Tumor suppressor p53 plays a key role in induction of both tristetraprolin and *let-7* in human cancer cells

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Running Title: p53 enhances *TTP* and *let-7* expression

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Supplemental Materials and Methods

Cell culture

Human cancer cell lines, PA1, MCF7, MDA-MB231, NIHOVCAR3, Colo205, A549, H1299, AGS, SNU16, SNU668, SNU750, HCT116, HepG2, HT29, and SW480 were purchased from the Korean Cell Line Bank (KCLB-Seoul, Korea). PA1 cells were cultured in Eagle's Minimum Essential Medium (EMEM). A549, AGS, Colo205, MCF7, and MDA-MB231 cells were cultured in RPMI 1640 media. HepG2 and HCT116 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). All cell lines were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (WELGENE, Korea) and were maintained at 37 °C in a humidified atmosphere of 5% CO_2. For the induction of genotoxic stress, cells were treated with DOX (Sigma, D1515), 5-FU (F6627 Sigma), or etoposide (E1383 Sigma) at concentration ranging aigfrom 0.1 to 0.5 μg/ml. Cells were harvested at 6, 12, and 24 h after DOX treatment and analyzed for mRNA by RT-PCR, protein by Western blotting, and cell viability by MTS assay.

Cell viability/proliferation

For the MTS cell proliferation assay, cells were plated in triplicate at 1.0×10^4 cells/well in 96-well culture plates in culture media. At 24 h after plating, CellTiter 96® AQueous One Solution reagent (Promega) was added to each well according to the manufacturer's instructions, and absorbance at 490 nm was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

Immunohistochemistry

Immunostaining for TTP and p53 was performed with deparaffinized tissue sections; these were incubated with 1:200 diluted anti-human TTP monoclonal antibody (sc-14030, Santa Cruz, CA) or anti-human p53 (1026-1, Epitomics). Primary antibodies were detected using EnVisionTMþ/HRP kits (DAKO, Carpinteria, CA). Peroxidase activity was visualized with 3-amino-9-ethyl carbazole (Sigma). The sections were counterstained with Mayer's hematoxylin. Negative controls, in which the primary antibody incubation step was omitted, were also included for each staining. The expression of TTP and mutant p53 was scored semiquantitatively based on the staining intensity and proportion of staining. Staining intensity was subclassified as 0, negative; 1, weak; 2, moderate; and 3, strong. The proportion of staining was scored as 1, 0–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. Staining scores were obtained by multiplying the staining intensity by the proportion of staining. Two pathologists, who did not have any prior clinical or pathological information, scored the expression at 100X magnification under light microscopy. All available areas in the section were evaluated. We selected a median staining score of 6.0 to define low expression (0 or ≤6.0) and high expression (>6.0) for p53.

Annexin V staining

Annexin V staining was conducted using an Annexin-V-FLUOS staining kit according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals). Briefly, cells were washed twice with PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 $7H_20$; 1.4 mM KH_2PO_4; pH 7.2) and resuspended in binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$) containing Annexin V and propidium iodide. Cells were analyzed for fluorescence intensity using a FACS flow cytometer (Becton Dickinson, Inc.).

Cell cycle analyses

Cells were harvested and washed twice in PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 $7H_2O$; 1.4 mM KH_2PO_4, pH 7.2) at room temperature and resuspended at $2x10^6$ cells/ml in PBS. For propidium iodide staining, the washed cells were fixed in pure ethanol at -20°C overnight and then treated according to the manufacturer's procedure (Molecular Probes Inc.). Cells were washed twice in PBS, resuspended in FACS buffer (PBS supplemented with 0.2% BSA and 1% sodium azide), and then analyzed using a FACS flow cytometer (Becton Dickinson, Inc., San Jose, CA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from PA1 cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's manual. Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng/ml poly(dI-dC), 0.05% Nonidet P-40, 5 mM $MgCl_2$, 10 mM EDTA, and 2.5% glycerol in 1X binding buffer (LightShift™ Chemiluminescent EMSA Kit, Pierce) using 20 fmol of biotin-end-labeled target DNA and 4 μg of nuclear extract. For supershift assays, 2 μl of anti-p53 (1026-1. Epitomics) was added per 20 μl of binding reaction where indicated. Assays were loaded onto native 5% polyacrylamide gels pre-electrophoresed for 60 min in 0.5X Tris borate/EDTA and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane (Hybond™-N+) in 0.5X Tris borate/EDTA at 100 V for 30 min. Transferred DNAs were cross-linked to the membrane at 10 mJ/cm2 and detected using horseradish peroxidase-conjugated streptavidin (Lightshift™ chemiluminescent EMSA kit) according to the manufacturer's instructions.

TTP promoter constructs

Construct pGL3/TTPp-1343 contains the 1343 bp promoter region of the human TTP gene up to nucleotide +68 bp (i.e., downstream from the TTP mRNA capsite) inserted into the *Sac*I and *Xho*I sites of the pGL3 basic vector (Promega). The pGL3/TTPp-1343 plasmid construct was used as a template to synthesize deletion reporter gene constructs. Two mutants of the TTP promoter were generated by substituting the sequences within two putative p53 half-sites (-175, RRRCAAGYYY-3'; -134, 5'-RRRCCAGYYY-3') with 5'-RRRTCGAYYY-3' (-175) and 5'-RRRTCGCYYY-3 (-134), respectively, using a QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instruction. Nucleotide sequences of the primers used for various 5'-deletion constructs, internal deletion constructs, and substitution mutant constructs of putative p53 binding sites within the TTP promoter are listed in Supplemental Table 1.

Supplemental Table and Figures

Supplemental Table 1 PCR primers used in this study.

Name	Sequences (forward and reverse, 5′ to 3′)
hTTP P-1343-U	*GAGCTC*GGCAGGAGAGTAACTTCACTTTG
hTTP P+68-D	*CTCGAG*AGATCCATGGTGTAACGGTTGG
hTTP P-812-U	*GAGCTC*TAATCCACCTGCCTCATCCTCTG
hTTP P-419-U	*GAGCTC*TCTGCCTGTCTGCCTGTCTGTAC
hTTP P-211-U	*GAGCTC*ACGCATTCCCCGCTCGGTCAC
hTTP P-41-U	*GAGCTC*GCTTGCAGTTTCCTATAA
hTTP P-211-p53RE-175MUT-U	GGCTGTCCACCGGCTCGACTCAGGCGCGTC
hTTP P-211-p53RE-175MUT-D	GACGCGCCTGAGTCGAGCCGGTGGACAGCC
hTTP P-211-p53RE-134MUT-U	GCGGAAGGGAATCGCTCCAGGGCCAGCC
hTTP P-211-p53RE-134MUT-D	GGCTGGCCCTGGAGCGATTCCCTTCCGC
qLin28a	AGAGTAAGCTGCACATGGAAGGGT, TATGGCTGATGCTCTGGCAGAAGT
qTTP	CGCTACAAGACTGAGCTAT, GAGGTAGAACTTGTGACAGA
CDC34	TGACCAAGATGTGGCACCCTAACA, TGATGTCTGTGTACTCCCGATCCT
qGAPDH	ACATCAAGAAGGTGGTGAAG, CTGTTGCTGTAGCCAAATTC
p53	GCCTGTAAGTACGGGGACAA, CTCTTCAGCGTTGTGGATGA
p21	CGGACACATAGAAAGATAACGACGG, AAAAGCATAGCTGCTGTTCCTACGA
VEGF	CGAAGTGGTGAAGTTCATGGATGT, TCACCGCCTCGGCTTGTC
c-Fos	ACGCAGACTACGAGGCGTCA, TTCACAACGCCAGCCCTGGA
c-Myc	ACCACCAGCAGCGACTCTGA, TCCAGCAGAAGGTGATCCAGACT
COX2	TCTAGAGTCCATATCACATTGC, TCTAGACCTCTTTGCATCCATC
HMGA2	GTGAGATGCAACAACCCCTGCTTT, TGTGGCCTTTGAAACTACCTCCCT

Restriction sites used for subcloning are in italics

Supplemental Figures

Supplemental Figure 1 DOX induces expression of *TTP* mRNA and protein by a p53-dependent mechanism in human breast cancer cells. (**A-C**) DOX increases TTP levels in p53 wild-type human breast cancer MCF7 cells. MCF7 cells were treated with the indicated concentration of DOX for (**A**) the indicated length of time or (**B, C**) 24 h. The levels of *TTP*, p53, and p21 were measured by (**A** and **C**, top) semi-qRT-PCR, (**B**) quantitative real-time PCR, and (**A** and **C**, bottom) Western blotting. The level of phospho-p53 (pp53) was also measured by Western blotting (**A**, bottom). GAPDH was used as an internal control. Results shown represent the means ± SD of three independent experiments (***p*<0.01). (**D-G**) p53 is required for DOX-induced expression of TTP. (**D, E**) p53 mutant human breast cancer MDA-MB231 cells were treated with the indicated concentration of DOX for 24 h. The level of *TTP* was measured by (**D**, top) semi-qRT-PCR, (**E**) quantitative real-time PCR, and (**D**, bottom) Western blotting. Results shown represent the means ± SD of three independent experiments. ns, not significant. (**F**) MDA-MB231 cells were transfected with pCMV-p53WT. After treatment with 0.3 μg/ml of DOX for 24 h, the levels of TTP, p53, and p21 were measured by (top panel) semi-qRT PCR and (bottom panel) Western blotting. The level of phospho-p53 (pp53) was measured by Western blotting (bottom panel). (**G**) MCF7 cells were transfected with siRNA against p53 (p53-siRNA). After treatment with 0.3 μg/ml of DOX for 24 h, the levels of TTP, p53, and p21 were measured by (top) semi-qRT PCR and (bottom) Western blotting. The level of phospho-p53 (pp53) was measured by Western blotting (bottom).

Supplemental Figure 2 DOX induces expression of *TTP* mRNA and protein by a p53-dependent mechanism in human colon cancer cells. (**A, B**) Colo205 cells were treated with

the indicated concentration of DOX for 24 h. The level of *TTP* was measured by (**A**, top) semi-qRT-PCR, (**A**, bottom) Western blotting, and (**B**) qRT-PCR. The level of phospho-p53 (pp53) was also measured by Western blotting (**A**, bottom). GAPDH was used as an internal control. Results shown represent the means ± SD of three independent experiments (*p<0.05). (**C**) Colo205 cells were transfected with p53-specific (p53-siRNA) or scramble siRNA (scRNA). After treatment with 0.3 μg/ml of DOX for 24 h, the levels of TTP, p53, and p21 were measured by (top) semi-qRT PCR and (bottom) Western blotting. The level of phospho-p53 (pp53) was measured by Western blotting (bottom).

Supplemental Figure 3 Both 5-FU and etoposide induce expression of *TTP* mRNA and protein in human cancer cells. MCF7 cells were treated with 0.1 μg/ml 5-FU (left) or 50 μM etoposide (right) for the indicated length of time. The levels of *TTP*, p53, and p21 were measured by semi-qRT-PCR (top) and Western blotting (bottom). The level of phospho-p53 (p-p53) was measured by Western blotting (bottom).

Supplemental Figure 4 The levels of TTP protein in *p53* wild-type cancer cells depend on p53 and are lower than those in normal colon tissues. (A) MCF7 cells were transfected with p53-siRNA or scRNA. After 24 h, the levels of *p53* and *TTP* were measured by semi-qRT-PCR (top) and Western blotting (bottom). (B) Levels of TTP and p53 proteins were determined by Western blot analysis in colon cancer cells (Colo205 and HCT116) and human normal colon tissues.

Supplemental Figure 5 TTP expressed in cancer cells is active in promoting target gene decay. (A) A549 and H1299 cells were transfected with psiCHECK2 luciferase reporter

constructs containing fragments derived from the 3'UTR of *Lin28a* mRNA. (B) A549 cells were cotransfected with psiCHECK2 luciferase reporter constructs containing fragments derived from the 3'UTR of *Lin28a* mRNA and TTP-siRNA or scRNA. After 24 h incubation, cells were lysed and assayed for luciferase activity. The levels of firefly luciferase activity were normalized to *Renilla* luciferase activity. Luciferase activity obtained from(A) H1299 or (B) A549 cells transfected with psiCHECK2 alone was set to 1.0. Values are means ± SD (n = 3). *p<0.05. ns, not significant.

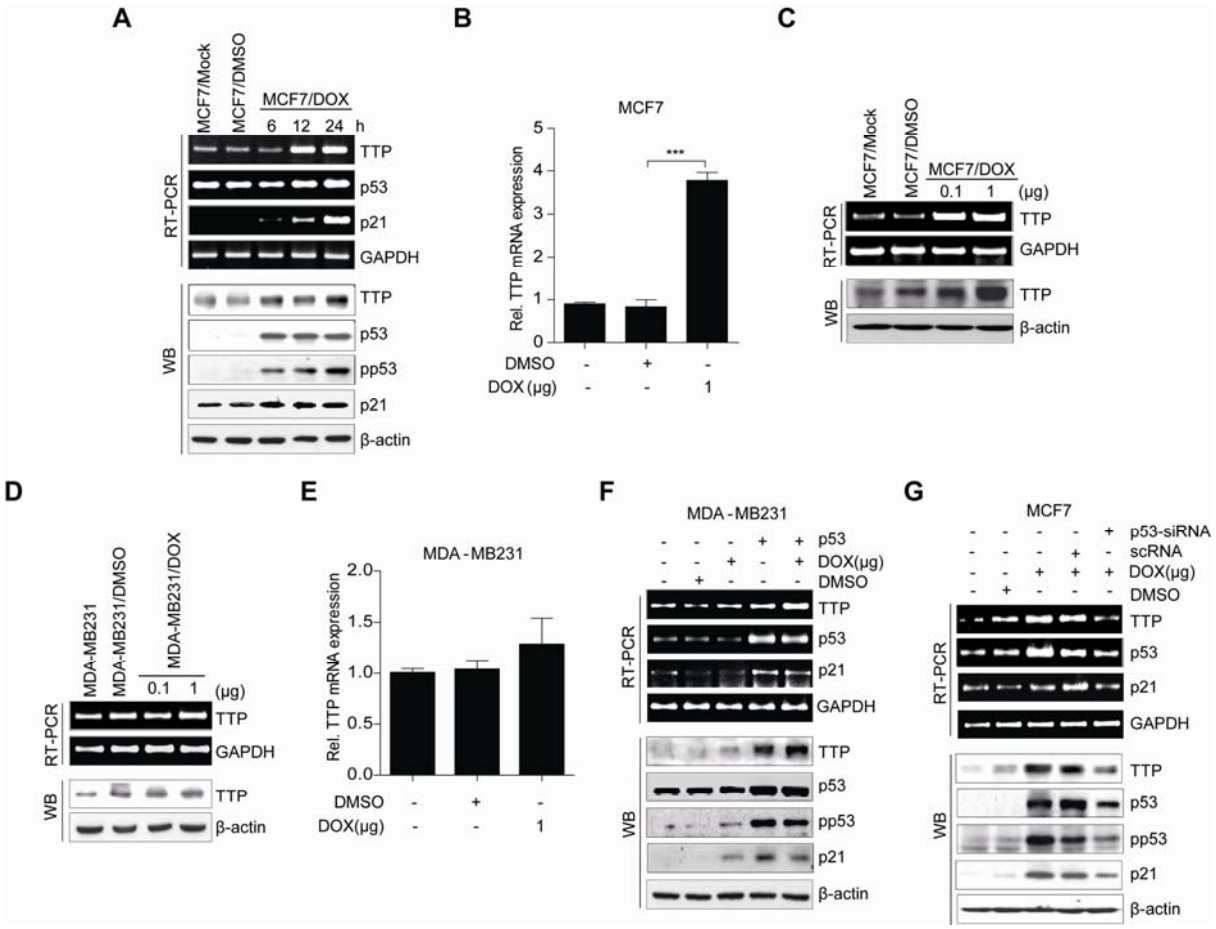
Supplemental Figure 6 DOX does not stabilizes *TTP* mRNA. After treatment with DOX for 24 h, expression of *TTP* mRNA in PA1 cells was determined by qRT-PCR at the indicated times after the addition of 5.0 μg/ml actinomycin D. Results shown on the graph represent the means ± SD (n = 3). ns, not significant.

Supplemental Figure 7 DOX enhances binding of p53 to the p21 promoter. ChIP analysis was performed to confirm the interaction of p53 with the *p21* promoter *in vivo* in PA1 cells. Formaldehyde cross-linked chromatin from PA1 cells was incubated with anti-p53 antibody or with nonspecific IgG. Total input DNA at a 1:10 dilution was used as positive control for the PCR reaction. Immunoprecipitated DNA was analyzed by PCR with primers specific for the p*21* promoter.

Supplemental Figure 8 DOX enhances TTP binding to the *Lin28a* mRNA 3'UTR. After treatment with DOX for 24 h, RNA EMSA was performed by mixing cytoplasmic extracts containing 3.0 μg of total protein from PA1 cells with 20 fmol biotinylated wild type (WT) or mutant (MUT) probe. Anti-TTP or control antibody was added to the reaction mixtures.

Position of the TTP containing bands (TTP) and super-shifted bands (SS) are indicated.

Supplemental Figure 9 Ectopic overexpression of p53 increases TTP and let-7 levels but decreases Lin28 level in p53 null mutant SKOV3 cells. SKOV3 cells were transfected with pCMV-p53WT or empty vector. After incubation for 24 h, the levels of TTP and Lin28a were measured by semi-qRT-PCR (top). The levels of mature-miRNA for *let-7b* and *let-7f* were determined by qRT-PCR. The levels obtained from untreated SKOV3 cells were set to 1. Values are means ± SD (n = 3). *$p<0.05$.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MCF7/Mock
MCF7/DMSO
MCF7/DOX
6 12 24 h
RT-PCR
TTP
p53
p21
GAPDH
WB
TTP
p53
pp53
p21
β-actin
B
MCF7
Rel. TTP mRNA expression
***
DMSO
DOX (μg)
C
MCF7/Mock
MCF7/DMSO
MCF7/DOX
0.1 1 (μg)
RT-PCR
TTP
GAPDH
WB
TTP
β-actin
D
MDA-MB231
MDA-MB231/DMSO
MDA-MB231/DOX
0.1 1 (μg)
RT-PCR
TTP
GAPDH
WB
TTP
β-actin
E
MDA-MB231
Rel. TTP mRNA expression
DMSO
DOX(μg)
F
MDA-MB231
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DOX(μg)
DMSO
RT-PCR
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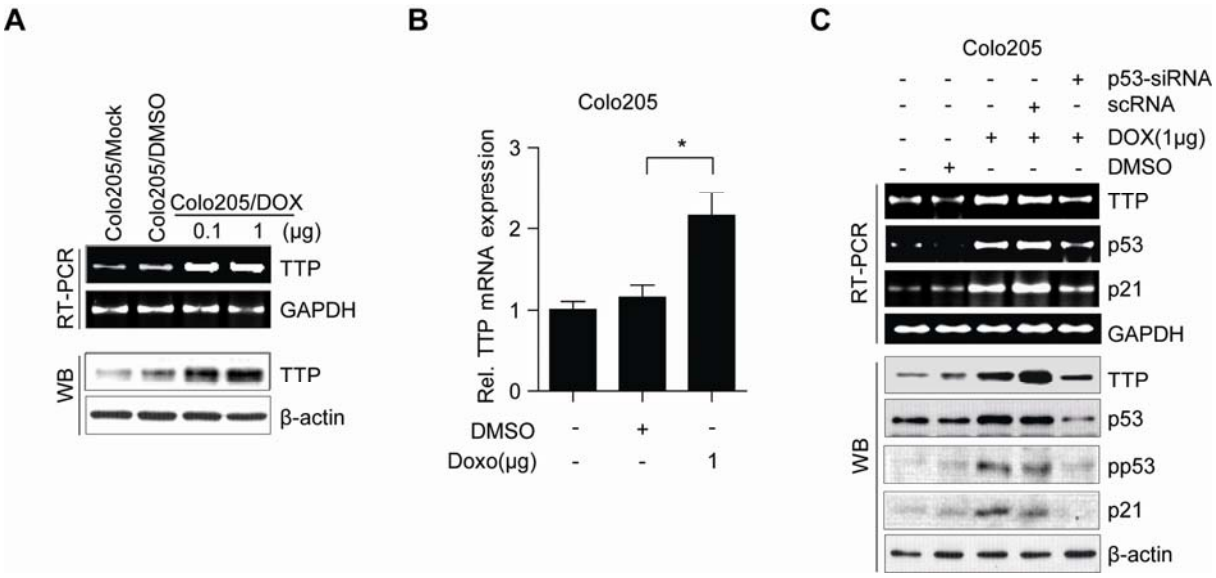
pp53
p21
β-actin

Fig. S1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Colo205/DMSO
Colo205/DOX
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1
(μg)
RT-PCR
TTP
GAPDH
WB
TTP
β-actin
B
Colo205
Rel. TTP mRNA expression
3
2
1
0
*
DMSO - + -
Doxo(μg) - - 1
C
Colo205
p53-siRNA - - - - +
scRNA - - - + -
DOX(1μg) - - + + +
DMSO - + - - -
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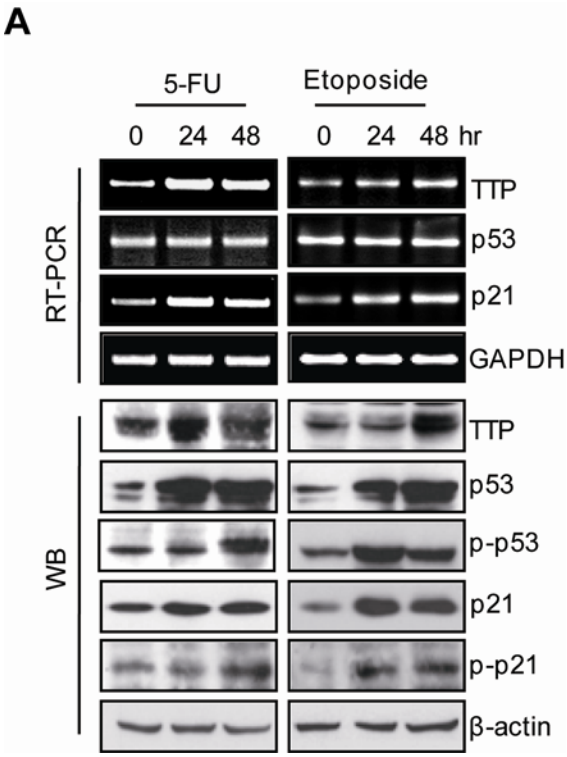
p21
GAPDH
WB
TTP
p53
pp53
p21
β-actin

Fig. S2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Etoposide
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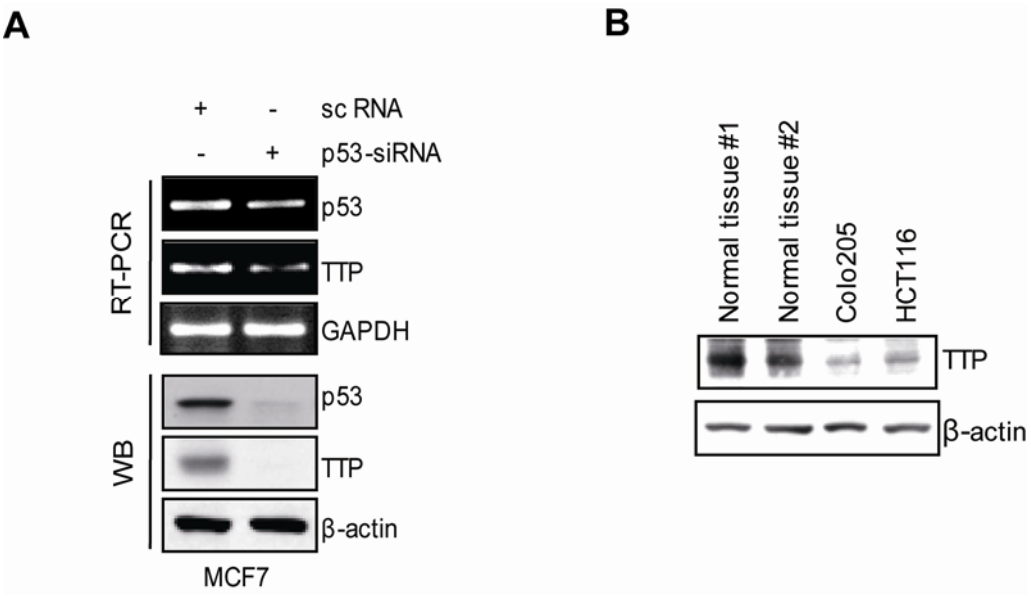
p21
p-p21
β-actin

Fig. S3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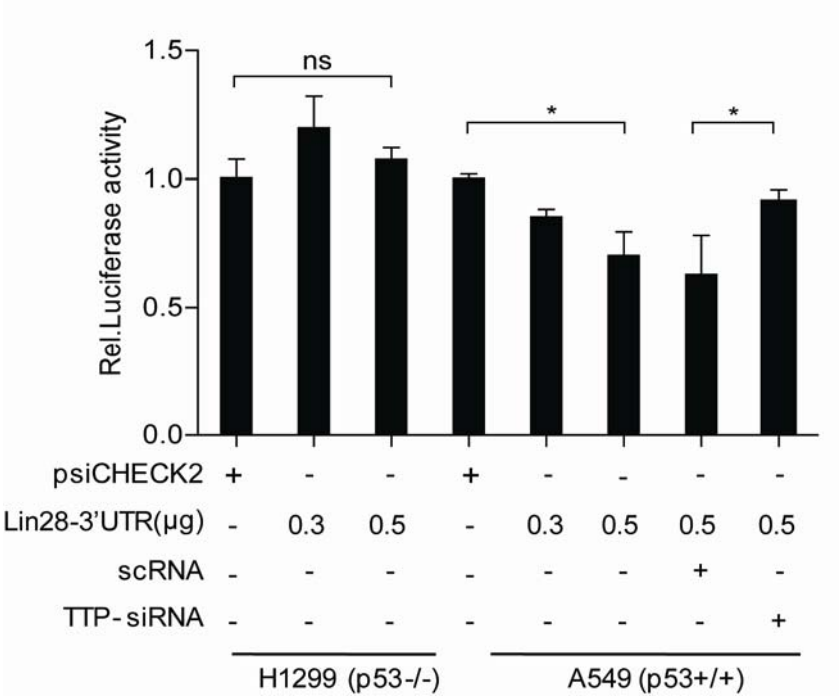
β-actin

Fig. S4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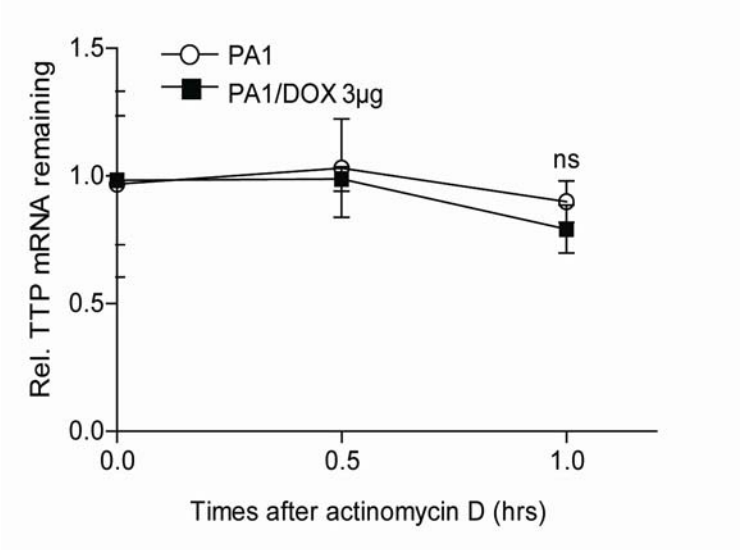
1.0
0.5
0.0
Rel.Luciferase activity
ns
*
*
psiCHECK2 + - - + - - - -
Lin28-3'UTR(μg) - 0.3 0.5 - 0.3 0.5 0.5 0.5
scRNA - - - - - - + -
TTP-siRNA - - - - - - - +
H1299 (p53-/-)
A549 (p53+/+)

Fig. S5


PA1
PA1/DOX 3μg
ns
Rel. TTP mRNA remaining
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1.0
0.5
0.0
0.0
0.5
1.0
Times after actinomycin D (hrs)

Fig. S6

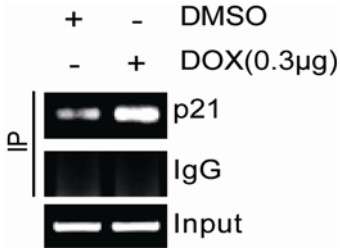


Fig. S7

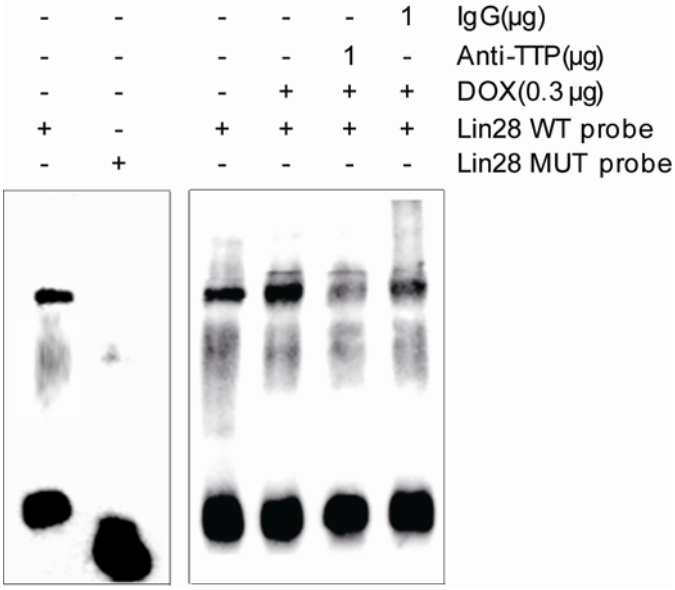
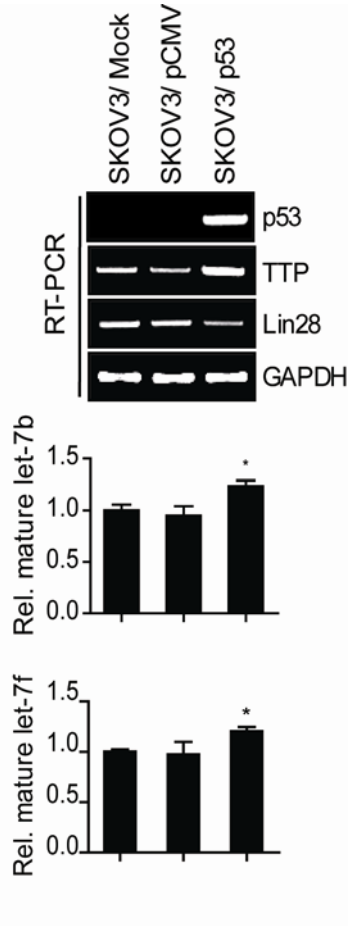


Fig. S8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SKOV3/ pCMV
SKOV3/ p53
RT-PCR
p53
TTP
Lin28
GAPDH
Rel. mature let-7b
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1.0
0.5
0.0
*
Rel. mature let-7f
1.5
1.0
0.5
0.0
*

Fig. S9