

Regulation of apoptosis by p53-inducible transmembrane protein containing sushi domain

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Abstract. The tumor suppressor p53 is a transcription factor that induces the transcription of various target genes in response to DNA damage and it protects the cells from malignant transformation. In this study, we performed cDNA microarray analysis and found that the transmembrane protein containing sushi domain (*TMPS*) gene, which encodes a putative type I transmembrane protein, is a novel p53-target gene. *TMPS* contains a sushi domain in the extracellular region, which is associated with protein-protein interaction. *TMPS* expression is induced by endogenous p53 under genotoxic stress in several cancer cell lines. Reporter assay revealed p53-dependent transactivation of the p53 binding-sites (BSs) located in the intron 1 of *TMPS*. Chromatin immunoprecipitation (ChIP) assay showed that p53 binds to these BSs *in vivo*. Overexpression of *TMPS* induced apoptosis through the activation of caspase-3, 8, and 9 in various cancer cell lines. Moreover, γ -irradiation induced the expression of *TMPS* mRNA in the spleen and colon of p53^{+/+} mice but not in those of p53^{-/-} mice. These data indicate that *TMPS* may play a role in p53-dependent apoptosis under DNA damage condition.

Introduction

p53 is called as the 'Guardian of the genome' because it is stabilized in response to DNA damage or critical cellular stress, and it prevents the malignant transformation of cells by inducing the expression of various kinds of target genes. Since p53 is mutated by genetic alterations such as mutations or deletions in more than half human cancers, p53 is indispensable for cancer research and has attracted the attention of many oncologists. Many target genes of p53 have been isolated; these target genes are involved in at least four main physiological functions such as cell cycle regulation, DNA

repair, apoptosis, and anti-angiogenesis (1,2). The *p21/waf1* gene is considered to be one of the most important p53 target genes because it is essential for p53-dependent cell cycle arrest at G1. p53R2, which supplies nucleotides for DNA synthesis, facilitates the repair of DNA damage. Several mitochondrial proteins including BAX, Noxa, and p53-regulated apoptosis-inducing protein 1 (p53AIP1) contribute to the release of cytochrome c from mitochondria. Other proteins such as Fas/ApoI and unc-5 homolog B (UNC5B) are also associated with apoptosis. Brain-specific angiogenesis inhibitor 1 (BAI1) and thrombospondin 1 (TSP1) suppress angiogenesis and inhibit tumor growth. In our previous studies, we have isolated and characterized additional p53 target genes by using cDNA microarray technology, and we have reported the p53-dependent transcription of genes encoding deafness, autosomal dominant 5 (DFNA5), Semaphorin 3F (SEMA3F), B cell linker protein (BLNK), and unc-5 homolog A (UNC5A) (3-6). More studies on these target genes would clarify the physiological function of p53 as a tumor suppressor gene.

Sushi domains are also known as complement control protein modules or short consensus repeats. Sushi domain contains approximately 60 amino acid residues and four cysteines (Cys); the 1st and 3rd, and the 2nd and 4th Cys residues in each repeat are linked by disulfide bonds (7-9). Proteins with these domains are included in a family which is a part of one of the largest protein superfamilies that includes proteins involved in the complement system such as complement factor H and complement receptor type I (10,11); blood coagulation factors such as coagulation factor XIII (12); adhesion proteins such as L-selectin (13); and cytokine receptors such as IL-2 or IL-15 receptors (14,15). Since the above mentioned proteins can bind to other proteins, it appears that the sushi domain often functions as a protein-binding module and endows the protein with different functions. The presence of ten sushi domain repeats in the β -subunit of the coagulation factor XIII (FXIII-B) allow for the binding of FXIII-B to the α -subunit of coagulation factor XIII or FXIII-B homodimer assembly (12). Furthermore, sushi domain is essential for binding the IL-15 receptor (IL-15R) to its ligand IL-15 (15).

In this study, we report a novel p53 target gene, namely, transmembrane protein containing sushi domain (*TMPS*), which encodes a sushi domain-containing protein in the

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extracellular region and regulates apoptosis with caspase activation.

Materials and methods

Cell lines. HepG2 (hepatoblastoma), H1299 (lung cancer), LS174T, HCT116 (colon cancer), HEK293 (human kidney cell) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), respectively. LC176 was a gift from Dr T. Takahashi (Aichi Cancer Center, Japan). All cell lines were cultured under the conditions recommended by their respective depositors.

Complementary DNA (cDNA) microarray. Total cellular RNA was extracted at the indicated time points (0, 6, 12, 24 and 48 h) from HepG2 cells after infection with adenoviral vectors at 30 MOI (multiplicity of infection) designed to express either wild-type p53 (Ad-p53-WT), p53-46F (Ad-p53-46F), in which Ser-46 is replaced with phenylalanine (16), or enhanced green fluorescent protein (EGFP) (Ad-EGFP). For gene expression profiling, GeneChip Human Genome U133A and U133B microarrays (Affymetrix, Santa Clara, CA, USA) were used that contain 22,215 and 22,577 probe sets, respectively, to examine a total of about 39,000 transcripts. Target cRNA for microarray hybridization was prepared from 5 μ g of total RNA according to the manufacturer's instructions using a BioArray RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY, USA). Hybridization to the microarrays, washing and staining with the antibody amplification procedure, and scanning was also carried out according to the manufacturer's instructions. The expression value of each gene was calculated and normalized using Affymetrix Microarray Suite software version 5.0 (3).

RNA interference. We established the p53-knock down (p53-KD) and the control (Cont) cell lines as described previously (3-6). Briefly, HepG2, LS174T, HCT116 and LC176 cells were infected with SI-MSCV-puro-H1R-p53Ri retrovirus for down-regulation of p53 expression and with SI-MSCV-puro-H1R-control retrovirus for negative control. Each stable cell line was established after selection with 1 μ g/ml puromycin.

Plasmids. The entire coding sequence of *TMPS* cDNA was amplified by PCR using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan) and inserted into pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, USA). After confirmation of sequencing, the EcoRI-digested DNA fragment was cloned into pcDNA 3.1(+) (pcDNA-*TMPS* sense/antisense) (Invitrogen) and pCMV-Tag2B (Tag2B-*TMPS*) (Stratagene, La Jolla, CA, USA).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). p53-KD and Cont cell lines were treated with adriamycin (1 μ g/ml, 2 h). Total RNAs were isolated at the indicated time (0, 6, 12, 24 and 48 h) using TRIzol reagent (Invitrogen). Furthermore total RNAs were also extracted from irradiated mouse tissues. A 5 μ g aliquot of total RNA was reverse-transcribed and the PCR was run in the exponential region (18-28 cycles) to allow semi-quantitative comparisons among cDNAs developed from identical reactions. Oligonucleotide primer sequences were as follows: Human-*TMPS*-

forward; 5'-GCTCAGGGAACCGCCAAC-3', Human-*TMPS*-reverse; 5'-AGGCAGATGGCGAGAATCT-3'. Mouse-*TMPS*-forward; 5'-GCAGGCAGTGTTCATCGAGTA-3', Mouse-*TMPS*-reverse; 5'-TTGGCTGCAGCAGTACAAAC-3'.

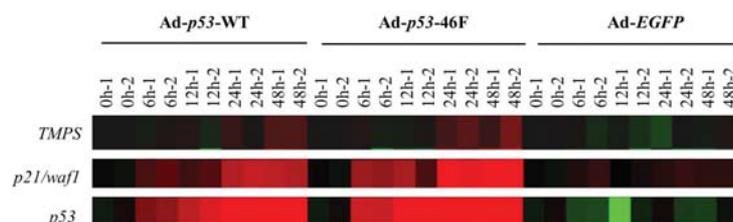
Western blot analysis. HEK293 cells were transfected with Tag2B-*TMPS*. Twenty-four hours later, the cells were collected and lysed in chilled Radio-immuno-protein-assay (RIPA) buffer [1% Nonidet-P40 (NP-40), 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Deoxycholate, 0.1% Sodium dodecyl sulfate (SDS), protease inhibitor cocktail]. Homogenates were subjected to SDS-polyacrylamide gel electrophoresis and blotted onto Hybond-P (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The blots were incubated with anti-Flag M2 antibody (Sigma, St. Louis, MO, USA) or anti- β -actin (Sigma), then visualized by ECL Western blotting detection reagents (GE Healthcare).

Gene reporter assay. The plasmid constructs containing the putative p53 BSs of *TMPS* were termed pGL-BS1, BS2, or BS3, respectively. Each genomic fragment containing putative p53BS was amplified by PCR and cloned into pGL3-promoter vector (Promega, Madison, WI, USA). H1299 cells were plated in 6-well culture dishes (1×10^5 cells/dish), then each pGL-BS vector containing BS1, BS2 or BS3 was co-transfected with either wild-type p53, mutant p53 (R175H) or empty expression vector (mock) in combination with pRL-CMV vector (Promega). Twenty-four hours after transfection, cells were lysed and subjected to the Dual Luciferase assay system (Promega). Quantification of luciferase activities was carried out manually with a luminometer.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using the ChIP assay kit (Upstate, Lake Placid, NY, USA) as recommended by the manufacture. H1299 cells (1×10^6 cells) were infected with Ad-p53-WT and Ad-EGFP at 30 MOI. After 24 h, genomic DNA and protein were cross-linked by adding formaldehyde (1% final concentration) directly into the culture medium and incubated for 15 min at 37°C. Cells were lysed in 200 μ l SDS lysis buffer with a protease inhibitor cocktail and sonicated to generate DNA fragments 200-1,000 bp long. After centrifugation, the cleared supernatant was diluted with ChIP assay buffer and incubated at 4°C with the specific antibody with rotation. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross links were reversed by heating to 65°C for 5 h. DNA was phenol-extracted, ethanol-precipitated and resuspended in 50 μ l of water. Two microliters of each sample were subjected to PCR amplification with 30 cycles. PCR was done using the following specific primers containing p53 BSs, 5'-ACCCTGAAGGTCTGGGAGA-3' (BS1, forward), 5'-CCTATGCAGATCTGTCCATC-3' (BS1, reverse), 5'-CATAGGAGTTACGCACACAC-3' (BS2, forward), 5'-GCACAAAGGTTGGTACTCAG-3' (BS2, reverse), 5'-CTGCTGTTAATGGACCATACT-3' (BS3, forward), 5'-GGGCTGTGGTTCTTATACCT-3' (BS3, reverse).

Recombinant adenovirus. The adenovirus expressing *TMPS* was prepared using adenovirus expression vector kit (Takara,

A



B

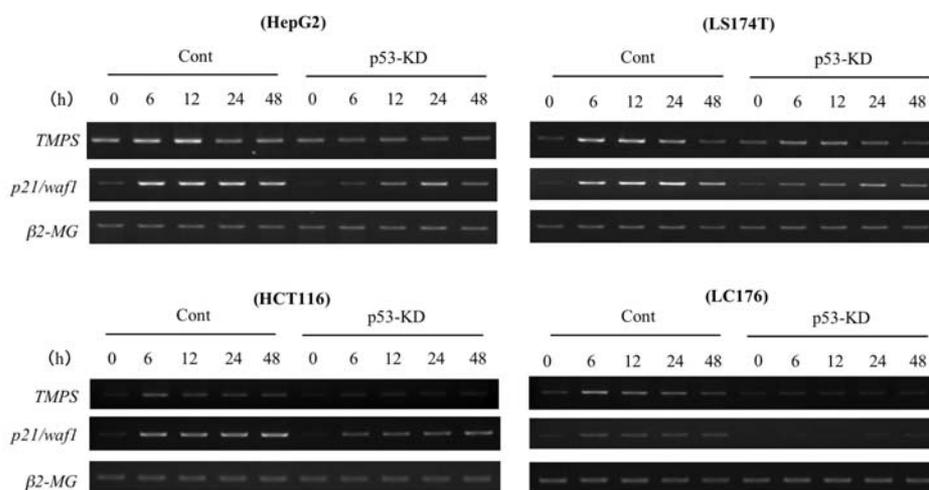


Figure 1. Identification of *TMPS* as a p53-inducible gene. (A) Microarray analysis of *TMPS* expression. The green and red signals on the microarray indicate the intensity of expression of *TMPS*, *p21/waf1*, and *p53* mRNA in HepG2 cells at the indicated times after infection with Ad-*p53*-WT, Ad-*p53*-46F, and Ad-*EGFP*. Red signal indicates upregulation and green signal indicates downregulated expression of genes. (B) Induction of *TMPS* by endogenous p53. Adriamycin-treated p53-KD and Cont cells (HepG2, LS174T, HCT116, and LC176 cells) were subjected to RT-PCR. *p21/waf1* and $\beta 2$ -MG were used as positive control and quantity control, respectively.

Otsu, Japan) according to the manufacturer's instructions. In brief, blunt-ended full length *TMPS* were also cloned into the *Sma*I site of the cosmid pAxCawtit, which contains the CAG promoter and the entire genome of type 5 adenovirus except for E1 and E3 regions, then transfected to HEK293. Viruses were propagated in the 293 cells and purified as described previously (16). Expression level of *TMPS* mRNA was evaluated by RT-PCR at 24 h post infection to H1299 cells at the indicated MOIs.

Apoptosis assay. Various cancer cell lines were infected with Ad-*TMPS* at indicated MOIs (0, 30, 60 and 100 MOI). After 72 h of infection, the cells were collected and fixed with 70% ethanol. Fixed samples were centrifuged, treated with RNase (0.2 mg/ml), and resuspended in propidium iodide (50 μ g/ml). The stained cells were analyzed on a FACScan flow cytometer (Beckton-Dickinson, Franklin Lakes, NJ, USA). The subG1 fraction of the cells was counted as the apoptotic cells, and the proportion of the apoptotic cells to the total cells was indicated as percentage.

Caspase activity. The labeled synthetic substrates N-acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) for caspase 3, N-acetyl-Ile-Glu-Pro-Asp p-nitroanilide (Ac-IEPD-pNA) for caspase 8 and N-acetyl-Leu-Glu-His-Asp-7-amido-4-fluoromethylcoumarin (Ac-LEHD-AFC) for caspase 9 were purchased from Sigma. Cell lysates were prepared from

LS174T cells 36 or 60 h after infection with Ad-*TMPS*, Ad-*EGFP* or Ad-*p53*, and reacted with each substrate in the assay buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 100 mM NaCl, 0.1% 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 5 mM dithiothreitol] at 37°C. The free pNA cleaved from the substrate was detected as the caspase activity by monitoring the optical density at 405 nm. In this system, caspase activity was presented as the ratio compared with the control from the cells infected with Ad-*EGFP*.

Immunocytochemistry. Tag2B-*TMPS* was transfected into H1299 cells in an 8-well chamber slide. Twenty-four hours later, the cells were fixed with methanol at -20°C for 15 min, then blocked with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h at room temperature (RT). Anti-Flag M2 antibody (1:250) was applied for 1 h at RT, followed by goat anti-mouse fluorescein isothiocyanate (FITC) (1:500, Vector Laboratories, Burlingame, CA, USA) and mounted with propidium iodide (PI), then visualized using Radiance 2000 confocal microscope (Bio-Rad Laboratories, Hercules, CA, USA).

p53-knockout mice. p53-deficient mice were a gift from Dr S. Aizawa, Center for Developmental Biology, RIKEN, Japan (17). *p53*^{+/+} and *p53*^{-/-} mice (8-12 w) were irradiated by

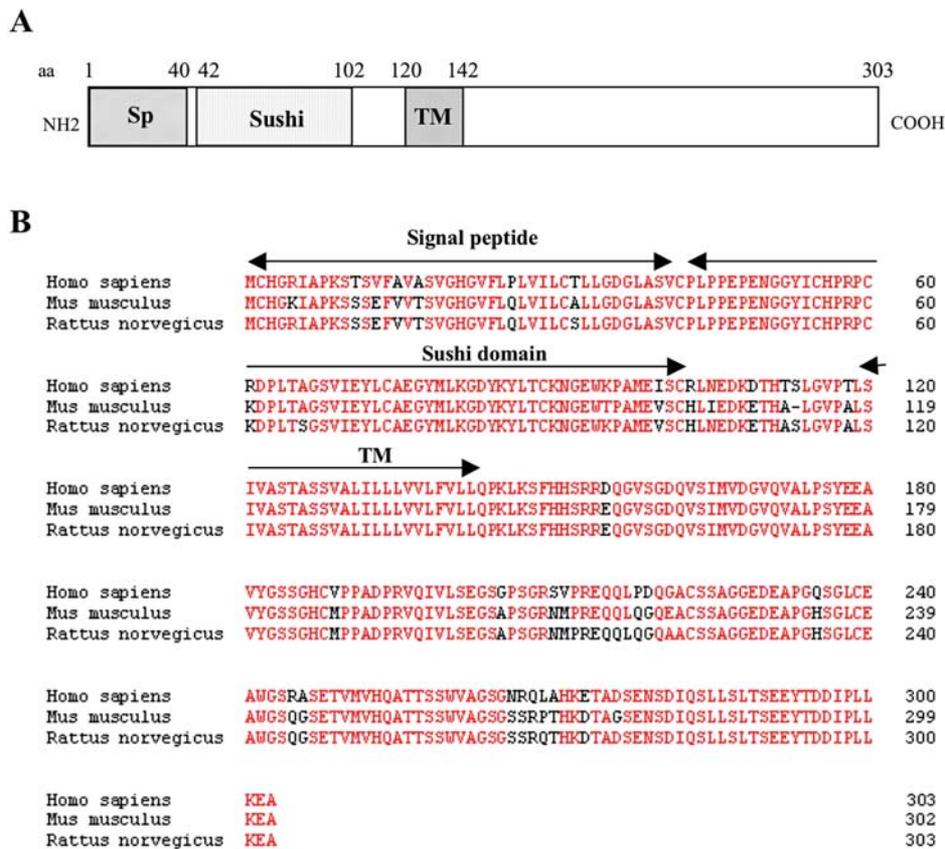


Figure 2. (A) Structure of TMPS. TMPS contains 303 amino acids. The structure of TMPS includes a few motifs or domains: Sp, signal peptide motif; Sushi, sushi domain and TM, transmembrane domain. Each number indicates the amino acid number. (B) Comparison between TMPS amino acid sequences of humans and other organisms. Alignment of human, mouse, and rat homologs of TMPS is shown. Consensus sequences are indicated in red. The arrows indicate either signal peptide motif, sushi domain, or transmembrane domain (TM), respectively.

10 Gy. Then the mice were euthanized and harvested for RNA extraction at indicated times (0, 4, 24 and 48 h) after irradiation. Total RNAs purified from the homogenized tissues (spleen, colon, thymus and brain) were reverse-transcribed and subjected to RT-PCR analysis as described above. All mouse procedures were carried out according to the recommendations of the Institutional Animal Care and Use Committee of the National Cancer Center at Tsukiji, Japan.

Results

Identification of TMPS as a novel p53-inducible gene. To identify additional transcriptional targets of p53 in the human genome, we used cDNA microarrays and screened p53-inducible transcripts according to previously described methods (3,5). In these experiments, *p21/waf1*, whose expression was clearly induced by p53, was used as the positive control; the results indicated that p53 upregulated the expression of approximately 100 genes. We selected one of these genes, namely, *TMPS*, whose expression level was especially elevated by either adenovirus vector expressing wild-type p53 (Ad-p53-WT) or adenovirus vector expressing mutant p53 (Ad-p53-46F), in comparison with adenovirus vector expressing green fluorescent protein (Ad-EGFP) in a time-dependent manner (Fig. 1A). p53-46F was previously demonstrated to be an active form of p53 with an enhanced ability to induce apoptosis (16). The microarray results were confirmed by

RT-PCR. We evaluated the induction of *TMPS* mRNA by endogenous p53. p53-knock down (p53-KD) and control (Cont) cell lines of HepG2, A549, HCT116, and LC176 were treated with adriamycin, and then the cells were harvested for total RNA isolation. Adriamycin treatment induced the expression of *TMPS* mRNA, which was stronger in Cont cells than in p53-KD cells (Fig. 1B); hence, the expression of *TMPS* mRNA was induced in response to DNA damage in a p53-dependent manner.

Structure of TMPS. The *TMPS* gene is located at chromosome 14q24.1; its sequence has been deposited in the National Center for Biotechnology Information (NCBI) under the accession number NM_014734 with unknown function. Basic Local Alignment Search Tool (BLAST) analysis revealed that TMPS contains a signal peptide (Sp) motif at the N-terminus; a sushi domain (42-102 aa), which functions as a protein-binding module; and a transmembrane domain (120-142 aa) (Fig. 2A). The identity values of TMPS amino acid sequence reveal that the protein sequence is well conserved between human and rodents (human vs. mouse, 89% identity; human vs. rat, 90% identity) (Fig. 2B).

TMPS as a direct transcriptional target of p53. We identified three putative p53 binding sites in the genomic region of *TMPS*. One binding site (BS1) was in the promoter region and the remaining two (BS2 and BS3) were in intron 1

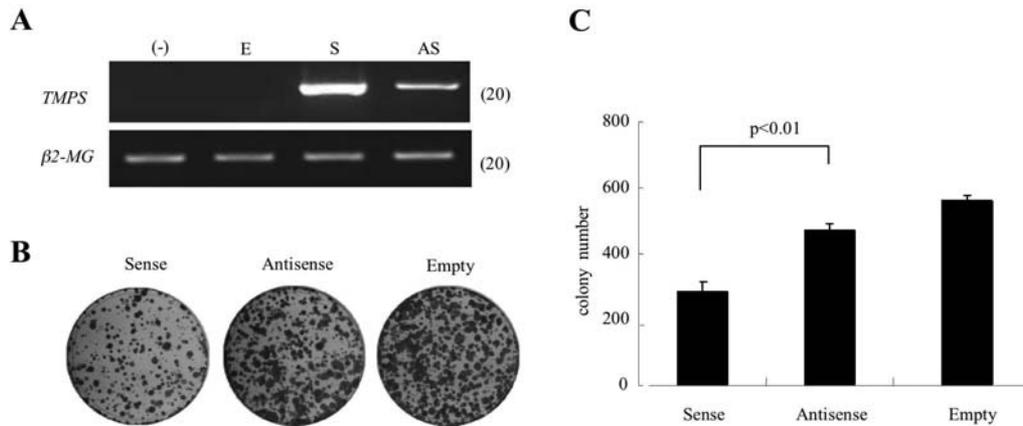


Figure 4. Colony formation assay. (A) The expression of *TMPS* in H1299 cells was analyzed by RT-PCR. The results indicate the expression levels of *TMPS* mRNA in H1299 cells 24 h after transfection with expression vectors. (-) indicates no transfection, (+), transfection with empty (E)/pcDNA3.1; S, transfection with pcDNA-*TMPS*-sense; and AS, transfection with pcDNA-*TMPS*-antisense. The numbers in parenthesis indicate the number of PCR cycles. (B) Representative G418-resistant colonies of H1299 cells transfected with each plasmid are shown. (C) The average number of colonies in triplicate samples is indicated with error bars indicating one standard deviation (SD) on histograms.

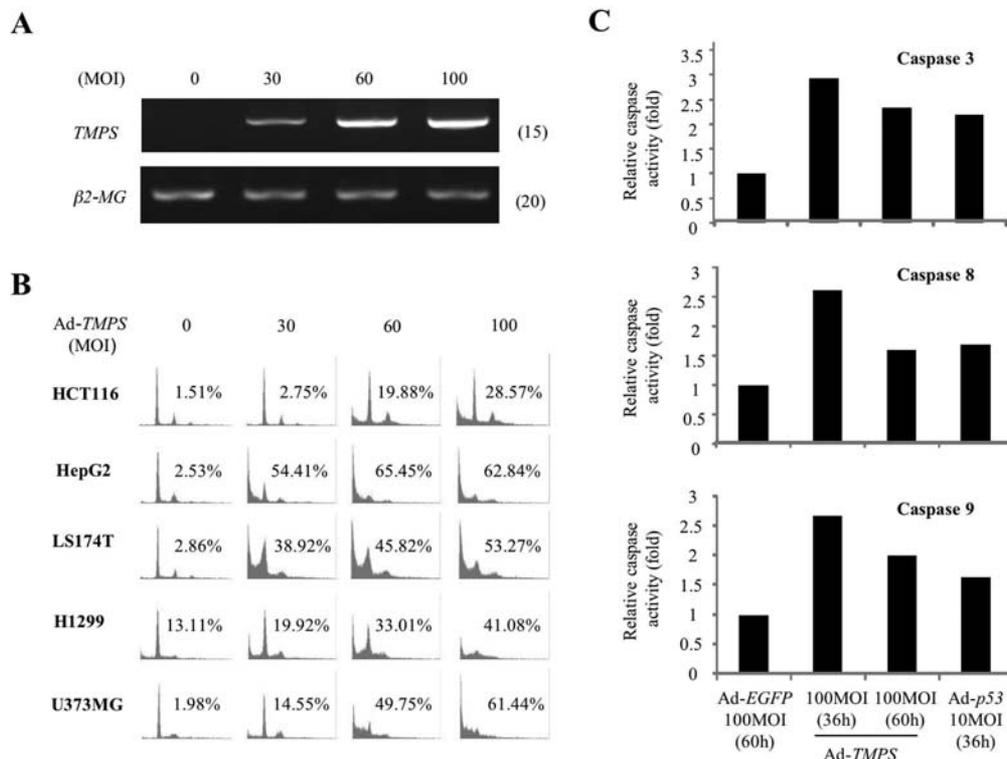


Figure 5. Apoptosis by *TMPS*. (A) The expression level of *TMPS* mRNA in H1299 cells at 24 h after infection with Ad-*TMPS*. The numbers in parenthesis indicate the number of PCR cycles. (B) Apoptotic cell death caused by Ad-*TMPS*. HCT116, HepG2, LS174T, H1299, and U373MG cells were infected with Ad-*TMPS* (0, 30, 60 and 100 MOI) and analyzed by FACS scan 72 h after infection. DNA histogram for each cell type is shown and the percentage of the respective subG1 fraction is also shown. (C) Caspase activity. LS174T cells were infected with either Ad-*TMPS*, Ad-*p53*, or Ad-*EGFP* at the indicated MOIs. The cells were collected and the activities of caspase 3, 8, and 9 were determined after 36 or 72 h after infection. The relative ratio of each sample was calculated in comparison with the caspase activity of Ad-*EGFP*-infected cells.

cells were already killed by then. These results suggest that *TMPS* caused apoptotic cell death with caspase activation.

Localization of *TMPS*. BLAST analysis suggested that *TMPS* is a membrane protein. To determine the localization of *TMPS*, we performed immunofluorescent analysis. We performed

Western blotting to confirm the expression of *TMPS* in HEK 293 cells transfected with Tag2B-*TMPS* (Fig. 6A). Then, we transfected this plasmid into H1299 cells and subjected the cells to immunofluorescent analysis. As predicted, fluorescent signals were detected on the surface of the cells, indicating that *TMPS* was a membrane protein (Fig. 6B).

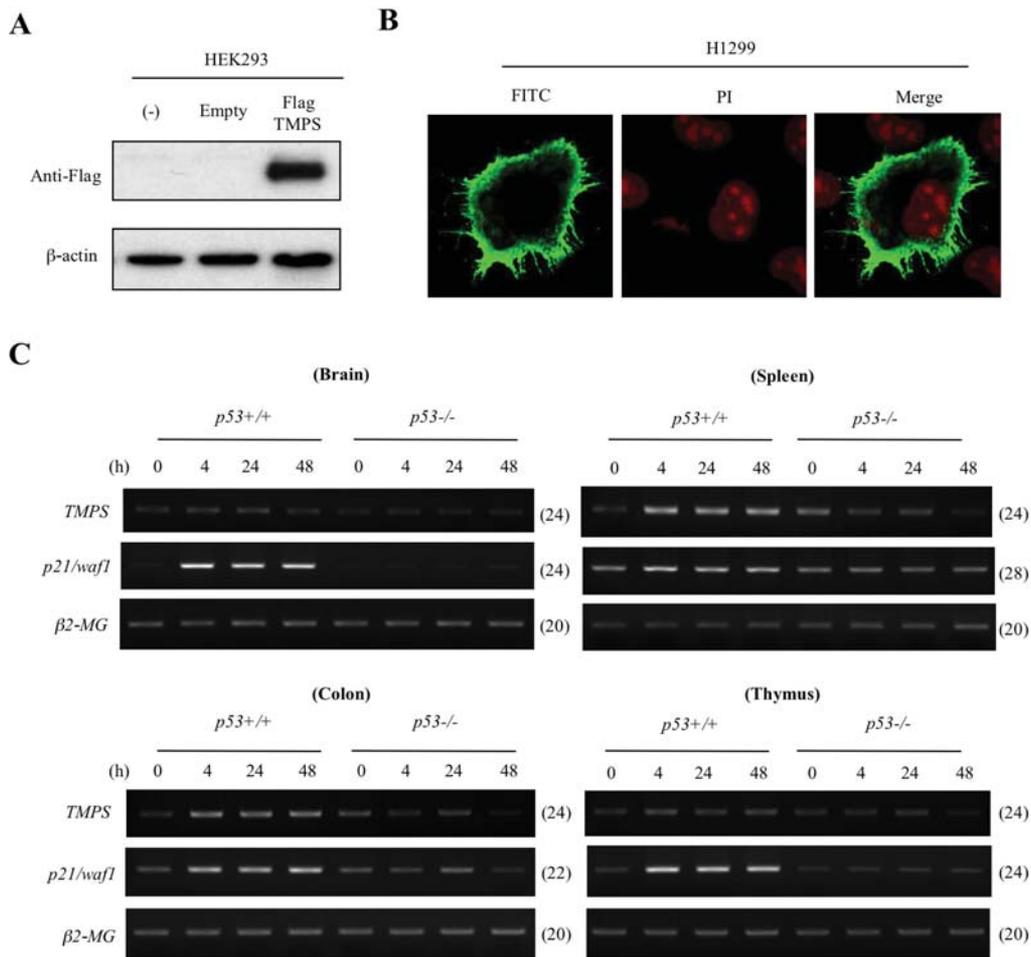


Figure 6. Localization and potential *in vivo* role of TMPS. (A) Expression of Flag-tagged TMPS was confirmed by Western blot analysis. β -actin was used as the loading control. (B) Tag2B-TMPS expressing Flag-tagged TMPS was transfected into H1299 cells. The cells were stained with anti-Flag antibody (FITC), counterstained by propidium iodide (PI), and visualized by confocal microscopy. (C) Irradiated $p53^{+/+}$ or $p53^{-/-}$ mice were euthanized at the indicated time points. cDNAs from various tissues including the spleen, colon, thymus and brain were analyzed by RT-PCR. *p21/waf1* was used as positive control and $\beta 2$ -MG as quality control. The numbers of RT-PCR cycles for each sample are indicated in parentheses.

TMPS functions as an *in vivo* mediator of p53 response after DNA damage. To assess whether TMPS truly functions as an *in vivo* mediator of p53 action, we determined the p53-dependent inducibility of TMPS by using $p53$ -knockout mice. We irradiated $p53^{+/+}$ and $p53^{-/-}$ mice with 10 Gy of γ -rays, and isolated total RNA from the spleen, colon, brain, and thymus of the irradiated mice at the indicated times. Interestingly, we observed a strong p53-dependent induction of TMPS expression in the spleen and colon. Further, the expression of TMPS mRNA in the brain and thymus was also slightly increased. These results suggest that TMPS plays an important role in DNA damage-induced p53-dependent apoptosis *in vivo* (Fig. 6C).

Discussion

p53 mainly prevents the development of cancer by inducing apoptosis. In response to DNA damage, p53 suppresses cell growth through the cell cycle and allows DNA repair. However, in the event of severe damage, p53 induces apoptosis and eliminates seriously damaged cells. Among the various p53 target genes isolated so far, several genes appear to be apoptosis-related genes. These genes are classified into at

least 3 groups as follows: genes in the mitochondrial pathway (*Bax*, *Puma*, *Noxa*, *p53AIP1*, *PIG3*, *POX*, *ALDH4*), death receptor pathway (*Killer/DR5*, *FAS*), and dependence receptor pathway (*UNC5B* termed *p53RD11*, *UNC5A*) (2). Several additional target genes that are not directly involved in these pathways also mediate p53-dependent apoptosis. Several specific domains in the proteins encoded by these genes are known to play a critical role in apoptosis. For example, the BH3 domain in Bcl-2 family proteins, including Bax and Puma, triggers a mitochondrial apoptotic event and affects the release of cytochrome c from mitochondria (18). Killer/DR5 and FAS receptor, which contain the death domain, bind to their ligands and induce cell death with the recruitment of an adaptor molecule and procaspase 8 (19). The death domain in dependence receptors such as UNC5A or UNC5B is also involved in caspase-activated apoptosis in the absence of ligand netrin-1 (6,20). In this study, we report that TMPS, which is encoded by a novel p53 target gene, contains a sushi domain in its extracellular region and is involved in apoptosis with caspase-activation. This is the first report of a sushi domain-containing p53-target that plays a role in apoptosis.

It was believed that sushi domain functions as a protein-binding module and is involved in receptor-ligand interactions

(7-9). Interestingly, emerging evidence suggests that sushi domain may also be involved in apoptosis. Sushi domain in the extracellular region of IL15-R α is strongly associated with cell survival and apoptosis (15). Amino acid substitution of the 1st or 4th cysteine in the sushi domain abolished the IL-15-binding activity of IL15-R α , and resulted in the loss of IL-15-induced proinflammatory cytokine production and anti-apoptotic activity (15). Further, the gene encoding tumor suppressor drs, which contains three sushi domain repeats, induces apoptosis in several human cancer cells with caspase activation (21). The deletion of these repeats apparently decreased apoptosis, indicating that sushi domains may be the key functional domains in apoptosis; however, the binding partners of sushi domain are unknown. Drs-induced apoptosis involved sequential activation of caspases 12, 9, and 3; however, neither cytochrome-c release nor activation of caspase 8 was observed, suggesting that mitochondrial pathway does not mediate drs-induced apoptosis (22). In contrast, in the present study, TMPS activated not only caspase 9 and 3 but also caspase 8, indicating that TMPS-induced apoptosis may involve the participation of various kinds of apoptotic pathways. Since TMPS localizes in cytoplasmic membrane, caspase 8 could also be activated by TMPS, similarly to death receptor pathway. These results indicate that TMPS is one of the apoptotic regulators induced by p53.

Indeed, apoptosis is one of the core functions regulated by p53, and it is very important to clarify its mechanism, including the role of TMPS. Further, detailed investigation is necessary to reveal the role of this interesting molecule in tumorigenesis.

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