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# The expression of phosphatidic acid phosphatase 2a, which hydrolyzes lipids to generate diacylglycerol, is regulated by p73, a member of the p53 family

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**Abstract**

p73, a p53-related gene, is essential for a development of animals, while p53 is important for tumor formation. And little is known about the target genes specifically regulated by p73. Identifying the specific targets of p73 is important to understand the physiological roles of p73. To identify the genes specifically regulated by p73, we conducted serial analysis of gene expression to quantitatively evaluate messenger RNA populations. We found that the gene for phosphatidic acid phosphatase 2a (PAP2a), an enzyme that hydrolyzes lipids to generate diacylglycerol, was specifically upregulated by ectopic production of p73β. The promoter region of this gene contains an element that is functionally responsive to p73β. And the quantity of PAP2a protein was upregulated by ectopic production of p73β. These results suggest that the expression of *PAP2a* is directly regulated by p73.


*Keywords:* p53; p73; Transcription; SAGE; PAP2a

p73 is a gene product sharing a structural homology with p53, which plays an important role in suppressing tumorigenic growth by transactivating target genes [1–5]. When overexpressed in cultured cells, both proteins transactivate target genes by binding to p53 responsive elements (p53REs). Despite the similarities, p53 and p73 perform divergent physiological functions [6–9]. Although p53-deficient mice develop normally, they are susceptible to spontaneous tumor formation [10]. In contrast, p73-deficient mice have neurological, pheromonal, and inflammatory defects, but show no increased susceptibility to spontaneous tumorigenesis [11]. But little is known about the target genes specifically regulated by p73 [12–14]. Identifying the specific targets of p73 is important to understand the physiological roles of p73.

To identify genes specifically induced by p73, we performed serial analysis of gene expression (SAGE), a technique that allows the quantitative evaluation of messenger RNA (mRNA) populations. Using mRNAs isolated from cultured cells producing exogenous p53 or p73β, an isoform of p73, we identified phosphatidic acid (PA) phosphatase 2a (PAP2a) as a p73 specific target gene. PAP2a is an integral membrane glycoprotein that hydrolyzes extracellular phosphatidic acid and causes the intracellular accumulation of diacylglycerol (DG) and its metabolites [15].

## Materials and methods

*Plasmid construction.* Construction of the expression plasmids pcDNA3-FLAG-p73β, pcDNA3-FLAG-p73βM, and pcDNA3-FLAG-p53 were described previously [16]. The reporter plasmids for the measurement of *PAP2a* promoter activity were constructed as follows. First, human genomic DNA was isolated from the blood of a healthy volunteer using the DNeasy Tissue System (Qiagen). The fragments synthesized by

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the PCR using human genomic DNA and appropriate primers were inserted into the *Xho*I–*Hin*dIII site of the pGL3-Basic vector (Promega) to obtain the reporter plasmids.

*Recombinant adenovirus.* The recombinant adenoviral vectors for the expression of p73β and p53 were prepared using the Adeno-X Expression System (Clontech) according to the manufacturer's protocol. DNA fragments for p73β and p53 were synthesized by PCR using pcDNA3-FLAG-p73β and pcDNA3-FLAG-p53, respectively, as templates. After digestion with *Xho*I and *Hin*dIII, the DNA fragments were subcloned into the *Xho*I–*Hin*dIII site of the pShuttle vector (Clontech) to obtain pAd-p73β and pAd-p53. The plasmids were transfected into 293 cells using FuGENE6 transfection reagent (Boehringer–Mannheim), and the transfectants were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum (FBS) for 1 week to generate recombinant adenovirus vectors. The adenovirus vector for the expression of LacZ was prepared as described above using the pAd-LacZ plasmid supplied by the manufacture. This adenovirus vector was named Ad-LacZ. The viral titers of the culture supernatants were estimated by plaque forming assays using 293 cells.

*Northern blot analysis.* Total RNA was isolated from Saos-2 cells infected by Ad-LacZ, Ad-p53, or Ad-p73β for 48 h using an RNeasy mini Kit (Qiagen). Total RNA was analyzed by Northern blot hybridization using ULTRAhyb (Ambion) according to the manufacturer's protocol. Partial DNA fragments representing PAP2a or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared with a One Step RT-PCR Kit (TAKARA) using total RNA from Saos-2 cells infected by Ad-p73β as a template. Two oligonucleotide pairs, pair 1 (5′-CCATTGCGCCCTGGGCTTGG-3′ and 5′-GGAAAACGAAGAGTGGCCTG-3′) and pair 2 (5′-ATGGGGAAGGTGAAGGTCGG-3′ and 5′-TGGAGGGATCTCGCTCCTGG-3′) were used as primers for PAP2a and GAPDH, respectively, during the reverse transcription-polymerase chain reaction (RT-PCR). Probes for the detection of PAP2a and GAPDH mRNAs were prepared by PCR in the presence of [$^{32}$P]dCTP (Amersham Biosciences) and these DNA fragments.

*Antibodies.* The anti-Tubulin was a monoclonal antibody from Oncogene Research Products. The polyclonal antibodies to PAP2a were generated by MBL, Japan by inoculating a rabbit with a peptide ETPTTGNHYPSNHQP corresponding to amino acids 271–285 of PAP2a.

*Immunoblot analysis.* Saos-2 cells were infected with Ad-LacZ, Ad-p53, or Ad-p73β for 48 h. The preparation of the cell lysates, sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as described previously [16]. The antibodies used in these experiments were anti-Tubulin or anti-PAP2a.

*SAGE.* Total RNA was isolated from Saos-2 cells infected by Ad-LacZ, Ad-p53, or Ad-p73β for 48 h. These RNA samples were analyzed using the I-SAGE Kit (Invitrogen) according to the manufacturer's protocol.

*Cell culture and transfection.* Saos-2 cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% FBS and L-glutamine. For plasmid transfection into the cells, we used the FuGENE6 transfection reagent (Boehringer–Mannheim) according to the manufacturer's protocol.

*Luciferase assay.* Saos-2 cells ($5 \times 10^4$ cells) were transfected with 0.01 μg of a reporter plasmid containing the firefly luciferase gene downstream of a portion of the transcriptional promoter region of *PAP2a* (see Fig. 2A), together with 0.1 μg of pcDNA3 vector, or with 0.05 μg of pcDNA3 vector and 0.05 μg of the expression plasmids encoding p53 or p73β. As for Fig. 2B, the amounts of plasmids used in the transfection were indicated in Fig. 2B. Forty-eight hours after transfection, the cells were lysed and the luciferase activity was quantified with a luciferase assay kit (Promega) as recommended by the manufacturer.

*DNA–protein complex immunoprecipitation assay.* 293T cells ($1 \times 10^6$ cells) were transfected with 1.0 μg of the reporter plasmid pp16008 (see Fig. 2A), together with 9.0 μg of the expression plasmid encoding p53 or p73β, or with 9.0 μg of pcDNA3. After cross-linking with 5% formaldehyde for 10 min, cells were lysed, sonicated, and subjected to immunoprecipitation using anti-FLAG tag antibodies or normal mouse Immunoglobulin G (IgG). After incubation at 65 °C for 8 h, the recovered immunocomplexes were digested with proteinase K for 1 h. DNA was extracted from the immunocomplexes by phase separation with phenol, precipitated with ethanol, and dissolved in 50 μl TE. Each sample (1 μl) was used as a template for PCR amplification. The sequences of the PCR primer pairs used for detection of the promoter regions of *PAP2a*, *p21$^{waf\text{-}1}$*, and *GAPDH*, were 5′-CTGGGACTGCAGCCCAGGTTTTC-3′ and 5′-AACTAGGACCACAAGTGTAC-3′, 5′-ACCTTTCACCATTCCCCTAC-3′ and 5′-GCCCAAGGACAAAATAGCCA-3′, and 5′-GTATTCCCCCAGGTTTACAT-3′ and 5′-TTCTGTCTTCCACTCACTCC-3′, respectively.

## Results

### *p73 induces the accumulation of mRNA and protein of PAP2a*

To identify genes specifically regulated by p73, we compared the gene expression profiles of human osteosarcoma-derived cells (Saos-2 cells) using SAGE [17]. In brief, this method relies on short sequence 'tags' composed of 11 base pairs, which are generated from defined positions within each mRNA molecule. Expression profiles are deduced from the abundance of individual tags.

The total number of SAGE tags required to quantitatively evaluate mRNA populations is estimated to be more than 100,000. If 100,000 SAGE tags from each of two samples are analyzed, 44% and 90% of the transcripts that make up 0.01% of the total transcripts and differ between the two samples by 2- and 3-fold, respectively, can be detected [18]. In this study, we identified 105,032 and 105,029 independent SAGE tags that were representative of the transcripts isolated from Saos-2 cells infected with adenovirus carrying p53 (Ad-p53) or p73β (Ad-p73β), respectively. Approximately 92% of the SAGE tags were identified between a few and twenty in number. Therefore, 92% of the transcripts we analyzed were expressed at a level of about 0.01% of the total number of transcripts. Moreover, we detected at least 40% of the transcripts that differed between the two samples in their expression levels by at least 2-fold. Furthermore, we detected at least 83% of the transcripts that differed between the two samples in their expression levels by at least 3-fold. We found 6886, 3748 independent transcripts that were more than twice, less than half as abundant in p53-producing Saos-2 cells compared to p73β-producing cells, respectively. Among the transcripts, we focused on *PAP2a*, which encodes a protein that may be related to signal transduction. We found 4 and 11 SAGE tags (TATGTAATAT) for PAP2a transcripts in samples from the p53- and p73β-producing Saos-2 cells, respectively, suggesting that the level of PAP2a mRNA was increased in the p73β-transfected cells. To confirm this result, we analyzed total RNA from the cells by northern blot analysis. As shown in Fig. 1A, the amount of PAP2a mRNA increased in Saos-2 cells infected with Ad-p73β (74-fold compared to control samples), but not in cells infected with Ad-p53 (0.4-fold compared to control samples). These results were con-

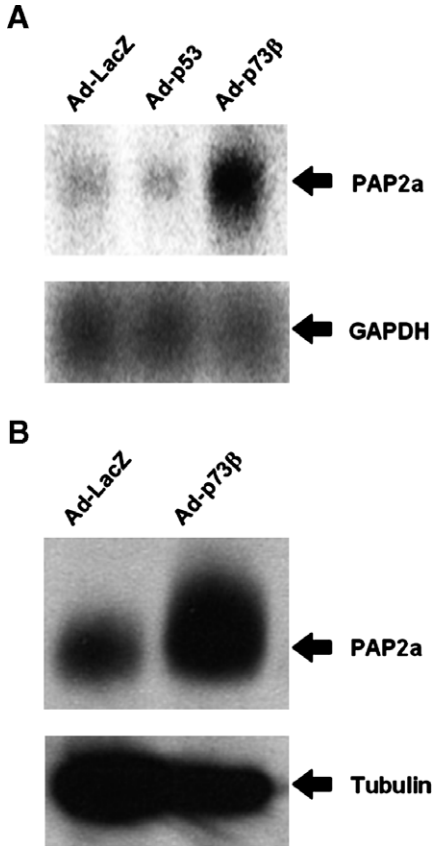


Fig. 1. (A) Upregulation of PAP2a mRNA in Saos-2 cells producing exogenous p73β. The results of northern blot analysis using 10 μg of total RNA isolated from Saos-2 cells infected with Ad-LacZ, Ad-p53, or Ad-p73β are shown. The bands representing the mRNAs of PAP2a (upper panel) and GAPDH (lower panel) are shown. (B) Upregulation of PAP2a protein in Saos-2 cells producing exogenous p73β. The cell lysates from Saos-2 cells infected with Ad-LacZ or Ad-p73β were analyzed by immunoblotting with the antibody for PAP2a. The bands in the polyacrylamide gels representing PAP2a (upper panel) and Tubulin (lower panel) are shown.

firmed by repeating the analysis with a human hepatocyte-derived cell line (Huh-7 cells, data not shown). This data suggested that p73β specifically modulates the expression level of *PAP2a*. Then, we analyzed the protein quantity of PAP2a from Saos-2 cells infected with Ad-p73β by western blot analysis. As shown in Fig. 1B, the amount of PAP2a protein increased in Saos-2 cells infected with Ad-p73β (4.5-fold compared to control samples) (Fig. 1B). This data suggested that p73β production resulted in the upregulation of PAP2a protein in the cells.

### *The transcriptional promoter region of PAP2a includes a functional p53RE*

Among the 5′ upstream region of *PAP2a*, we found several p53RE-like sequences that p73 may bind to (Fig. 2A).

To test whether the expression of *PAP2a* is induced by p73β, we performed a reporter assay using p73β to measure the promoter activity of the region upstream of *PAP2a*. Because the putative promoter region spans more than 16 kb, we divided the region into five fragments and each fragment was subcloned into the promoterless luciferase reporter vector pGL3-basic. The resulting reporter plasmids were called pp555, pp5121, pp8563, pp12482, and pp16008 (Fig. 2A). We found that Saos-2 cells co-transfected with Ad-p73β and pp16008 showed significantly higher luciferase activity (a 39.7-fold increase compared to control cells) than cells co-transfected with Ad-p53 and pp16008 (a 4.2-fold increase), whereas the other reporter plasmids did not respond to the transcription factors (Fig. 2A).

These results indicated that the 5′ upstream region spanning from nt positions −16,008 to −13,005 from the transcriptional initiation site of *PAP2a* includes at least one potent p73 responsive element (p73RE).

To identify the minimum p73RE, deletion analysis of the region between nt positions −16,008 and −13,005 upstream of *PAP2a* was performed (Fig. 2A del-1 through del-8). As shown in Fig. 2A, only del-4 and del-7 did not respond to p73β, while the others did. These results narrowed our search to the region between −13,793 and −13,693, which contained the p53RE-like sequence (from −13,761 to −13,730) (Fig. 2A).

To examine whether the p53RE-like sequence located from −13,761 to −13,730 was responsive to p73β, we analyzed the responsiveness of several mutant plasmids (from del-10 to del-15), in which the p53RE-like sequences were serially deleted, to p73β (Fig. 2A). We found that only del-13 was not responsive to p73β, which lacked the p53RE in the region from −13,761 to −13,730 (Fig. 2A), suggesting that this region is required for p73-dependent *PAP2a* expression.

To analyze whether this region is sufficient for the p73β-dependent induction of *PAP2a* expression, we performed reporter assays using the reporter plasmid del-9, which included the region from −13,792 to −13,693 and the *PAP2a* basal promoter (Fig. 2A). As shown in Fig. 2B, the del-9 hardly respond to the p53-expression plasmid (lanes 2, 6, and 10) or p73βM mutated in DBD (lanes 4, 8, and 12), while the del-9 respond to p73β-expression plasmid dose-dependently (lanes 3, 7, and 11). This result implies that the p53RE in the region from −13,792 to −13,693 is sufficient for the p73-dependent induction of *PAP2a* expression and that p73β binding to DNA is essential for *PAP2a* expression.

### *p73β binds the p53RE in the PAP2a promoter*

To examine whether p73β directly binds to the promoter region of *PAP2a*, we performed DNA–protein immunoprecipitation assays (Fig. 3). The promoter region of *PAP2a* in the reporter plasmid was detected in complexes precipitated by antibodies against p73β or p53 expressed in 293T cells (Fig. 3, upper panel). These results indicated that p73β is a component of the complex formed on the *PAP2a* promoter. From these results, we concluded that p73β transcriptionally regulates *PAP2a* through binding

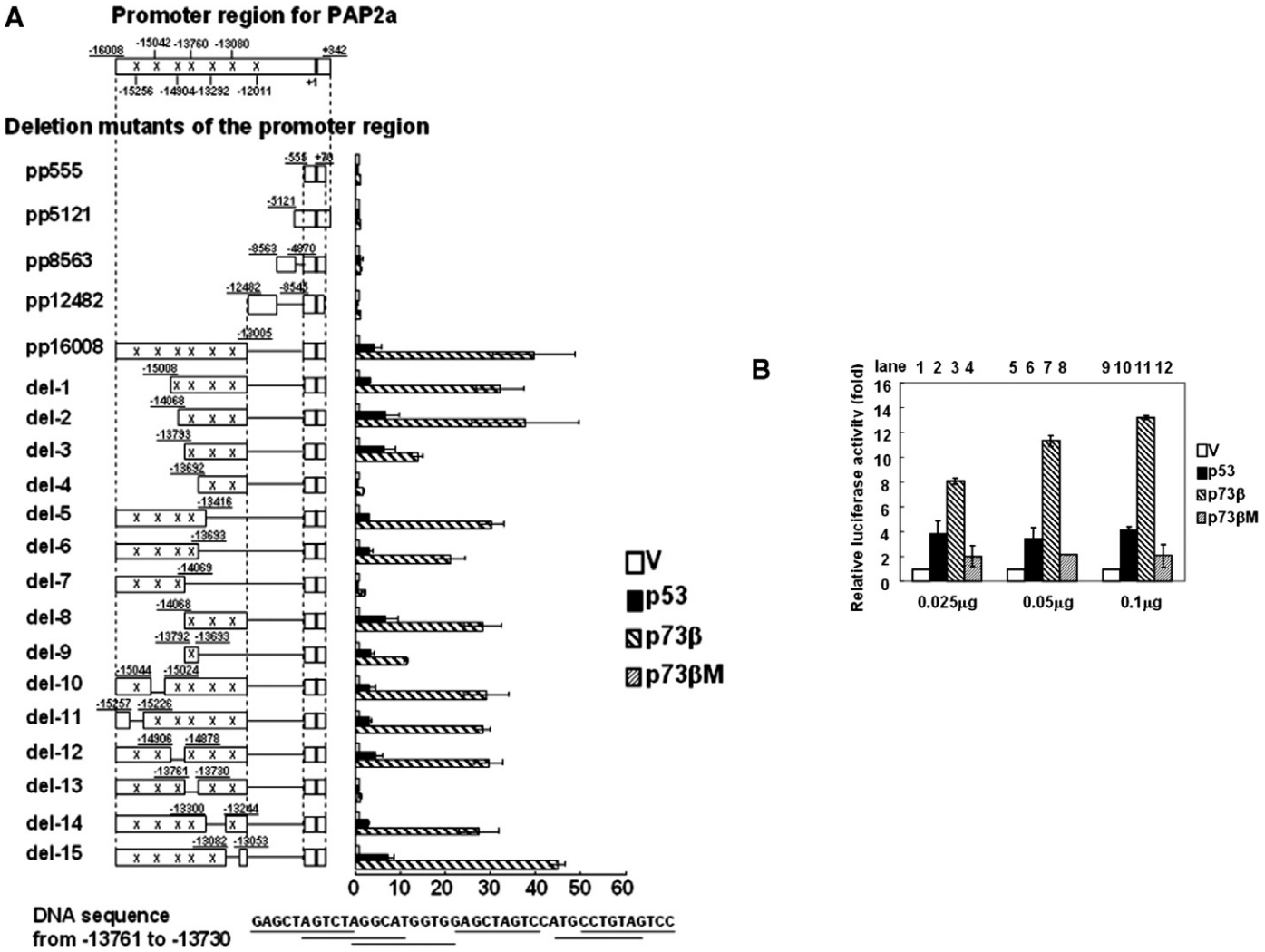


Fig. 2. (A) The potential p53RE between nt positions −16,008 and −13,005 in the PAP2a promoter was responsive to p73, but not to p53. Schematic representations of the transcriptional promoter region of *PAP2a* and the deletion construct are shown. The upper box represents the *PAP2a* promoter spanning from nt positions −16,008 to +342 (the underlined positions on each side of the upper box). The transcriptional initiation site, represented by a thick line on the right side of this figure, was designated as nt position +1. The positions of the potential p53REs proposed by homology search analysis are indicated only for the promoter subregion from −16,008 to −12,000 (Xs). The numbered positions of the most upstream residue in each potential p53RE are shown above or below the upper box. The structure of the deletion constructs (left) and the responsiveness of each construct to p73β and p53 analyzed by luciferase reporter assays (right) are shown below the upper box. The promoter was divided into four pieces that include multiple p53REs as well as the basal promoter region from −555 to +70. pp555 included only the basal promoter region, whereas pp5121, pp8563, pp12482, and pp16008 included −5121 to +342, −8563 to −4870, −12,482 to −8545, and −16,008 to −13,005, respectively. The deletion constructs, del-1 through 9 and del-10 through 15 are the 5′- and 3′-terminal and the internal deletion mutants of the promoter subregion from −16,008 to −13,005. The deletion constructs, del-1 through 15 contain the basal promoter region, respectively. The underlined numbers denote the nucleotide positions located at the end(s) of those regions in the deletion constructs. The luciferase activity from each reporter construct in cells expressing p73β or p53 was measured in comparison to cells transfected with the empty effector plasmid pcDNA3. The relative luciferase activity (vertical axis) is shown. Values represent the average of three independent experiments. Error bars represent the standard error. "DNA sequence from −13,761 to −13,730" represents the sequence the *PAP2a* promoter spanning from nt positions −13,761 to −13,730 (underlines represent homologous sequences to p53RE). The consensus p53RE is RRRCYYGYYYn(0–23)RRRCYYGYYY. R represents purine, Y pyrimidine, and W adenine or thymidine. (B) The responsiveness of the region from −13,761 to −13,730 (del-9) to p53, p73β or 73βM was analyzed as described in panel A. The amount of the expression plasmids encoding p53, p73β or 73βM transfected in Saos-2 cells were as indicated. Total amount of expression plasmid DNA used for transfection was adjusted to be 0.1 μg by adding appropriate amount of empty vector.

an upstream particular p53RE-like element in the promoter region of the gene.

## Discussion

We identified *PAP2a* as a gene specifically regulated by p73β. PAP2a hydrolyzes lipids to generate DG. Although both p53 and p73β bind to p53REs and transactivate various target genes, there are genes specifically responsive to either p53 or p73β [6–9]. This implies that p73 and p53 have some distinct physiological roles. For example, p73-deficient mice have neurological, pheromonal, and inflammatory defects but do not develop spontaneous tumors, in contrast to p53-deficient mice. This indicates that p73 is

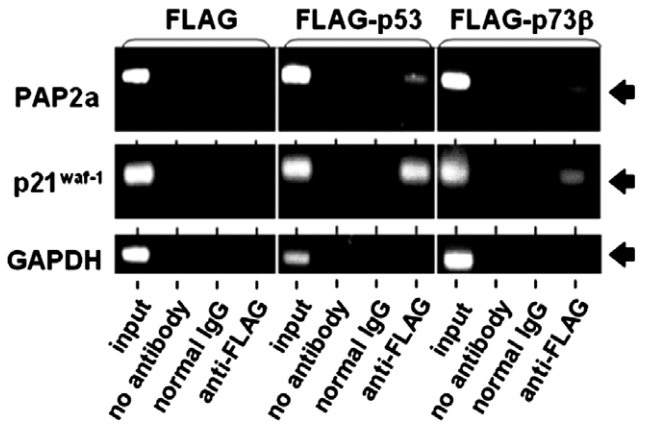


Fig. 3. p73β associates with p53RE in the *PAP2a* promoter. The association of p73β or p53 with the p53RE in the *PAP2a* promoter was analyzed by a DNA–protein complex immunoprecipitation assay using pp16008-transfected 293T cells that overproduced either FLAG tag alone (FLAG), FLAG-tagged p53 (FLAG-p53), FLAG-tagged p73β (FLAG-p73β). Formaldehyde-cross-linked DNA–protein complexes were immunoprecipitated with anti-FLAG antibodies (anti-FLAG), normal mouse IgG (normal IgG), or in the absence of antibodies (no antibody). Before immunoprecipitation, the sonicated chromatin fractions were directly analyzed (input) as a positive control to show the presence of the DNA fragment from pp16008 in the fraction. The presence of the promoter regions of *PAP2a*, $p21^{waf\text{-}1}$, or *GAPDH* in each immunoprecipitated fraction was examined by the PCR after extraction of DNA from the fraction. The bands detected by ethidium bromide staining in the agarose gel are indicated by arrows.

essential for a development of the mouse and p53 cannot compensate for p73. A possible explanation of this is the different expression patterns of p73 and p53. In addition, it is well known that activation of the transactivational function of p53 is regulated by several post-translational events, including phosphorylation, acetylation, and ubiquitination. For example, phosphorylation of p53 is induced after DNA damage and results in the stabilization and activation of p53. The mechanisms underlying p73 activation, however, will be different. For example, p73 activation was not responsive to DNA damage caused by treatment with p53 activators, such as UV irradiation, actinomycin D, doxorubicin, or mitomycin C [1,19]. These results indicate that the functional divergence between p73 and p53 is at least partially due to different post-translational regulatory mechanisms. Although we found that both p53 and p73 interacted with the p53REs in the promoter region of *PAP2a*, p73 produced a higher level of gene activation in our reporter assay (Fig. 3), suggesting that there is likely an additional mechanism supporting the p73-dependent activation of transcription. We have not identified an obvious difference between the nucleotide sequences specifically bound by p73 and p53. The transcription of a gene, however, requires a complex composed of some factors. Therefore, other factors, which bind to p53 protein, p73 protein or other DNA elements in the *PAP2a* promoter, may act together with p73 and p53 to modulate the transcriptional activity of *PAP2a*. The expression of these factors may also be induced by p73 or p53. Tumor protein p53 induced nuclear protein 1 (*TP53INP1*) was a p73 target gene [20]. Two isoforms of TP53INP1, α and β, enhance the p53- and p73α-dependent transcription of *murine double minute clone 2 oncoprotein* (*Mdm2*). TP53INP1β, however, does not enhance p73α-dependent transcription of $p21^{waf\text{-}1}$, whereas TP53INP1α does. On the other hand, these isoforms inhibit both p53- and p73-dependent transcription of *TP53INP1*. This implies that the p53 family, including p73, regulates its transcriptional activation potential through the induction of particular target genes. These factors may determine the specificity of the p73β-dependent gene induction.

To clarify the physiological roles of p73, it is essential to know the functions of the genes regulated by p73. *Aquaporin3* (*AQP3*) is transcriptionally regulated by p73 [12]. Because p73-deficient mice have hydrocephalus caused by defects in the reabsorption of cerebrospinal fluid [11], p73 may play an essential role in maintaining cerebral spinal fluid homeostasis through the induction of *AQP3* expression. PAP2a hydrolyzes extracellular PA, followed by DG spontaneously transversing the lipid bilayer and the intracellular accumulation of DG and its metabolites [15]. Once entering in the cell, DG can be metabolized to other products or act as a regulatory molecule for other proteins, such as protein kinase C (PKC) [21]. As PKC activity is required for the differentiation of various cells [22,23], the abnormal differentiation caused by p73 deficiency may be at least partially due to a lack of PAP2a production, which in turn leads to a reduced PKC activation. PAP2a knockout mice may provide important information about this hypothesis.

Further studies to reveal the identity and functions of other p73-specific target genes will help clarify the physiological roles of p73.

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