Regulation of myo-inositol biosynthesis by p53-ISYNA1 pathway

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Abstract. In response to various cellular stresses, p53 exerts its tumor suppressive effects such as apoptosis, cell cycle arrest, and senescence through the induction of its target genes. Recently, p53 was shown to control cellular homeostasis by regulating energy metabolism, glycolysis, antioxidant effect, and autophagy. However, its function in inositol synthesis was not reported. Through a microarray screening, we found that five genes related with myo-inositol metabolism were induced by p53. DNA damage enhanced intracellular myo-inositol content in HCT116 p53+/+ cells, but not in HCT116 p53−/− cells. We also indicated that inositol 3-phosphate synthase (ISYNA1) which encodes an enzyme essential for myo-inositol biosynthesis as a direct target of p53. Activated p53 regulated ISYNA1 expression through p53 response element in the seventh exon. Ectopic ISYNA1 expression increased myo-inositol levels in the cells and suppressed tumor cell growth. Knockdown of ISYNA1 caused resistance to adriamycin treatment, demonstrating the role of ISYNA1 in p53-mediated growth suppression. Furthermore, ISYNA1 expression was significantly associated with p53 mutation in bladder, breast cancer, head and neck squamous cell carcinoma, lung squamous cell carcinoma, and pancreatic adenocarcinoma. Our findings revealed a novel role of p53 in myo-inositol biosynthesis which could be a potential therapeutic target.

Introduction

p53 is one of the most frequently mutated tumor suppressor genes (1, 2). In response to various cellular stresses, ATM-Chk2 cascade stabilizes p53 protein through the phosphorylation of its N-terminal domain (3). Activated p53 functions as a transcription factor and exerts its tumor suppressive effects such as apoptosis, cell cycle arrest, and senescence through the induction of its target genes (1,2). In addition to genes related with cell proliferation, regulation of glycolysis (4), energy metabolism, antioxidant effect (5), autophagy (6), and respiration with mitochondria are reported as novel functions of p53. Thus, p53 regulates not only tumor cell growth but also pathways related with cellular homeostasis. Since inactivation of p53 is the most common feature of cancer cells, the elucidation of p53 signaling pathways would contribute to the understanding of tumor cells as well as for drug development.

Myo-inositol is water-soluble vitamin found in a variety of food products, and are also synthesized in cells (7). Previous studies indicated that myo-inositol has various functions including glucose and lipid metabolism (8,9), neurotropic effect (10), and tumor suppression (11-13). However, the regulation of myo-inositol biosynthesis in cancer tissues has not been disclosed yet. Through a cDNA microarray screening using mRNAs isolated from HCT116 p53+/+ and HCT116 p53−/− cells, we identified ISYNA1 which encodes an enzyme essential for myo-inositol biosynthesis as a novel p53 target.

Materials and methods
cDNA microarray. Gene expression analysis was performed using SurePrint G3 Human GE 8x60K microarray (Agilent, Santa Clara, CA, USA) according to the manufacturer’s protocol. Briefly, HCT116 p53+/+ or HCT116 p53−/− cells were treated with 2 µg/ml of adriamycin (ADR) for 2 h and incubated at 37˚C until harvest. At 12, 24 and 48 h after treatment, total RNA was isolated from the cells using standard protocols. Each RNA sample was labeled and hybridized to array slides.

Cell culture and treatment. Human embryonic kidney cells HEK293T were obtained from Riken Cell Bank. Human cancer cell lines U373MG (astrocyloma), HepG2 (hepatocellular carcinoma), and HCT116 (colorectal adenocarcinoma) were purchased from American Type Culture Collection. HCT116 p53+/+ and HCT116 p53−/− cells were treated with B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). HEK293T, HCT116, and HepG2 cells were transfected with plasmids using FuGENE 6 (Promega, Madison, WI, USA). U373 MG cells were transfected with plasmids using FuGENE 6 or Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). Small interfering RNA (siRNA) oligonucleotides,
commercially synthesized by Sigma Genosys, were transfected with Lipofectamine RNAiMAX reagent (Invitrogen). Sequences of siRNA oligonucleotides are shown in Table I. We generated and purified replication-deficient recombinant viruses expressing p53 (Ad-p53) or LacZ (Ad-LacZ) as described previously (14). U373MG (p53-mutant) cells were infected with viral solutions at various amounts of multiplicity of infection (MOI) and incubated at 37°C until the time of harvest. For treatment with genotoxic stress, cells were incubated with 2 µg/ml of ADR for 2 h.

**Plasmid construction.** The entire coding sequence of ISYNA1 isoforms 1 and 4 were amplified by PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan), and inserted into the p21WAF1 monoclonal antibody (OP64) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p53 and anti-p53 monoclonal antibody were purchased by incubation with Alexa Fluor 488-conjugated secondary IgG (Molecular Probes) for 1 h. Cells were subjected to 4′,6-diamidino-2-phenylindole (DAPI) staining to visualize cell nuclei. Immunofluorescence was visualized and recorded on an Olympus FV1000D laser confocal microscope. Images were processed using Olympus FV10-ASW software and Adobe Photoshop CS3.

**Gene reporter assay.** DNA fragments, including the potential p53-response elements (REs), were amplified and subcloned into the pGL4.24 vector (Promega). Point mutations ‘T' were inserted at the 4th and the 14th nucleotide ‘C' and the 7th and the 17th nucleotide ‘G' of each RE by site-directed mutagenesis. Reporter assays were performed using the Dual Luciferase assay system (Promega) as described previously (15). Primers for amplification and mutagenesis are shown in Table I.

**Chromatin immunoprecipitation (ChIP) assay.** ChIP assay was performed using EZ-Magna ChIP G Chromatin Immunoprecipitation kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's protocol. In brief, U373MG cells infected with Ad-p53- or Ad-LacZ at a MOI of 10 were cross-linked with 1% formaldehyde for 10 min, washed with PBS, and lysed in nuclear lysis buffer. The lysate was then sonicated using Bioruptor UCD-200 (CosmoBio) to shear DNA to ~200-1,000 bp. Supernatant from 1x10⁶ cells was used for each immunoprecipitation with anti-p53 antibody (OP140, Merck Millipore) or normal mouse IgG (sc-2025, Santa Cruz). Column-purified DNA was quantified by qPCR. Primer sequences are shown in Table I.

**Western blot analysis.** To prepare whole cell extracts, cells were collected and lysed in chilled RIPA buffer (50 mmol/l Tris-HCl at pH 8.0, 150 mmol/l NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP40) containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM DTT and 0.1% Calbiochem Protease Inhibitor Cocktail Set III, EDTA-Free (EMD Chemicals Inc., Merck KGaA, Darmstadt, Germany). Samples were sonicated for 15 min with a 30 sec on/30 sec off cycle using Bioruptor UCD-200 (Cosmobio, Tokyo, Japan). After centrifugation at 16,000 x g for 15 min, supernatants were collected and boiled in SDS sample buffer (Bio-Rad, Hercules, CA, USA). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for each sample, and the proteins were then transferred to a nitrocellulose membrane (Hybond™ ECL™, Amersham, Piscataway, NJ, USA). Protein bands on western blots were visualized by chemiluminescent detection (ECL, Amersham and Immobilon, Millipore). Anti-β-actin monoclonal antibody (AC-15) was purchased from Abcam (Cambridge, UK). Anti-ISYNA1 monoclonal antibody (sc-271830) and anti-p53 monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p21WAF1 monoclonal antibody (OP64) was purchased from Merck Millipore (Darmstadt, Germany).

**Immunocytochemical analyses.** Cells were seeded on coverslips in 24-well plates. After each treatment indicated in the text, cells were washed in phosphate-buffered saline (PBS) before fixation in 4% paraformaldehyde. Cells were immunostained overnight with primary antibodies followed by incubation with Alexa Fluor 488-conjugated secondary
maintained under specific pathogen-free conditions and were handled in accordance with the Guidelines for Animal Experiments of the University of Tokyo. 
p53+/+ and p53--mice at 6 weeks of age were irradiated with 10 Gy of X-ray. At 24 h after irradiation, mice were sacrificed for liver extraction.

Database analysis. ISYNA1 expression and p53 mutation status in clinical samples were obtained from the TCGA project via data portal on 15 May 2015 (17). The association between ISYNA1 expression and the presence of the p53 gene mutation was determined by using the Student’s t-test.

Results

p53 regulates genes related with myo-inositol metabolism. To screen novel p53 target genes, we conducted cDNA microarray analysis using mRNAs isolated from HCT116 p53+/+ and HCT116 p53-- cells that were treated with 2 µg/ml of adriamycin (ADR). Fig. 1A shows a schematic representation of inositol phosphate metabolism pathway. The result of cDNA microarray analysis indicated that five genes related with myo-inositol metabolism were induced by p53 (Fig. 1B). We selected inositol 3-phosphate synthase (ISYNA1) for further analysis, because ISYNA1 showed the highest expression among the five genes.

To validate the result of cDNA microarray analysis, we performed quantitative real-time PCR (qPCR) analysis and western blotting of ISYNA1 using HCT116 p53+/+ and HCT116 p53-- cells treated with ADR. As a result, we found dose-dependent induction of ISYNA1 mRNA and protein only in HCT116 p53+/+ cells in response to ADR treatment (Fig. 1C). We also confirmed the induction of ISYNA1 mRNA and protein by ADR treatment in HepG2 (Fig. 1D). Moreover, transfection with siRNA against p53 remarkably inhibited

Table I. Sequence of primers and oligonucleotides.

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<tr>
<th>siRNA</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td>siISYNA1-A</td>
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<td>AAUCACCUCACAGAGCCGCTT</td>
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<tr>
<td>siISYNA1-B</td>
<td>GGGCUGAGCAGGAGGGGUGAANATTT</td>
<td>AUGCACUGUCCUAGGAGGCTT</td>
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| siISYNA1-C         | UCAUGUCAGGCGAGCCAGCAGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
**Figure 1. Regulation of ISYNA1 by p53.** (A) Schematic representation of the inositol phosphate metabolism pathway. PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P3, inositol 1,4,5-trisphosphate; Ins(1,4)P2, inositol 1,4-bisphosphate; Ins(4)P, inositol 4-phosphate; Ins(1,3,4,5)P4, inositol 1,3,4,5-tetrakisphosphate; Ins(1,3,4)P3, inositol 1,3,4-trisphosphate; Ins(3,4)P2, inositol 3,4-bisphosphate; Ins(3)P, inositol 3-phosphate; Ins(1,3)P2, inositol 1,3-bisphosphate; Ins(1)P, inositol 1-phosphate; G6P, glucose 6-phosphate; DAG, diacylglycerol; PA, phosphatidate; CDP-DAG, CDP-diacylglycerol. (B) Induction of genes related with myo-inositol biosynthesis by p53. HCT116 p53+/+ and HCT116 p53−/− cells were treated with 2 µg/ml of adriamycin (ADR) for 2 h. mRNAs isolated from these cells were subjected to microarray analysis. Five genes related with inositol phosphate metabolism were shown to be induced by p53. (C) qPCR analysis (upper) and western blotting (lower) of ISYNA1, p53, and WAF1 in HCT116 p53+/+ and HCT116 p53−/− cells at 36 h after treatment with ADR for 2 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used for the normalization of expression levels. Error bars represent SD (n=3). (D) qPCR analysis (upper) and western blotting (lower) of ISYNA1, p53, and WAF1 in HepG2 cells at 36 h after treatment with ADR for 2 h. GAPDH and β-actin were used for the normalization of expression levels. Error bars represent SD (n=3). (E and F) qPCR analysis of ISYNA1 mRNA in HepG2 (E) or U373MG (F) cells. At 24 h after transfection of each siRNA, HepG2 cells were treated with 2 µg/ml of ADR for 2 h. At 40 h after treatment, cells were harvested for qPCR analysis. U373MG cells were harvested at 36 h after infection with Ad-p53. siEGFP or Ad-LacZ were used as controls. GAPDH was used for the normalization of expression levels. Error bars represent SD (n=3).
the induction of ISYNA1 (Fig. 1E). p53-mediated induction of ISYNA1 was also observed in U373MG glioblastoma cells that were infected with adenovirus designed to express wild-type p53 (Ad-p53) (Fig. 1F). These results clearly indicated that ISYNA1 was regulated by p53.

**Expression and subcellular localization of ISYNA1.** There are three major variants of human ISYNA1, namely isoform 1, 2, and 4. All isoforms are similar in domain structure as shown in Fig. 2A. We constructed plasmids expressing each isoform. Result of western blotting indicated that isoform 1 is the major

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**Figure 2.** Expression and localization of ISYNA1. (A) Upper, genomic structure of ISYNA1 variants. Black boxes indicate the locations and relative sizes of exons. Lower, domain structure of ISYNA1 isoforms. (B) Western blotting of ISYNA1, p53, and WAF1 at 36 h after treatment with 2 µg/ml of adriamycin (ADR) for 2 h in HCT116 p53+/+ and HCT116 p53-/− cells. HEK293T cells transfected with plasmid designed to express ISYNA1 isoforms 1, 2 and 4 were used for molecular weight estimation of endogenous ISYNA1 protein. β-actin was used for the normalization of expression levels. (C and E) At 24 h after transfection of each siRNA, HepG2 cells were treated with 2 µg/ml of ADR for 2 h. At 40 h after treatment, ISYNA1 expression was evaluated by (C) western blotting or (E) immunocytochemistry with an anti-ISYNA1 antibody (Alexa Fluor 488; green). Expression of p53, WAF1, and β-actin is also shown. DAPI was used to visualize the nuclei (blue). (D) Immunocytochemical analysis of ISYNA1 with an anti-ISYNA1 antibody (Alexa Fluor 488; green) at 36 h after treatment with 2 µg/ml of ADR for 2 h in HCT116 p53+/+ and HCT116 p53−/− cells. DAPI was used to visualize the nuclei (blue).
Identification of ISYNA1 as a novel p53 target. To investigate whether ISYNA1 is a direct target of p53, we searched for p53 response element (RE) (18) within the ISYNA1 genomic region which is located on chromosome 19p13. We found putative p53 RE in the promoter region (RE1) and the seventh exon (RE2) (Fig. 3A). We subcloned DNA fragments including the RE1 or RE2 into pGL4.24 vector (pGL4.24/RE1 and pGL4.24/RE2) and performed gene reporter assay using U373MG cells. As a result, U373MG cells transfected with pGL4.24/RE1 or pGL4.24/RE2 showed enhanced luciferase activity only in the presence of plasmid expressing wild-type p53 (Fig. 3B). In addition, base substitutions within the RE1 and RE2 (pGL4.24/RE1mt and pGL4.24/RE2mt) completely abolished the enhancement of luciferase activity (Fig. 3B). To investigate whether p53 could directly bind to RE2 which showed higher transcriptional activity, we performed chromatin immunoprecipitation (ChIP) assay using U373MG cells that were infected with Ad-p53 or Ad-Lacz. qPCR analysis of the immunoprecipitated DNA indicated that the p53 protein bound to the genomic fragment that included the RE2 (Fig. 3C). Taken together, p53 directly regulated ISYNA1 expression through binding to the RE2 in the seventh exon.

Growth suppressive effect of ISYNA1. ISYNA1 is the rate-limiting enzyme of myo-inositol de novo synthesis (7) which is conserved among eukaryotes (19-25). To evaluate the biosynthesis of myo-inositol by ISYNA1, we performed myo-inositol (MI) assay using HEK293T cells that were transfected with mock or plasmid expressing mock or ISYNA1 isoform 1 (Fig. 4A). The results showed that intracellular myo-inositol content in cells expressing ISYNA1 isoform 1 was significantly higher than those in control cells. In addition, DNA damage significantly increased intracellular myo-inositol content in HCT116 p53+/+ cells, but did not affect the myo-inositol content in HCT116 p53−/− cells (Fig. 4B). Thus, our results indicated that p53 could regulate intracellular myo-inositol levels in response to DNA damage.

We also evaluated the effect of p53-ISYNA1 pathway on cancer cell growth. The result of colony formation assay using HCT116 and HepG2 cells indicated that ISYNA1 overexpression suppressed cell proliferation (Fig. 4C). We then designed three siRNAs (siA, siB and siC) and found that siRNAs effectively suppressed ISYNA1 mRNA and protein (Fig. 4D). We performed ATP assay using HCT116 p53+/+ cells and found that ISYNA1-silencing caused resistance to ADR treatment (Fig. 4E). These results indicated that p53 could regulate intracellular myo-inositol levels in response to DNA damage.
from liver tissues. qPCR analysis revealed that mouse *Isyna1* mRNA was induced by DNA damage only in p53 wild-type mice (Fig. 5A). Screening of p53 RE within *Isyna1* genomic region identified a putative RE (mRE) at ~10 kb upstream of the *Isyna1* gene (Fig. 5B). We subcloned a DNA fragment including mRE into the pGL4.24 vector (pGL4.24/mRE) and performed gene reporter assay using U373MG cells (Fig. 5C). Luciferase activity was strongly enhanced by co-transfection...
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with wild-type p53, but not with mutant p53. In addition, base substitutions within mRE diminished the enhancement of luciferase activity, demonstrating regulation of Isyna1 by p53 through mRE.

We also analyzed whether p53 regulates ISYNA1 in human cancer tissues. Correlation between p53 mutation and ISYNA1 expression was analyzed by using omics data of various tumor tissues released from the TCGA database (17). Interestingly, ISYNA1 mRNA expression in bladder cancer, breast cancer, head and neck squamous cell carcinoma, lung squamous cell carcinoma, and pancreatic adenocarcinoma was significantly decreased in tumor tissues with p53 mutation compared with those without p53 mutation (Fig. 5D). These findings indicate that p53 regulates ISYNA1 expression in vivo.

Discussion

We identified ISYNA1 as a novel p53 target. ISYNA1 is a key enzyme which affects myo-inositol de novo synthesis (7,26,27). In addition, p53 induced INPP1 and INPP5 (28) that are involved in myo-inositol salvage pathway. Myo-inositol is one of the chemical compounds which is essential for living organisms (29), and myo-inositol depletion affects cell survival and growth (30). Myo-inositol was also reported to

Figure 5. Regulation of ISYNA1 by p53 in vivo. (A) qPCR analysis of Isyna1 in mouse livers. Mice were divided into four groups; p53 wild-type mice without irradiation (W), p53 wild-type mice with irradiation (WX), p53 knockout mice without irradiation (K), p53 knockout mice with irradiation (KX) (n=6 per group). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used for the normalization of expression level. Top bar represents maximum observation, lower bar represents minimum observation, the top side of the box represents the third quartile, and the bottom side, the first quartile. The middle bar represents the median value. The P-value was calculated by Student's t-test. (B) Upper, genomic structure of mouse Isyna1. Black boxes indicate the locations and relative sizes of exons. The white box indicates the location of the p53 response element (mRE). Lower, comparison of mRE to the consensus p53RE. R, purine; W, A or T; Y pyrimidine. (C) Luciferase assay of mRE in U373MG with or without mutation of the RE. Luciferase activity is indicated relative to the activity of the mock vectors. The plasmid expressing mouse p53 carrying a missense mutation (R172H) served as a negative control. Error bars represent the SD (n=3). (D) Box plot of ISYNA1 expression in bladder cancer, breast cancer, head and neck squamous cell carcinoma, lung squamous cell carcinoma, and pancreatic adenocarcinoma tissues from the TCGA database. The vertical axis indicates the normalized expression level of ISYNA1, top bar represents maximum observation, lower bar represents minimum observation, the top side of the box represents the third quartile, and the bottom side, the first quartile. The middle bar represents the median value. The P-value was calculated by Student's t-test.
suppress tumor growth in vitro and in vivo (31-38). Previous studies indicated that myo-inositol suppresses phosphorylation of Akt and Erk by inhibiting PI3K activity (12,13), p53 was also shown to suppress PI3K-Akt pathway by inducing PTEN (39) and Phlda3 (40). Our results suggested a novel mechanism whereby p53 negatively regulates PI3K-Akt pathway by inducing ISYNA1.

Epidemiological studies indicate that myo-inositol prevents progression of dysplasia in smokers (11-13), and decreases tumorigenesis in chronic hepatitis patients (33). These findings suggested that p53 would suppress tumorigenesis by inducing biosynthesis of myo-inositol. We also found that ISYNA1 was induced in mouse liver tissue by DNA damage. To evaluate the chemopreventive effect of myo-inositol, we fed p53 knockout mice with myo-inositol in drinking water. However, oral myo-inositol did not suppress tumor development (data not shown). Although, myo-inositol was shown to suppress liver cancer (32,33), liver cancer is relatively rare for p53 knockout mice compared with lymphoma of thymus or spleen (41). In addition, although induction of Isynal was observed in liver tissues, Isynal was not induced in thymus and spleen (data not shown). Therefore, to evaluate the chemopreventive effect of myo-inositol or ISYNA1 in vivo, liver cancer model would be appropriate.

Taken together, ISYNA1 was shown to be a mediator of p53-dependent growth suppression, and ISYNA1 expression was reduced in several types of cancers with p53 mutations. Therefore, myo-inositol could be a potential anticancer agent for cancer cells with p53 mutation. Our findings revealed a novel role of p53 in myo-inositol biosynthesis which could be a possible therapeutic target.

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References


