Delta40p53 suppresses tumor cell proliferation and induces cellular senescence in hepatocellular carcinoma cells

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Abbreviations:

AAV: adeno-associated virus
BAX: BCL2-associated X protein
CCNB1: cyclin B1
CRISPR: clustered regularly interspaced shot palindromic repeats
FACS: Fluorescence-activated cell sorting
FAS: Fas cell surface death receptor
FISH: fluorescence in situ hybridization
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
HCC: Hepatocellular carcinoma
IL-8: Interleukin 8
MAPK: Mitogen-activated protein kinase
MDM2: MDM2 oncogene, E3 ubiquitin protein ligase
MOI: multiplicity of infection
p21WAF1/CIP1: p21 wild-type p53-activated fragment/cyclin-dependent kinase interacting protein 1
p53: tumor protein 53
PUMA: p53 upregulated modulator of apoptosis
SASP: senescence-associated secretory phenotype
sgRNA: small guide RNA
shRNA: small hairpin RNA
TAD: transactivation domain
Δ40p53: delta40p53
Summary statement

Δ40p53 exerts tumor suppressor activity and induces senescence in HCC cell lines.

ABSTRACT

Splicing isoforms of certain genes impact on genetic biodiversity in mammals. The tumor suppressor TP53 gene plays an important role in the regulation of tumorigenesis in hepatocellular carcinoma (HCC). Delta40p53 (Δ40p53) is a naturally occurring p53 isoform that lacks N-terminal transactivation domain, yet little is known about the role of Δ40p53 in the development of HCC. Here, we first report the role of Δ40p53 in HCC cell lines. In the p53+/Δ40 cell clones, clonogenic activity and cell survival dramatically decreased, while the percentage of SA-β-gal positive cells as well as p21WAF1/CIP1 expression significantly increased. These observations were clearly attenuated in the p53+/Δ40 cell clones after Δ40p53 knockdown. In addition, exogenous Δ40p53 expression significantly suppressed cell growth in TP53wt, TP53mt, and TP53-/- HCC cells. Notably, Δ40p53-induced tumor suppressor activity was markedly attenuated in cells expressing the hot-spot mutant, Δ40p53/R175H, which lacks transcriptional activity of p53. Moreover, Δ40p53 expression was associated with increased full-length p53 protein expression. These findings enhance the understanding of the molecular pathogenesis of HCC; Δ40p53 acts as an important tumor suppressor in HCC cells.
INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most frequent malignancies worldwide (Lozano et al., 2012), commonly develops in response to continuous microenvironmental stresses, including chemical exposure, chronic inflammation from hepatitis viral infection, tissue remodeling in the liver, and a high fat diet (Liu et al., 2014; Nishida and Goel, 2011). As an initiating oncogenic event in HCC, the disruption of the p53 tumor suppressor gene has been shown to be closely associated with hepatocarcinogenesis. Deletion of the Trp53 gene resulted in the development of liver tumors in a significant number of mice (Katz et al., 2012; Morris et al., 2012), while restoring p53 in a murine liver carcinoma model limited tumor cell growth by mediating cellular senescence (Xue et al., 2007). Thus, accumulating evidence implicates TP53 gene dysfunction in the development of HCC.

In general, splicing isoforms of certain genes play an important role in biodiversity. It has known that TP53 gene potentially encodes at least twelve p53 isoforms, in which four different N-terminal p53 forms (full length, Δ40, Δ133, and Δ160) are combined with three different C-terminal domains (alpha, beta, and gamma) (Marcel et al., 2012). Full-length (FL)-p53 protein (also called TAp53alpha) is the canonical p53 protein, while Δ40p53alpha (also known as p53/47), a p53 isoform that lacks the 39 N-terminal amino acids corresponding to the first transactivation domain (TAD-I) of FL-p53, is translated from an in-frame second AUG at nucleotides 252-254 of p53 mRNA through a second internal ribosome entry site (Olivares-Illana and Fåhraeus et al., 2010; Wei et al., 2012). Recent studies demonstrated the biological effects of Δ40p53alpha in both humans and mice. Transgenic mice overexpressing p44, the mouse homolog of Δ40p53alpha, showed obvious signs of aging and a shorter lifespan (Maier et al., 2004; Qian and Chen, 2013). It has been reported that Δ40p53alpha exerts anti-cancer properties in human lung cancer and melanoma cells (Yin et al., 2002; Candeias et al., 2006; Takahashi et al., 2014). In contrast,
Courtois et al reported that Δ40p53alpha counteracts growth suppression via FL-p53 in mouse fibroblasts (Courtois et al., 2002). Thus, the biological function of Δ40p53alpha potentially varies according to cell type. Although accumulating evidence has implicated Δ40p53alpha in aging and/or tumor suppression, little is known about the involvement of Δ40p53alpha in the development of HCC.

In the present study, we are the first to report the tumor suppressor role of Δ40p53alpha (hereafter called Δ40p53) in the development of HCC. We also discuss a possible molecular mechanism underlying Δ40p53-induced tumor suppression and senescence.
MATERIALS AND METHODS

Cell culture

Human hepatocellular carcinoma cell lines HuH-1 (TP53 WT), HepG2 (TP53 WT), Hep3B (TP53 -/-), HuH-7(TP53 Y220C), and PLC/PRF/5(TP53 R249S) were obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). HCC cell lines were maintained in DMEM medium supplemented with 10% FBS and penicillin-streptomycin at 37°C in 5% CO2 humidified air. Adherent cells were dissociated from a 90-mm dish using trypsin and then seeded in 96-well, 12-well, or 6-well plates for the experiments.

Reagents

Dulbecco’s modified Eagle’s medium (DMEM), β-galactosidase, crystal violet, trypsin-EDTA, and penicillin-streptomycin solution were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glutaraldehyde was obtained from Sigma-Aldrich Japan (Tokyo Japan). Fetal bovine serum (FBS) was obtained from Nichirei Biosciences Inc. (Tokyo, Japan). The mouse anti-human p53 monoclonal antibody (DO-1) was obtained from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA USA). Annexin V (AxV)-fluorescein isothiocyanate (FITC) was obtained from MBL (Nagoya, Japan). Propidium iodide (PI) was obtained from Abcam Inc. (Cambridge, MA USA). Apo-ONE™ Homogeneous Caspase-3/7 Assay Kits were obtained from Promega KK (Tokyo, Japan). All other primary and secondary antibodies used for western blot analysis were obtained from Cell Signaling Technology (CST) Japan, K.K. (Tokyo, Japan). The Cell Proliferation Kit I was purchased from Roche (Tokyo, Japan).

Targeted deletion of TP53 exon 2 in the HepG2 cell line

To remove each allele of TP53 exon 2, a vector targeting exon 2 of TP53 (a generous gift from the laboratory of Dr. Fred Bunz) and the targeted clones were created as previously described.
(Topaloglu et al., 2006; Weiss et al., 2010). Knockout of exon 2 of TP53 was performed using an adeno-associated viral (AAV) vector, as previously described. The targeting vector was transduced into cells and antibiotic selection was performed with 800 μg/mL of G418 (Life Technologies) in 96-well plates. Neomycin-resistant colonies were expanded, replicated and pooled, and PCR-based screening was performed as previously described (Konishi et al., 2007). Targeted cells were infected with an adenovirus encoding Cre recombinase to remove the selection cassette. This was followed by single-cell dilutions and then screening by PCR for successful Cre recombination. The primer sequences for PCR are shown in Supplementary Table S1. To create cell clones carrying homozygous deletions of TP53 exon 2, p53+/Δ40 clones were infected with the same AAV targeting vector as shown in Fig. S1A, and concurrently selected with neomycin. During each attempt, approximately 1000 drug-resistant cell clones were screened for homologous recombination (HR) events by applying the protocol described above.

**Quantitative reverse transcription (qRT)-PCR analysis**

HepG2 cells (2 × 10^5 cells/well) were seeded in 6-well plates and incubated for 48 h. Total RNA was then extracted using a PureLink® RNA Mini Kit (Life Technologies Japan, Tokyo Japan), and 2 μg of total RNA was used for cDNA synthesis, which was performed using High Capacity cDNA Reverse Transcription Kits (Life Technologies Japan). qRT-PCR analysis was performed using SYBR Green I, as previously described (Takahashi et al., 2013). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used in this study are described in Supplementary Table S2.

**Western blot analysis**

HepG2 cells were seeded in a 6-well culture plate (2 × 10^5 cells/well) and incubated for 48 h. Preparation of cell extracts and western blot analysis were performed, as previously described.
(Hossain et al., 2015). Immune complexes were detected using ImmunoStar LD (Wako) with a LAS-4000 image analyzer (GE Healthcare, Tokyo, Japan). Band intensity was measured using ImageQuant TL software (GE Healthcare). Relative protein levels were calculated after normalization against an internal control, β-actin.

**Retrovirus**

A fragment containing the Δ40p53 open reading frame (ORF) was amplified from HepG2 cDNA using KOD PlusNeo polymerase (TOYOBO; Tokyo Japan) and the following primer set: forward, 5’-GGATCCCAAGCAATGGATGAT and reverse, 5’-TCAGTCTGAGTCAGGCCCTT. A fragment of the Δ40p53 ORF carrying the R175H or R273H mutation was amplified from pCMV-Neo-Bam p53 R175H (plasmid 16436, obtained from Addgene) or pCMV-Neo-Bam p53 R273H (plasmid 16439, obtained from Addgene), respectively. Each fragment was inserted into a pBabe-puro vector or a pLXSN vector (Clontech Laboratories, Inc., Mountain View, CA, USA). The retroviral plasmids were packaged in 293T cells using the pCL10A vector. Viral supernatants were harvested 96 h after transfection and filtered before infection. The cells were infected with retroviruses in the presence of 8 μg/mL Polybrene (Sigma-Aldrich). Antibiotic selection (puromycin; 2 μg/mL for pBabe-puro or neomycin; 800 μg/mL for pLXSN) was begun 48 h after infection and continued for at least 3 days.

**Knockdown of p53**

pMKO.1-puro p53 shRNA-2 and pMKO.1-puro GFP shRNA were gifts from William Hahn (Addgene plasmids # 10671 and # 10675, respectively) (Masutomi et al., 2003). To obtain cell clones that exhibited stably decreased p53 expression, either the p53 shRNA vector or the control GFP shRNA vector was introduced into HepG2/p53+/Δ40 #1 cells. The retroviral plasmids and
supernatants were prepared as described above. Following infection and antibiotic selection, single colonies were manually selected, expanded, and examined for the expression of p53 at either the mRNA or protein level using qRT-PCR analysis or western blot analysis, respectively. Transient p53 knockdown was performed using p53 siRNA (CST Japan).

**Clonogenic assay**

HepG2 cell clones were seeded in a 12-well culture plate (200 cells/well) and incubated for 14 days to form colonies, as described elsewhere (Attardi et al, 2004). For retroviral vector-based clonogenic assays, HepG2 cells were seeded into a 6-well culture plate (1 × 10^5 cells/well) one day before infection. qRT-PCR analysis was performed to quantitate the level of retroviral RNA by measuring the copy number of the *puromycin N-acetyl-transferase* (*PAC*) gene with a primer set (Supplementary Table S2). The cells were then infected with same multiplicity of infection (MOI) for 48 h, which was followed by antibiotic selection, as described above. After incubation for 14 days, the cells were fixed and then stained with 0.1% crystal violet in PBS. The number of stained colonies was counted manually.

**Cell proliferation assay (MTT assay)**

HepG2 cells were plated in a 96-well culture plate (1 × 10^3 cells/well) and incubated at 37°C for the indicated durations. After incubation, cell proliferation was assessed using Cell Proliferation Kit I (Roche). Absorbance was measured at 595 nm using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

**Annexin V and cellular caspase-3/7 activity assay**

HepG2 cells were stained with AxV-FITC and PI (10 µg/mL) at room temperature for 15 min. Fluorescent intensities were determined using fluorescence-activated cell sorting (FACS) in
a FACSCantoII (BD, Franklin Lakes, NJ, USA), which analyzed 10,000 events (determined by forward and side scatter). The caspase -3/7 assay was performed using an Apo-ONE™ Homogeneous Caspase-3/7 Assay Kit (Promega) according to the manufacturer’s instructions. Fluorescence intensity (499 nm excitation and 521 nm emission) was measured using a SpectraMax M5 spectrophotometer (Molecular Devices).

**Cell cycle analysis**

HepG2 cells were seeded in a 6-well culture plate (1 × 10⁵ cells/well) and incubated for 24 h. To synchronize the cell cultures, the cells were rinsed in PBS and replaced with serum-free DMEM. After serum starvation for 48 h, the cells were released into the cell cycle by the addition of FBS. For FACS analysis, the cells were detached using trypsin at 24 h after serum treatment and fixed in ice-cold 70% ethanol overnight. After fixation, the cells were treated with RNase A (100 μg/mL) and stained with PI (100 μg/mL). The percentages of cells in the sub-G1, G1, S, and G2-M phases were measured using FlowJo software (Tree Star Inc.; Ashland, OR USA).

**TP53 knockout using the clustered regularly interspaced shot palindromic repeats (CRISPR)-Cas9 system**

CRISPR-Cas9 system was used to disrupt the expression of the TP53 gene, as described elsewhere (Ran et al., 2013). pSpCas9(BB)-2A-Puro (PX459) was a gift from Feng Zhang (Addgene plasmid # 48139) (Ran et al., 2013). In brief, a single guide RNA (sgRNA) sequence was selected using Optimized CRISPR Design (http://crispr.mit.edu/). The sgRNA sequence for TP53 was 5’- ATCCATTGCTTGGGACGGCA (hereafter called TP53 sgRNA). The plasmid expressing hCas9 and the TP53 sgRNA were prepared by ligating oligonucleotides into the BbsI site of PX459 (TP53/PX459). To examine the efficacy of the sgRNA, we used pCAG-EGxxFP, which was a gift from Masahito Ikawa (Addgene plasmid # 50716) (Mashiko et al., 2013). An
approximately 500 bp long genomic fragment containing the sgRNA target sequence was amplified using PCR and cloned between the EGFP fragments of pCAG-EGxxFP (TP53/pCAG-EGxxFP). We then co-transfected 293T cells with TP53/PX459 and TP53/pCAG-EGxxFP. After 4 days of transfection, the efficacy of HR of the EGFP gene was evaluated using fluorescence microscopy analysis (BZ-9000, KEYENCE, Osaka, Japan). To establish a TP53-/- clone, HepG2 cells (1 × 10^6 cells/dish) were seeded in a 10 cm dish. The cells were then transfected with 10 μg of TP53/PX459 using Lipofectamine 3000 (Life Technologies). Antibiotic selection (puromycin; 2 μg/mL) was begun 72 h after infection and continued for at least 3 days. A single clone was selected, expanded, and then used for biological assays. For sequence analysis of the TP53 gene, the following primer set was used: 5’- CAGCCATTCTTTTCCTGCTC and 5’- TGCCCTGGTAGGTTTTCTGG.

**Statistical analysis**

At least 3 independent experiments and 3 replications per experiment were performed. The results are expressed as the mean ± SE. Statistical significance between groups was determined using one-way ANOVA and Dunnett’s comparison. Statistical analyses were performed using SPSS 23.0 (SPSS Inc; Chicago, IL, USA).
Results

Establishment of p53<sup>+/Δ40</sup> HepG2 Cell Clones Expressing Δ40p53

We first performed gene targeting of wild-type (WT) p53 in the human HepG2 hepatoma cell line and generated isogenic cell clones harboring exon 2 deletions of p53 to induce endogenous Δ40p53 expression using AAV-based methodology (Fig. S1A), as previously observed in colon cancer HCT116 p53<sup>−/−</sup> cell clones previously (Thomas et al., 2013). We established two independent heterozygous exon 2-deleted p53 HepG2 cell clones (p53<sup>+/Δ40</sup> #1 and #2). Gene targeting was successfully confirmed by PCR amplification of the targeted genomic locus (Fig. S1B). In addition, we isolated cell clones that underwent random integration (RI) of the targeting vectors within their genomes (RI #1 and #2); these clones were used as controls for the p53<sup>+/Δ40</sup> clones. Figure 1A shows a schematic of the FL-p53 and Δ40p53 protein domains, illustrating the lack of an N-terminal TAD-I domain (corresponding to FL-p53 residues 1-39) in Δ40p53. We next examined the protein expression of the p53 isoforms by western blot analysis and determined that an anti-p53 pAb that recognizes both isoforms clearly detected Δ40p53 protein in the p53<sup>+/Δ40</sup> clones but not in the RI clones, whereas an anti-p53 monoclonal antibody (DO-1) that recognizes residues 11-25, which are present only in FL-p53, did not detect Δ40p53 protein in the p53<sup>+/Δ40</sup> clones (Fig. 1B). We confirmed that the molecular weight of Δ40p53 in the p53<sup>+/Δ40</sup> clones was almost identical to that of Δ40p53 exogenously expressed via retrovirus in HepG2 cells (Fig. S1C). These results indicate that the shorter p53 isoform in the p53<sup>+/Δ40</sup> clones is most likely the Δ40p53 protein. We next attempted to create p53<sup>Δ40/Δ40</sup> cell clones by targeting the remaining WT allele in p53<sup>+/Δ40</sup> clones. However, despite several attempts, we failed to obtain p53<sup>Δ40/Δ40</sup> cells after gene targeting in p53<sup>+/Δ40</sup> cells; all the candidate clones were genotyped as p53<sup>+/Δ40</sup> by genomic PCR amplification (Supplementary Table S3). Because the lack of the TAD-I domain enable Δ40p53 to avoid MDM2-induced protein degradation (Hafsi et al., 2013), it is a
reasonable that an increase in Δ40p53 isoform expression potentially robustly induces cell death and/or growth arrest in null clones.

**Impact of Δ40p53 on Tumor Cell Growth and Senescence**

To address the biological function of Δ40p53 in the HepG2 cell clones, we performed clonogenicity and MTT assays. As shown in Figures 1C and 1D, both colony formation and cell survival were significantly reduced in the $p53^{+/Δ40}$ clones compared to the RI clones, suggesting that tumor cell growth was suppressed in the $p53^{+/Δ40}$ clones. Transgenic mice overexpressing Δ40p53 were reported to exhibit a striking growth defect with a reduced lifespan and accelerated aging (Maier et al., 2004; Qian and Chen, 2013). These reports prompted us to utilize a β-galactosidase assay to investigate whether cellular senescence is accelerated in $p53^{+/Δ40}$ clones. As shown in Fig. 1E, the percentage of SA-β-gal-positive cells was significantly higher in the $p53^{+/Δ40}$ clones compared to the RI clones. The increase in SA-β-gal-positive cells was continuously observed in the $p53^{+/Δ40}$ clones. In addition, western blot analysis revealed that protein expression of $p21^{WAF1/CIP1}$, but not those of BAX and PUMA, significantly increased in $p53^{+/Δ40}$ clones (Fig. 1F). We also found that caspase-3/7 activity and the percentage of apoptotic cells did not significantly increase in the $p53^{+/Δ40}$ clones compared to the RI clones (Figs. S2A and S2B), suggesting that the growth suppression was not a result of increased apoptosis. Thus, we next examined that mRNA expression of *IL-8*, one of the prominent components of the senescence-associated secretory phenotype (SASP), using qRT-PCR analysis. As shown in Fig.1G, mRNA expression of *IL-8* significantly increased in $p53^{+/Δ40}$ clones. Furthermore, the mRNA levels of p53-induced genes, including $p21^{WAF1/CIP1}$, *MDM2* and *FAS* were significantly increased in $p53^{+/Δ40}$ clones compared to the RI clones (Figs. S2C and S2D). These results indicate that cellular senescence is accelerated in the $p53^{+/Δ40}$ clones.
Promising Tumor Suppressor Activity of Δ40p53 in TP53 Knockdown Conditions

To further investigate the tumor suppressor activity of Δ40p53 in HepG2 clones, we examined clonogenic activity, cellular growth, and SA-β-gal activity in p53+/Δ40 cells after knocking down the TP53 gene using retrovirus-mediated RNAi. We established TP53 knockdown clones (p53sh #1, #2, and #3) and control (GFP) clones (GFPsh #1, #2, and #3) from the parental p53+/Δ40 #1 cell clone. We confirmed that the expression of p53 isoforms at both the mRNA and protein levels was markedly reduced in all the p53sh clones compared to the GFPsh clones (Fig. S3A and Fig. 2A). Next, we investigated the effect of p53 knockdown on clonogenic activity using a colony formation assay. Colony formation was significantly increased in the p53sh clones compared to the GFPsh clones (Fig. 2B). Similarly, cell survival was significantly increased in the p53sh clones compared to GFPsh clones (Fig. 2C). In addition, p53 knockdown significantly decreased the percentage of SA-β-gal-positive cells (Fig. 2D) and p21WAF1/CIP1 mRNA and protein expression, but not BAX and PUMA protein expression (Fig. S3B and Fig. 2E). qRT-PCR analysis revealed that the mRNA levels of MDM2, GADD45A, BAX, and FAS, but not of BAX, PUMA and CCNB1, were significantly decreased in the p53sh clones compared with the GFPsh clones (Fig. S3C). Based on these results and data demonstrating that the induction of p21WAF1/CIP1 gene expression results in G1-phase arrest, we examined the cell cycle distribution in p53sh clones using PI staining-based cell cycle analysis (Ozturk et al., 2009; Larsson, 2011). The population of cells in G1 decreased in response to p53 knockdown (Fig. S3D). Furthermore, we rescued Δ40p53 expression in the p53sh #1 and #3 clones using a retroviral expression vector. Rescuing Δ40p53 expression reversed the increase in colony formation and the decrease in the percentage of SA-β-gal positive cells (Fig. 2G). We observed that rescuing Δ40p53 expression did not affect the result of colony formation in the GFPsh clones (data not shown). These results strongly suggest that Δ40p53 suppresses tumor cell growth and promotes cellular senescence.
**Exogenous Expression of Δ40p53 Suppresses Cell Growth and Enhances Senescence and G\(_1\) Arrest**

Given our experimental results in \(p53^{+/Δ40}\) cells, we next examined Δ40p53 protein expression in HCC cells by western blot analysis. We found that an anti-p53 poAb, but not a DOI-1 moAb, detected Δ40p53 protein expression in HepG2, HuH-7, and PLC/PRF/5 cells; these protein levels were further up-regulated after treatment with doxorubicin (Fig. 3A, left panel). Therefore, we examined the endogenous Δ40p53 protein expression in HCC cell lines using western blot analysis. We found that Δ40p53 protein expression was detected in all the HCC cell lines except for a TP53-deleted cell line, Hep3B (Fig. 3A, right panel). Thus, we examined whether the exogenous expression of Δ40p53 affects tumor cell growth and/or cellular senescence. In the colony formation assay, HepG2 cells expressing Δ40p53 formed fewer colonies than those expressing the control (pBabe) or FL-p53 (Fig. 3B). Similarly, clonogenicity was significantly suppressed in both PLC/PRF/5 and HuH-7 cells (Fig. 3B). We established two independent cell clones that exogenously expressed Δ40p53 protein (Δ40p53 #1 and #2) as well as vector control clones (pBabe #1 and #2) from parental HepG2 cells. An MTT assay revealed that cell survival was significantly suppressed in the Δ40p53 clones compared to the pBabe clones (Fig. 3C). In addition, the percentage of SA-β-gal-positive cells was significantly higher for the Δ40p53 clones compared to the pBabe clones (Fig. 3D). These results provide further experimental evidence that Δ40p53 plays a pivotal role in tumor cell growth and senescence. We next examined the effect of Δ40p53 on cell cycle progression. Cell cycle analyses revealed that the population of cells in G1 was larger for the Δ40p53 clones (Fig. 3E), and these clones were desensitized to serum-induced cell cycle progression (data not shown). These results suggest that Δ40p53 may block cell cycle progression at G1. We then examined the effect of Δ40p53 on \(p53\) transcriptional activity and \(p53\)-induced gene expression, and found that the mRNA and protein expression of p21\(^{WAF1/CIP1}\) as
well as $p53$ transcriptional activity was significantly increased in the Δ40p53 cell clones (Fig. 3F and Figs. S4A-B). Similarly, mRNA expression level of IL-8 significantly increased in Δ40p53 cell clone (Fig. 3G). Unexpectedly, FL-p53 protein expression was found to be higher in the Δ40p53 clones compared to the pBabe clones (Fig. 3F, see below). qRT-PCR analysis showed that the mRNA expression of MDM2 and FAS, but not of BAX, PUMA, GADD45A and CCNB1, was significantly increased in the Δ40p53 clones compared to the pBabe clones (Fig. S4C), indicating that Δ40p53 exerts tumor suppressor activity by mediating $p53$-inducible gene expression.

**Tumor Suppressor Activity of Δ40p53 and Its Mutants**

It has been reported that TP53 gene mutations impair $p53$-mediated apoptosis and/or $p53$ transcriptional activity (Serrano et al., 1997; Cui et al., 2002). Thus, we decided to ascertain the effect of Δ40p53 harboring a TP53 hot-spot missense mutation, R175H, on clonogenic activity and senescence in HepG2 cells. The clonogenicity assay revealed that cells expressing WT-Δ40p53 formed fewer colonies; this reduction was significantly attenuated in cells expressing Δ40p53/R175H (Fig. 4A). In addition, the percentage of SA-β-gal-positive cells was significantly higher among those expressing WT-Δ40p53; this increase was attenuated in cells expressing Δ40p53/R175H (Fig. 4B). We confirmed that both $p53$ transcriptional activity and p21\textsuperscript{WAF1/CIP1} protein expression were markedly increased in cells expressing WT-Δ40p53 compared to control cells, but was not increased in those expressing Δ40p53/R175H (Figs 4C and 4D). Furthermore, qRT-PCR analysis demonstrated that Δ40p53/R175H failed to increase the mRNA levels of MDM2 and FAS (Fig. 4E). These results suggest that Δ40p53-induced transcription is closely associated with the roles of Δ40p53 in tumor suppression and/or cellular senescence.
Δ40p53 Itself Exerts Tumor Suppressor Activity in TP53-Deleted Cells

Because our experimental cell model was generated in the presence of FL-p53, it was difficult to exclude the possibility that Δ40p53 exerts tumor suppressor activity dependent upon the expression of FL-p53. To investigate whether Δ40p53 independently exerts tumor suppressor activity, we established a p53−/− HepG2 cell clone using the CRISPR/Cas9 system (Fig. 5A). Western blot analysis revealed that FL-p53 was undetectable in the p53−/− HepG2 cell clone, whereas it was detected in parental HepG2 cells after 24 h of incubation in the presence or absence of doxorubicin (Fig. 5B). We also confirmed that p53 transcriptional activity was minimal in the p53−/− cell clone (Fig. 5C). Thus, we investigated the effect of exogenous Δ40p53 expression on clonogenic activity in the absence of FL-p53. Cells expressing Δ40p53 formed fewer colonies than those expressing control (pBabe) or Δ40p53/R175H (Fig. 5D). Similar results were observed in p53-deleted Hep3B cells (Fig. 5D). Furthermore, the percentage of SA-β-gal-positive cells was significantly higher among those expressing WT-Δ40p53; this increase was partly attenuated in cells expressing Δ40p53/R175H (Fig. 5E). We then examined the effect of Δ40p53 on expression of p53 target genes under TP53 knockdown. We found that p21WAF1/CIP1 mRNA and protein expression of significantly increased in the Δ40p53/pBabe/HepG2/p53−/− cells compared with the pBabe/HepG2/p53−/− cells; this increase was attenuated after knockdown of Δ40p53 (Figs. 5F and 5G). Similarly, mRNA expression level of IL-8, MDM2, and FAS significantly increased in Δ40p53 cell clones; these increases were reversed after knockdown of Δ40p53 (Fig. 5F). In addition, we examined the effects of mutant Δ40p53/R175H on p21WAF1/CIP1, CCNB1, and IL-8 gene expression. We then compared the effects with those obtained in the cells expressing control (pBabe) and WT-Δ40p53. qRT-PCR analysis revealed that mRNA expression of CCNB1 and IL-8 but not p21WAF1/CIP1 significantly increased in the cells expressing Δ40p53/R175H (Fig. S4D). These results strongly suggest that Δ40p53 itself exerts tumor suppressor activity.
Δ40p53 Increases the Protein Levels of FL-p53

Finally, we examined the effect of Δ40p53 on FL-p53 protein expression using western blot analysis. FL-p53 protein levels were significantly increased in the Δ40p53#1 cells compared to the pBabe#1 cells (Figs 6A and 6B). Moreover, the protein levels of FL-p53 were reduced in a time-dependent manner after cycloheximide (CHX) treatment in both pBabe#1 and Δ40p53#1 cells; this reduction was clearly delayed in the Δ40p53#1 cells (Fig. 6B), as indicated by the longer half-life of FL-p53 protein in Δ40p53#1 cells (t_{1/2} > 4 h) compared to pBabe#1 cells (t_{1/2} = 1.26 h). In addition, the reduction in FL-p53 protein levels after CHX treatment was partly attenuated by treatment with the proteasome inhibitor MG132, suggesting that Δ40p53 may preserve the protein levels of FL-p53 by affecting its proteasomal degradation. To further investigate the effect of Δ40p53 on FL-p53-induced anti-tumor activity, we performed clonogenic assay in the HepG2/p53−/− cells expressing pBabe, Δ40p53, FL-p53, and FL-p53+Δ40p53 (co-expression). The clonogenic assay revealed that cells expressing FL-p53 formed fewer colonies; this reduction was significantly augmented in cells co-expressing of FL-p53 and Δ40p53 (Fig. S4F).

Collectively, these results strongly suggest that Δ40p53 exerts growth suppressive activity and promotes senescence in HCC cells (Fig. 6C).
Discussion

In the present study, we demonstrate for the first time that Δ40p53 exerts tumor suppressor activity in HCC cells. To investigate the biological function of Δ40p53, we established TP53 gene-modified cell models, including p53+/Δ40 HepG2 cells expressing endogenous Δ40p53, parental HepG2 cells expressing exogenous Δ40p53, and CRISPR/Cas9-mediated p53-/- HepG2 cells harboring a disrupted TP53 gene. Takahashi et al reported that Δ40p53 elicits apoptosis in human melanoma cells (Takahashi et al., 2014). Our data showed that caspase-3/7 activity and the AxV+/PI+ population were not increased in the p53+/Δ40 cell clones, indicating that the tumor suppressor activity of Δ40p53 may not be mediated by apoptosis.

HepG2 cells express WT p53, whereas HuH-7 and PLC/PRF/5 cells harbor mutated p53 (HuH-7, TP53Y220C; PLC/PRF/5, TP53R249S) (Hsu et al., 1993). Retroviral-induced Δ40p53 expression suppressed colony formation in all 3 HCC cell lines, in p53+/ Hep3B cells, and in genetically engineered p53-/- HepG2 cells. These results strongly suggest that Δ40p53 exerts tumor suppressor activity regardless of the FL-p53 status. Furthermore, both endogenous and exogenous Δ40p53 expression significantly suppressed clonogenicity, induced cellular senescence, and up-regulated p53-target gene expression in the presence of FL-p53 with or without the TP53 mutation, strongly suggesting that Δ40p53, which lacks the MDM2-interactive TAD-I domain, may exhibit higher tumor suppressor activity than FL-p53. We detected endogenous expression of Δ40p53 and its up-regulation after doxorubicin treatment in both HepG2 and PLC/PRF/5 cells, indicating that Δ40p53 potentially has important anti-tumor effects in HCC cells. Given that Δ40p53 exerted promising anti-tumor activity in HCC cells, it would be interesting to examine whether changes in Δ40p53 expression are related to liver carcinogenesis in vitro and/or in vivo.
Senescence is a recognized barrier to cellular proliferation (Maier et al., 2004). In this study, there were significantly more SA-β-gal positive cells among the HepG2 \( p53^{+/Δ40} \) and \( Δ40p53/pBabe \) cell clones compared to the control cell clones. In addition, the increased number of SA-β-gal-positive cells was clearly suppressed by \( p53 \) knockdown, suggesting that \( Δ40p53 \) may play a pivotal role in cellular senescence. \( p21^{WAF1/CIP1} \), a well-known \( p53 \)-target gene, is up-regulated during replicative senescence and functions as an inhibitor of proliferation (Lanigan et al., 2011; Brugarolas et al., 1995). We found that the expression levels of both \( p21^{WAF1/CIP1} \) and \( p53 \)-inducible genes including \( MDM2 \) and \( FAS \) were significantly up-regulated in HepG2 \( p53^{+/Δ40} \) and \( Δ40p53/pBabe \) cell clones compared to the control cell clones. These changes in gene expression were abrogated by \( p53 \) knockdown. Moreover, the tumor suppressor activity of \( Δ40p53 \) was partly, but significantly, attenuated in cells expressing mutant \( Δ40p53/R175H \), although the mRNA levels of \( p21^{WAF1/CIP1} \) and \( p53 \)-inducible genes including \( MDM2 \) and \( FAS \) did not change, providing further experimental evidence that the transcriptional activity of \( Δ40p53 \) is closely associated with its anti-tumor effects. We also observed that cells expressing mutant \( Δ40p53/R175H \) protein formed fewer colonies and exhibited increased SA-β-gal positive staining without up-regulation of \( p21^{WAF1/CIP1} \); however, these levels were reduced compared with cells expressing wild type \( Δ40p53 \). Moreover, we found that \( Δ40p53/R175H \) significantly increased mRNA expression of \( CCNB1 \) and/or \( IL-8 \), components of SASP. These results suggest that up-regulation of \( p21^{WAF1/CIP1} \) is not always responsible for cellular senescence induced by \( Δ40p53 \). In addition, \( Δ40p53/R175H \) may exhibit anti-tumor activity by modulating \( p53 \) target genes, which are similar to but different from those modulated by wild-type \( Δ40p53 \). Ohki et al reported that \( Δ40p53 \) induces some, but not all, \( p53 \)-inducible genes in \( p53^{-/} \) Saos2 cells (Ohki et al., 2007). Thus, further studies, including comprehensive microarray and/or RNA sequencing analyses, may contribute to a better understanding of the molecular basis underlying the role of \( Δ40p53 \) and its
mutants in tumor suppression and senescence in HCC cells.

Thus far, Δ40p53 has been shown to regulate p53-inducible gene expression in both a positive and negative manner (Yin et al., 2002). Although we did not clarify the interaction of Δ40p53 with FL-p53 or MDM2, our observations that Δ40p53 increased the protein half-life of FL-p53 and augmented the FL-p53-induced anti-tumor activity suggest that Δ40p53 may positively regulate FL-p53 activity in our proposed HCC cell model.

In conclusion, to the best of our knowledge, this study is the first to demonstrate that Δ40p53 exerts tumor suppressor activity and promotes cellular senescence, at least in part, by up-regulating p53-target gene expression in HCC cells. Our findings enhance the understanding of the molecular pathogenesis of HCC. Further studies, including in vivo experiments, are warranted to investigate the role of Δ40p53 in the pathogenesis of HCC, and such studies should consider the effects of Δ40p53 to advance current therapeutics for patients with HCC.
Competing interests

No competing interests declared.

Author contributions

Conception and design: A.O., H.K., and H.N.
Development of methodology: A.O., H.K., and Y.H.
Acquisition of data (provided cells, provided facilities, etc.): A.O., Y.S., S.K., and M.W.
Writing, review, and/or revision of the manuscript: A.O., H.K., and H.N.
Administrative, technical, or material support (i.e., reporting or organizing data, preparing vectors): S.K., M.W., T.I., Y.K., N.I., Y.S., Y.N., T.Y., T.O., K.S., K.I., and H.N.
Study supervision: H.K., Y.H., and H.N.

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References


Katz, S.F., Lechel A, Obenauf, A.C., Begus-Nahrmann, Y., Kraus, J.M., Hoffmann, E.M.,
Duda, J., Eshraghi, P., Hartmann, D., Liss, B., et al. (2012). Disruption of Trp53 in livers of
mice induces formation of carcinomas with bilineal differentiation. *Gastroenterology* **142**, 1229–
1239.

Konishi, H., Lauring, J., Garay, J.P., Karakas, B., Abukhdeir, A.M., Gustin, J.P., Konishi, 


Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J.,
of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of 


Maier, B., Gluba, W., Bernier, B., Turner, T., Mohammad, K., Guise, T., Sutherland, A.,
Thorner, M., Scrable H. (2004). Modulation of mammalian life span by the short isoform of

Marcel, V., Dichtel-Danjoy, M.L., Sagne, C., Hafsi, H., Ma, D., Ortiz-Cuaran, S., Olivier, M.,
evolution: lessons from animal and cellular models. *Cell Death 
Differ.* **18**, 1815–1824.


Figures

A

B

C

Figures
Fig. 1. The cellular phenotype of p53<sup>+</sup>/Δ40 HepG2 cells. (A) A schematic of the domain structures of the human p53 protein and the Δ40p53 isoform. A monoclonal antibody, DO-1, recognizes the first TAD domain in p53 that is not present in Δ40p53. TAD, transactivation domain; PrD, proline-rich domain; DBD, DNA binding domain; NLS, nuclear localization signal; OD, oligomerization domain; Reg, regulatory domain. (B) p53 protein levels were examined by western blot analysis. We analyzed 5 μg of cell lysate by western blotting to detect p53 isoforms with an anti-p53 polyclonal antibody or an anti-p53 monoclonal antibody, DO-1. The HepG2 parental cells (P), RI clones (#1 and #2), and p53<sup>+/Δ40</sup> clones (#1 and #2) are shown. β-actin was used as an internal control. (C) A representative colony formation assay with HepG2/RI and HepG2/p53<sup>+/Δ40</sup> clones. Two hundred cells were seeded in a 6-well plate. After 14 days, the cells were stained with crystal violet and imaged. Bar graphs represent the number of stained colonies. Data are presented as the mean ± SE (n = 6). (D) MTT analysis of the growth rate of HepG2/RI and HepG2/p53<sup>+/Δ40</sup> clones. The optical density (595 nm) at each time point (day 0, 1, 3, and 5) is presented as the mean ± SE (n = 4). Asterisks (** or *) indicate significant differences at \( P < 0.005 \) and \( P < 0.05 \), respectively, compared to RI clone #1. (E) Analysis of cellular senescence using the SA-β-gal assay. Twenty thousand cells were seeded in 12-well plates, and then incubated for 48 h. After incubation, the cells were stained with SA-β-gal to determine the β-gal activity. Representative result of SA-β-gal staining is shown above. Magnification: ×100. Bar graphs represent the percentage of SA-β-gal-positive cells. Data are presented as the mean ± SE (n = 3). (F) Protein expression of p21<sup>WAF1/CIP1</sup>, BAX, and PUMA. We analyzed 5 μg of the protein by western blotting with an antibody specific to p21<sup>WAF1/CIP1</sup>, BAX, and PUMA. β-actin was used as an internal control. After normalization to β-actin protein levels, the protein levels of p21<sup>WAF1/CIP1</sup> are expressed relative to the protein expression in the HepG2/RI #1 cells, which was arbitrarily defined as 1. The data are represented as the mean ± SE of 3 separate experiments. (G)
qRT-PCR analysis of the gene expression levels of IL-8 in RI clone and HepG2/p53^{+/-Δ40} clones.

The primers used for qRT-PCR are shown in Supplementary Table S3. Relative gene expression levels are shown after normalization to GAPDH mRNA expression. The data are expressed relative to the mRNA levels found in the corresponding sample of HepG2/RI clone #1 cells, which was arbitrarily defined as 1. The values shown represent the mean ± SE (n = 3).
Fig. 2. Effect of TP53 gene knockdown on cell growth and senescence in HepG2/p53Δ40 cells.
(A) Confirmation of TP53 gene knockdown in HepG2/p53+/Δ40 cells. Protein levels of p53 isoforms were examined by western blotting with an anti-p53 polyclonal antibody. The HepG2/p53+/Δ40/GFPsh clones (#1 to #3) and HepG2/p53+/Δ40/p53sh clones (#1 to #3) are shown. β-actin was used as an internal control. (B) Effect of TP53 gene knockdown on clonogenicity. Colony formation assays were performed as described in the legend of Fig. 1C. Bar graphs represent the number of stained colonies (n = 6). (C) Effect of TP53 gene knockdown on cell survival. An MTT assay was performed as described in the legend of Fig. 1D. The Optical density (595 nm) at each time point (day 0, 1, 3, and 5) is expressed as the mean ± SE (n = 4). (D) Effect of TP53 gene knockdown on cellular senescence. SA-β-gal assay were performed as described in the legend of Fig. 1E. Bar graphs represent the percentage of SA-β-gal-positive cells (n = 3). (E) Protein expression of p21WAF1/CIP1, BAX, and PUMA were examined by western blotting as described in the legend of Fig. 1F. After normalization to β-actin protein levels, the data are expressed relative to the protein expression in the HepG2/p53+/Δ40/GFPsh #1 cells, which was arbitrarily defined as 1. (F) qRT-PCR analysis for the gene expression levels of IL-8 in HepG2/GFPsh and HepG2/p53sh clones. Relative gene expression levels are shown after normalization to GAPDH mRNA expression. The data are expressed relative to the mRNA level found in the corresponding sample of HepG2/GFPsh clone #1, which was arbitrarily defined as 1. (G) Effect of Δ40p53 on clonogenicity and senescence in the HepG2/p53+/Δ40/p53sh clones. pLXSN, Δ40p53/PLXSN and FL-p53/PLXSN retroviruses were generated using 293T cells. After the viral supernatants were prepared, the HepG2/p53+/Δ40/p53sh clones (#1 and #3) were infected at the same MOI with pLXSN, Δ40p53/PLXSN, and FL-p53. After infection for 48 h, the cells were treated with neomycin (800 μg/mL) for 14 days, followed by staining with either crystal violet or SA-β-gal. The upper and lower bar graphs represent the number of colonies and the percentage of SA-β-gal-positive cells, respectively (n = 3).
Fig. 3. Effect of exogenous Δ40p53 expression on cell growth and senescence. (A) Protein expression levels of Δ40p53 in HCC cells. Left panel, inducible protein expression of Δ40p53. HepG2, PLC/PRF/5, and HuH-7 cells were incubated in medium containing the indicated concentration (0, 0.5, 1, 2 μM) of doxorubicin (Dox) for 12 h; right panel, endogenous protein expression of Δ40p53 in HCC cell lines including HuH-1 (TP53WT), HepG2 (TP53WT), PLC/PRF/5 (TP53R249S), HuH-7 (TP53Y220C), and Hep3B (TP53−/−) cells. The cells were lysed with lysis buffer. Five microgram or 1 μg of protein lysate was then subjected to western blot analysis to detect p53 or β-actin protein, respectively. An anti-p53 polyclonal antibody and an anti-p53 monoclonal antibody (DO-1) were used to detect both FL-p53 and Δ40p53. C, control (HepG2 p53+/Δ40 cells). (B) Effect of Δ40p53 on clonogenicity. pBabe, Δ40p53/pBabe, and FL-p53/pBabe retroviruses were generated using 293T cells. After the viral supernatants were prepared, HepG2 cells, PLC/PRF/5, and HuH-7 cells were infected at the same MOI with pBabe, Δ40p53/pBabe, or FL-p53/pBabe. After infection for 48 h, the cells were treated with puromycin (2 μg/mL) for 14 days followed by staining with crystal violet and imaging. Bar graphs represent the number of stained colonies (n = 6). Asterisks (** or *) indicate statistically significant differences (at p < 0.005 or p < 0.05, respectively). (C) The growth rate of the pBabe and Δ40p53/pBabe HepG2 cell clones were determined using an MTT assay. Each single clone was picked from a dish after 14 days of puromycin treatment, expanded, and then utilized for the following assay. The optical density (595 nm) at each time point (day 0, 2, 4, and 6) is presented as the mean ± SE (n = 4). (D) The cellular senescence of the pBabe and Δ40p53/pBabe clones was examined as described in the legend of Fig. 1E. Bar graphs represent the percentage of SA-β-gal-positive cells (n = 3). (E) Cell cycle analysis of pBabe and Δ40p53/pBabe clones. Each cell clone (1 × 10⁵ cells/well) was incubated in serum-free medium for 48 h to synchronize the cell cycle. After 48 h of serum
starvation, the cells were further cultured in medium containing 10% FBS for 24 h. The cells were detached, fixed, and stained with propidium iodide (PI, 100 μg/mL) after RNase (1 mg/mL) treatment. The cell cycle populations were measured using FACSCanto II (BD Biosciences, San Jose, CA, USA) and FlowJo software (Tree Star, Inc., Ashland, OR, USA). A representative FACS histogram and bar graphs of the cell cycle ratios are shown. (F) Protein levels of p21WAF1/CIP1, BAX, PUMA, Δ40p53, and FL-p53 in the pBabe and Δ40p53/pBabe clones were determined by western blot analysis as described in the legend of Fig. 1F. (G) qRT-PCR analysis of relative IL-8 gene expression level in pBabe and Δ40p53 clones. The data are expressed relative to the mRNA levels found in the corresponding sample of pBabe clones, which was arbitrarily defined as 1.
Fig. 4. Involvement of transcriptional activity in the tumor suppressor activity of Δ40p53.

(A) Colony formation assay. pBabe, Δ40p53 (WT)/pBabe, and Δ40p53 (R175H)/pBabe retroviruses were generated using 293T cells. After the viral supernatants were prepared, HepG2 cells were infected as described in the legend of Fig. 3B. Bar graphs represent the number of stained colonies (n = 6). (B) SA-β-gal assay. pBabe/HepG2, Δ40p53 (WT) HepG2, and Δ40p53 (R175H)/HepG2 cells (2 × 10^4 cells/well) were seeded in 12-well plates. The cells were incubated for 48 h and then stained. Bar graphs represent the percentage of SA-β-gal-positive cells (n = 3). (C) p53-dependent transactivation was examined using a p53 luciferase reporter assay. Each clone was co-transfected with 0.15 μg of the TG13-Luc vector (containing the WT p53 DNA binding site; p53 firefly activity) and 0.03 μg of the phRL-TK vector (internal control; Renilla luciferase activity). Luciferase activity was measured 48 h after the transfection. After normalization to Renilla luciferase activity, the data are expressed relative to the p53-dependent luciferase activity in the HepG2/RI #1 cells, which was arbitrarily defined as 1 (n = 4). (D) Protein levels of p21^{WAF1/CIP1} and p53 isoforms were examined by western blot analysis as described in Fig. 1F. β-actin was used as an internal control. (E) qRT-PCR analysis of MDM2 and FAS gene expression. The relative gene expression levels are shown after normalization to GAPDH mRNA expression. Data are presented relative to the mRNA expression in pBabe/HepG2 cells, which was arbitrarily defined as 1 (n = 3).
Figure 5. Effect of Δ40p53 on p53−/− cell growth and expression of p53 target genes. (A) An sgRNA (arrow) sequence was designed against the TP53 loci to excise an in-frame second ATG (bold) that initiates the transcription of Δ40p53. The sgRNA sequence and the PAM sequence are indicated by an underline or overbar, respectively. The sequences of the parental and p53−/− (null) cell clones (bi-allelic modification) were analyzed, and the results are shown below. A junction in the p53−/− cell clone is indicated by the arrowhead. (B) p53 protein expression was determined by western blot analysis. Parental HepG2 and HepG2/p53−/− cells were incubated for 24 h in the presence or absence of doxorubicin (500 nM) and then lysed with lysis buffer. Five micrograms of lysate was subjected to western blot analysis to detect p53 protein. β-actin was used as an internal control. (C) p53-dependent transactivation was examined using a p53 luciferase reporter assay as described in Fig. 4C. Luciferase activity was measured 48 h after the transfection. After normalization to Renilla luciferase activity, the data are expressed relative to the p53-dependent luciferase activity in the parental HepG2 cells, which was arbitrarily defined as 1 (n = 6). (D) Colony formation assay. pBabe, Δ40p53 (WT)/pBabe, and Δ40p53 (R175H)/pBabe retroviruses were prepared using 293T cells. After the viral supernatants were prepared, HepG2/p53−/− cells and Hep3B (p53 null) cells were infected as described in the legend of Fig. 4B. Bar graphs represent the number of stained colonies (n = 3). (E) The cellular senescence of the pBabe, Δ40p53/pBabe, and Δ40p53/R175H/pBabe was examined with HepG2/p53−/− cells as described in the legend of Fig. 1E. Bar graphs represent the percentage of SA-β-gal-positive cells (n = 3). (F and G) pBabe/HepG2/p53−/− cells and Δ40p53 (WT)/pBabe/HepG2/p53−/− cells were transfected with 50 nM of siRNA specific to TP53 or nonspecific control siRNA. (F) After 72 h, total RNA was extracted from the cells and mRNA expression levels of p21WAF1/CIP1, IL-8, BAX, PUMA, MDM2, FAS, GADD45A, and CCNB1 were examined using SYBR Green I. The primers used for qRT-PCR are presented in Supplementary Table S3. Relative gene expression levels are presented...
after normalization to GAPDH mRNA expression. The data are expressed relative to the mRNA levels found in the corresponding sample of pBabe/HepG2/p53-/- cells, which was arbitrarily defined as 1. The values shown represent the mean ± SE (n = 3). (G) After 72 h, protein expression of p21\textsuperscript{WAF1/CIP1}, BAX, PUMA and Δ40p53 were examined by western blot as described in Fig. 1F. After normalization to β-actin protein levels, p21\textsuperscript{WAF1/CIP1} protein levels are expressed relative to the protein expression in pBabe/HepG2/p53-/- cells, which was arbitrarily defined as 1. β-actin was used as an internal control. Asterisks (** and *) indicate significant differences at $P < 0.005$ and $P < 0.05$, respectively.
**Figure A**

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<td>MG132 (10 μM)</td>
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**Figure B**

Relative FL-p53 protein levels over time with and without MG132 (10 μM).

**Figure C**

Diagram showing the interaction between MDM2, FL-p53, Δ40p53, and their effects on proteasomal degradation, cytoplasmic and nuclear localization, growth suppression, and cellular senescence.

**Notes:**
- *t_{1/2} (h)*: pBabe 1.79, Δ40p53 > 4
- MDM2, FL-p53, Δ40p53
- Proteasomal degradation
- Cytoplasm
- Nucleus
- Growth suppression
- Cellular senescence
Figure 6. The effect of Δ40p53 on FL-p53 protein expression. (A) The pBabe #1 and Δ40p53 #1 (1 × 10^5 cells/well) clones were treated with cycloheximide (CHX; 50 μM) in the presence or absence of the proteasome inhibitor MG132 (10 μM). After treatment for the indicated duration (0, 0.5, 1, 2, and 4 h), the cells were lysed, and the protein lysates were subjected to western blot analysis to detect the p53 isoforms. A representative western blot result is shown. (B) After normalization to β-actin protein expression, the data are presented relative to FL-p53 protein expression in the pBabe #1 clone (at 0 h after CHX treatment), which was arbitrarily defined as 1. β-actin was used as an internal control. Asterisks (**) and (*) indicate significant differences at P < 0.005 and P < 0.05, respectively. The protein half-life (t_{1/2}) of FL-p53 after CHX treatment is indicated at the right side of the bar graph. (C) A molecular mechanism by which Δ40p53 exerts tumor suppressor activity in HCC cells is proposed. The interaction of Δ40p53 with FL-p53 potentially disturbs the binding of FL-p53 to MDM2, which negatively regulates p53 transcriptional activity through the ubiquitin-proteasome pathway. Thus, Δ40p53 can promote the expression of p53 target genes including MDM2, p21WAF1/CIP1, and IL-8, which suppresses tumor growth and induces cellular senescence in HCC cells.
**Fig. S1.** Establishment and confirmation of TP53+/Δ40 in HepG2 cells. (A) A schematic of the TP53+/Δ40 gene. The endogenous TP53 gene locus surrounding exon 2, the targeting vector containing the neomycin resistance gene (NeoR) cassette flanked by loxP sites, homologous sequences to the intronic regions located 5’ and 3’ of exon 2 of TP53, and the targeted allele before and after Cre infection are described. Dashed lines indicate areas of homology between the targeting vector and the endogenous sequences surrounding exon 2 of the TP53 gene. A filled box and open triangles represent TP53 exon 2 and the loxP sites, respectively. Primers F1 and R1, indicated in the targeted allele, were used in the first PCR screen for TP53 gene targeting before Cre infection, whereas primers F2 and R2, indicated at the bottom, were used for PCR amplification in B. (B) DNA electrophoresis showing the PCR products resulting from using the genomic DNA (post-Cre infection) of parental HepG2 cells. Random integration (RI) clones (#1, #2), and p53+/Δ40 clones (#1, #2) are indicated. The primers used for PCR are shown in A, and their sequences are shown in Table S2. (C) The protein expression levels of p53 isoforms were examined using western blot analysis. RI clones (#1 and #2), p53+/Δ40 clones (#1 and #2), control (pBabe) cells, and cells expressing Δ40p53 protein (Δ40p53) were lysed with lysis buffer, and 5 μg of cell lysate was subjected to western blot analysis to detect p53 protein using a rabbit anti-p53 polyclonal antibody or a mouse anti-p53 monoclonal antibody (DO-1). β-actin was used as an internal control.
Fig. S2. (A) The ratio of apoptotic HepG2/RI and HepG2/p53<sup>Δ40</sup> clones is shown. Flow cytometry analyses was performed using double staining with Annexin V (AxV)-FITC and propidium iodide (PI). Twenty thousand cells were seeded in 6-well plates and then incubated for 48 h. Bar graphs represent the percentages of cells undergoing apoptosis (AxV<sup>+</sup>/PI<sup>+</sup> cells). The data are expressed as the means ± SE (n = 3). N.S., not significant. (B) Caspase-3/7 activity in HepG2/RI and HepG2/p53<sup>Δ40</sup> clones. Ten thousand cells were seeded in 96-well plates and then incubated for 24 h. The data are expressed as the means ± SE (n = 4). (C-D) qRT-PCR analysis of the gene expression levels of <i>p21</i><sup>WAF1/CIP1</sup> (C), <i>MDM2</i>, <i>GADD45A</i>, <i>PUMA</i>, <i>BAX</i>, <i>FAS</i>, and <i>CCNB1</i> (D) in RI clones and HepG2/p53<sup>Δ40</sup> clones. The primers used for qRT-PCR are shown in Table S3. Relative gene expression levels are shown after normalization to <i>GAPDH</i> mRNA expression. The data are expressed relative to the mRNA levels found in the corresponding sample of HepG2/RI clone #1 cells, which was arbitrarily defined as 1. The values shown represent the mean ± SE (n = 3). N.S., not significant.
Fig. S3. (A-B) qRT-PCR analysis of p53 (A) and p21WAF1/CIP1 (B) gene expression. Relative mRNA expression levels are shown after normalization to GAPDH mRNA expression. The data are expressed relative to the mRNA levels found in the corresponding sample of HepG2/p53+/Δ40/GFPsh#1 cells, which was arbitrarily defined as 1. The values shown represent the mean ± SE (n = 3). (C) qRT-PCR analysis for the gene expression levels of MDM2, GADD45A, PUMA, BAX, FAS, and CCNB1 in HepG2/GFPsh and HepG2/p53sh clones. The primers used for qRT-PCR are shown in Table S3. Relative gene expression levels are shown after normalization to GAPDH mRNA expression. The data are expressed relative to the mRNA level found in the corresponding sample of HepG2/GFPsh clone #1, which was arbitrarily defined as 1. The values shown represent the mean ± SE (n = 3). N.S., not significant. (D) Cell cycle analysis in HepG2/GFPsh and HepG2/p53sh clones. Representative FACS histograms of HepG2/GFPsh and HepG2/p53sh clones are shown. Ten thousand cells of each clone were seeded in 6-well plates. After 48 h of serum starvation, the cells were cultured in a medium containing 10% FBS for 24 h. The cells were then detached, washing, re-suspended in PBS, and fixed in 70% ethanol. The cells were stained with PI (100 μg/mL) after treatment with RNase (100 μg/mL). The cell cycle ratios in HepG2/GFPsh and HepG2/p53sh clones are shown as a bar graph.
Fig. S4. Effect of exogenous Δ40p53 on p53-target gene expression in HepG2 cells. (A) Analysis of p53-dependent transactivation in pBabe and Δ40p53/pBabe clones. Luciferase activity was measured as described in the legend of Fig. 4C. After normalization to Renilla luciferase activity, the fold-change induced by p53 promoter activity was expressed relative to that found in the corresponding sample of pBabe#1 cells, which was arbitrarily defined as 1 (n = 4). (B) qRT-PCR analysis of relative p21WAF1/CIP1 gene expression levels. The relative p21WAF1/CIP1 mRNA expression levels are shown after normalization to GAPDH mRNA expression. The data are expressed relative to the mRNA levels found in the corresponding sample of pBabe#1 cells, which was arbitrarily defined as 1. (C) qRT-PCR analysis of the gene expression levels of MDM2, GADD45A, PUMA, BAX, FAS, and CCNB1 in HepG2/pBabe and HepG2/Δ40p53/pBabe clones. The relative gene expression levels are shown as described in C. The values shown represent the mean ± SE (n = 3). N.S., not significant. (D) qRT-PCR analysis for the gene expression levels of p21WAF1/CIP1, CCNB1, and IL-8 in the cells expressing pBabe (control), Δ40p53, and Δ40p53/R175H in HepG2/p53−/− cells. The primers used for qRT-PCR are presented in Table S3. Relative gene expression levels are presented after normalization to GAPDH mRNA expression. The data are expressed relative to the mRNA level observed in the corresponding sample of pBabe#1 cells, which was arbitrarily defined as 1. The values shown represent the mean ± SE (n = 3).
Table S1. Primers applied for genotyping PCR of TP53-targeting clones

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<td>R2 primer</td>
<td>5' - TAG CAG AGA CCT GTG GGA AG</td>
<td>loxP allele: 180 bp</td>
</tr>
</tbody>
</table>

Table S2. Primers applied for qRT-PCR

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>sequences (5')</th>
<th>size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>Sense 5' - AGA CTG CCT TCC GGG TCA CT</td>
<td>434</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - TCA TCT GGA CCT GGG TCT TC</td>
<td></td>
</tr>
<tr>
<td>p21WAF1/CIP1</td>
<td>Sense 5' - GCA GAC CAG CAT GAC AGA TTT</td>
<td>296</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - AAT GCC CAG CAC TCT TAG GA</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>Sense 5' - GTG CAG TTT TGC CAA GGA GT</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - CTC TGC ACC CAG TTT TCC TT</td>
<td></td>
</tr>
<tr>
<td>MDM2</td>
<td>Sense 5' - GGT GGG AGT GAT CAA AAG GA</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - ACA CAG AGC CAG GCT TTC AT</td>
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<tr>
<td>GADD45A</td>
<td>Sense 5' - ACG AGG AGC AGC ACA GAG AT</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - GCA GGA TCC TTC CAT TGA GA</td>
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</tr>
<tr>
<td>FAS</td>
<td>Sense 5' - CAA GGG ATT GGA ATG GA</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - TGG AAG AAA AAT GGG CTT TG</td>
<td></td>
</tr>
<tr>
<td>BAX</td>
<td>Sense 5' - GGG TTG TCG CCC TTT TCT AC</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - GGA GGA AGT CCA ATG TCC AG</td>
<td></td>
</tr>
<tr>
<td>PUMA</td>
<td>Sense 5' - GAC GAC CTC AAC GCA CAG TA</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - CAC CTA ATT GGG CTC CAT CT</td>
<td></td>
</tr>
<tr>
<td>CCNB1</td>
<td>Sense 5' - CGG GAA GTC ACT GGA AAC AT</td>
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<tr>
<td></td>
<td>Antisense 5' - AAA CAT GGC AGT GAC ACC AA</td>
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</tr>
<tr>
<td>PAC</td>
<td>Sense 5' - GTC ACC GAG CTG CAA GAA CT</td>
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<tr>
<td></td>
<td>Antisense 5' - CAG ACC CTT GCC CTG GTG GT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense 5' - GAG TCA ACG GAT TTT GTC GT</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' - GAC AAG CTT CCC GTT CTC AG</td>
<td></td>
</tr>
</tbody>
</table>

Table S3. Summary for results of gene targeting in p53+/Δ40 HepG2 cell clones

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Single cell clones screened</th>
<th>p53+/Δ40 clones (re-targeted)</th>
<th>p53ΔΔ40 clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53+/Δ40 #1</td>
<td>360</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>TP53+/Δ40 #2</td>
<td>371</td>
<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>