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Tumor and Stem Cell Biology

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FOXO3a-Dependent Mechanism of E1A-Induced Chemosensitization

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Abstract

Gene therapy trials in human breast, ovarian, and head and neck tumors indicate that adenovirus E1A can sensitize cancer cells to the cytotoxic effects of paclitaxel *in vitro* and *in vivo*. Resistance to paclitaxel has been reported to occur in cells expressing low levels of the Forkhead transcription factor FOXO3a. In this article, we report that FOXO3a is critical for E1A-mediated chemosensitization to paclitaxel. RNA interference–mediated knockdown of FOXO3a abolished E1A-induced sensitivity to paclitaxel. Mechanistic investigations indicated that E1A indirectly stabilized FOXO3a by acting at an intermediate step to inhibit a ubiquitin-dependent proteolysis pathway involving the E3 ligase βTrCP and the FOXO3a inhibitory kinase IKKβ. E1A derepressed this inhibitory pathway by stimulating expression of the protein phosphatase 2A (PP2A)/C protein phosphatases, which by binding to the TGF-β–activated kinase TAK1, inhibited its ability to activate IKKβ and, thereby, to suppress βTrCP-mediated degradation of FOXO3a. Thus, by stimulating PP2A/C expression, E1A triggers a signaling cascade that stabilizes FOXO3a and mediates chemosensitization. Our findings provide a leap forward in understanding paclitaxel chemosensitization by E1A, and offer a mechanistic rational to apply *E1A* gene therapy as an adjuvant for improving therapeutic outcomes in patients receiving paclitaxel treatment. *Cancer Res; 71(21); 6878–87. ©2011 AACR.*

Introduction

Adenovirus type 5 E1A (E1A) was originally recognized as an oncogene that could facilitate oncogenic transformation by other viral and cellular oncogenes. However, E1A has not been associated with human malignancies despite extensive efforts to identify such a link (1). Instead, E1A was shown to have antitumor activities by reversing the transformed phenotype, inhibiting metastasis, and inducing apoptosis in multiple transformed rodent cells and human cancer cell lines (2–6). In addition to the tumor suppressor activities, expression of the *E1A* gene in stably transfected normal fibroblasts and human cancer cells has been shown to induce sensitization among different categories of anticancer drugs *in vitro*, including etoposide, cisplatin, doxorubicin, gemcitabine, TNF–related apoptosis-inducing ligand (TRAIL), histone deacetylase (HDAC) inhibitors, and paclitaxel in normal fibroblasts and in sarcoma, non–small cell lung, hepatocellular, ovarian, and breast cancer cells (7–12). Furthermore, animal studies showed that the combination of systemic *E1A* gene therapy with paclitaxel significantly enhanced paclitaxel-induced apoptosis and prolonged survival rates in the animal orthotopic model *in vivo* (11, 13). Therefore, *E1A* is, at present, considered a tumor suppressor gene and has been tested in multiple clinical trials in a gene therapy setting for patients with breast (14, 15), ovarian (2, 14), and head and neck cancers (15, 16). A clinical study using *E1A* gene therapy combined with paclitaxel has been initiated for treatment of ovarian cancer. Thus, it is critical and timely to understand the detailed molecular mechanisms that are associated with E1A-mediated chemosensitization, and future clinical trials using the combination of chemotherapy with *E1A* gene therapy can be further improved.

One of the molecular mechanisms by which E1A induces chemosensitization is downregulation of Her-2/neu overexpression (8, 11). Recently, inhibition of Akt and activation of p38 was reported to provide a general cellular mechanism for E1A-mediated chemosensitization (9, 17). Regulation of some critical tumor suppressors was also proposed as being involved in E1A-induced chemosensitization, such as p53 and p19ARF (18), the proapoptotic protein Bax, caspase 9, and a yet-unidentified

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inhibitor that ordinarily provides protection against cell death (11, 19–22).

Forkhead box O–class (FOXO) transcription factors include FOXO1 (FKHR; Forkhead in rhabdomyosarcoma), FOXO3a (FKHRL1; FKHR-like 1), and FOXO4 (AFX; acute lymphocytic leukemia–fused gene from chromosome X). The FOXOs activate and/or repress transcription of genes involved in metabolism, apoptosis, DNA damage repair, and cell-cycle progression (23). For example, FOXO3a has been shown to enhance $p27^{kip}$ expression and induce cell-cycle arrest (24). Furthermore, FOXO3a and FOXO4 have been shown to inhibit the cell cycle through downregulation of cyclin D by a $p27^{kip}$-independent mechanism (25, 26). In breast cancer, FOXO3a has been shown to upregulate Bim, a proapoptotic BH3-only protein (25, 27). The activity of the FOXOs can be inhibited by activating the phosphoinositide 3-kinase (PI3K)/Akt pathway. FOXO3a can be phosphorylated by Akt at 3 conserved serine/threonine residues (Thr-32, Ser-253, and Ser-315), and it subsequently translocates from the nucleus to the cytoplasm, where it is retained by binding to the 14-3-3 protein (28). FOXO3a activity can also be inhibited by the IκB kinase (IKK) signaling pathway. IKK physically interacts with and phosphorylates FOXO3a independently of Akt, which causes nuclear exclusion of FOXO3a and, subsequently, proteolysis of FOXO3a via the βTrCP-mediated ubiquitin (Ub)-dependent proteasome pathway (29). Recently, extracellular signal-regulated kinase (Erk) was also shown to phosphorylate FOXO3a at Ser-294, Ser-344, and Ser-425 sites, which enhance interaction with the E3 Ub ligase MDM2, resulting in FOXO3a degradation (30). However, the biologic function and detailed molecular mechanism of FOXO3a proteolysis in E1A-mediated chemosensitization are still unclear.

In an attempt to understand the molecular mechanism of E1A-mediated chemosensitization, we found that FOXO3a is critical to that process. E1A stabilizes FOXO3a by preventing βTrCP-mediated Ub-dependent proteolysis through inhibiting the phosphorylation of FOXO3a at Ser-644 by IKKβ. E1A induces the expression of protein phosphatase 2A (PP2A; a protein phosphatase involved in multiple cellular functions, including chemosensitization), which inhibits TGF-β–activated kinase 1 (TAK1)-activated IKK signaling, thus stabilizing FOXO3a and inducing chemosensitization.

Materials and Methods

Cell lines, DNA constructs, and antibodies

Cells of the cell lines MDA-MB-231, HeLa, and MDA-157 were purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% FBS. The human breast cancer cell line MDA-MB-231 and its E1A/vector-stable transfectants have been described previously (31). The transfectants were grown under the same conditions as the controls, except that G418 was added to the culture medium. Cell lines have been characterized using DNA analysis by STR fingerprinting (HeLa, March 2009; MDA-MB-231, December 2010; MDA-MB-157, ongoing). Cell lines were frozen after they were received from the ATCC and had not been passed for more than 6 months in culture when the experiments were carried out.

Plasmids E1A (2), IKKβ (29), βTrCP siRNA plasmids (kindly provided by Dr. Serge Y. Fuchs, University of Pennsylvania, Philadelphia, PA), PP2A/A, PP2A/C (17), and TAK1-HA (32) were described previously. FOXO3a siRNA plasmids were kindly provided by Dr. Alex Toker (Harvard Medical School, Boston, MA).

The monoclonal antibody used against the E1A protein was M58 (Pharmingen). The following were obtained as indicated: HA (11666606001; Roche), FOXO3a (SC-11351; Santa Cruz Biotechnology), IKKβ (2684; Cell Signaling Technology, or SC-7607; Santa Cruz Biotechnology), and pIKKβ (S181; 2681; Cell Signaling Technology). Rabbit anti-human PP2A/A and PP2A/C antibodies were purchased from CalBiochem. In addition, we purchased the following from the suppliers indicated: Ub (3936; Cell Signaling Technology), βTrCP (37-3400; Zymed, or SC-15354; Santa Cruz Biotechnology), TAK1 (SC-7967; Santa Cruz Biotechnology), pTAK1 (4531S; Cell Signaling Technology), and α-tubulin (T-5168; Sigma). Recombinant human TNFα was purchased from Roche. MG132 was purchased from Sigma.

Immunoprecipitation and Western blotting

Cells were washed twice with PBS, scraped into 500 μL of lysis buffer, and incubated on ice for 20 minutes. After centrifugation at 14,000 × *g* for 10 minutes, 1.5 mg of each supernatant was preincubated with 2 μg of immunoglobulin G (IgG) and 50 μL of protein G for 1 hour at 4°C. Immunoprecipitation was carried out overnight with 2 μg antibody and 50 μL of protein G. The immunocomplex was washed 5 times with lysis buffer, dissolved in loading buffer, subjected to SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween-20 and incubated with primary antibodies, followed by secondary antibodies (Jackson ImmunoResearch Laboratories). The immunoblots were visualized with enhanced chemiluminescence (Amersham).

Paclitaxel-induced cell death

Cells were treated with 20 nmol/L paclitaxel and incubated for 24 hours. Aliquots of 1×10^6 cells were collected and washed once with ice-cold PBS and then fixed with ice-cold 70% ethanol overnight. After fixation, cells were washed with PBS to remove residual ethanol, pelleted, and resuspended in PBS containing 50 μg/mL of propidium iodide (PI; Sigma). Staining was done at 4°C for at least 30 minutes, and samples were analyzed using an Epics PROFILE flow cytometer (Coulter) in the Core Facility at The University of Texas MD Anderson Cancer Center (Houston, TX).

Orthotopic breast tumor growth assay

Six-week-old female severe combined immunodeficient (SCID) mice were orthotopically inoculated with tumor cells into the mammary fat pad and treated with vehicle or paclitaxel as described previously (9). Tumor development was followed in individual animals (8 per group) by measuring tumor length (L) and width (W) with calipers every 3 days. Tumor volume was calculated with the formula $LW^2/2$. All

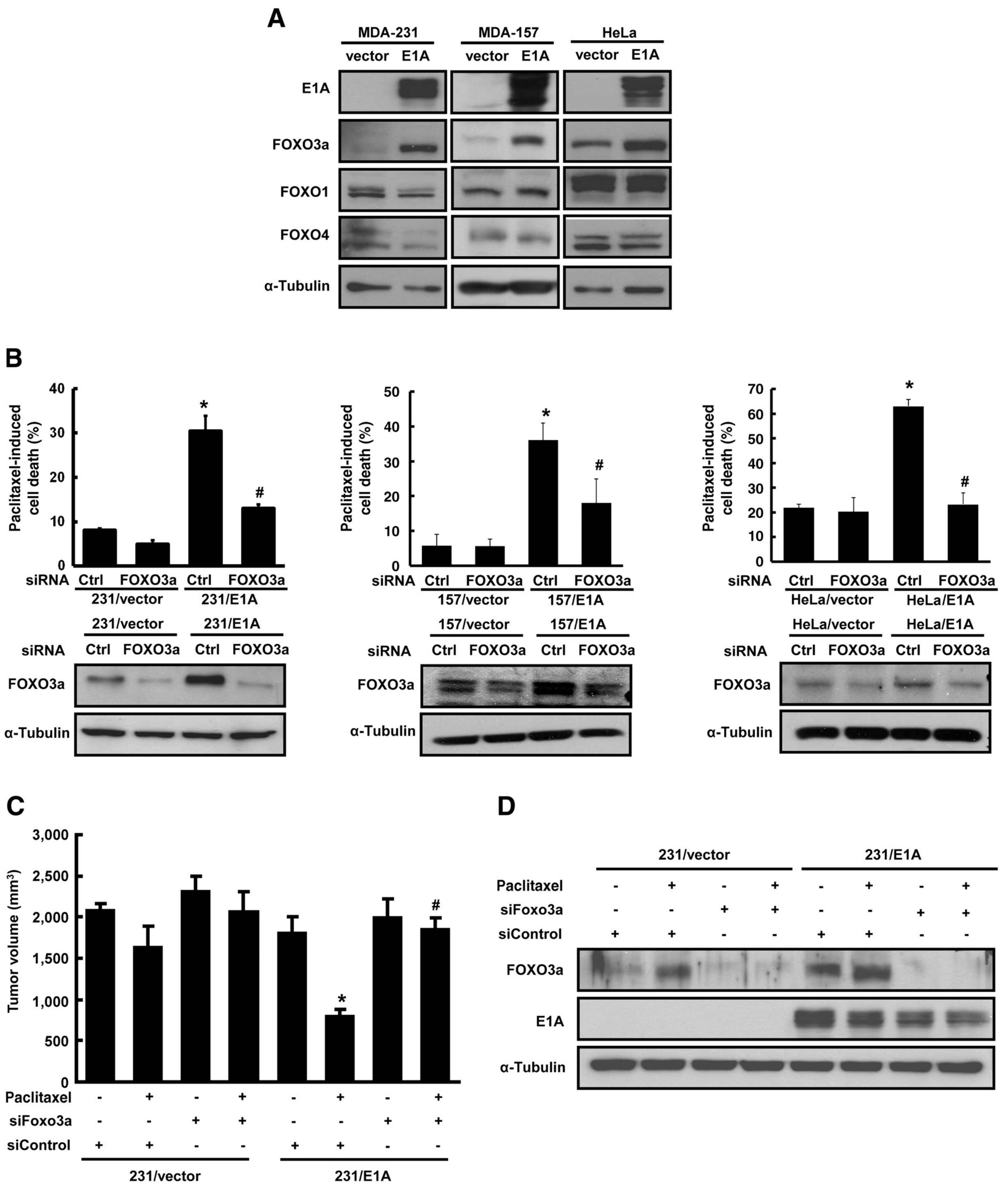

Figure 1. FOXO3a is critical for E1A-mediated chemosensitization. A, E1A-expressing vector (E1A) or control vector (vector) was transfected into different types of cells, followed by analysis of E1A, FOXO1, FOXO4, and FOXO3a protein expression using Western blot analysis. α-Tubulin was used as the internal protein loading control. B, E1A-induced FOXO3a expression was required for E1A-mediated chemosensitization. Top, chemosensitization of E1A-expressing cells or vector control cells transfected with siFOXO3a or control (Ctrl) siRNA as analyzed by the DNA flow cytometric assay. Each type of transfected cell was treated with 20 nmol/L paclitaxel for 24 hours. The columns are the mean values from 3 independent experiments. Bars indicate means ± SE. *, statistically significant difference compared with values of column 1 (*, $P < 0.05$, the 2-tailed Student *t* test). E1A-dependent chemosensitization was overturned by siFOXO3a to a significant degree, as indicated by the # symbol. Bottom, expression of FOXO3a was analyzed by Western blotting. C, tumor volume of orthotopic xenograft tumors formed by MDA-MB-231/vector cells or MDA-MB-231/E1A cells stably transfected with either control siRNA

animal work and care were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of China Medical University (Taichung, Taiwan).

Results

FOXO3a is critical for E1A-mediated chemosensitization

E1A gene therapy has been shown to induce chemosensitization among different chemotherapeutic agents, including paclitaxel in breast and ovarian cancers (33). It has been shown that resistance to paclitaxel occurs in cells expressing low levels of FOXO3a (34). We, therefore, speculated whether FOXO3a might contribute to E1A-mediated chemosensitization. To this end, we examined the effects of E1A on FOXO3a expression in various types of cancer cells including MDA-MB-231, HeLa, and MDA-MB-157 and found that expression of FOXO3a was significantly increased in *E1A*-transfected cells (Fig. 1A). We found that FOXO3a-regulated apoptotic genes, such as *FasL* and *p27*, were increased in *E1A*-transfected cells and decreased by FOXO3a knockdown (Supplementary Fig. S1A) and involved in E1A-mediated chemosensitization (Supplementary Fig. S1B). More importantly, E1A-induced chemosensitization of paclitaxel was abolished by knockdown of FOXO3a expression using FOXO3a-specific small interfering RNA (siFOXO3a) in *E1A*-transfected MDA-MB-231, HeLa, and MDA-MB-157 cancer cell lines (Fig. 1B). Furthermore, E1A-induced chemosensitization of doxorubicin and cisplatin was reduced by knockdown of FOXO3a (Supplementary Fig. S1C). Using the established stable transfectants, we further investigated the effects of FOXO3a on E1A-mediated chemosensitization in a xenograft tumor model in which mice were injected orthotopically with stably transfected cell clones. The results indicated that E1A induces the chemosensitization of paclitaxel *in vivo*, in that the tumor volume in 231/E1A-bearing mice treated with paclitaxel was significantly less than that in 231/vector-bearing mice treated with paclitaxel (810.7 ± 73.2 mm^3 vs. $1{,}648.7 \pm 237.4$ mm^3; Fig. 1C, lane 6 vs. lane 2). E1A-induced chemosensitization to paclitaxel was abolished by knockdown of the expression of FOXO3a by stable expression of siFOXO3a in 231/E1A cells (810.7 ± 73.2 mm^3 vs. $1{,}855.1 \pm 135.8$ mm^3; Fig. 1C, lane 6 vs. lane 8). Increased tumor volumes by siFOXO3a treatment in 231/E1A correlated well with reduced FOXO3a expression in the tumors (Fig. 1D). We, therefore, concluded that FOXO3a is required for the E1A-mediated chemosensitization to paclitaxel.

E1A prevents Ub-dependent proteolysis of FOXO3a

Posttranslational modification and regulation of FOXO3a protein stability are critical for FOXO3a activity (29). Therefore, we attempted to determine the stability of FOXO3a protein in response to E1A in breast cancer cells. For this analysis, we treated control vector and E1A-expression vector-stable transfectants (231/vector and 231/E1A) with cycloheximide for various times to block *de novo* protein synthesis and found that the half-life of FOXO3a protein was more than 7 hours for E1A-transfected cells but less than 1.5 hours for control cells by using Western blot analysis (Fig. 2A). TNFα-mediated FOXO3a polyubiquitination (29) was significantly decreased in 231/E1A cells compared with that in 231/vector cells (Fig. 2B). These results indicate that E1A increases FOXO3a protein expression by preventing Ub-dependent proteolysis of FOXO3a.

βTrCP is involved in E1A-induced FOXO3a induction

βTrCP oncogenic Ub E3-ligase interacts with FOXO3 and induces its Ub-dependent degradation in an IKKβ phosphorylation dependent manner (27). Thus, we asked whether βTrCP was involved in E1A-mediated FOXO3a protein stabilization. To this end, we first asked whether βTrCP physically interacted with FOXO3a. We analyzed proteasome inhibitor MG132-treated 231/vector and 231/E1A cell lysates by reciprocal co-immunoprecipitation (IP) followed by immunoblotting (IB) using antibodies against FOXO3a and βTrCP. Our results showed that endogenous FOXO3a was associated with endogenous βTrCP *in vivo* in 231/vector cells and this interaction was stimulated by TNFα treatment. Interestingly, TNFα-induced binding between FOXO3a and βTrCP was significantly reduced in E1A-expressing cells (Fig. 2C). In addition, βTrCP was shown to be required for maintenance of low FOXO3a expression by using siRNA of βTrCP. Transfection with siβTrCP increased FOXO3a expression in 231/vector but not in 231/E1A cells (Fig. 2D). In addition, knockdown of βTrCP abolished the association between FOXO3a and βTrCP (Fig. 2D). Taken together, these results indicate that E1A inhibits interaction of FOXO3a and βTrCP, which may prevent FOXO3a degradation.

Inhibition of TAK1-IKK signaling is required for E1A-mediated prevention of βTrCP/FOXO3a interaction and chemosensitization

It is known that βTrCP interacts with FOXO3 and induces its Ub-dependent degradation in an IKKβ–phosphorylation-dependent manner (29). To define whether E1A-mediated FOXO3a stabilization is attributable to prevention of FOXO3a phosphorylation by IKKβ and subsequent recognition by βTrCP, we investigated the association between FOXO3a and βTrCP in 231/vector and 231/E1A cells transfected with IKKβ-expression plasmid or control vector. Notably, transfection with the IKKβ expression vector reestablished the association between FOXO3a and βTrCP in 231/E1A cells (Fig. 3A, left). To further investigate whether inactivation of IKKβ by E1A is required for E1A-induced chemosensitization, we transiently transfected E1A expression vector with or without IKKβ expression vector into MDA-MB-231 cells and determined the effects of paclitaxel-induced cell death. E1A-induced chemosensitization was strikingly suppressed by transfection with IKKβ expression vector (Fig. 3A, right), supporting the notion that E1A-repressed IKKβ activity is required for E1A-mediated

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(siControl) or FOXO3a siRNA (siFOXO3a). Each column represents the mean ± SD of 8 primary tumors. *, $P < 0.05$ versus column 2 values, by the 2-tailed Student *t* test. E1A-dependent chemosensitization was overturned by siFOXO3a to a significant degree, as indicated by the # symbol. D, expression of FOXO3a protein and E1A was examined by immunoblotting assays using MDA-MB-231/vector tumors or MDA-MB-231/E1A tumors stably transfected with either control siRNA (siControl) or FOXO3a siRNA (siFOXO3a) in combination with paclitaxel treatment.

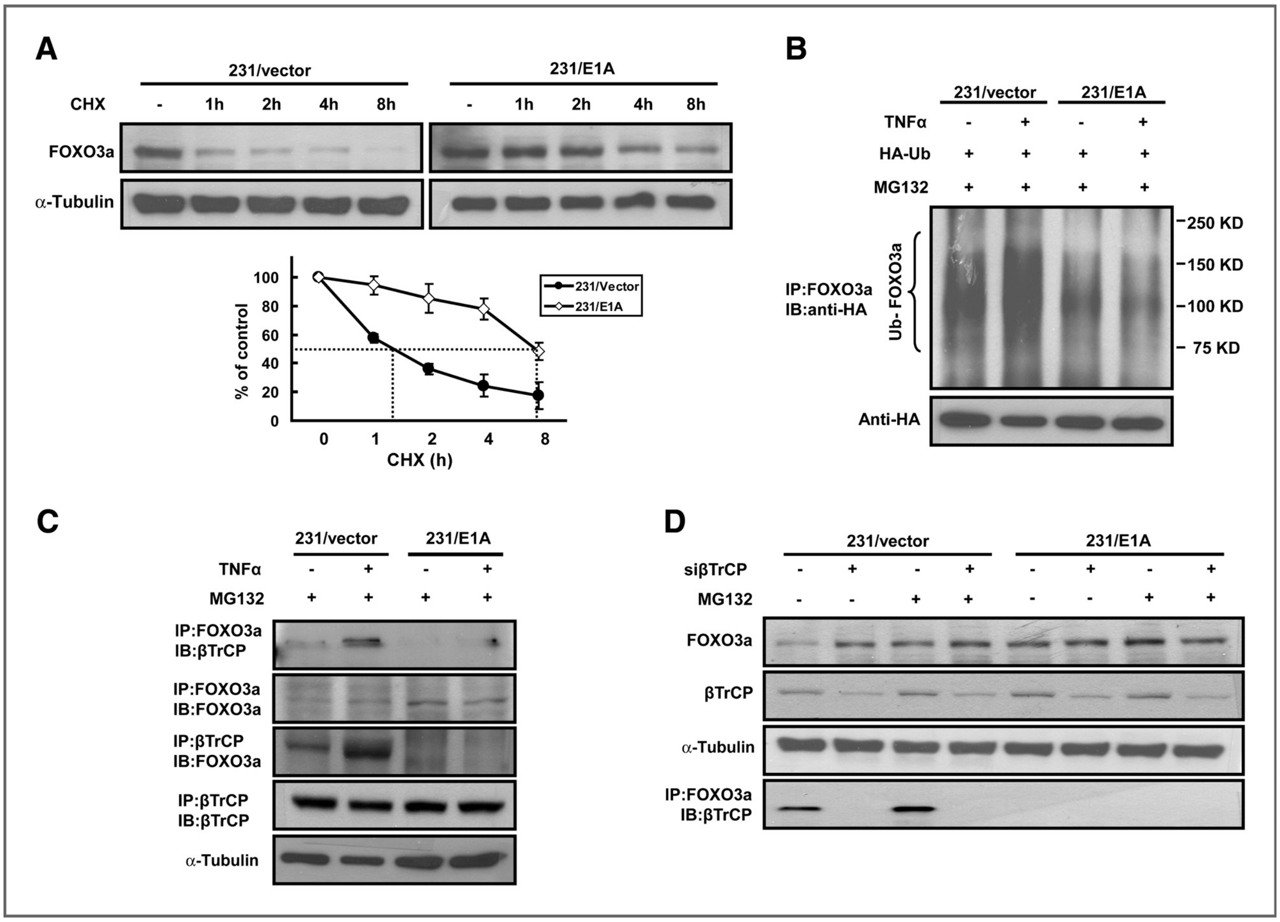

Figure 2. E1A prevents βTrCP-mediated ubiquitin-dependent proteolysis of FOXO3a. A, determination of the protein stability of FOXO3a in MDA-MB-231/vector cells and MDA-MB-231/E1A cells. The 231/vector cells and 231/E1A cells were treated with 100 μg/mL cycloheximide (CHX) for the indicated times. Total protein was isolated, and expression of FOXO3a was analyzed by Western blot assay and quantified (bottom). The results are representative of at least 3 independent experiments. Error bars, SD. B, top, 231/vector or 231/E1A cells transfected with HA-ubiquitin (HA-Ub) were treated with the proteasome inhibitor MG132 with or without TNFα (50 ng/mL), and the lysates of these cells were analyzed using immunoprecipitation (IP)/immunoblotting (IB). Bottom, lysates of 231/vector or 231/E1A cells transfected with HA-Ub were subjected to Western blotting. C, 231/vector or 231/E1A cells were treated with the proteasome inhibitor MG132 with or without TNFα (50 ng/mL), and the lysates of these cells were analyzed by IP/IB. D, knockdown of βTrCP expression by βTrCP-specific siRNAs increased FOXO3a expression and disrupted the interaction between βTrCP and FOXO3a. The 231/vector cells and 231/E1A cells were transfected with siβTrCP or control siRNA. Forty-eight hours after transfection, total proteins were isolated, and expression of FOXO3a and βTrCP was analyzed by Western blotting. Lysates of 231/vector or 231/E1A cells transfected with siβTrCP or control siRNA in the presence of MG132 (5 μmol/L) were analyzed by IP/IB (anti-FOXO3a/anti-βTrCP).

paclitaxel chemosensitization. To determine whether the Ser-644–phosphorylated FOXO3a is capable of reestablishing the association between FOXO3a and βTrCP in 231/E1A cells, we transfected the GFP-tagged Ser-644 phosphorylation-mimic mutant FOXO3a, GFP-FOXO3a-S644E, into 231/vector and 231/E1A cells. Expression of GFP-FOXO3a-S644E reestablished the association between FOXO3a and βTrCP in 231/E1A cells (Supplementary Fig. S2). The above-described data indicated that phosphorylation of FOXO3a at Ser-644 is critical for the association between FOXO3a and βTrCP. To further define whether Akt and ERK signaling pathways are involved in E1A-mediated FOXO3a expression and paclitaxel chemosensitization, we modulated these 2 kinases by a specific inhibitor or genetic modulation. On one hand, E1A-induced FOXO3a expression and chemosensitization were suppressed by transfection with constitutively activated Akt (Myr-Akt) expression vector (Supplementary Fig. S3). On the other hand, we found that treatment with a MAP/ERK kinase (MEK) inhibitor, U0126, slightly increased FOXO3a expression and paclitaxel chemosensitization in 231/vector cells but not in 231/E1A cells (Supplementary Fig. S4). The above-described data indicate that the Akt but not ERK signaling pathway may also be involved in E1A-mediated FOXO3a regulation and paclitaxel chemosensitization.

To explore the mechanism(s) through which inhibition of IKK activity participates in the cellular responses to E1A, we determined the phosphorylation of IKK in 231/vector and 231/E1A cells. Consistent with a previous report (35), treatment with TNFα increased the phosphorylation of IKK in 231/vector cells, but this activation was abolished in 231/E1A cells (Fig. 3B). These data indicated that E1A-induced inhibition of IKK signaling may target the upstream kinase of IKK. Recent

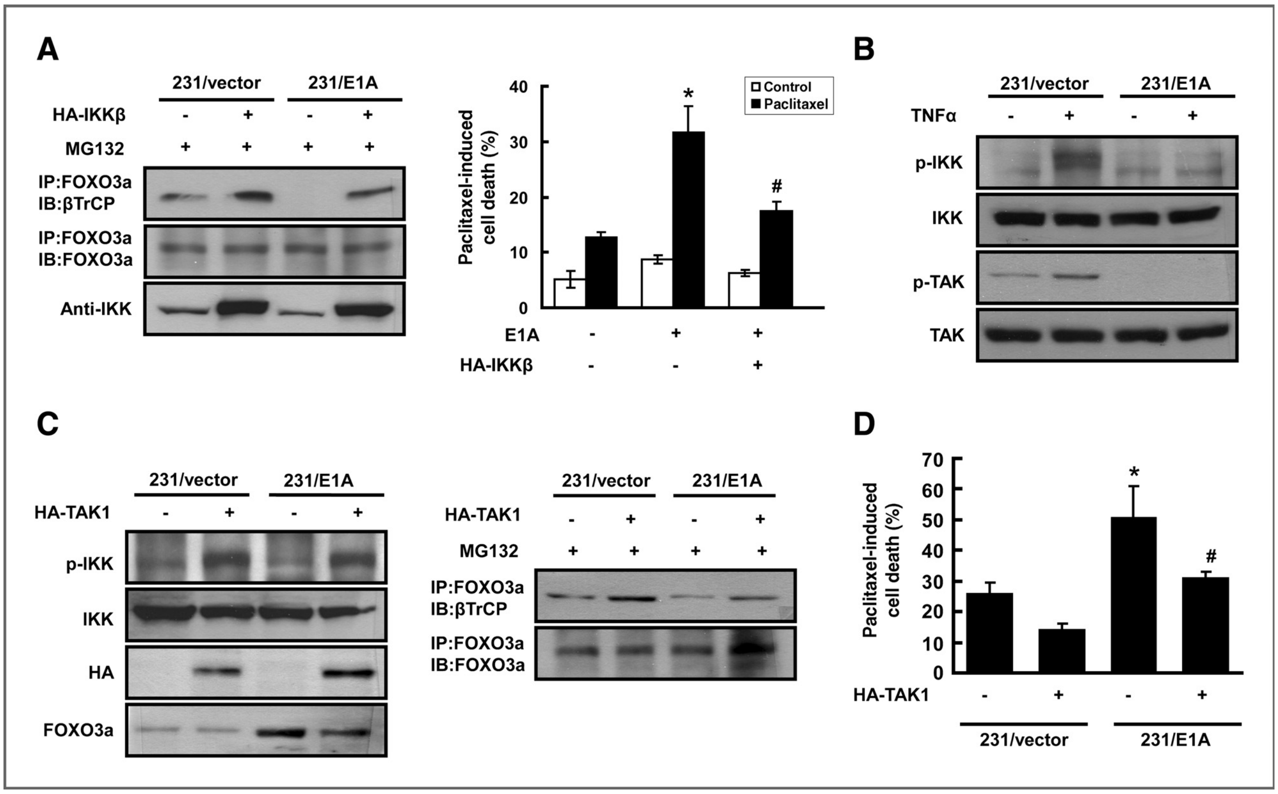

Figure 3. Inhibition of TAK1-IKKβ signaling is required for E1A-mediated prevention of βTrCP/FOXO3a interaction and chemosensitization. A, IKKβ was required for E1A-mediated prevention of interaction between βTrCP and FOXO3a and chemosensitization. Left, lysates of 231/vector cells and 231/E1A cells transfected with the HA-IKKβ expression vector in the presence of MG132 (5 μmol/L) were analyzed by IP/IB (anti-FOXO3a/anti-βTrCP, anti-FOXO3a). Right, chemosensitization of 231/E1A and 231/vector cells transfected with HA-IKKβ expression plasmid or control vector as analyzed by the DNA flow cytometric assay. Each type of transfected cell was treated with 20 nmol/L paclitaxel for 24 hours. The columns are the mean values from the 3 independent experiments. Bars indicated means ± SE. *, statistically significant difference compared with values of group 1 (*, $P < 0.05$, the 2-tailed Student *t* test). E1A-dependent chemosensitization was overturned by HA-IKKβ to a significant degree, as indicated by the # symbol. B, lysates of MDA-MB-231/vector and MDA-MB-231/E1A cells left untreated or treated with TNFα (50 ng/mL) were subjected to Western blotting to analyze the phosphorylation of IKK and TAK1. C, lysates of 231/vector and 231/E1A cells transfected with or without HA-TAK1 expression plasmid were subjected to Western blotting to analyze the expression of FOXO3a and phosphorylated IKK protein (left). The interaction between βTrCP and FOXO3a was determined by IP and Western blotting (right). D, 231/E1A and 231/vector cells were transfected with HA-TAK1 expression plasmid or control vector and then analyzed for paclitaxel-induced cell death by DNA flow cytometry. Each type of transfected cells was treated with 20 nmol/L paclitaxel for 24 hours. The columns are the mean values from the 3 independent experiments. Bars indicate means ± SE. *, statistically significant difference compared with values of column 1 (*, $P < 0.05$, the 2-tailed Student *t* test). E1A-dependent chemosensitization was overturned to a significant degree by overexpression of HA-TAK1, as indicated by the # symbol.

evidence indicates that TAK1 is essential for the activation of IKK in multiple signaling pathways (26). Therefore, we investigated the possible involvement of TAK1 in E1A-mediated inhibition of IKK signaling, FOXO3a stabilization, and chemosensitization. We found that treatment with TNFα increased the phosphorylation of TAK1 in 231/vector cells, and this TNFα-induced phosphorylation was diminished by expression of E1A (Fig. 3B). Furthermore, transfection with the HA-TAK1 expression vector significantly increased phosphorylation of IKK, and the E1A-mediated downregulation of p-IKK was overcome by the forced expression of HA-TAK1 (Fig. 3C, left). Experiments were also carried out to ascertain whether TAK1 is involved in E1A-mediated FOXO3a interaction with βTrCP and chemosensitization. Forced expression of HA-TAK1 significantly increased the interaction between FOXO3a and βTrCP in 231/vector cells, and the E1A-mediated inhibition effect was also recovered by exogenous expression of HA-TAK1 (Fig. 3C, right). Consistently, E1A-mediated chemosensitization to paclitaxel was significantly impaired by expression of TAK1 (Fig. 3D). Taken together, these data indicate that TAK1, the upstream kinase of IKK, is a critical regulator for E1A-repressed FOXO3a interaction with βTrCP and is required for E1A-mediated chemosensitization.

E1A-induced PP2A expression is required for regulation of TAK1-IKK signaling, βTrCP/FOXO3a interaction, and chemosensitization

Phosphorylation of protein kinases is tightly regulated by related protein phosphatases, and it has been reported that E1A increases the expression of PP2A/C, the catalytic subunit of PP2A (14). Because E1A inhibits TAK1 phosphorylation, we asked whether PP2A might be involved in E1A-mediated dephosphorylation of TAK1 and IKK and FOXO3a stabilization. To this end, 231/vector and 231/E1A cells were

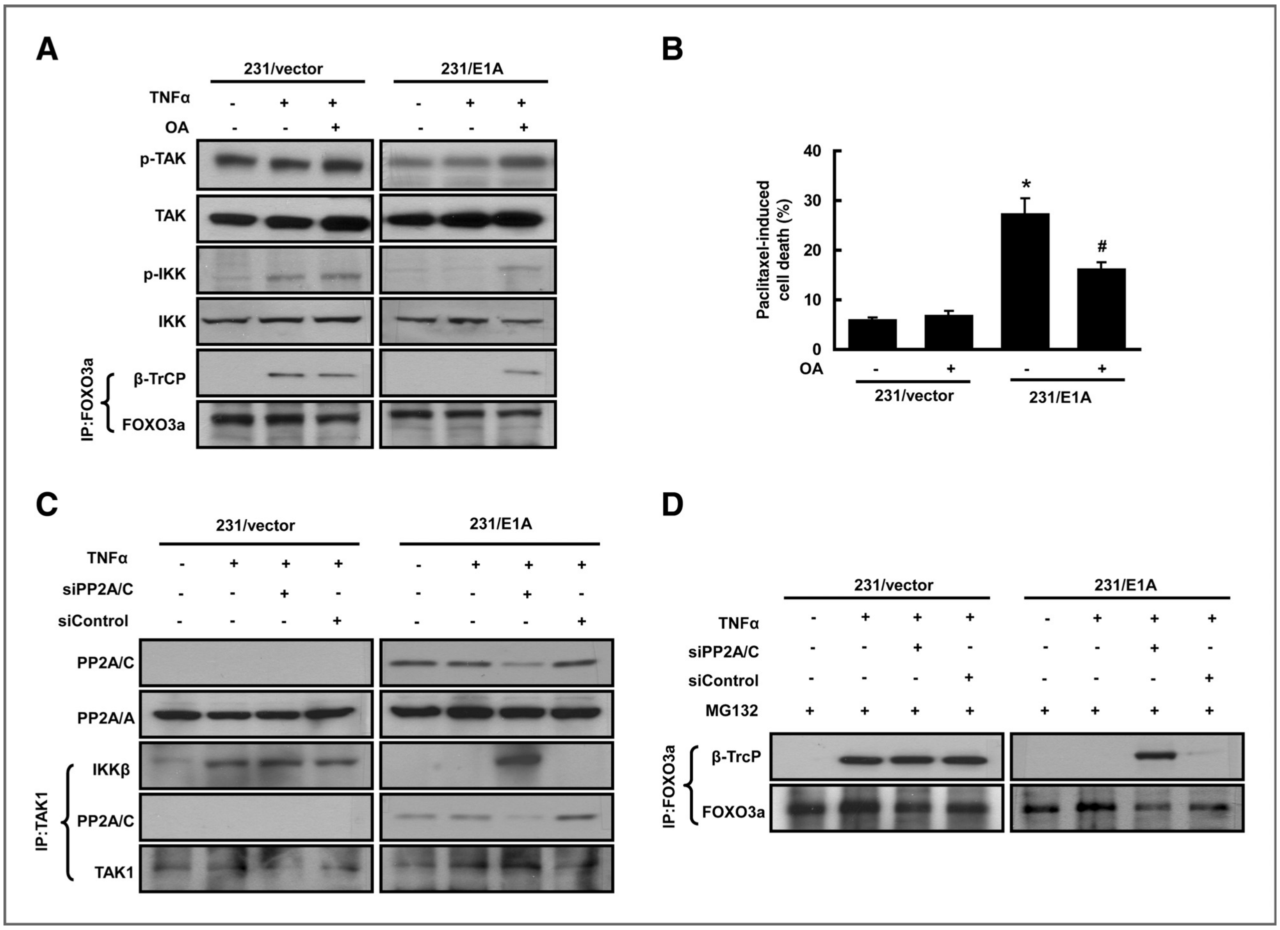

Figure 4. E1A-induced PP2A expression is required for regulation of TAK1-IKK signaling, βTrCP/FOXO3a interaction, and chemosensitization. A, Western blot analyses of the phosphorylation of TAK1 and IKK and the interaction between FOXO3a and βTrCP in MDA-MB-231/vector and MDA-MB-231/E1A cells left untreated or treated with TNFα (50 ng/mL) and 10 nmol/L OA. Equal amounts of cell lysates were subjected to Western blotting with specific antiphosphorylated TAK1 and IKK antibodies and anti-TAK1 and IKK antibodies. The results are representative of at least 3 independent experiments. The cell lysates were also subjected to IP/IB (anti-FOXO3a/anti-βTrCP) analysis. B, 231/E1A and 231/vector cells were untreated or treated with OA (10 nmol/L) combined with 20 nmol/L paclitaxel for 24 hours and then analyzed for chemosensitization by DNA flow cytometry. The columns are the means of 3 independent experiments. Bars indicate means ± SE. *, statistically significant difference compared with values of column 1 (*, $P < 0.05$, the 2-tailed Student *t* test). E1A-dependent chemosensitization was overturned to a significant degree by treatment with OA, as indicated by the # symbol. C, E1A-induced PP2A/C expression was required for E1A-mediated signaling. Lysates of 231/vector and 231/E1A cells transfected with siPP2A/C or control siRNA were subjected to IP/IB (anti-TAK1/anti-IKK and anti-PP2A/C) analysis and Western blotting (PP2A/C and PP2A/A). D, lysates of 231/vector and 231/E1A cells transfected with siPP2A/C or control siRNA were subjected to IP/IB (anti-FOXO3a/anti-FOXO3a and anti-βTrCP) analysis.

treated with the phosphatase inhibitor okadaic acid (OA). We found that E1A-mediated inhibition of TNFα-induced TAK1 phosphorylation was restored by OA treatment, as were E1A-mediated inhibition of IKK phosphorylation and the interaction between FOXO3a and βTrCP (Fig. 4A). Consistently, E1A-induced chemosensitization was also decreased in 231/E1A cells by treatment with OA (Fig. 4B). We next examined whether PP2A binds to TAK1. As shown in Fig. 4C, treatment with TNFα notably increased the interaction between TAK1 and IKK in 231/vector cells but not in 231/E1A cells. Moreover, PP2A/C formed a complex with TAK1 in 231/E1A cells and severely impaired the TNFα-induced interaction between TAK1 and IKK (Fig. 4C).

To confirm this novel binding between PP2A and TAK1, we transfected 231/vector and 231/E1A cells with siRNA for catalytic subunit of PP2A, PP2A/C, (siPP2A/C) to target knockdown of PP2A/C protein and then measured the binding preference of TAK1. Transfection with siPP2A/C, but not with control siRNA, decreased E1A-induced PP2A/C expression (Fig. 4C), and accordingly affected the binding complex of TAK1 and PP2A/C in 231/E1A cells. Interestingly, the formation of TAK1/IKKβ complex was significantly increased by siPP2A/C (Fig. 4C, lane 7). E1A-mediated inhibition of the interaction between FOXO3a and βTrCP was also restored by knockdown of siPP2A/C (Fig. 4D, lane 7). These findings indicate that E1A-induced PP2A/C expression is required for regulation of TAK1/IKK signaling, βTrCP/FOXO3a interaction, and chemosensitization.

In summary, we found that FOXO3a is critical for E1A-mediated chemosensitization. E1A stabilizes FOXO3a by inducing the expression of PP2A/C and results in enhanced PP2A phosphatase activity. The enhanced PP2A/C interacts

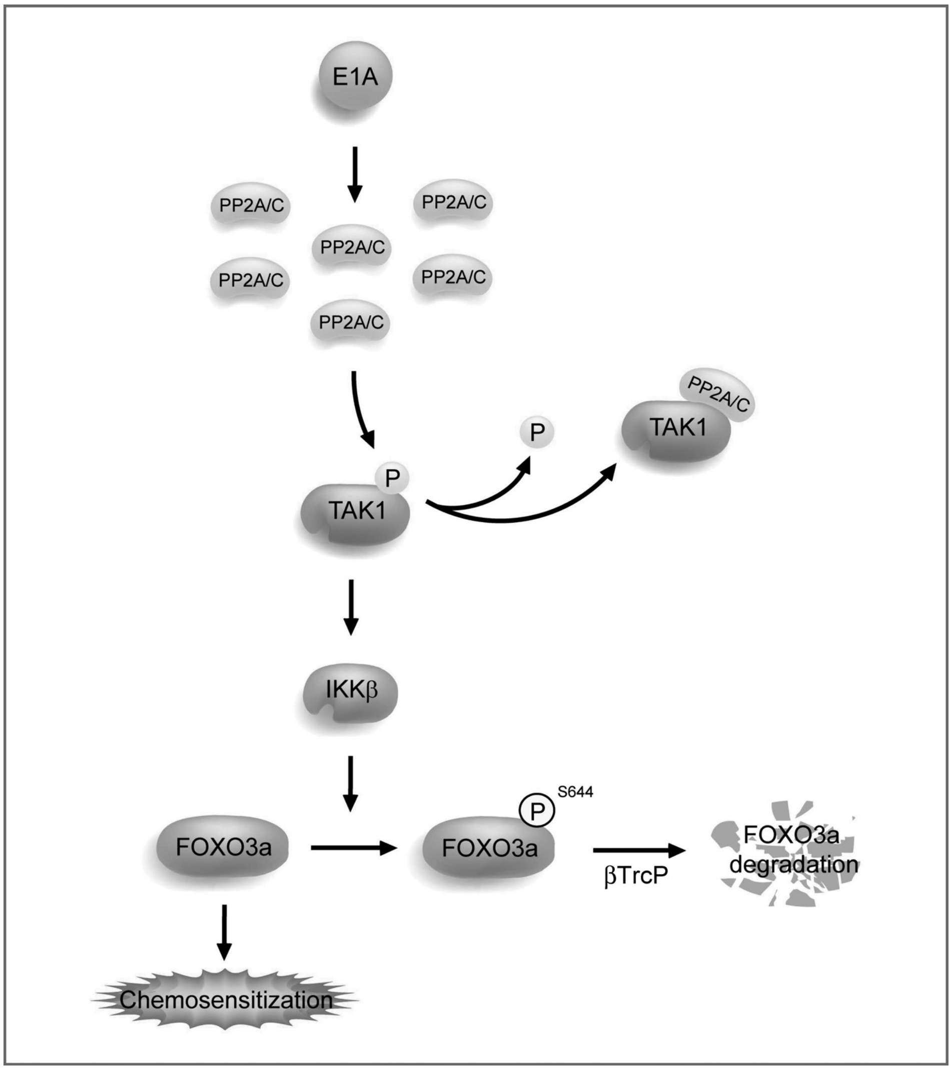

Figure 5. A model of molecular mechanisms involved in E1A-mediated chemosensitization. In this model E1A stabilizes FOXO3a by inducing the expression of PP2A/C, which inhibits the activation of IKKβ through binding and inactivation of TAK1, thus inhibiting IKKβ-mediated FOXO3a phosphorylation at Ser-644 and preventing βTrCP-induced FOXO3a degradation and, thereby, inducing chemosensitization.

and dephosphorylates TAK1 to inactivate TAK1, which also renders inactivation of IKKβ, thus inhibiting IKKβ-mediated interaction between βTrCP and FOXO3a and preventing βTrCP-induced FOXO3a degradation (Fig. 5).

Discussion

E1A is associated with many antitumor activities and has been tested in multiple clinical trials. Studies have shown that, although *E1A* gene therapy is safe and well tolerated, the tumor response to it is only modest (14–16). However, E1A has been shown to induce sensitization to apoptosis induced by different categories of anticancer drugs; therefore, one improvement that might render E1A more useful as an anticancer therapy is the combination of *E1A* gene therapy with conventional chemotherapy. Paclitaxel is a front-line chemotherapeutic agent for the treatment of human breast and ovarian cancer. One of the mechanisms that paclitaxel uses to induce apoptosis in cancer cells is through increasing Bim (proapoptotic BH3-only protein) expression by activated FOXO3a activity (34). In the present study, we found that E1A can stabilize FOXO3a, thus sensitizing MDA-MB-231 breast cancer cells to paclitaxel-induced apoptosis both *in vitro* and *in vivo*. This result provides a molecular mechanism for stronger antitumor strategy by combination of *E1A* gene therapy and paclitaxel chemotherapy (11, 13, 33). It should be mentioned that the identified molecular mechanism for E1A-induced FOXO3a expression was not observed in the MDA-MB-468 cell line, indicating that this mechanism might be cell-type specific.

We found that E1A can protect FOXO3a from degradation by inhibiting its ubiquitination, although previous studies showed that FOXO3a can be targeted by the proteasome pathway after being phosphorylated by Akt or IKK (28, 29). It is worth mentioning that FOXO3a was also shown to be phosphorylated by Erk at different sites and the phospho-FOXO3a by Erk can be degraded by an E3-Ub ligase MDM2 (30). Our data indicate that the ERK signaling pathway may not be involved in E1A-mediated FOXO3a regulation and paclitaxel chemosensitization. Although the mechanism is not yet clear, a possible reason that the ERK signaling may not be involved could be that ERK-mediated degradation of FOXO3a, unlike Akt and IKK, occurs through MDM2 (30), and E1A can regulate members of the

MDM2 family. For instance, it is known that E1A can bind to MDM4 to inhibit MDM2-induced degradation of p53 (36). Recently, a study has indicated that βTrCP1 oncogenic E3-Ub ligase interacts with FOXO3 and induces its Ub-dependent degradation in an IKKβ phosphorylation–dependent manner (37). In our study, we found that βTrCP can physically bind to FOXO3a and mediate its degradation and that E1A stabilizes FOXO3a by inhibiting the binding of FOXO3a to βTrCP. βTrCP is the substrate-recognition subunit of the Skp1 Cullin1 F-box protein E3-Ub protein ligase that can recognize specifically phosphorylated substrates and confer their ubiquitination. βTrCP plays a key role in the NF-κB signaling pathway by recognizing IKK-phosphorylated IκB and mediating its degradation (38). Our findings revealed a new substrate of βTrCP that requires phosphorylation by IKK. Previous studies showed that IKK can phosphorylate FOXO3a at Ser-644 and cause FOXO3a nuclear exclusion (29). Consistent with the report by Tsai and colleagues (37), we found that Ser-644 phosphorylation mediated by IKK is also required for FOXO3a binding to βTrCP and for further degradation induced through βTrCP. E1A prevents the binding of βTrCP to FOXO3a by inhibiting the IKK-mediated FOXO3a phosphorylation at Ser-644.

IKK activation requires its phosphorylation by upstream kinases, including TAK1 (39), and phosphorylation plays a significant role in TAK1 activation (40). We found, in this study, that E1A inhibits FOXO3a binding to βTrCP by preventing TAK1 activation and its effect on IKK activation. It was previously shown that TRAF6 and RIP1 can activate TAK1 and lead to IKK phosphorylation and activation (41, 42). However, overexpression of TRAF6 or RIP1 in E1A-stable cell lines did not restore TAK1 activation and mediate FOXO3a degradation (data not shown), indicating that prevention of TAK1 activation by E1A is not mediated by these 2 upstream activators. It is known that PP2A phosphatase activity is enhanced in E1A-expressing cells through E1A-mediated upregulation of PP2A/C expression, which results in repression of Akt activation (17). A previous study indicates that PP2A functions as a negative regulator in TGF-β1–induced TAK1 activation (43). Therefore, E1A-mediated upregulation of PP2A/C is involved in TAK1 inactivation and inhibits the binding of TAK1 to IKK, which abolishes the function of IKK in phosphorylating FOXO3a, resulting in the stabilization of FOXO3a.

The activities of protein kinases are finely regulated by phosphorylation and dephosphorylation; however, little is known about the dephosphorylation and respective protein phosphatase involved in the regulation of TAK1. PP2A is a ubiquitously expressed protein serine/threonine phosphatase that accounts for the tumor suppression activity in eukaryotic cells. Mutation of PP2A was found in human breast, colon, and lung cancers, and in melanoma (44). In addition, a variety of mechanisms for inactivating PP2A were found to be involved in transformed cells. PP2A can be inhibited by the small T antigen of the DNA tumor virus SV40 (45), by upregulation of the *c*-Myc-specific inhibitor CIP2A (46), or through the upregulation of SET protein by the *BCR/ABL* oncogene (47). It was previously shown that PP2A can suppress Akt (17) and RalA (48) activation, thus inhibiting both PI3K/Akt and ERK signaling pathways (49). We found, in this study, that TAK1 is a target of PP2A/C in another important signaling pathway—the IKK pathway (50). Therefore, PP2A may inhibit the 3 major oncogenic kinase pathways, PI3K/Akt, ERK, and IKK, to exert its tumor suppressor activity. E1A, through upregulation of PP2A/C to stimulate PP2A phosphatase activity, may share these same pathways to suppress tumor development.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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