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Overexpression of TTRAP inhibits cell growth and induces apoptosis in osteosarcoma cells

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TTRAP is a multi-functional protein that is involved in multiple aspects of cellular functions including cell proliferation, apoptosis and the repair of DNA damage. Here, we demonstrated that the lentivirus-mediated overexpression of TTRAP significantly inhibited cell growth and induced apoptosis in osteosarcoma cells. The ectopic TTRAP suppressed the growth and colony formation capacity of two osteosarcoma cell lines, U2OS and Saos-2. Cell apoptosis was induced in U2OS cells and the cell cycle was arrested at G2/M phase in Saos-2 cells. Exogenous expression of TTRAP in serum-starved U2OS and Saos-2 cells induced an increase in caspase-3/-7 activity and a decrease in cyclin B1 expression. In comparison with wild-type TTRAP, mutations in the 5'-tyrosyl-DNA phosphodiesterase activity of TTRAP, in particular TTRAP^{E152A}, showed decreased inhibitory activity on cell growth. These results may aid in clarifying the physiological functions of TTRAP, especially its roles in the regulation of cell growth and tumorigenesis. [BMB Reports 2013; 46(2): 113-118]

INTRODUCTION

Cancer is a malignant neoplasm that is associated with unregulated cell growth (1). The growth of healthy cells is strictly and intricately controlled by multiple genes involved in various functions, including cell proliferation, differentiation and apoptosis. Recently, a cellular protein, TRAF- and TNF receptor-associated protein (TTRAP), has stimulated interest because of its pleiotropic functions in the regulation of cell growth and tumorigenesis (2).

As a ubiquitously expressed protein, TTRAP has multiple effects on cell growth and tumorigenesis. It is involved in multiple signaling pathways associated with the inhibition of cell growth and the induction of apoptosis. Originally, TTRAP was reported to interact with members of the TNF receptor family and TNF receptor-associated factors (TRAFs) (3). In FOXO3a-induced apoptosis, it is upregulated together with signal proteins downstream of the TNF receptor (4). TTRAP was also denoted as ETS1- associated protein II (EAPII) for its inhibitory effects on ETS1 transcriptional activity and ETS1-dependent cell migration (5). In mammalian cells, TTRAP has been associated with the TGF-β receptor-TRAF6-TAK1 signaling module and is involved in TGF-β-mediated apoptosis (6). TTRAP also promotes apoptosis in cytotoxic agent (hydroquinone)-treated HL-60 cells (7). In zebrafish, TTRAP might work as a component of the Nodal/activin signaling pathway and an important regulator of embryogenesis (8). However, several lines of evidence have indicated that TTRAP promotes cell growth and oncogenesis. TTRAP protects neuroblastoma cells from apoptosis induced by proteasome impairment (9). Recently, Li *et al*. reported that TTRAP facilitated the oncogenesis of non-small-cell lung carcinoma (NSCLC) cells through the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK-ERK) signaling pathway both *in vitro* and *in vivo* (10). Furthermore, TTRAP plays a key role in the maintenance of genomic stability. In 2009, TTRAP, also known as tyrosyl DNA phosphodiesterase-2 (TDP2), was shown to be the first 5′- tyrosyl DNA phosphodiesterase necessary for the repair of DNA double-strand breaks (11). Proteins derived from exogenous pathogens, such as Hantaan hantavirus (HTNV) nucleocapsid proteins, ΦC31 and HIV-1 integrases, interact with TTRAP directly (12-14). TTRAP has also been identified as a novel promyelocytic leukemia nuclear body (PML NB) protein involved in the regulation of ribosome biogenesis (15, 16).

The maintenance of genome stability is central to cell survival. As the only 5'- tyrosyl DNA phosphodiesterase identified, TTRAP should play a role in cell growth and tumorigenesis. Although TTRAP has been reported to promote cell proliferation in NSCLC cells, its function may be different in other tumor cells. Here, we expressed TTRAP in two osteosarcoma cell lines, U2OS and Saos-2, through lentiviral infection. The profile of cell growth and apoptosis upon the ectopic expression of TTRAP was investigated. The relationship between mutations in the 5′- tyrosyl DNA phosphodiesterase motif of TTRAP and their effects on cell growth was also evaluated.

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RESULTS

The overexpression of TTRAP inhibits the colony formation and cell growth of U2OS and Saos-2 cells

TTRAP has been reported to express ubiquitously. When detected with anti-TTRAP antibodies, the endogenous expression level of TTRAP varied widely among different cell lines (supplementary Fig. 1). U2OS, a human osteosarcoma cell line that has a relatively low level of endogenous TTRAP expression, was selected to evaluate the effects of TTRAP overexpression on cell growth and tumorigenesis *in vitro*. Saos-2, another human osteosarcoma cell line that shows deficient expression of p53, was also tested (17).

Exogenous TTRAP was introduced into U2OS and Saos-2 cells using a lentivirus expression system. The expression profile of EGFP and the TTRAP-EGFP fusion protein (derived from pWPXL and pWPXL-TTRAP, respectively) after viral infection was detected with FACS from day 1 to day 6. The proportion of EGFP-positive cells was 100% on day 2 (Supplementary Fig. 2). The maximum expression was maintained from day 3 (Supplementary Fig. 3). To abolish the potential cytotoxicity from EGFP expression, the open reading frame (ORF) of EGFP was removed from the parent lentivirus plasmid pWPXL to generate pWPXL-NE. Empty viruses (lenti-vector) and viruses expressing TTRAP without the EGFP tag (lenti-TTRAP) were produced with pWPXL-NE and pWPXL-NE-TTRAP, respectively. U2OS and Saos-2 cells were infected with lenti-vector and lenti-TTRAP. The improved expression of TTRAP in lenti-TTRAP-infected U2OS and Saos-2 cells was identified with Western blotting (Fig. 1A).

The inhibitory effect of TTRAP on the growth of osteosarcoma cells was first evaluated with a colony formation assay. The stable infection with lenti-TTRAP resulted in an obvious decrease in the number and size of colonies of U2OS and Saos-2 cells (Fig. 1B). Compared with the control, the colony count per well was reduced by 26.6% in U2OS cells and 34.4% in Saos-2 cells in the TTRAP overexpression groups (Fig. 1C). A variety of factors may result in the suppression of colony formation, such as the inhibition of cell growth or migration. Pei *et al.* and Zucchelli *et al.* reported that cells stably transfected with TTRAP showed no difference in cell growth in the absence of exogenous stress (5, 9). To determine whether TTRAP inhibited cell growth, we plated U2OS and Saos-2 cells three days after lentiviral infection and incubated the cells for 1-6 days. The cell viability was measured with MTT. Compared with the lenti-vector-infected control, the number of viable cells in the lenti-TTRAP-infected groups was reduced by 34.0% in U2OS cells (Fig. 1D) and 31.1% in Saos-2 cells (Fig. 1E) at the end of the incubation. These results indicated that TTRAP significantly inhibited cell growth in both cell lines.

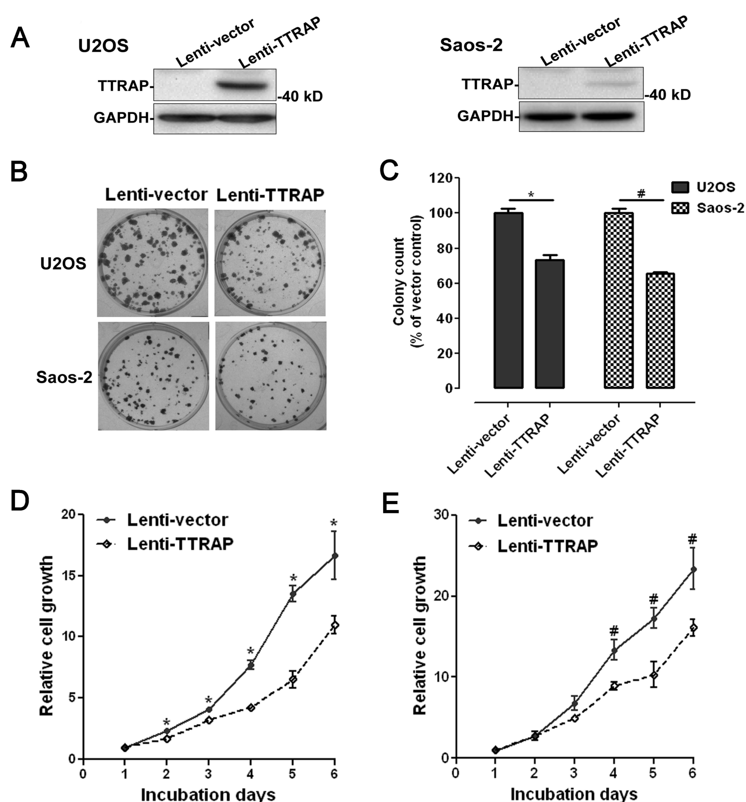

Fig. 1. The overexpression of TTRAP inhibited the colony formation and cell growth of U2OS and Saos-2 cells. (A) Western blot analysis showing TTRAP overexpression in U2OS and Saos-2 cells. (B) Representative images of crystal violet-stained colonies are shown for U2OS and Saos-2 cells. Following the infection with lentiviruses, the cells were seeded in 6-well plates and incubated for the indicated times (500 cells/well for 12 days for U2OS cells and 1,000 cells/well for 21 days for Saos-2 cells) before fixation, staining and counting. (C) Relative colony counts for U2OS and Saos-2 cells. The amount of colonies of the vector control cells was set as 100%. (D, E) Cell growth curves for U2OS and Saos-2 cells. The cells were plated in 96-well plates at a density of 500 cells per well for U2OS (D) and 1,000 cells per well for Saos-2 cells (E) and incubated for 1-6 days. The cell growth was measured with MTT at the indicated time. The values are the relative cell growth compared with the absorbance of cells detected 16 h after cell plating. The data are presented as the means ± SD. $^{*,\#}P < 0.05$ compared with the vector control in U2OS and Saos-2 cells, respectively.

Effect of mutations in the 5′-tyrosyl DNA phosphodiesterase activity of TTRAP on cell growth

As a 5′-tyrosyl DNA phosphodiesterase, TTRAP restores the 5′-phosphate termini at DNA double-strand breaks in preparation for DNA ligation. To determine the functional domains associated with the inhibitory effect of TTRAP on cell growth, we constructed two plasmids expressing inactive TTRAP phosphodiesterase mutants, $TTRAP^{E152A}$ and $TTRAP^{D262A}$, using the plasmid pcDNA-myc as a parent backbone. These plasmids and the control plasmid pcDNA-myc were introduced into U2OS cells through transient transfection. Anti-myc antibody was used to evaluate the expression of TTRAP and its mutants with Western blot assay. The levels of $TTRAP^{E152A}$ and $TTRAP^{D262A}$ expression were comparable, but both were lower than that of the wild type one (Fig. 2A). All of the transfected cells were incubated under neomycin selection, and the resulting colonies were counted. The ectopic $TTRAP^{wt}$ inhibited colony formation by 43.6% compared with vector-treated cells. The two inactive 5'- tyrosyl DNA phosphodiesterase mutants of TTRAP, $TTRAP^{E152A}$ and $TTRAP^{D262A}$, showed variable effects on the growth of U2OS cells (Fig. 2B and 2C). Ectopic $TTRAP^{E152A}$ did not affect colony formation while $TTRAP^{D262A}$ decreased the clones' growth by 17%. Recombinant lentiviruses expressing $TTRAP^{E152A}$ were also prepared. In consistent with the results obtained in the transient transfection assay, $TTRAP^{E152A}$ lost the capacity of inhibiting colony formation in U2OS cells (Supplementary Fig. 4).

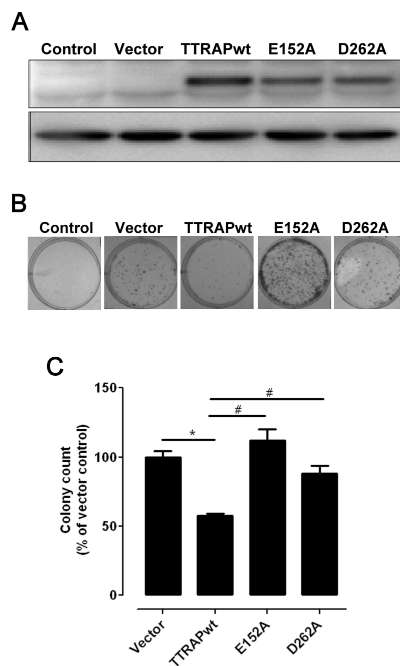

Fig. 2. The inhibitory effect of TTRAP on cell growth was associated with the $TTRAP^{E152A}$ and $TTRAP^{D262A}$ mutations. (A) U2OS cells were cultured overnight and transfected with vehicle control, pcDNA-myc empty vector, pcDNA-TTRAP or the vectors containing cDNAs of two mutants of TTRAP, $TTRAP^{E152A}$ and $TTRAP^{D262A}$. The expression of TTRAP-myc was detected with Western blotting 48 h after transfection. (B) The transfected cells were selected with 800 μg/ml G418 for 12 days. The colonies were stained with crystal violet and photographed. (C) The relative colony counts of each well of the transient-transfected cells. The amount of colonies of the vector control groups was set as 100%. The data represent the means ± SD. $^{*,\#}P < 0.05$ compared with the vector control and $TTRAP^{wt}$-expressing cells, respectively.

TTRAP induces cell cycle arrest and apoptosis in osteosarcoma cells

The growth inhibition of cells may be caused by a reduced cell proliferation rate, increased apoptosis or cell cycle arrest. We first investigated the effect of exogenous TTRAP on cell cycle distribution. Virus-infected cells were collected 24 h after plating, and the cell cycle profile was analyzed after PI staining. In U2OS cells, approximately 5.3% of the TTRAP-expressing cells were dead compared with 1.3% of the control cells, but no significant difference in the cell population in G2/M or G1 phase was detected between these two groups (Fig. 3A). In Saos-2 cells, approximately 6.8% of the TTRAP-expressing cells were dead compared with 4.0% of the control cells. Furthermore, ectopic TTRAP expression induced a significant increase in the cell population at G2/M phase and a decrease in the cell population at G1 phase compared with the control cell groups, which indicated a G2/M-phase cell cycle arrest (Fig. 3B).

To confirm whether TTRAP-induced cell death was caused by apoptosis, virus-infected cells were further evaluated through the detection of caspase-3/-7 activity after being serum-starved in 0.2% FBS for 2 days. The caspase-3/-7 activity in untreated cells was arbitrary set as the standard. The lenti-TTRAP infection increased the caspase-3/-7 activity by 2.3 fold in U2OS cells and

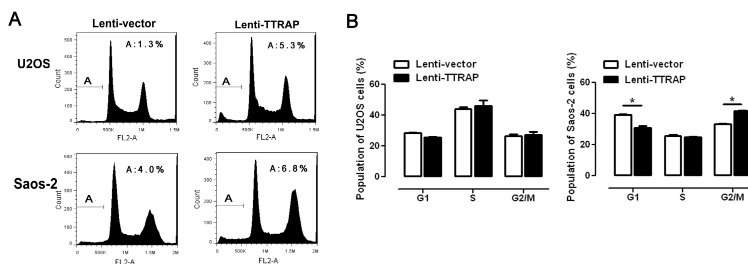

Fig. 3. TTRAP induced apoptosis and arrested the cell cycle at G2/M phase. (A) The cell cycle distribution of U2OS and Saos-2 cells 24 h after cell plating. The cells were fixed with 70% ethanol and stained with PI before FACS analysis. (B) The cell population in each phase of the cell cycle in shown. $^{*}P < 0.05$ compared with the vector control.

1.2 fold in Saos-2 cells compared with the lenti-vector-treated cells (Fig. 4A). In addition, the cell survival of lenti-TTRAP-treated cells was significantly decreased (from 95.1% to 66.3% in U2OS cells and from 94.4% to 79.4% in Saos-2 cells), as shown in Fig. 4B, which indicates a significant increase in caspase activity and apoptosis in TTRAP-overexpressing cells. The mutant TTRAP^{E152A} obviously lost the activity of TTRAP^{wt} to induce apoptosis in both cell lines, which induced caspase-3/-7 activity comparable with the vector control.

TTRAP inhibits cyclin B1 expression in both U2OS and Saos-2 cells

The inhibitory effects of exogenous TTRAP expression on cell growth and colony formation might be caused by both cell cycle arrest and apoptosis. Cyclin B1 is a key regulator of G2/M transition of cell cycle. Exogenous TTRAP^{wt} decreased the expression of cyclin B1 protein by 28.6% in U2OS cells (Fig. 4C and 4E) and 40.4% in Saos-2 cells (Fig. 4D and 4F), respectively. Exogenous TTRAP^{E152A} lost the activity of TTRAP^{wt} in inhibiting the expression of cyclin B1 in both U2OS and Saos-2 cells.

DISCUSSION

TTRAP is a multi-functional protein that controls a variety of cellular activities, including cell proliferation, apoptosis and DNA repair (2). Many studies have indicated that TTRAP may be responsible for aberrant cell growth and tumorigenesis. Although it has been reported that TTRAP facilitates the oncogenesis of non-small-cell lung carcinoma both *in vitro* and *in vivo*, its expression and function in osteosarcoma cells has not been explored. In the present study, we infected osteosarcoma cell lines with a recombinant lentivirus containing the ORF of TTRAP. The results demonstrated that exogenous TTRAP significantly inhibited the growth and colony formation capacity of osteosarcoma cells. Ectopic TTRAP induced cell apoptosis in both U2OS and Saos-2 cells. TTRAP also arrested cells in G2/M phase in Saos-2 cells. Various studies have shown the involvement of cell cycle regulation-mediated apoptosis as a mechanism of cell growth inhibition. Both in U2OS and Saos-2 cells, ectopic TTRAP decreased the expression of cyclin B1 protein, indicating that cell cycle arrest may account for the apoptosis in both cell lines. The discrepancy of TTRAP's effects in different tumor cell lines can result from the complexity of the cell microenvironment and the TTRAP protein interaction network. The effect of TTRAP expression on the tumorigenesis of osteosarcoma in vivo was not clarified in this study. A breakthrough regarding TTRAP knockout animal models in the future may shed new light on TTRAP's physiological functions, including its roles in the regulation of cell growth and tumorigenesis.

Many pathways can influence cell fate decisions upon exogenous stress. The data on TTRAP functions imply that it is involved in the response to different types of stress associated with chromosomal instability, such as DNA damage caused by irradiation, chemical reagents or viral infection. Being a member of the PML NB protein family, TTRAP is a relatively small protein compared with the classical members in the family, such as PML (18) and DAXX (19). Some structural properties may account for different activities among TTRAP's multiple functions. The two

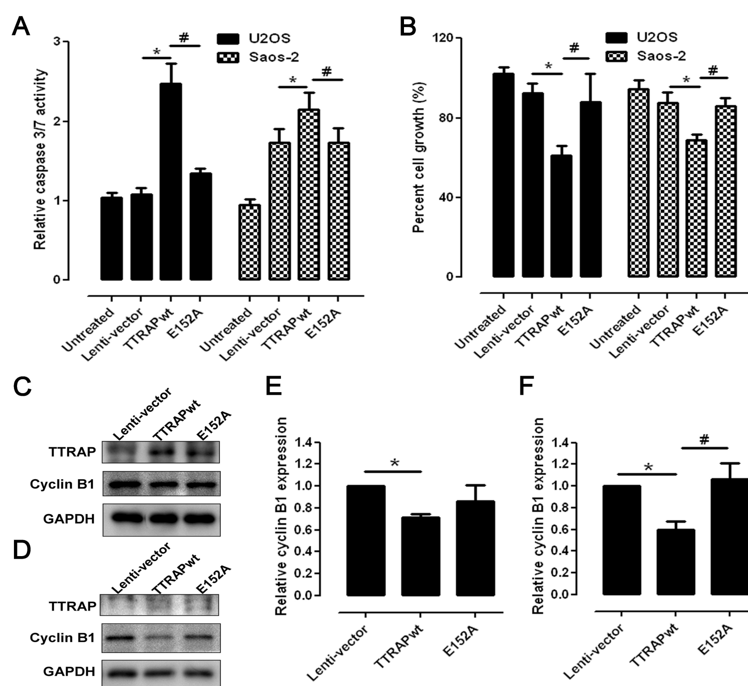

Fig. 4. Ectopic TTRAP induced caspase-3/-7 activity and inhibited cyclin B1 expression. (A) The caspase-3/-7 activity of lenti-vector- and lenti-TTRAP-infected U2OS and Saos-2 cells. The cells were plated at a density of 500/well in a 384-well plate and incubated in medium containing 0.2% FBS for 2 days before analysis with Caspase-Glo 3/7 assay kits. (B) The percentage of cell growth of U2OS and Saos-2 cells. The cell viability was assayed with CCK-8. Average values of untreated cells were set as 100%. (C-F) Western blot analysis of cyclin B1 expression in recombinant lentivirus-infected U2OS (C, E) and Saos-2 (D, F) cells. The data represent the mean ± SD of at least three independent experiments. $^{*,\#}P < 0.05$ compared with the vector control and TTRAP^{wt}-expressing cells, respectively.

mutations in TTRAP's 5′-tyrosyl-DNA phosphodiesterase activity, $TTRAP^{E152A}$ and $TTRAP^{D262A}$, showed decreased inhibitory actions on cell growth. $TTRAP^{E152A}$ also lost the activity of $TTRAP^{wt}$ in inducing the increase of caspase-3/-7 activity and the decrease of cyclin B1 expression. This result demonstrated that the DNA repair activity of TTRAP was associated with its cell growth modulation capability in osteosarcoma cells.

MATERIALS AND METHODS

Plasmids

The ORF of TTRAP was amplified from a human fetal brain cDNA library (Clontech, Mountain View, CA). The mammalian expression vector pcDNA3.1-TTRAP was constructed as described previously (14). The cDNA sequences for the mutants $TTARP^{E152A}$ and $TTRAP^{D262A}$ were generated with PCR using pcDNA3.1-TTRAP as a template and subcloned into the vector pcDNA3.1/myc-His(-) A (Life Technologies, Carlsbad, CA) between the *Eco*R I and *Bam*H I sites. To express TTRAP with a lentiviral expression system, the ORF of TTRAP was inserted into pWPXL (Addgene, Cambridge, MA) between the *Bam*H I and *Mlu* I sites to produce pWPXL-TTRAP, which expressed a TTRAP-EGFP fusion protein. The ORF of EGFP between the *Sma* I and *Pme* I sites was removed from pWPXL in order to produce pWPXL-NE. The TTRAP cDNA was inserted into pWPXL between *Bam*H I and *Eco*R I to generate pWPXL- NE-TTRAP, which expressed the TTRAP protein without the EGFP tag. The primers for constructing these plasmids are shown in Supplementary information Table S1. All of the constructs were verified with DNA sequence analysis.

Cell culture and transfection

HEK293T and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with 100 units/ml of penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Saos-2 cells were grown in DMEM/F-12 supplemented with 15% FBS. The cells were transfected with Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's instructions.

Lentivirus preparation, infection and flow cytometry analysis

The corresponding pWPXL vectors, the packaging plasmid psPAX2 and the envelope plasmid pMD2.G (Addgene) were co-transfected into HEK293T cells using Lipofectamine 2000 reagent. The virus particles were harvested 48 h after transfection. The cells (1×10^5) were infected at a multiplicity of infection (MOI) of 10 with 6 μg/ml of polybrene (Sigma-Aldrich, St. Louis, MO). The expression of EGFP or TTRAP-EGFP after lentivirus infection was detected with fluorescence-activated cell sorting (FACS) with an Accuri C6 cytometer (BD Biosciences, Franklin Lakes, NJ). The data were analyzed with FlowJo flow cytometry analysis software (Tree Star, Inc., Ashland, OR).

Cell proliferation and colony formation assays

To examine the effect of TTRAP on cell growth, U2OS and SAOS-2 cells were infected with either lentivirus containing the TTRAP gene (lenti-TTRAP) or empty virus (lenti-vector). The infected cells were seeded in 96-well plates and incubated for 1 to 6 days. Subsequently, 20 μl of a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml) was added to each well 3 h before the end of incubation. The crystals were dissolved in 150 μl dimethyl sulfoxide (DMSO), and the absorbance at 570 nm was measured with a SPECTRAmax 340PC (Molecular Devices, Sunnyvale, USA). When the assays were performed in 384-well plates, a Cell Counting Kit-8 (CCK-8, Dojindo Corporation, Japan) was used instead of MTT. Ten μl of CCK-8 was added to the cells 3 h before the end of cell culture, and the absorbance was measured at 450 nm with a 690 nm reference.

To evaluate the colony formation capacity of the lenti-TTRAP or lenti-vector infected cells, cells were seeded in a six-well plate at a density of 500 or 1,000 cells per well. After incubation at 37°C for 12-21 days, the colonies were fixed and stained in a dye solution containing 0.1% crystal violet (Sigma-Aldrich) and 20% methanol. The number of colonies per well was counted. For growth suppression studies using transient transfection, U2OS cells were transfected with either a TTRAP expression vector (pcDNA3.1-TTRAP) or a control empty vector (pcDNA3.1) for 24 h and then seeded at 4×10^4 per well in a six-well plate. The number of stable colonies formed after selection in 800 μg/ml G418 (Sigma-Aldrich) for 12 days was counted.

Western blotting

Cells were washed with cold phosphate-buffered saline (PBS) and lysed in ice-cold buffer. The protein concentration was determined with the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Protein extracts were resolved through 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked in 5% fat-free milk and incubated with anti-human TTRAP polyclonal antibodies (Aviva Systems Biology, San Diego, CA), anti-myc (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-cyclin B1 and anti-GAPDH (Cell Signaling Technology, Danvers, MA) antibodies at 4°C overnight. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. The protein signals were visualized with an enhanced chemiluminescence immunoblotting detection kit (GE Healthcare, Piscataway, NJ). GAPDH was used as an equal loading control.

Cell cycle analysis

Cells grown in regular growth medium for 24 h were collected, fixed in 70% cold ethanol overnight and stained with PBS containing 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A (Tiangen Biotech, Beijing, China) for 30 min at 37°C. The DNA content of the labeled cells was measured using the Accuri C6 flow cytometry system (BD Biosciences).

Detection of caspase-3/-7 activity

The lenti-TTRAP- or lenti-vector-infected cells were seeded at a density of 500/well in triplicate wells in a 384-well plate. After an overnight incubation, the medium was replaced with DMEM supplemented with 0.2% FBS and incubated for another 48 h. Caspase activity was subsequently measured with a Caspase-Glo 3/7 Assay System (Promega, Fitchburg, WI) according to the manufacturer's protocols. Briefly, an equal volume of caspase substrate was added to the cells, and the samples were incubated at room temperature for 1 h. The luminescence was measured using an EnVision 2103 Multilabel Reader (PerkinElmer, Waltham, MA). The luminescence of the untreated control cells was set as the standard.

Statistical analysis

The experiments were repeated at least three times, and the results were expressed as the mean $\pm$ SD. Student's t-test was used to compare the values of the TTRAP overexpression samples with the vector control samples. A value of $P < 0.05$ was considered to be statistically significant.

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