Oncogene (2003) 22, 4333–4347

www.nature.com/onc

Characterization of p73 functional domains necessary for transactivation and growth suppression

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p73, a p53 family member, is highly similar to p53 in both structure and function. Like p53, the p73 protein contains an N-terminal activation domain, a DNA-binding domain, a tetramerization domain, and several PXXP motifs. Previously, we and others have shown that some functional domains in p53, such as the DNA-binding and tetramerization domains, are required for inducing both cell cycle arrest and apoptosis whereas others, such as the second activation domain, the proline-rich domain, and the C-terminal basic domain, are only required for inducing apoptosis. To determine the activity of p73 functional domains, we have generated stable inducible cell lines that express p73β and various mutants deficient in one or more functional domains. We found that in addition to the DNA-binding domain, p73-mediated growth suppression requires the N-terminal activation domain and the tetramerization domain. However, unlike p53, p73-mediated apoptosis does not require the region adjacent to the activation domain or the entire C-terminal region. Interestingly, while the N- or the C-terminal PXXP motifs are dispensable for p73 function, deletion of both the N- and the C-terminal PXXP motifs renders p73 inactive in transactivation. In addition, we found that substitution of two conserved tandem hydrophobic residues with two hydrophilic ones, which can abrogate the activity of the first activation domain in p53, has no effect on p73 transcriptional activity. Together, we showed that the p73 protein has its own unique determinants for transactivation and growth suppression.
Oncogene (2003) **22,** 4333–4347. doi:10.1038/sj.onc.1206470

Keywords: p73; p53 family; apoptosis; cell cycle

Introduction

The p73 gene is localized to chromosome 1p36.3 and consists of 14 exons (Kaghad *et al*., 1997). Interestingly, the p73 gene is regulated by two distinct promoters, that is, an upstream promoter located 5′ of the exon 1 and an intron promoter located within intron 3 (Irwin and Kaelin, 2001; Moll *et al*., 2001; Yang *et al*., 2002). When the p73 gene is transcribed from the upstream promoter, alternative splicing can generate at least seven transcripts that differ in their 3′ ends. These transcripts encode seven polypeptides, called TA variant. These include p73α (exons 1–14; 636 amino acids), p73β (exons 1–12 and 14; 499 amino acids), p73γ (exons 1–10, 12, and 13; 475 amino acids), p73δ (exons 1–10; 404 amino acids), p73ε (exons 1–10, 12, and 14; 555 amino acids), p73ζ (exons 1–10, 13, and 14; 540 amino acids), and p73η (exons 1–12 and an extended exon 13 containing an alternate stop codon; 571 amino acids). Similarly, when the p73 gene is transcribed from the intron promoter, at least seven additional alternatively spliced transcripts are produced. These transcripts encode seven polypeptides, called ΔN variant, with 13 unique residues in their N-termini. The ΔN variant lacks 62 residues that constitute the N-terminal activation domain in the TA variant. In addition, a third group of p73 transcripts has been found, which is transcribed from the 5′ upstream promoter but are aberrantly spliced to either exclude exon 2 (p73Δex2), exons 2 and 3 (p73Δex2/3), or include an additional exon, exon 3′ (ΔN′p73) (Ng *et al*., 2000; Fillippovich *et al*., 2001; Stiewe *et al*., 2002a, b). Since a translational start site exists within both exons 2 and 3′, these transcripts encode several p73 polypeptides, called ΔTA variant. Like the ΔN variant, the ΔTA variant lacks the N-terminal activation domain of the TA variant but contains its own unique N-terminal residues (Ng *et al*., 2000; Fillippovich *et al*., 2001; Stiewe *et al*., 2002a, b).

Despite the differences in their respective amino and carboxy termini, all p73 isoforms contain two N- and one C-terminal PXXP motifs, a DNA-binding domain, and a tetramerization domain (Chen, 1999; Yang and McKeon, 2000). The TA variant contains an N-terminal activation domain (Chen, 1999; Irwin and Kaelin, 2001; Moll *et al*., 2001; Yang *et al*., 2002). Among these domains, p73 shares a considerable sequence identity with p53 in the N-terminal activation domain, the DNA-binding domain, and the tetramerization domain (Chen, 1999; Yang and McKeon, 2000). In addition, all alpha isoforms contain a sterile alpha motif (SAM) in their C-termini (Chi *et al*., 1999; Thanos and Bowie,

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Received 13 November 2002; revised 3 February 2003; accepted 3 February 2003

1999). In some signaling proteins, the SAM domain is involved in protein–protein interaction (Schultz *et al*., 1997). However, it is not clear whether the SAM domain in p73 has similar activity. Furthermore, a potential second activation domain has been identified that is located within residues 380–513 in p73α (Takada *et al*., 1999). Like the activation domain in some transcription factors, this region is rich in glutamine and proline residues (Takada *et al*., 1999).

Of all the p73 isoforms, p73β appears to be the most potent form in transactivation and growth suppression (Chen, 1999; Irwin and Kaelin, 2001; Moll *et al*., 2001; Yang *et al*., 2002). p73α, p73δ, and p73η are less active than p73β, whereas p73γ and p73ε are inert (De Laurenzi *et al*., 1998; Zaika *et al*., 1999; Ishimoto *et al*., 2002). The activity of p73ζ has not yet been characterized. The ΔN variant appears to be incapable of activating transcription from a p53 reporter gene and can inhibit the ability of the TA variant, and possibly p53, to activate transcription, and thus is potentially dominant negative (Pozniak *et al*., 2000; Yang *et al*., 2000; Ishimoto *et al*., 2002).

In this report, we have used the tetracycline-inducible expression system and generated stable cell lines that express various forms of the p73β protein to further determine the functional domains of p73. We have found that in addition to the DNA-binding domain, the N-terminal activation domain and the tetramerization domain are necessary for p73 activity. However, unlike p53, p73-mediated apoptosis does not require the region adjacent to the activation domain or the entire C-terminal region. Interestingly, while the N- or the C-terminal PXXP motifs are dispensable for p73 function, deletion of both the N- and the C-terminal PXXP motifs renders p73 inactive in transactivation. In addition, we found that substitution of two conserved tandem hydrophobic residues with two hydrophilic ones, which can abrogate the activity of the first activation domain in p53, has no effect on p73 transcriptional activity.

Results

*p73*β *suppresses cell growth*

Previously, we and others have shown that p73 can suppress cell growth and induce apoptosis (Jost *et al*., 1997; Kaghad *et al*., 1997; Zhu *et al*., 1998a). In this study, we chose the H1299 cell line, which is p53-null and expresses an undetectable level of p73, and the tetracycline-regulated expression system to generate stable cell lines that inducibly express human p73β. Western blot analyses of two representative cell lines, p73β-22 and p73β-24, are shown in Figure 1a. To determine the transcriptional activity of HA-p73β, we monitored the level of endogenous p21, a known target of p53 and p73. As shown in Figure 1a, HA-p73β induced the expression of p21.

One of the striking similarities between p53 and p73 is their shared ability to suppress cell growth when overexpressed (Jost *et al*., 1997; Kaghad *et al*., 1997; Zhu *et al*., 1998a). To determine whether p73β suppresses cell proliferation in our system, the growth rate of HA-p73β-producing cells was determined over a 5-day period. As shown in Figure 1b, cells failed to multiply in the presence of p73β, suggesting that p73β is capable of inhibiting cell growth. Next, we performed trypan blue dye exclusion assays and found that the number of trypan blue dye positive cells, that is, dead cells, increased from 4 to 12% in the presence of p73β (Figure 1c), suggesting that in our system, HA-p73β is functional and capable of inducing cell death. Furthermore, we performed DNA histogram analysis and found that the percentage of cells exhibiting sub-G1 DNA content increased from 1.8 to 8.8% when cells were induced to express HA-p73β (Figure 1d). We also found that in the presence of a low level of HA-p73β, H1299 cells underwent cell cycle arrest, primarily in G1 (data not shown). Taken together, we showed that the characteristics of p73 are recapitulated in the H1299 cell lines inducibly expressing human p73β.

The N-terminal activation domain is necessary for the activity of p73 in transactivation and growth suppression

Previously, we and others have shown that p53 contain two tandem activation domains in its N-terminus (Candau *et al*., 1997; Zhu *et al*., 1998b). Both activation domains are required for efficient growth suppression by p53 (Zhu *et al*., 2000). In p73, the N-terminal 54 residues are homologous to the first activation domain in p53 (Chen, 1999; Yang and McKeon, 2000). To characterize the activity of the N-terminal activation domain and to determine whether p73 contains a second activation domain, we generated stable cell lines that inducibly express Flag-tagged p73β(Δ1–54). Two representative cell lines, p73β(Δ1–54)-26 and -30, are shown in Figure 2a. Western blot analysis showed that p73β(Δ1–54) was expressed at a level comparable to that of wild-type p73β in p73β-22 and -24 cells, but p21 was not induced by p73β(Δ1–54), indicating that lack of the N-terminal activation domain renders p73 incapable of transactivating the p21 gene. It should be mentioned that like HA-p73β, Flag-tagged wild-type p73β is active in transactivation and growth suppression (data not shown). Thus, this finding suggests that p73 contains only a single functional activation domain, or that the potential C-terminal activation domain (Takada *et al*., 1999), which is still intact in p73β(Δ1–54), may be involved in regulating a different set of target genes.

To determine whether the transcriptional activity is necessary for p73 to induce growth suppression, we measured the growth rate of p73β(Δ1–54)-producing cells over a 5-day period. As shown in Figure 2b, the growth rate of cells expressing p73β(Δ1–54) was indistinguishable from that of cells not expressing p73β(Δ1–54). To confirm this, we performed trypan blue dye exclusion assay and found that the number of dead cells was not significantly increased upon the expression of p73β(Δ1–54) (Figure 2c). These data indicate that the transcriptional activity is required for p73β-mediated growth suppression.

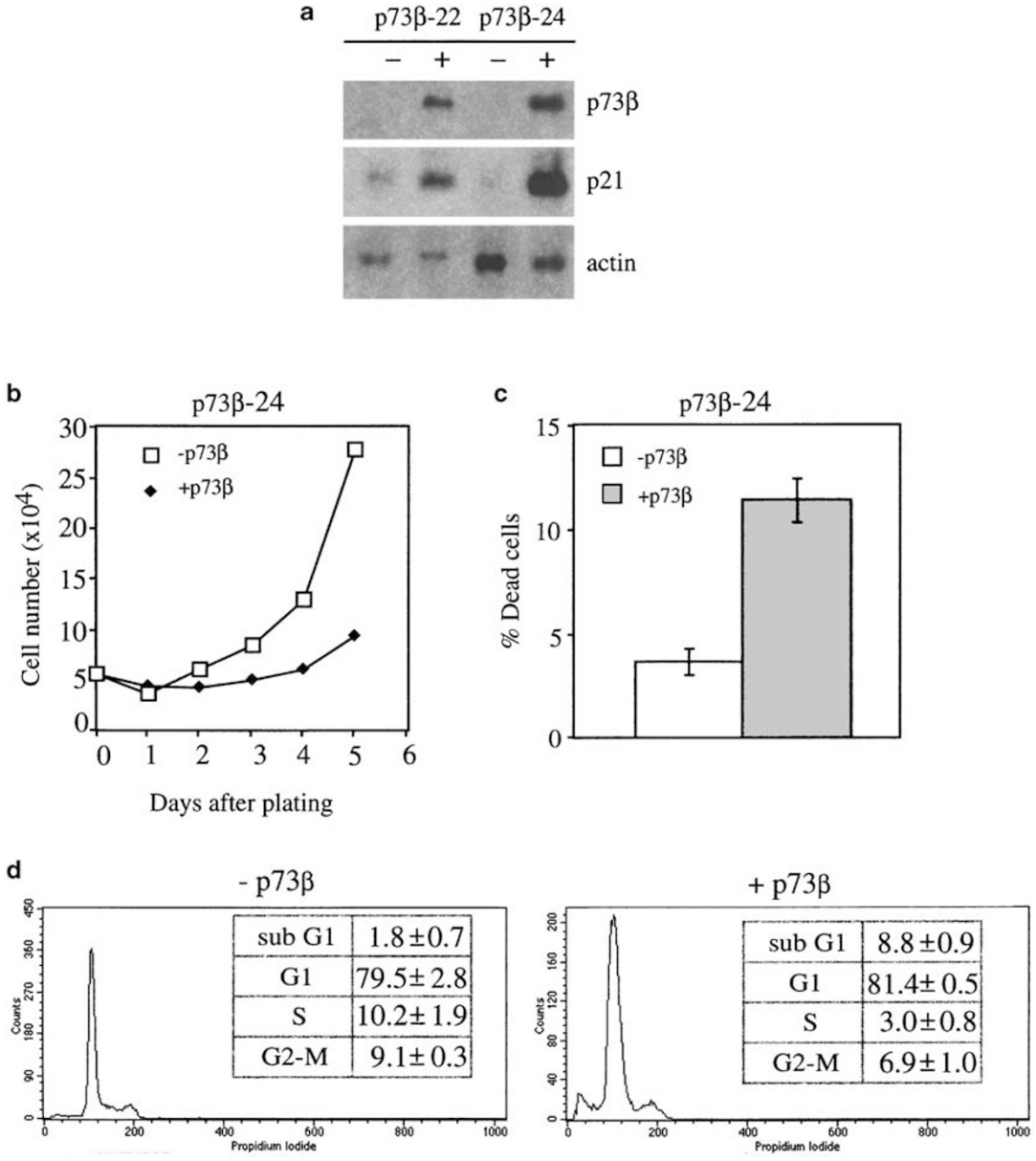

Figure 1 Wild-type p73*β* suppresses cell growth. (**a**) The level of p73*β*, p21, and actin was assayed by Western blot analysis in cell lines as shown above the blots. Cell extracts were prepared from uninduced cells (−) and cells induced to express p73*β* (+) for 24 h. HA-tagged p73*β* was detected with 12CA5 antibody. p21 was detected with anti-p21 polyclonal antibodies (C-19). Actin was detected with antiactin polyclonal antibodies. (**b**) Growth rates of cells in the absence (□) or presence (◆) of p73*β* over a 5-day period. (**c**) Trypan blue dye exclusion assay of cells grown in the absence (−) or presence (+) of p73*β* for 3 days. (**d**) DNA content was quantified by propidum iodide staining of fixed cells that were uninduced (−) or induced (+) to express p73*β* for 3 days

p73 is a transcription factor that functions only in the nucleus. To determine whether deletion of residues 1–54 alters the subcellular localization of p73*β*, which may provide another explanation for the loss of function exhibited by p73*β*(Δ1–54), we performed immunofluorescence assay. As shown in Figure 2d, both p73*β* and p73*β*(Δ1–54) exhibited nuclear localization, indicating that the loss of function by p73*β*(Δ1–54) is not because of altered subcellular localization. Hence, we conclude that p73-mediated transactivation and growth suppression require an intact N-terminal activation domain.

*Two conserved tandem hydrophobic residues in the N-terminal activation domain are not critical for the transcriptional activity of p73*β

The N-terminal activation domain 1 in p53 contain two hydrophobic residues, Leu-22 and Trp-23, which were previously shown to be critical for p53-mediated transactivation (Lin *et al*., 1995). By sequence alignment between the residues in the N-terminal activation domains of p53 and p73, we found that p73 contains two analogous hydrophobic residues, Leu-18 and Trp-19. To determine whether the analogous residues in p73

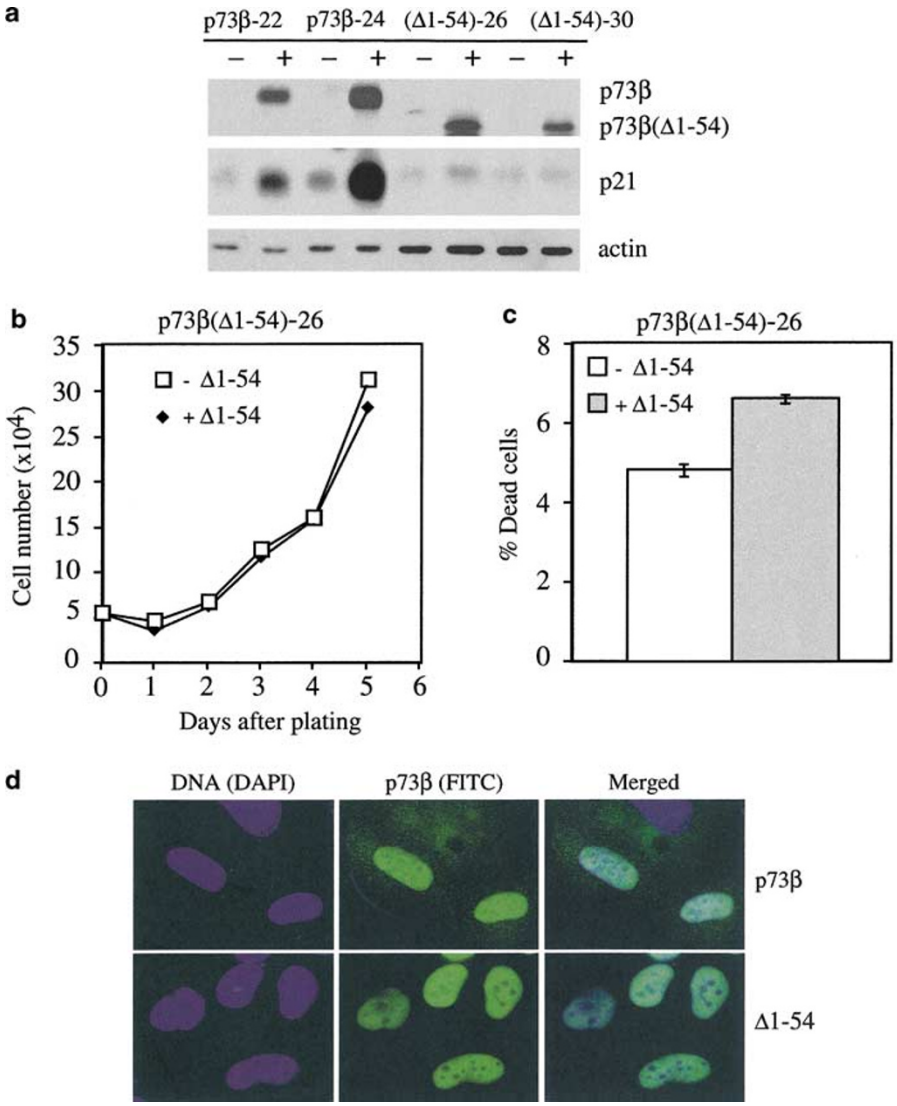

Figure 2 The N-terminal activation domain in p73β is required for transactivation and growth suppression. (**a**) The level of p73β, p21, and actin was assayed by Western blot analysis in cell lines as shown in the absence (–) or presence (+) of p73β for 24 h. p73β was detected with anti-p73β monoclonal antibody (Ab-3). Other antibodies used were as described in the legend to Figure 1. (**b**) Growth rates of cells in the absence (□) or presence (◆) of p73β(Δ1–54) over a 5-day period. (**c**) Trypan blue dye exclusion assay of cells grown in the absence (–) or presence (+) of p73β(Δ1–54) for 3 days. (**d**) Subcellular localization of p73β(Δ1–54). Cells stably expressing p73β and p73β(Δ1–54) were doubly stained with DAPI (blue) and anti-p73 (Ab-3) (green). The stained cells were analysed by fluorescent microscopy

are required for the transcriptional activity, we generated stable cell lines that inducibly express HA-p73β(AD$^-$), wherein the hydrophobic residues Leu-18 and Trp-19 are substituted with hydrophilic residues Gln and Ser, respectively. Two representative cell lines, HA-p73β(AD$^-$)-1 and -15, are shown in Figure 3a. Western blot analysis showed that HA-p73β(AD$^-$) was expressed at a level comparable to that of wild-type p73β in p73β-22 and -24 cells and that p21 was efficiently induced by HA-p73β(AD$^-$). This suggests that Leu-18 and Trp-19 are not critical for p73-mediated transactivation. Next, we performed growth rate analysis and found that HA-p73β(AD$^-$) was still capable of inhibiting cell growth (Figure 3b). To confirm this, we performed trypan blue dye exclusion assays and found that the number of dead cells increased from 2 to 8.5% when p73β(AD$^-$) was expressed (Figure 3c). We also performed DNA histogram analysis (Figure 3d) and found that when cells were induced to express HA-p73β(AD$^-$), the number of cells exhibiting apoptosis increased from 4.1 to 16.1%, which is more than that induced by wild-type p73β. Nevertheless, these data suggest that Leu-18 and Trp-19 are not critical for transactivation and growth suppression.

*p73*β *does not require the region adjacent to the N-terminal activation domain for transactivation and growth suppression*

The second activation domain in p53 is located next to its N-terminal activation domain 1 (Candau *et al*., 1997;

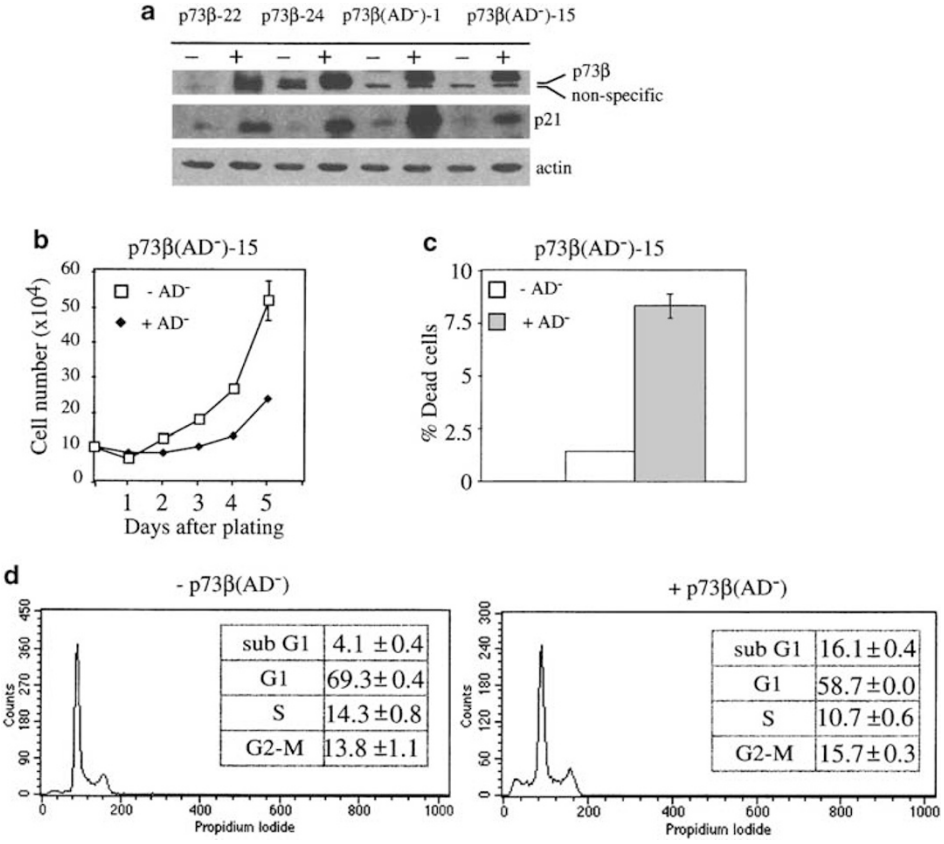

Figure 3 Two tandem hydrophobic residues within the N-terminal activation domain are not critical for the transcriptional activity of p73*β*. (**a**) The level of p73*β*, p21, and actin was assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p73*β* for 24 h. Antibodies used were as described in the legend to Figure 1. (**b**) Growth rates of cells in the absence (□) or presence (◆) of p73*β*(AD⁻) over a 5-day period. (**c**) Trypan blue dye exclusion assay of cells grown in the absence (−) or presence (+) of p73*β*(AD⁻) for 3 days. (**d**) DNA content was quantified by propidum iodide staining of fixed cells that were uninduced (−) or induced (+) to express p73*β*(AD⁻) for 3 days

Zhu *et al*., 1998b). Previously, we have shown that the second activation domain in p53 is required for efficient induction of apoptosis (Zhu *et al*., 1998b, 2000). To determine whether the region adjacent to the N-terminal activation domain may constitute another activation domain, we generated stable cell lines that inducibly express p73*β*(Δ55–82), which lacks 27 amino acids between residues 55 and 82. Two representative cell lines, HA-p73*β* (Δ55–82)-14 and -15, are shown in Figure 4a. Western blot analysis showed that the level of p73*β*(Δ55–82) is expressed at a level comparable to that of wild-type p73*β* in p73*β*-22 and -24 cells and that p21 was highly induced by p73*β*(Δ55–82). To determine whether HA-p73*β*(Δ55–82) can suppress cell proliferation, we measured the growth rate of HA-p73*β*(Δ55–82)-producing cells. As shown in Figure 4b, cells grown in the presence of HA-p73*β*(Δ55–82) grew more slowly than cells grown in the absence of HA-p73*β*(Δ55–82). To confirm this, we performed trypan blue dye exclusion assay and DNA histogram analysis. We found that the number of trypan blue dye positive cells increased from 2.5 to 8% when HA-p73*β*(Δ55–82) was expressed (Figure 4c). Furthermore, as shown in Figure 4d, the percentage of cells exhibiting sub-G1 DNA content increased from 4.7 to 17.2% when HA-p73*β*(Δ55–82) was induced. These data indicate that residues 55–82, which are not critical for p73-mediated growth suppression, are unlikely to constitute an activation domain.

Deletion of the two N-terminal PXXP motifs between residues 84 and 117 does not interfere with p73-mediated apoptosis

Between residues 84–87 and 103–106, p73 contain two N-terminal PXXP motifs, where P represents proline and X represents any amino acid. Sandwiched between these two PXXP motifs is Tyr-99, which can be phosphorylated by c-abl in response to *γ*-irradiation (Yuan *et al*., 1999). Phosphorylation of Tyr-99 correlates with the induction of p73 target genes and apoptosis (Agami *et al*., 1999; Yuan *et al*., 1999). Previously, we and others have shown that the proline-rich domain (PRD) in p53, which consists of five PXXP motifs, is required for p53-mediated apoptosis (Walker and Levine, 1996; Ruaro *et al*., 1997; Sakamuro *et al*., 1997; Venot *et al*., 1998; Zhu *et al*., 1999; Baptiste *et al*., 2002). To determine the activity of the PXXP motifs and Tyr-99, we generated stable cell lines that inducibly express HA-p73*β*(Δ84–117), which lacks Tyr-99 and both PXXP motifs. Two representative cell lines,

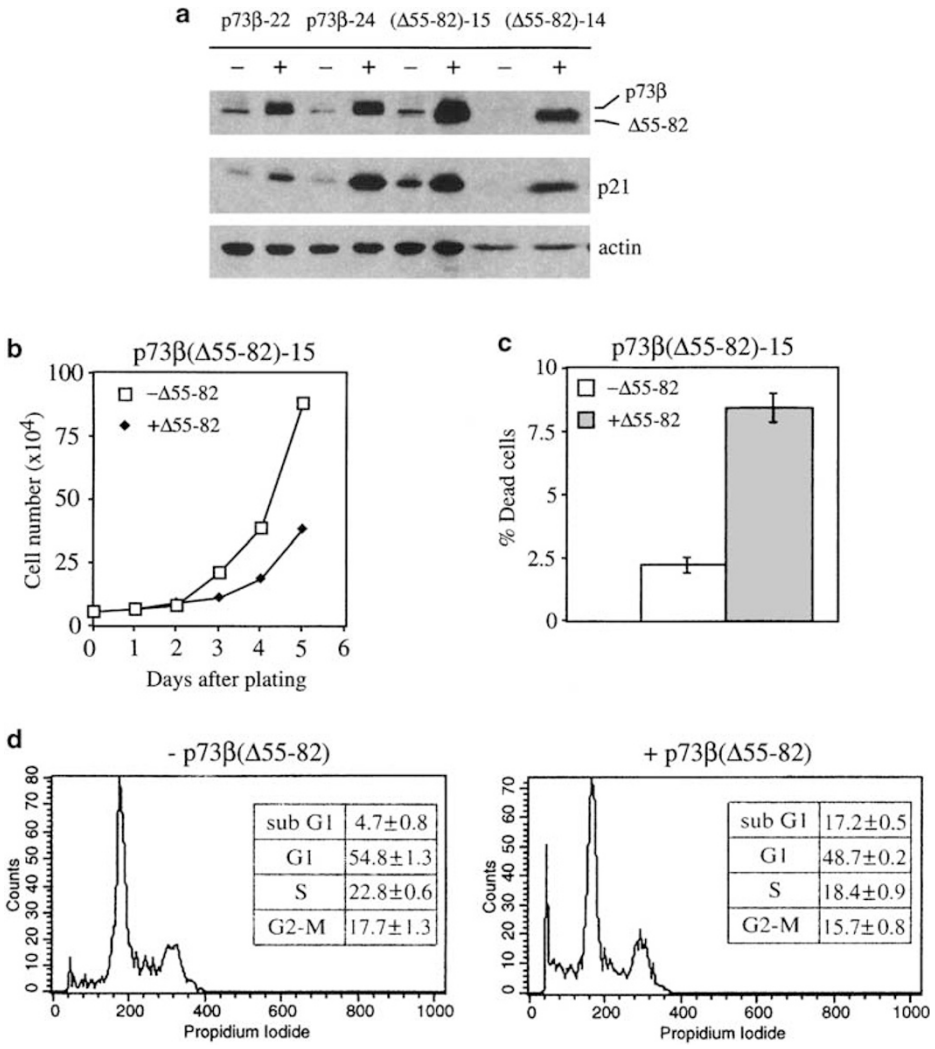

Figure 4 The region adjacent to the N-terminal activation domain in p73β is not required for transactivation or growth suppression. (**a**) The level of p73β, p21, and actin was assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p73β for 24 h. Antibodies used were as described in the legend to Figure 1. (**b**) Growth rates of cells in the absence (□) or presence (♦) of p73β(Δ55–82) over a 5-day period. (**c**) Trypan blue dye exclusion assay of cells grown in the absence (−) or presence (+) of p73β(Δ55–82) for 3 days. (**d**) DNA content was quantified by propidium iodide staining of fixed cells that were uninduced (−) or induced (+) to express p73β(Δ55–82) for 3 days

HA-p73β(Δ84–117)-4 and -23, are shown in Figure 5a. Western blot analysis showed that HA-p73β(Δ84–117) was expressed at a level comparable to that of wild-type p73β in p73β-22 and -24 cells and that p21 was highly induced by HA-p73β(Δ84–117), suggesting that Tyr-99 and the N-terminal PXXP motifs are not critical for the transcriptional activity of p73.

Since a previous study showed that mutation of Tyr-99 to Phe makes p73 less active in inducing apoptosis in response to ionizing radiation by transient transfection assay (Yuan *et al*., 1999), we wanted to determine whether HA-p73β(Δ84–117) is still capable of inhibiting cell growth. To do this, we performed growth rate analysis and found that in the presence of HA-p73β(Δ84–117), cells grew more slowly than did cells in the absence of HA-p73β(Δ84–117) (Figure 5b). To confirm this, we performed trypan blue dye exclusion assays and found that the number of dead cells in the control group was 3% (Figure 5c). However, the number of dead cells increased to 7% when p73β(Δ84–117) was expressed. Additionally, we performed DNA histogram analysis (Figure 5d) and found that when cells were induced to express HA-p73β(Δ84–117), the number of apoptotic cells dramatically increased from 2.4 to 28.5%, which is even slightly higher than that by wild-type p73β (Figure 1d). These data suggest that Tyr-99 and the N-terminal PXXP motifs are not critical for p73-mediated apoptosis.

The PXXP motif between residues 322 and 339 is not required for the activity of p73 in transactivation and growth suppression

Previous studies have shown that the PXXP motif between residues 322 and 339 in p73 directly interacts with the SH3 domain in c-abl (Agami *et al*., 1999; Yuan

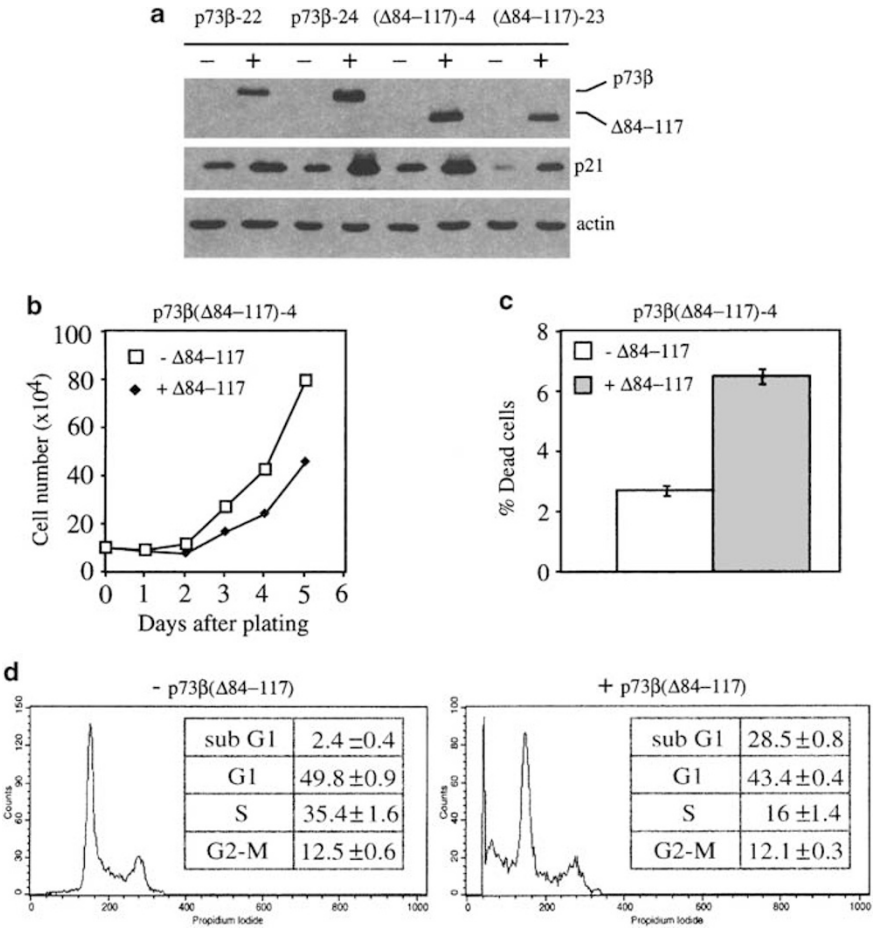

Figure 5 Deletion of the PXXP motifs between residues 84 and 117 has no effect on p73-mediated apoptosis. (**a**) The level of p73*β*, p21, and actin was assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p73*β* for 24 h. Antibodies used were as described in the legend to Figure 1. (**b**) Growth rates of cells in the absence (□) or presence (◆) of p73*β*(Δ84–117) over a 5-day period. (**c**) Trypan blue dye exclusion assay of cells grown in the absence (−) or presence (+) of p73*β*(Δ84–117) for 3 days. (**d**) DNA content was quantified by propidum iodide staining of fixed cells that were uninduced (−) or induced (+) to express p73*β*(Δ84–117) for 3 days

et al., 1999). To further determine the significance of the c-abl/p73 interaction, we generated stable cell lines that inducibly express HA-p73*β*(Δ322–339). Two representative cell lines, HA-p73*β*(Δ322–339)-18 and -19, are shown in Figure 6a. Western blot analysis showed that HA-p73*β*(Δ322–339) was expressed at a level comparable to that of wild-type p73*β* in p73*β*-22 and -24 cells and that p21 was highly induced by HA-p73*β*(Δ322–339). This suggests that the C-terminal PXXP motif, and hence the p73/c-abl interaction, are not required for induction of the p21 gene.

To determine the effect of the C-terminal PXXP motif on cell proliferation, we performed growth rate analysis (Figure 6b) and found that cells completely failed to grow when HA-p73*β*(322–339) was expressed. To confirm this, we performed both trypan blue dye exclusion assay and DNA histogram analysis. As shown in Figure 6c, the number of dead cells increased from 4.5 to 10.3% when grown in the presence of HA-p73*β*(Δ322–339). Moreover, as shown in Figure 6d, in the presence of p73*β*(Δ322–339), the number of cells undergoing apoptosis increased from 2.0 to 20.6%, and the number of cells arrested in G2–M increased from 12.8 to 17.2%. Together, these data indicate that the C-terminal PXXP motif within residues 322–339 is not required for p73-mediated growth suppression.

The tetramerization domain is necessary for the activity of p73

Like p53, p73 has been shown to function as a homotetrameric protein (Davison *et al*., 1999). To characterize the function of the tetramerization domain within residues 346–394, we generated stable cell lines that inducibly express HA-p73*β*(Δ346–394). Two representative cell lines, p73*β*(Δ346–394)-7 and -9, are shown in Figure 7a. Western blot analysis showed that HA-p73*β*(Δ346–394) was expressed at a level comparable to that of wild-type p73*β* in p73*β*-22 and -24 cells, but p21 was not induced by HA-p73*β*(Δ346–394). This suggests that this domain, and hence tetramerization, is required for p73 to function as a transcription factor. Next, we performed growth rate analysis and found that the rates of cell growth in the absence or presence of

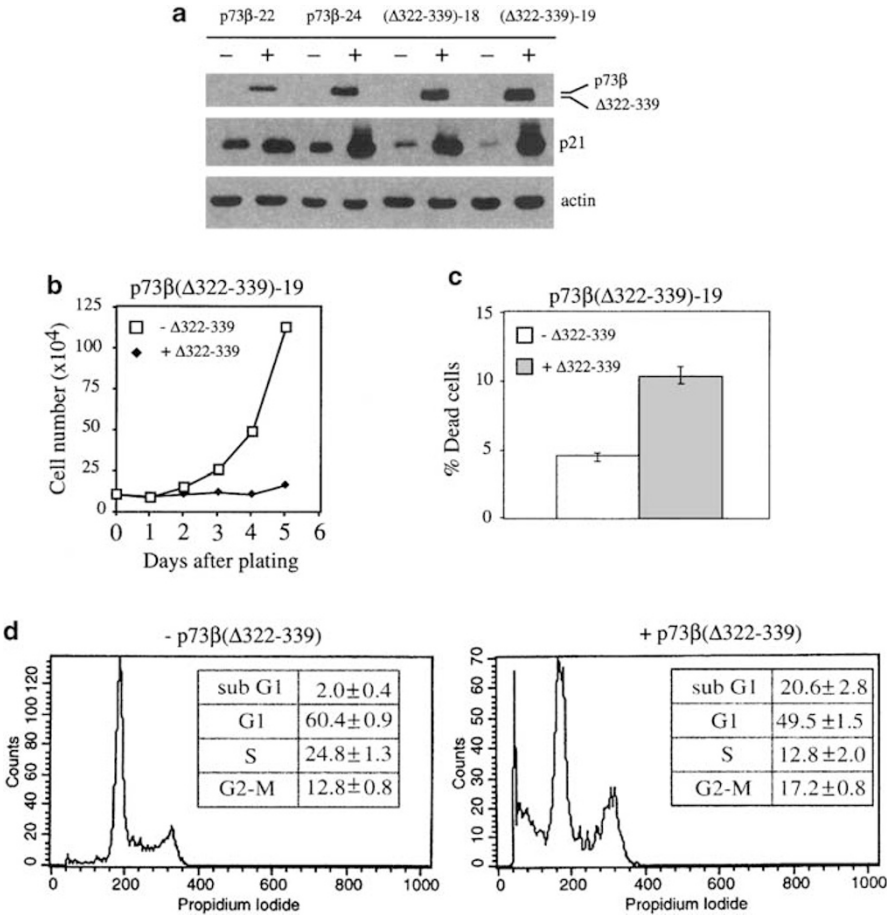

Figure 6 The PXXP motif between residues 322 and 339 is not required for transactivation or growth suppression. (**a**) The level of p73*β*, p21, and actin was assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p73p*β* for 24 h. Antibodies used were as described in the legend to Figure 1. (**b**) Growth rates of cells in the absence (□) or presence (◆) of p73*β*(Δ322–339) over a 5-day period. (**c**) Trypan blue dye exclusion assay of cells grown in the absence (−) or presence (+) of p73*β*(Δ322–339) for 3 days. (**d**) DNA content was quantified by propidum iodide staining of fixed cells that were uninduced (−) or induced (+) to express p73*β*(Δ322–339) for 3 days

HA-p73*β*(Δ346–394) were identical (Figure 7b), suggesting that lack of the tetramerization domain renders p73*β* functionally inert. To confirm this, we performed trypan blue dye exclusion assay (Figure 7c) and found that no significant difference in the number of dead cells was detected in the absence or presence of HA-p73*β*(Δ346–394) expression.

Although the most likely explanation for the loss of function is the inability of HA-p73*β*(Δ346–394) to form a homotetramer, it is also possible that this domain may affect the subcellular localization of p73. To test this, we performed immunofluorescence assay (Figure 7d) and found that while wild-type p73*β* exhibited nuclear localization, HA-p73*β*(Δ346–394) was primarily expressed in the cytoplasm. This result suggests that lack of nuclear localization also contributes to the loss of function for HA-p73*β*(Δ346–394).

To further support the requirement of the tetramerization domain for p73 activity, we compared the induction of the p21 promoter by p73*β* and p73*β*(Δ346–394) using the dual luciferase reporter assay. Luciferase activity was measured in H1299 cells that were transiently transfected with an empty pcDNA3, or pcDNA3 expressing p73*β* or p73*β*(Δ346–394) (Figure 7e), and in stable H1299 cell lines that inducibly express p73*β* or p73*β*(Δ346–394) (Figure 7f). As shown in Figure 7e, we found that the luciferase activity was highly increased by p73*β*. Although the luciferase activity was also increased by p73*β*(Δ346–394), the extent of induction by this mutant is much less than by wild-type p73*β*. When the luciferase activity was measured in stable H1299 cell lines, p73*β*, but not p73*β*(Δ346–394), significantly increased the luciferase activity (Figure 7f). This is consistent with the level of induction of endogenous p21 by p73*β* and p73*β*(Δ346–394) (Figure 7a, p21 panel). It should be mentioned that transient overexpression of p73*β*(Δ346–394) may allow a small percentage of the mutant protein into the nucleus, and thus, leading to expression of the luciferase under the control of the p21 promoter. Taken together, our data showed that the tetramerization domain is necessary for transcriptional activity.

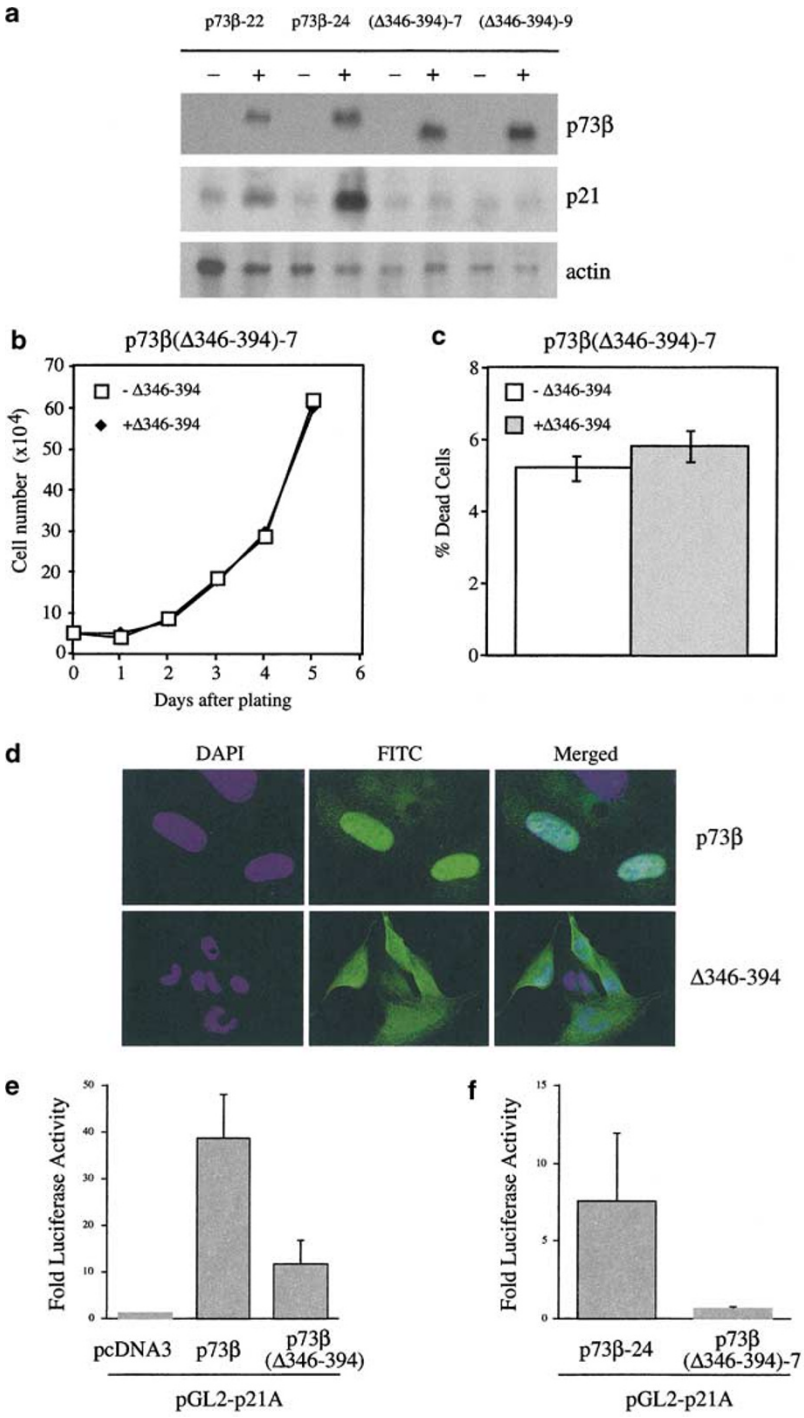

Figure 7 The tetramerization domain within residues 346–394 in p73β is necessary for transactivation, growth suppression, and nuclear localization. (**a**) The level of p73β, p21, and actin was assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p73β for 24 h. Antibodies used were as described in the legend to Figure 1. (**b**) Growth rates of cells in the absence (□) or presence (◆) of p73β(Δ346–394) over a 5-day period. (**c**) Trypan blue dye exclusion assay of cells grown in the absence (−) or presence (+) of p73β(Δ346–394) for 3 days. (**d**) Subcellular localization of p73β(Δ346–394). Cells stably expressing p73β and p73β(Δ346–394) were doubly stained with DAPI (blue) and anti-HA (12CA5) (green). The stained cells were analysed by fluorescent microscopy. (**e**) The luciferase activity under the control of the p21 promoter was increased by p73β and, to a lesser extent, p73β(Δ346–394) using transient transfection assay. (**f**) The luciferase activity under the control of the p21 promoter was increased by p73β, but not p73β(Δ346–394), in stable cell lines that inducibly express p73β and p73β(Δ346–394), respectively

p73 is functional without the entire C-terminal region

To determine the significance of the C-terminal region, we generated stable cell lines that inducibly express HA-p73β(Δ391–499), which lacks the last 109 amino acids in p73β. Two representative cell lines, HA-p73β(Δ391–499)-1 and -7, are shown in Figure 8a. Western blot analysis showed that HA-p73β(Δ391–499) was ex-

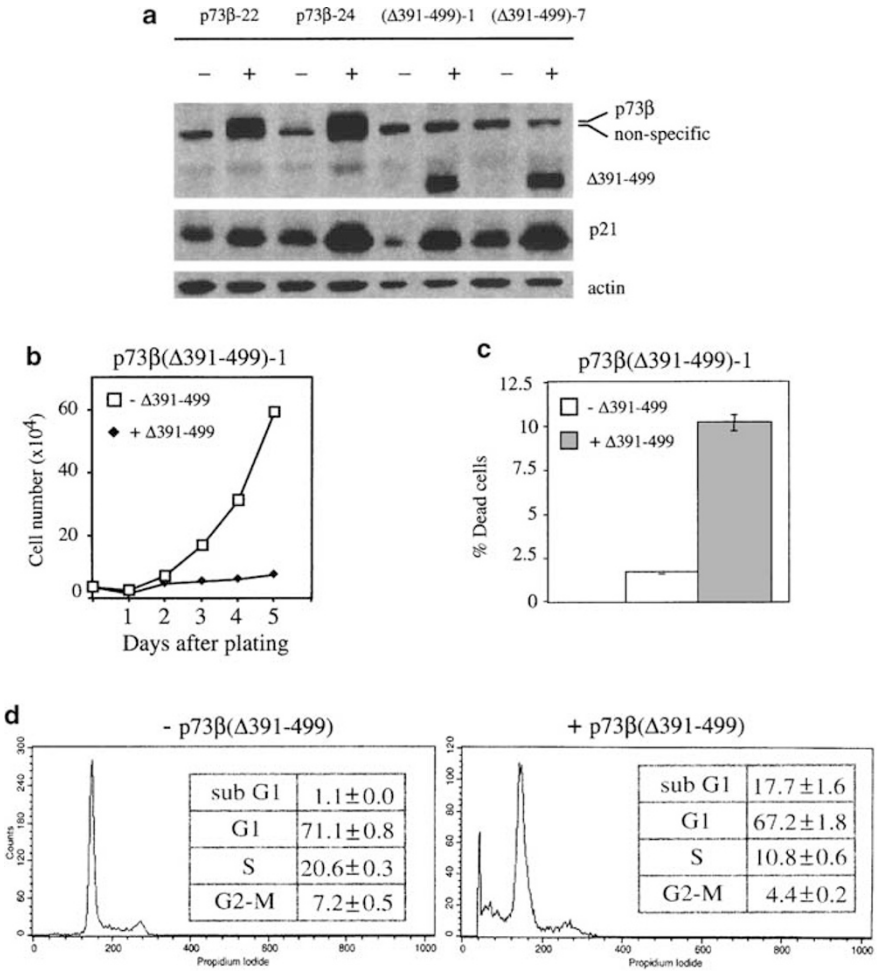

Figure 8 Deletion of the entire C-terminal domain has no effect on p73-mediated growth suppression. (**a**) The level of p73*β*, p21, and actin was assayed by Western blot analysis in cell lines as shown in the absence (−) or presence (+) of p73*β* for 24 h. Antibodies used were as described in the legend to Figure 1. (**b**) Growth rates of cells in the absence (□) or presence (◆) of p73*β*(Δ391–499) over a 5-day period. (**c**) Trypan blue dye exclusion assay of cells grown in the absence (−) or presence (+) of p73*β*(Δ391–499) for 3 days. (**d**) DNA content was quantified by propidum iodide staining of fixed cells that were uninduced (−) or induced (+) to express p73*β* (Δ391–499) for 3 days

pressed at a level comparable to that of wild-type p73*β* in p73*β*-22 and -24 cells and that p21 was highly induced by HA-p73*β*(Δ391–499). Since HA-p73*β*(Δ391–499) is 37 amino acids smaller than the previously characterized p73α(Δ208), which is unable to induce apoptosis (Ozaki *et al*., 1999), we wanted to determine whether HA-p73*β*(Δ391–499) affects cell proliferation. To do this, we measured the rate of cell growth (Figure 8b) and found that in the presence of HA-p73*β*(Δ391–499), cells completely failed to grow. This suggests that HA-p73*β*(Δ391–499) is still capable of negatively regulating cell proliferation. To confirm this, we performed trypan blue dye exclusion assay (Figure 8c) and found that when HA-p73*β*(Δ391–499) was expressed, the number of dead cells increased from 2.4 to 10%. In addition, we performed DNA histogram analysis and found that the number of cells exhibiting a sub-G1 DNA content increased from 1.1 to 17.7% upon the induction of HA-p73*β*(Δ391–499) (Figure 8d). Together, these data indicate that p73*β*(Δ391–499) is capable of inducing apoptosis.

*The p53 responsive element B (Box B) in IGFBP3 is responsive to wild-type p73*β *and some mutants that are competent in inducing apoptosis*

Insulin-like growth factor binding protein 3 (IGFBP3) is a target of p53 and has been implicated in p53-dependent apoptosis (Buckbinder *et al*., 1995; Grimberg, 2000). Within the IGFBP3 gene, one p53-binding site is located in intron 1, called Box A and the second p53-binding site is located in intron 2, called Box B. To determine whether IFGBP3 can be induced by p73, we analysed the luciferase activity under the control of the Box A or B. We found that Box B, but not Box A, was responsive to wild-type p73*β* (Figure 9a, b). We also found that the Box B was responsive to the p73 mutants that are active in inducing apoptosis, that is, p73(AD1$^-$), p73*β*(Δ55–82), p73*β*(Δ84–117), p73*β*(Δ322–339), and p73*β*(Δ391–499) (Figure 9b). However, p73*β*(Δ346–394), which is inactive in inducing apoptosis (Figure 7), was incapable of increasing the luciferase activity (Figure 9b). Since both p73*β*(Δ84–117) and

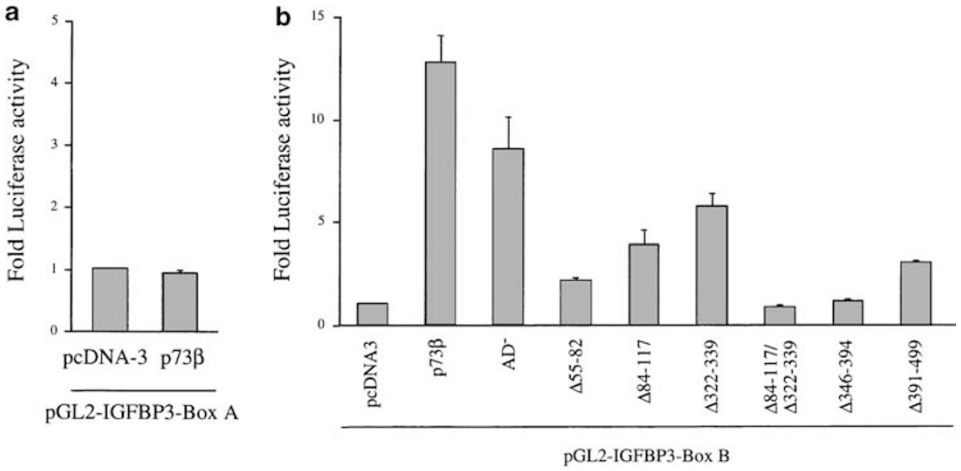

Figure 9 p73β activates the apoptosis gene IGFBP3 through Box B, the p53-binding site within intron 2. (**a**) The p53-binding site Box A within intron 1 of the IGFBP3 gene was not responsive to p73β. The luciferase reporter under the control of Box A was cotransfected with an empty pcDNA3 or pcDNA3 vector expressing wild-type p73β. (**b**) The p53-binding site Box B in IGFBP3 was responsive to wild-type p73β and certain mutants that are active in inducing apoptosis. The luciferase activity under the control of Box B was cotransfected with an empty pcDNA3 or pcDNA3 vector that expresses wild-type p73β or one of the p73β mutants as indicated below the figure

p73β(Δ322–339), which lack the N- and C-terminal PXXP motifs, respectively, are active in inducing apoptosis (Figures 5 and 6) and in increasing the luciferase activity under the control of the Box B from the IGFBP3 gene (Figure 9b), we determined whether p73β(Δ84–117/Δ322–339), which lacks both the N- and the C-terminal PXXP motifs, is transcriptionally active. We found that p73β(Δ84–117/Δ322–339) is inactive in increasing the luciferase activity under the control of the Box B from the IGFBP3 gene (Figure 9b). Additionally, we found that this mutant was inactive in activating the p21 promoter under the control of two p53-binding sites (data not shown). Thus, deletion of both the N- and C-terminal PXXP motifs abrogates the transcriptional activity of p73.

Discussion

In this study, we have analysed the functional domains of p73 by generating a number of stable cell lines that inducibly express p73 and various p73 mutants using the tetracycline-inducible system. Except p73(Δ1–54), which was tagged with a Flag epitope, all other p73 proteins were tagged with an HA epitope and the levels of the p73 proteins expressed in the inducible stable cell lines were compared by Western blot analysis with anti-p73 antibody or anti-HA antibody. Therefore, the activity of various p73β mutants can be compared with wild-type p73β in the cell lines that express a comparable level of p73 proteins. By measuring the effect of various domains on the p73 characteristics, that is, growth suppression, transactivation, and nuclear localization, we have made several novel observations as summarized in Table 1. On the basis of these findings, we conclude that the activity of p73 requires the N-terminal activation domain, the DNA-binding domain, and the tetramerization domain.

N-terminal activation domain

The region of the N-terminal 54 residues in p73 has a considerable similarity to the first activation domain in p53 and thus serves as an activation domain (Chen, 1999; Yang and McKeon, 2000). Here, we showed that deletion of this activation domain completely abolishes the activity of p73 in transactivation and in inducing growth suppression. We also showed that unlike the first activation domain in p53, substitution of two conserved tandem hydrophobic residues with two hydrophilic ones has no effect on the activation domain in p73. In addition, the region adjacent to the activation domain is dispensable for p73 activity, whereas in p53 this region is a part of the second activation domain necessary for inducing apoptosis (Candau *et al*., 1997; Venot *et al*., 1999; Zhu *et al*., 2000). This suggests that the activation domains in p53 and p73 are different, which may be at least in part responsible for differential gene regulation of these two transcription factors (Chen, 1999; Moll *et al*., 2001; Melino *et al*., 2002).

PXXP motifs

Unlike the PXXP motifs in p53 that are necessary for inducing apoptosis (Sakamuro *et al*., 1997; Zhu *et al*., 1999), we have found that the N- or C-terminal PXXP motifs are not required for p73 in transactivation and growth suppression. Our data are different from, but do not necessarily contradict, previous findings (Agami *et al*., 1999; Yuan *et al*., 1999). p73 can be stabilized by DNA damage in a c-abl-dependent manner when cells are treated with cisplatin (Gong *et al*., 1999) and phosphorylated at a tyrosine residue (Tyr-99) by c-abl when cells are γ-irradiated (Agami *et al*., 1999; Yuan *et al*., 1999). In addition, it appears that c-abl directly transduces the DNA damage signals to p73 through its Src homology 3 domain that interacts with the

Table 1 Characterization of p73 functional domains

	AD[1]	*PXXP*[2]	*DBD*[3]	*PXXP TD*[4]	*Transactivation*	*Growth suppression*	*Nuclear localization*
	1 54	84 116		310 345 390 499			
P73β					+	+	+
Δ1–54					–	–	+
AD[5]					+	+	+
Δ55–82					+	+	+
Δ84–117					+	+	+
Δ322–339					+	+	+
Δ84–117/ Δ322–339					–	ND[6]	ND
Δ346–394					–	–	–
Δ391–499					+	+	+

[1]AD, activation domain; [2]PXXP, P is proline and X is any amino acid; [3]DBD, DNA-binding domain; [4]TD, tetramerization domain; [5]AD$^-$, double-point mutation in L18Q and W19S; [6]ND, not done

C-terminal PXXP motif in p73 (Agami *et al*., 1999; Yuan *et al*., 1999). Both stabilization and tyrosine phosphorylation of p73 by c-abl can enhance the transcriptional and proapoptotic activity of p73 (Agami *et al*., 1999; Gong *et al*., 1999; Yuan *et al*., 1999). It should be mentioned that Tyr-99 is sandwiched between two N-terminal PXXP motifs. Thus, we postulate that although the PXXP motifs are potentially required for p73 stabilization and subsequent apoptosis in response to cisplatin or γ-irradiation (Agami *et al*., 1999; Gong *et al*., 1999; Yuan *et al*., 1999), they are dispensable when p73 is overexpressed or activated by other signals. For example, we and other have shown that DNA damage and other stresses can activate p73 transcriptionally (Chen *et al*., 2001; Zaika *et al*., 2001; Nakagawa *et al*., 2002; Vossio *et al*., 2002). Additionally, several other studies showed clearly that p73 is not stabilized in response to various DNA-damaging agents (Fang *et al*., 1999; Zeng *et al*., 1999). Furthermore, it has been shown that the Yes-associated protein (YAP) interacts with, and then activates, p73, but the YAP-p73 interaction is not affected by either c-abl or Tyr-99 phosphorylation (Strano *et al*., 2001). Interestingly, we showed that p73β, which lacks both the N- and C-terminal PXXP motifs, is transcriptionally inert (Figure 9b). Thus, further studies are needed to determine whether such a mutant is also defective in inducing cell cycle arrest and apoptosis.

Tetramerization domain and nuclear localization signal

The region between residues 346 and 394 is 38% identical to p53 tetramerization domain (Chen, 1999; Yang and McKeon, 2000) and has been shown to be necessary for p73 tetramerization (Davison *et al*., 1999). Here, we showed that p73 tetramerization is necessary for p73 activity since deletion of this domain completely abolishes the ability of p73 in transactivation and growth suppression. In addition, we found that this domain is also necessary for the nuclear localization of p73. A recent study showed that a nuclear localization signal exists within residues 344–348 (Inoue *et al*., 2002), which is consistent with our finding. Thus, the tetramerization domain also contains, or overlaps with, the nuclear localization signal.

C-terminal region

The C-terminal region in p73 is much longer than that in p53 (Chen, 1999; Yang and McKeon, 2000). Within this region, a stretch of 21 amino acids within residues 450–470 is 33% identical to the C-terminal basic domain in p53. In the α isoforms of p73, the C-terminal region also contains an SAM domain. In addition, a recent study showed that the region between residues 382–413 and 425–491 has the property of an activation domain (Takada *et al*., 1999). In this study, we showed that deletion of the entire C-terminal region between residues 391 and 499 has no adverse effect on the activity of p73β. Since p73β lacks the SAM domain, our results suggest that the SAM domain, the potential basic domain, and the putative C-terminal activation domain are not required for the activity of p73 in transactivation and in growth suppression. Furthermore, since p73β(Δ1–54) is not competent in transactivation, it

suggests that the potential C-terminal activation domain cannot functionally compensate for the loss of the N-terminal activation domain.

Previously, we and others have shown that the C-terminal basic domain in p53 is necessary for inducing apoptosis (Chen *et al*., 1996; Wang *et al*., 1996). When the first activation domain is deleted, the C-terminal basic domain is not required for p53 in inducing apoptosis (Zhu *et al*., 2000). This has prompted us to hypothesize that there is a functional interaction between these two domains and the C-terminal basic domain is required for maintaining the activation domain competent in transactivation (Zhu *et al*., 2000). In this study, we showed that only one functional activation domain exists in p73 and the two conserved tandem hydrophobic residues in the activation domain are not critical for transactivation. These data indicate that the characteristics of the activation domain in p73 are different from that in p53. Thus, we hypothesize that there is no functional interaction between the activation domain and the C-terminal region, and the C-terminal region does not regulate p73 transcriptional activity, especially when p73 is overexpressed or activated independently of the C-terminal region. However, we cannot rule out the possibility that the C-terminal region in the α isoform may negatively regulate p73α since its activity is always less than p73β activity (Chen, 1999; Irwin and Kaelin, 2001; Moll *et al*., 2001; Yang *et al*., 2002). Therefore, further studies are needed to identify and characterize a potential negative domain in p73α.

In summary, we have found that various domains in p73 function in a manner both similar to, and different from, other p53 family members. Combined with the expression profile of p73 primarily in epithelial cells, the unique characteristics of p73 functional domains may be responsible for its differential target gene regulation and biological activity in neuronal development and the pheromonal and inflammatory response. The unique p73 functional domains may also be responsible for its distinctive regulation by various viral and cellular proteins and stress signals. Therefore, our findings in this study will provide an insight to further characterize the function and regulation of the p73 protein.

Materials and methods

Plasmids and mutagenesis

Mutant p73β constructs were generated by polymerase chain reaction using the full-length wild-type p73β cDNA as a template. The p73β(Δ1–54) protein is tagged at its N-terminus with a FLAG peptide recognizable by anti-Flag antibody. All other p73 proteins are tagged at their N termini with an influenza hemagglutinin (HA) peptide recognizable by anti-HA antibody 12CA5. HA-tagged wild-type p73β was generated using 5′ end primer p73HA (5′ AAG GAT CCA CCA TGT ACC CAT ACG ATG TTC CAG ATT ACG CTG CCC AGT CCA CCG CCA CC 3′) and 3′ end primer p73-3 (5′ GGT GTT GGA GGG GAT GAC AGG 3′). To generate Flag-p73β(Δ1–54), a cDNA fragment lacking amino acids 1–54 was amplified using 5′ end primer Flag-p73(Δ1–54) (5′ AA CCG CGG ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG GAG GGC ATG ACT ACA TCT GAG GGC ATG ACT ACA TCT GTC ATG 3′) and 3′ end primer p73-3. To generate HA-p73β(AD$^-$), a cDNA fragment encoding amino acids 1–19 with two point mutations (L18Q and W19S) was amplified and ligated through an internal *Sac*I site to a cDNA encoding amino acids 20–499. The cDNA fragment encoding amino acids 1–19 was amplified using 5′ end primer p73HA and 3′ end primer p73β(ADptmt)-3 (5′ GCT GCT TGG TTC CAG AGA GCT CGA CTG GTG 3′). To generate HA-p73β(Δ55–82), a cDNA fragment encoding amino acids 48–499 but lacking amino acids 55–82 was generated using 5′ end primer ΔAD2 (5′ AGC ATG GAC GTC TTC CAC CTG AGC CCC TAC ACC CCA GAG CAC 3′) and 3′ end primer p73-3. This fragment was digested with *Bbs*I and *Eco*RI and used to replace the corresponding region in HA-p73β. To generate HA-p73β(Δ84–117), a cDNA fragment encoding amino acids 68–251 but lacking amino acids 84–117 was generated using 5′ end primer Δ84–5 (5′ GCT GAG CAG CAC CAT GGA CCA GAT GAG CAG CCG CGC GGC CTC GGC CAG CAA CAC CGA CTA CCC CGG A 3′) and 3′ end primer Δ84–3 (5′ GGT GGT GAA TTC CGT CCC CAC 3′). This fragment was digested with *Dde*I and *Eco*RI and used to replace the corresponding region in HA-p73β. To generate HA-p73β (Δ322–339), cDNA fragments encoding amino acids 1–322 and 339–499 were amplified independently and ligated through an internal *Hind*III site. The cDNA fragment encoding amino acids 1–322 was generated using 5′ end primer p73HA and 3′ end primer C322 (5′ GGA AGC TTG GCG GAG CTC TCG TTC AG 3′). The cDNA fragment encoding amino acids 339–499 was generated using 5′ end primer C339 (5′ AAA AGC TTG GTG CAG GTG TGA AGA AGC GG 3′) and 3′ end primer p73-3. To generate HA-p73β(Δ346–394), a cDNA fragments encoding amino acids 1–346 and 394–499 were amplified independently and ligated through an internal *Hind*III site. The cDNA fragment encoding amino acids 1–346 was generated using 5′ end primer p73HA and 3′ end primer C346 (5′ AAA AGC TTC AGA CCG GCA CCA AGG GCG 3′). The cDNA fragment encoding amino acids 394–499 was generated using 5′ end primer C394 (5′ AAA AGC TTC TAC AGA GGC CGA GTC AC 3′) and 3′ end primer p73-3. To generate HA-p73β(Δ391–499), a cDNA fragment encoding amino acids 1–391 was generated using 5′ end primer p73HA and 3′ end primer C391 (5′ AAG GAT CCT CAC GGA TAG GAG TCC ACC AG 3′). Mutations were confirmed by DNA sequencing. cDNAs were cloned separately into a tetracycline-regulated expression vector, pUHD10-3, at its *Bam*HI site and the resulting plasmids were used to generate stable cell lines.

Cell lines

The culture, transfection, and generation of H1299 cell lines were performed as previously described (Chen *et al*., 1996). Individual clones were screened for inducible expression of the p73 protein by Western blot analysis using monoclonal antibodies against the HA or Flag epitope.

Western blot analysis

Cells were collected from plates in phosphate-buffered saline, resuspended with 2$\times$ sample buffer, and boiled for 5 min. Western blot analysis was performed as described previously (Nozell and Chen, 2002). The affinity-purified monoclonal antibody against p73 (p73-3) was purchased from Oncogene Science (Cambridge, MA, USA). Affinity-purified anti-p21 (C-19) polyclonal antibodies was purchased from Santa Cruz (Santa Cruz, CA, USA). The monoclonal antibody against the Flag epitope (anti-Flag) and the affinity-purified anti-actin

polyclonal antibodies were purchased from Sigma (St Louis, MO, USA). The monoclonal antibody used to detect the hemagluttinin epitope, 12CA5, was purchased from Boehringer Mannheim (Germany).

Growth rate analysis

To determine the rate of cell growth, cells were seeded at approximately 6×10^4 cells/60-mm plate in the presence or absence of tetracycline (2 μg/ml) to regulate the expression of the protein of interest. The medium was replaced every 72 h. At times indicated, two plates were rinsed twice with PBS to remove dead cells and debris. Live cells on the plates were trypsinized and collected separately. Cells from each plate were counted four times using the Coulter cell counter. The average number of cells from two plates was used for growth rate determination.

DNA histogram analysis

Cells were seeded at 2×10^5 per 90-mm plate in the presence or absence of tetracycline. At 72 h after plating, both floating and dead cells in the medium and live cells on the plate were collected and fixed with 2 ml of 100% ethanol for at least 2 h and then centrifuged and resuspended in 1 ml of PBS solution containing 50 μg/ml each of RNase A (Sigma) and propidium iodide (Sigma). The stained cells were analysed in a fluorescence-activated cell sorter (FACS) (FACSCaliber, Becton Dickinson) within 4 h. The percentage of cells in sub-G1, G0–G1, S, and G2–M phases was determined using the Cell Quest and ModFit programs. The percentage of cells in sub-G1 phase was used as an index for the degree of apoptosis.

Trypan blue dye exclusion assay

Cells were seeded at approximately 6×10^4 cells/60-mm plate in the presence or absence of tetracycline for 3 days. At 72 h after plating, both floating cells in the medium and live cells on the plate were collected and concentrated by centrifugation. After staining with trypan blue (Sigma) for 15 min, both live (unstained) and dead (stained) cells were counted two times in a hemocytometer. The percentage of dead cells was calculated as the number of dead cells divided by the total number of cells counted.

Fluorescent microscopy

Cells were seeded on eight-well chamber slides and grown overnight in the absence or presence of tetracycline. Cells were washed with PBS, fixed with 10% formalin, and permeabilized with 1% NP-40. The cells were blocked with 15% bovine serum albumin (BSA) and incubated with mouse anti-p73 antibody (p73-3) (Oncogene) or mouse anti-HA antibody (Sigma, St Louis, MO, USA) followed by FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 30 min at room temperature. Nuclei were visualized by labeling with 4′, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Inc., Eugene, OR, USA). For all experiments, coverslips were mounted with Prolong (Molecular Probes, Inc., Eugene, OR, USA) and analysed by fluorescent microscopy.

Luciferase assay

HA-p73β was cloned independently into pcDNA3 at the *Bam*HI site. The cDNA fragments encoding HA-p73 β (AD$^-$), HA-p73β (Δ55–82), HA-p73β (Δ84–117), HA-p73β (Δ322–339), HA-p73β (Δ346–394), and HA-p73β (Δ391–499) were cleaved from their respective pUHD10-3 plasmids with *Bam*HI and cloned into pcDNA3 at the *Bam*HI site. HA-p73β (Δ84–117/Δ322–339), which lacks both the N- and C-terminal PXXP motifs, was cloned into pcDNA3 using the 459-bp *Bam*HI/*Bgl*I fragment from the pUHD10-3 plasmid encoding the N-terminal 153 amino acids with the deletion of Δ84–117 and the 987 bp *Bgl*I/*Bam*HI fragment from the pUHD10-3 plasmid encoding the C-terminal 329 amino acids with the deletion of Δ322–339. The dual luciferase assay was performed using H1299 cells or stable H1299 cell lines inducibly expressing HA-p73β or HA-p73β(Δ346–394). The luciferase reporter constructs used were pGL2/p21-A (Chinery *et al*., 1997), which is regulated by the p21 promoter with two p53-binding sites; pGL2/IGFBP3-Box A (Buckbinder *et al*., 1995), which contains the p53-binding site within intron 1 of the IGFBP3 gene; or pGL2/IGFBP3-Box B (Buckbinder *et al*., 1995), which contains the p53-binding site within intron 2 of the IGFBP3 gene. In all, 1 μg of a luciferase reporter, 1 μg pcDNA3 control vector or pcDNA3 vector that expresses HA-p73β or various p73β mutants, and 25 ng of renilla luciferase assay vector pRL-CMV (Promega, Madison, WI, USA) were cotransfected into H1299 cells. Dual luciferase assay was performed in triplicate according to the manufacturer's instructions (Promega, Madison, WI, USA). The fold increase in relative luciferase activity is a product of the luciferase activity induced by p73β or various p73β mutants divided by that induced by an empty pcDNA3 vector. For measuring the luciferase activity in H1299 stable cell lines, 1 μg of pGL2/p21-A reporter and 25 ng of pRL-CMV were cotransfected into p73β-24 that inducibly expresses wild-type p73 or p73β(Δ346–394)-7 that inducibly expresses p73β(Δ346–394) in the presence of tetracycline. At 24 h post-transfection, the cells were split 1 : 2 to be grown in the absence or presence of tetracycline (p73β or p73β(Δ346–394)). At 24 h postinduction, the dual luciferase assay was performed. The fold increase in relative luciferase activity is a product of the luciferase activity in the presence of p73β or p73β(Δ346–394) divided by the luciferase activity in the absence of p73β or p73β(Δ346–394).

Acknowledgements
We thank Ebbie Shafizadeh for cloning human p73β, Steve Anander and Rob Fang for generating some p73 mutant constructs, and Yiman Zheng for technical assistance. This work is supported in part by NIH Grant CA 81237.

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