochemical analysis using a human TRAF2 antibody. As shown in Fig. 4A (*right*), whereas normal lung tissues showed no TRAF2 staining, lung tumor tissues showed significant staining of TRAF2 in the cytoplasm of cancer cells. These data show that TRAF2 is not expressed in normal lung tissue but overexpressed in lung cancer. We further determined whether TRAF2 is also overexpressed in lung cancer cell lines compared with immortalized bronchial epithelial cells (NL20). As shown in Fig. 4B, TRAF2 is detectable but low in NL20 cells. TRAF2 was found to be overexpressed in the majority of lung cancer cell lines tested.

TRAF2 silencing has a minimal effect on normal bronchial epithelial NL20 cells. Silencing of TRAF2 siRNA inhibited growth or survival of cancer cells and sensitized cancer cells to radiation, suggesting that TRAF2 is a promising cancer and radiosensitizing target. To gain the therapeutic index, however, inhibition of a potential cancer target should have a minimal effect on normal cells. To this end, we tested the effects of TRAF2 silencing on the growth and radiosensitization of normal bronchial epithelial NL20 cells. Compared with the control siRNA, TRAF2 siRNA-mediated knockdown (Fig. 4C) has no effect on normal cell growth nor effect on cellular sensitivity to radiation up to 5 Gy (Fig. 4D). We were unable to assess the effect on radiosensitization using the clonogenic assay because NL20 cells are nontransformed cells and cannot form colonies.

TRAF2 siRNA enhances radiation-induced G₂-M arrest and apoptosis. To elucidate the nature of growth suppression and radiosensitization by TRAF2 silencing, we performed fluorescence-activated cell sorting profiling analysis to reveal TRAF2 silencing-

or radiation-induced cell cycle redistribution as well as apoptosis as evidenced by appearance of a sub-G₁ population. As shown in Table 1, for untreated U251 cells, the majority of cells were in the G₁ phase of the cell cycle, regardless of TRAF2 silencing. Radiation treatment of the control U251 cells induced apoptosis as well as a G₂-M arrest. This was more pronounced after TRAF2 silencing, which resulted in a further increase of the apoptotic population (from 11.5% to 18.9%) and corresponding G₂-M arrest (from 45.9% to 59.4%). In H1299 cells, radiation caused a moderate induction of apoptosis, independent of TRAF2 silencing, but a significant induction of the G₂-M arrest. This radiation-induced G₂-M arrest was much more pronounced in TRAF2-silenced cells (from 11.5% to 19.2%).

We further confirmed the regulation of TRAF2 on radiation-induced G₂-M arrest using MEF cells derived from WT and TRAF2 knockout mice. Indeed, TRAF2 deletion increased the levels of radiation-induced apoptosis (from 2.5% to 9.5%) and G₂-M arrest (from 29.4% to 39.6%; Table 1). Because cells in the different phases of the cell cycle have a different sensitivity to radiation, we synchronized MEFs in the G₁ phase by serum starvation (SS) for 72 h (leading to 80% cell population in G₁ in both cell types; data not shown) before radiation. Serum-starved MEF cells were resistant to radiation-induced apoptosis but sensitive to G₂-M arrest, which was increased remarkably in TRAF2-null cells (39.4% versus 18.3% in control; Table 1). Thus, TRAF2 silencing or knockout enhances radiation-induced G₂-M arrest.

TRAF2 silencing reduces RIP-1 levels and blocks radiation-induced IκBα degradation and NF-κB activation. TRAF2 is an adaptor protein that binds to antiapoptotic proteins cIAP-1, cIAP-2, and RIP-1 to mediate TNFα/TNFR signal pathway, leading to NF-κB activation and cell survival (14, 16, 27), whereas ionizing radiation activates NF-κB (28) through degradation of IκB (29, 30). To elucidate the mechanism by which TRAF2 silencing induces growth suppression and radiosensitization, we determined the levels of cIAPs, RIP-1, as well as IκBα on TRAF2 silencing alone or in combination with radiation. As shown in Fig. 5A, TRAF2 levels in both H1299 and U251 cells were completely silenced on lenti-si-TRAF2 infection (*lanes 4-6 and 10-12*). Neither TRAF2 silencing nor radiation had any effects on the levels of cIAP-1 and

⁴ <http://www.oncomine.org>

ciAP-2. However, TRAF2 silencing significantly reduced the levels of RIP-1, particularly in H1299 cells, regardless of radiation (lanes 4–6 and 10–12). Furthermore, although TRAF2 silencing did not change the basal levels of I κ B α (lane 4 versus lane 1 and lane 10 versus lane 7), it did prevent radiation-induced degradation of I κ B α , particularly in U251 cells (lanes 2 and 3 versus lanes 5 and 6 and lanes 8 and 9 versus lanes 11 and 12). We further determined whether the failure to induce I κ B degradation, on TRAF2 silencing, would be translated to the loss of NF- κ B activation by a luciferase-based transactivation assay. As shown in Fig. 5B, in siControl-infected U251 cells, TNF α (serving as positive control for the assay) induced a 4-fold activation of NF- κ B, which is statistically significant ($P < 0.01$). In contrast, NF- κ B in TRAF2-silenced U251 cells was not activated by either TNF α or radiation. The results indicate that TRAF2 silencing blocks the activation of NF- κ B signaling pathway, which could contribute, at least in part, to observed growth suppression and radiosensitization.

TRAF2 siRNA silencing reduces the levels of several G₂-M checkpoint regulatory proteins. Because TRAF2 silencing enhances radiation-induced G₂-M arrest, we determined potential changes of proteins known to regulate G₂-M progression. As shown in Fig. 5C, in both H1299 and U251 cells, TRAF2 siRNA silencing reduced the levels of Wee1, cyclin B1, and Aurora-B (70–90%) and moderately reduced (20–50%) the levels of Cdc2, PLK-1, and Aurora-A. In H1299 cells, TRAF2 silencing also reduced the levels of Cdc25C but had little or no effect on the levels of Cdc25B and Chk2. We were unable to detect the expression of Cdc25C and Chk2 in U251 cells (Fig. 5C) and Chk1, 14-3-3 σ , and phosphorylated form of Chk1 and Chk2 in both lines (data not shown). Among these G₂-M checkpoint proteins, radiation seemed to induce cyclin B1, PLK-1,

and Aurora-B and the induction was largely independent of TRAF2 silencing. Thus, TRAF2 silencing caused the repression of several G₂-M checkpoint proteins, which likely contributes to the observed growth inhibition, enhanced G₂-M arrest on radiation, and radiosensitization.

Discussion

Through the siRNA-based screening, we identified the ubiquitin ligase TRAF2 as a candidate radiosensitizing target. Further characterization revealed that TRAF2 silencing remarkably inhibited tumor cell growth and survival and moderately sensitized cancer cells, including glioblastoma U251, lung cancer H1299, and head and neck squamous carcinoma UM-SCC-1, to radiation. Our observations that TRAF2 confers resistance to radiation, plays a role in cell survival, and is an E3 ubiquitin ligase with druggable feature suggest that TRAF2 is an attractive target for anticancer therapy and radiosensitization.

TRAF2 seems to meet several criteria for an ideal anticancer target (31, 32). TRAF2 is a cellular survival protein that protects cells from apoptosis (see refs. 14–16 for review). Likewise, we have shown here that siRNA silencing of TRAF2 suppressed the growth and survival of U251 and H1299 cells. Similarly, transfection of U251 cells with a RING-less TRAF2 dominant-negative mutant (33) significantly inhibited cell growth (data not shown). In addition, whereas TRAF2 overexpression dramatically protected cancer cells from radiation-induced killing, TRAF2 inhibition by siRNA or dominant-negative mutant sensitized cancer cells to radiation in three independent human cancer cell lines, indicating that this effect is a general phenomenon. A greater radiosensitizing effect, with SER value increasing from 1.2 up to 1.4, was observed when TRAF2 was transiently silenced at the time of radiation to avoid

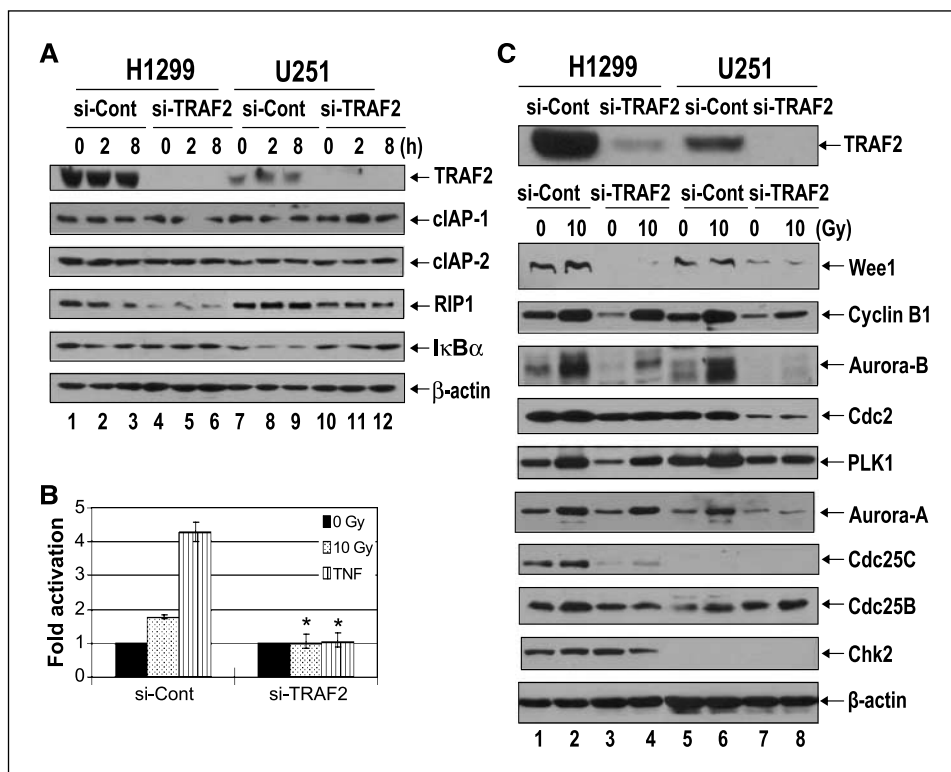


Figure 5. TRAF2 silencing reduced RIP-1 levels (A), blocked radiation-induced NF- κ B activation (B), and altered expression of several G₂-M checkpoint proteins (C). A, Western blot analysis. U251 and H1299 cells were infected with lenti-siRNA targeting TRAF2 (*si-TRAF2*), along with control (*si-Cont*). Seventy-two hours later, cells were exposed to irradiation at 10 Gy. Cells were harvested 2 or 8 h later, respectively, and subjected to Western blot analysis for indicated proteins with β -actin as the loading control. B, luciferase reporter assay for NF- κ B activity. U251 cells were infected with lentivirus targeting TRAF2, along with the control. Cells were then transfected with a luciferase reporter driven by NF- κ B consensus sequence, along with *Renilla* control for transfection efficiency, followed by radiation at 10 Gy or TNF α treatment for 3 h. Cells were harvested and subjected to luciferase activity assay. Columns, mean from three independent experiments; bars, SE. The paired Student's *t* test was performed to determine statistical difference. *, $P < 0.01$. C, Western blot analysis. H1299 and U251 cells were infected with lenti-si-TRAF2, along with lenti-si-Cont. Seventy-two hours after infection, cells were left untreated or exposed to radiation (10 Gy). Cells were harvested 24 h later and subjected to Western blot analysis with indicated antibodies. Representative blots were shown with β -actin as the loading control.

the high levels of cytotoxicity resulting from lenti-si-TRAF2-mediated constitutive silencing. Thus, TRAF2 silencing remarkably inhibits the growth and survival of cancer cells and to a lesser extent enhances radiation-induced cancer cell killing.

Importantly, we found that compared with normal lung tissues and nontransformed bronchial epithelial cells, the levels of TRAF2 were significantly higher in lung adenocarcinoma and in most lung cancer cell lines (Fig. 4). Few previous microarray profiling studies also showed that TRAF2 mRNA was overexpressed in lung cancer tissues (26, 34). Furthermore, TRAF2 overexpression was reported in pancreatic cancer, which protected cancer cells from apoptosis and promoted invasiveness (35, 36). Increased TRAF2 levels were also found in hepatocellular carcinoma, compared with nontumorous liver (37), in metastatic prostate cancer, compared with localized prostate carcinoma (38), or during melanoma progression (39). Thus, it seems that TRAF2 may be involved in carcinogenesis and/or tumor metastasis.

The E3 ubiquitin ligase activity of TRAF2 seems critical for its apoptosis-protecting function because RING-deleted TRAF2 mutant acts in a dominant-negative manner to block WT TRAF2 function (24, 39), consistent with our observation that TRAF2 dominant-negative mutant suppressed cancer cell growth and sensitized cancer cells to radiation. Importantly, WT TRAF2 protected cancer cells from radiation, whereas a RING mutant with minimal ligase activity lost such an activity. Thus, the inhibitors against TRAF2 E3 ligase could be identified and developed as a novel class of anticancer agent and radiosensitizer (31, 32, 40).

Mechanistically, prosurvival protein TRAF2 was found to bind to antiapoptotic protein cIAP-1 or cIAP-2 to block caspase-8 activation (41) or bind to TRADD and RIP, leading to I κ B kinase (IKK) activation, followed by NF- κ B activation (see refs. 13, 14, 16 for review). We found that TRAF2 silencing had no effect on the levels of cIAP-1 and cIAP-2 but did reduce the levels of RIP-1. Although it is not clear at the present time how TRAF2 silencing down-regulates RIP-1, the lower level of RIP-1 is likely to reduce IKK activation, followed by the lack of I κ B α degradation and of NF- κ B activation. This seems to be the case because in TRAF2-silenced U251 cells, radiation-induced I κ B α degradation was abolished and NF- κ B was no longer activated by either radiation or TNF α . Because NF- κ B is a predominant survival transcription factor (42), the lack of NF- κ B activation would likely contribute to less cellular viability and more radiosensitization, which is noted in TRAF2-silenced cells. Consistently, previous studies using MEF or thymocytes from TRAF2-null or TRAF2 dominant-negative mice also showed an increased sensitivity to TNF- α -induced apoptosis (17, 43). Moreover, expression of dominant-negative TRAF2 sensitized metastatic melanoma cells to UV-induced apoptosis via suppression of NF- κ B activity (39).

On DNA damage induced by ionizing radiation, mammalian cells are arrested at the G₂-M phase of cell cycle by checkpoint control proteins that allow time for damaged DNA to be repaired before cells enter the next cycle (see refs. 44, 45 for review). G₂-M progression is positively regulated by Cdc2/cyclin B1 and Cdc25B/Cdc25C and negatively regulated by Wee1, Chk1, and Chk2 kinases, whereas mitotic spindle checkpoints are controlled by PLK-1, Aurora-A, and Aurora-B kinases (44–47). In this study, we made several novel observations that (a) TRAF2 silencing has a profound inhibitory effect on the levels of both positive and negative regulators of G₂-M checkpoint proteins; (b) radiation induces expression of mitotic spindle checkpoint proteins, particularly Aurora-B (Fig. 5C); and (c) TRAF2 silencing enhances radiation-induced G₂-M arrest and cell killing (Table 1). It has been recently shown that targeting Aurora-B kinase via small molecular inhibitor, siRNA, or dominant-negative mutants sensitizes human cancer cells, particularly those with p53 deficiency, to radiation (48). Thus, radiation-induced Aurora-B expression could be a cellular protective response to ensure a mitotic arrest on radiation, whereas the elimination of Aurora-B via TRAF2 silencing would likely contribute to radiosensitization of p53-null (H1299) and p53-mutant (U251) cancer cells. On the other hand, the lack of obvious changes in cell cycle distribution on TRAF2 silencing may reflect a neutralizing consequence of simultaneous down-regulation of both positive and negative G₂-M checkpoint regulators. Moreover, the profound inhibition of G₂-M regulatory proteins on TRAF2 silencing would make it difficult to rescue the phenotypes of growth inhibition and radiosensitization by overexpression of one or two such proteins. Given the fact that many of these checkpoint proteins are well-characterized cancer targets and many of their inhibitors are currently being developed at the different phases of clinical trials (46, 47), the finding that TRAF2 silencing inhibits the expression of multiple checkpoint proteins makes TRAF2 a more appealing anticancer target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 2/19/2008; revised 6/27/2008; accepted 7/4/2008.

Grant support: National Cancer Institute grants CA111554, CA116982, and CA118762 and the University of Michigan Comprehensive Cancer Center MUNN Idea award (Y. Sun).

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We thank Dr. Tak Wah Mak for MEFs from TRAF-null mice, Dr. James Chen for the TRAF2 RING deletion mutant, Dr. Qingyang Gu for making TRAF2 RING mutant, Dr. Ted Lawrence for stimulating discussion, and Dr. Swaroop Bhojani for providing NF- κ B luciferase reporter and TNF α and for help in plotting Oncomine TRAF2 expression data.

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Cancer Res 2008;68:7570-7578.

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