Tumor Suppressors p53, p63TAα, p63TAy, p73α, and p73β Use Distinct Pathways to Repress Telomerase Expression*

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Background: Inactivation of p53 and reactivation of telomerase expression are two of the most common events in human cancer.

Results: Here we report the mechanisms used by p53, p63, and p73 to suppress telomerase expression.

Conclusion: p53, p63, and p73 suppress telomerase expression through distinct pathways that involve E2F, E-box, c-Myc, and NF-YB.

Significance: This study provides new insights into the cellular pathways used by p53, p63, and p73 to suppress hTERT expression.

The promoter of the telomerase catalytic subunit (TERT) is subject to tight regulation and remains repressed in somatic cells to ensure their limited life span and to prevent tumor initiation. Here we report that the hTERT promoter is strongly repressed by p53 and the related family members p63 and p73. We found that p53-mediated repression was different in human and mouse cells and occurred through p53-dependent transcription inhibition of c-Myc or through E-box/E2F pathways, respectively. Although p63TAy-mediated repression occurred through SP1, p63TAy-mediated repression occurred through E2F signaling. Finally, p73α- and p73β-mediated repression occurred through NF-YB2. Our results show a complex multifactorial mechanism used by p53 and its family members to keep hTERT expression under tight control.

Telomeres, which form the ends of eukaryotic chromosomes, help to preserve genome integrity and to prevent replicative senescence (1–3). Somatic cells have a finite proliferative capacity largely due to the inability of DNA polymerase to replicate the distal ends of chromosomes, which leads to a progressive shortening of the telomeres after each cell division. Activation of human telomerase, an RNA-dependent DNA polymerase that elongates telomeres through expression of its catalytic subunit hTERT,3 is essential for avoiding telomere shortening and replicative senescence (4, 5). Despite a critical role of telomerase activation in cancer initiation and progression, regulation of the hTERT promoter is still insufficiently characterized (6–9). A number of transcriptional factors and signaling pathways, often activated in cancer cells, have been shown to positively regulate telomerase expression by E2F, E-box, c-Myc, and NF-YB. Therefore, absence of these factors repress hTERT promoter expression. In this regard, c-Myc and SP1, two positive regulators (10), are involved in nuclear factor (NF)-κB-mediated activation of the hTERT promoter (11). Additional studies have demonstrated that upstream stimulatory factor, STAT3, PI3K, and nuclear factor of activated T-cells can also stimulate hTERT promoter expression (12–16). In contrast, Mad, histone deacetylases, E2F1, TAK1, WT1, Smad3, Menin, and E-box signaling can negatively regulate hTERT promoter expression (17–27). Increased expression of wild type p53 has also been shown to repress telomerase expression in various cancer cell lines independently of its effects on growth arrest and apoptosis (28). p53 operates as both a transcriptional activator and repressor. In response to cellular stress or DNA damage, activation of p53 expression leads to an increased expression of numerous target genes involved in the cell cycle, DNA repair, and survival (29). Several studies have also established a link between transcriptionally active p53 and activation of cellular senescence programs. p53 family members p63 and p73 are produced as several isoforms by alternative splicing and can be produced as full-length proteins containing the transactivation domain (TA) and ΔN proteins missing the TA (30). As opposed to p53 knock-outs, p63 and p73 knock-outs exhibit severe developmental abnormalities but no increased cancer susceptibility. Although p63 and p73 are rarely mutated in human cancers, frequent epigenetic silencing of p73 has been reported in leukemias, lymphomas, and brain tumors (31–33).

In this study, we examined the mechanistic aspects of p53-mediated repression of the hTERT promoter. Our results demonstrate that, in contrast to what has been reported previously, p53-mediated repression is largely independent of SP1 binding sites in the hTERT promoter. Instead, p53-induced repression of the hTERT promoter occurs through distinct indirect transcriptional and non-transcriptional mechanisms and is mediated by E-box and E2F pathways. We further extended our studies by investigating the role of other p53 family members, p63TAα, p63TAy, p73α, and p73β. We found that all these factors repress hTERT promoter expression. Promoter studies revealed that p63TAα-mediated repression requires SP1 because it had no effect in MEF sp1 KO cells and could no longer suppress an hTERT-luciferase reporter construct in

3 The abbreviations used are: hTERT, human telomerase catalytic subunit; MEF, mouse embryonic fibroblast; TSA, trichostatin A; NF, nuclear factor; TRAP, telomeric repeat amplification protocol; pRb, retinoblastoma protein; Luc, luciferase; TA, transactivation domain.

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which all SP1 binding sites had been mutated. In contrast, p63TAy-mediated repression was mediated by E2F signaling, and mutations of E2F binding sites in an hTERT-luciferase reporter construct prevented p63TAy repressive effects. Both p73/H9251- and p73/H9252-mediated repression was independent of SP1, E-box, or E2F sites in the hTERT promoter. We found that p73/H9251- and p73/H9252-mediated repression was rescued by trichostatin A (TSA) treatment. Similarly to what has been observed on the platelet-derived growth factor/H9252 receptor promoter, p73/H9251 and p73/H9252 required NF-YB2 to repress the hTERT promoter.

EXPERIMENTAL PROCEDURES

Plasmids—Wild type hTERT promoter-luciferase reporter construct p330-hTERT-Luc has been described previously. E-box-mutated hTERT promoter-luciferase reporters hTERT 24/25, hTERT 24/25 1A, hTERT 24/25 1B, and hTERT 24/25 1C were provided by N. Keith (University of Glasgow) (34). p63TAα-, p63TAγ-, p63ΔNα-, p63ΔNγ-, p73α-, and p73β-expressing vectors were provided by K. Engeland (University Leipzig) (35, 36) and C. Geisen (Dana-Farber Cancer Institute) (37). pGL13-Luc reporter vector was used for measuring the transcriptional activity of p53, p63, and p73 constructs. CMV-SP1 was purchased from Addgene, and CMV-SP3 was obtained from G. Suske (University of Marburg) (38). Alternatively, SP1 and SP3 were tagged with 3HA by cloning into the pMH vector. NF-YB1 and NF-YB2 shRNA expression vectors were provided by C. Imbriano (University of Modena) (39).

Cloning of hTERT Promoter with Five SP1 Site Mutations into PGL3-Basic and Site-directed Mutagenesis of Other Sites—The wild type and substituted sequence with five SP1 sites mutated (10) were chemically synthesized as duplex oligonucleotides by Integrated DNA Technologies. Sequences were amplified by PCR, cloned into the PGL3-Basic vector, and sequenced using GW2 primer. These vectors are referred to as hTERT-SP1-5M and hTERT (wild type sequence counterpart). hTERT-E2FM was constructed by site-directed mutagenesis disruption of the E2F binding site using wild type hTERT-SP1 as a template. hTERT-E-box/E2FM was constructed by site-directed mutagenesis of the proximal and distal E-boxes present in the hTERT-SP1 vector.

Cell Lines—MEF cell lines were maintained in complete Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and gentamicin. MEF p53 KO, p63 KO, and p73 KO were provided by Dr. E. Flore (M. D. Anderson Cancer Center). MEF sp1 KO and sp3 KO cell lines were provided by J. Boss (Emory Univer-
sity) (40) and G. Suske (University of Marburg) (38), respectively. The human HEK cell line (ATCC) was maintained in complete DMEM supplemented with 10% FBS, penicillin, streptomycin, and gentamicin. For c-myc transfection experiments, 20% serum was added to prevent toxicity.

Transfection and Luciferase Assays—MEF cell transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. HEK cells were transfected using Polyfect (Qiagen) or calcium phosphate (Invitrogen) according to the manufacturers’ instructions. Cells were inoculated onto 6-well plates 1 day before transfections, and cells were harvested after 24–48 h. Assays were performed using the Luciferase Assay system (Promega) and a Berthold Junior luminometer (Oak Ridge, TN). c-myc, sp1, and p21waf siRNAs were obtained from Ambion and transfected into HEK cells with Lipofectamine (Invitrogen).

Telomeric Repeat Amplification Protocol (TRAP) and Real Time PCR—To determine telomerase activity, cells were lysed on ice in CHAPS lysis buffer and used in TRAP assays using the Trapeze Telomerase Detection kit (Chemicon) as described by the manufacturer. For real time quantitative PCR, RNA was extracted with TRIzol reagent (Invitrogen), treated with DNase I (Roche Applied Science), and reverse transcribed using the RNA-to-cDNA kit (Invitrogen). cDNA was used in real time PCRs using SYBR Green Master Mix (Qiagen) using the StepOnePlus real time PCR machine (Applied Biosystems). The following primers were used: hTERT (F), 5′-TGACACCTCACCCTCACCCAC-3′; hTERT (R), 5′-CAGGTTCTCAGGCAAGTTCAC-3′; c-myc (F), 5′-CGTCTCCACATCACGAACCAAA-3′; c-myc (R), 5′-CTTTGGCCAGCAGGATAGTCTT-3′; GAPDH (F), 5′-GAAGAGTGAAGGTCGGAGTC-3′; and GAPDH (R), 5′-GAAGATGGTGATGGGATTTC-3′.

Western Blots—Cells were lysed on ice in passive lysis buffer (Promega) or radioimmune precipitation assay buffer. Protein concentrations were quantified using the Bradford protein assay (54) and run on SDS-acrylamide gels. Proteins were transferred onto PVDF membrane and incubated with the following antibodies: p53 (FL-393), SP1 (5931S, Cell Signaling Technology), myc ((E10, Roche Applied Science), HA (3F10, Roche Applied Science), c-Myc (A-14), p63 (4-A4), p21WAF (C-19), and Actin (C-11). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology unless stated otherwise. Proteins were visualized using Pierce chemiluminescent solutions.

RESULTS

Repression of hTERT Promoter by p53 Family Members Is Independent of SP1—We investigated the ability of p53, p63TAα, p63TAγ, p63∆Nα, p63∆Nγ, p73α, or p73β to repress hTERT promoter activity in the absence or presence of SP1 or SP3. Western blots were performed for each set of p53 family members to demonstrate the level of each transfected plasmid. C and D, hTERT promoter-reporter construct p330 was transfected in MEF cells along with plasmids expressing p53, p63TAα, p63TAγ, p63∆Nα, p63∆Nγ, p73α, or p73β in MEF sp1 KO or sp3 KO cells, respectively. Results are the average percentages derived from three independent transfections, and error bars represent S.D.
regulate the hTERT promoter and found that all p53 family members have strong repressive effects albeit to various extents (Fig. 1A). Repression of the hTERT promoter did not correlate with transcriptional activities as demonstrated using the reporter plasmid (pGL13-Luc) (Fig. 1B). In fact, consistent with previous results, p73α/H9251 and p73α/H9252 have very distinct transcriptional potentials, whereas their effect on the hTERT promoter was similar. We found no significant additive effect among p53, p63, or p73 members when used in various combinations (Fig. 1C). These results were validated in MEF p53 KO, p63 KO, and p73 KO cells to test for any cooperation requirement of different p53 family members in suppressing the hTERT promoter. Our results confirmed that p53 represses the hTERT promoter in MEF wild type, p63 KO, and p73 KO cells (Fig. 1D). Similarly, p63 and p73 did not require the presence of additional p53-related factors to exert their repressive effects on the hTERT promoter (Fig. 1, E and F).

Prior studies suggest that p53 and p73β repress the hTERT promoter by forming complexes with SP1 that are recruited onto SP1 binding sites within the hTERT promoter (41, 42). These studies relied largely on the observation that p53 and p73β had minimal effects on an hTERT-Luc reporter vector when mutation of all SP1 binding sites was introduced. The caveat to these experiments is that mutation of the SP1 sites leads to a drastic overall decrease in hTERT promoter activity close to background levels, and therefore repression by p53 or p73β from those levels can no longer be appreciated to their fullest. Indeed, in both of these studies, the authors still observed near 50% repression of the SP1-mutated hTERT promoter by either p53 or p73β despite the low luciferase levels. In general, our results do not support the involvement of SP1 binding sites in p53- or p73-mediated suppression of hTERT promoter expression. Although exogenously added SP1 may appear to prevent p53-mediated repression of the hTERT promoter (Fig. 2A), this was not specific and was explained by the 6-fold stimulation of the hTERT promoter by SP1 in the absence of p53 (Fig. 2B). In fact, we still observed a 4-fold repression of the hTERT promoter by p53 even in the presence

![Diagram](attachment://diagram.png)

**FIGURE 3.** p63TAα is unable to repress hTERT promoter mutated for SP1 binding sites. A, schematic representation of hTERT promoter construct with all five SP1 binding sites mutated (hTERT-SP1-5M). B, repression of hTERT promoter-reporter construct p330-hTERT-SP1 and mutated vector hTERT-SP1-5M was analyzed in MEF cells transfected with plasmids expressing p53, p63TAα, p63TAγ, p73α, or p73β. Results are representative of three independent transfections. Error bars represent S.D. C, Western blots indicate the expression level of each p53 family member used in the luciferase reporter assays in B. D, p63TAα represses endogenous hTERT expression. Two to 4 μg of p63TAα was transfected into HEK cells. To examine only the cells transfected with p63TAα, CD8 plasmid was also co-transfected, and positive cells were selected with CD8 Dynabeads. Real-time PCR was performed for hTERT expression using GAPDH amplification for normalization. E, p63TAα decreases telomerase activity in HEK cells. TRAP assays were performed on HEK cells transfected and selected as described in D. F, hTERT expression following p63TAα and sp1 siRNA transfection. HEK cells were transfected with p63TAα and CD8 expression plasmids. Three hours later, the cells were retransfected with 20 nm sp1 siRNA. Positively transfected cells were sorted as stated in D for hTERT expression. A Western blot indicates knockdown of SP1 expression in HEK cells following transfection of 20 nm sp1 siRNA. dH2O, distilled H2O.
of excess SP1 (Fig. 2B). SP3 had no significant effect on the hTERT promoter. Consistent with this observation, we found that p53, p73α, and p73β are able to repress the hTERT promoter even in the presence of a large excess of exogenously added SP1, suggesting that SP1 cannot rescue the repressive functions of either p53 or p73 (Fig. 2B). Also consistent with these results, we found that p53, p73α, and p73β are able to repress the hTERT promoter in sp1 and sp3 KO MEF cells (Fig. 2, C and D). Finally, mutations in all five SP1 binding sites in the hTERT promoter had a negligible impact on p53- or p73-mediated repression (Fig. 3, A and B). In contrast to the other p53 family members, p63TA/ was the only one for which transcriptional repression of the hTERT promoter occurred through SP1. In support of this conclusion, p63TA/ was able to repress the hTERT promoter in MEF wild type and sp3 KO cells but not in sp1 KO cells (Figs. 2, C and D, and 3B). Similarly, exogenously expressed p63TA/ had no effect on an hTERT promoter-reporter with mutated SP1 binding sites (Fig. 3B). To verify that p63TAα could repress not only the exogenously added hTERT promoter but also the human endogenous hTERT promoter, we performed quantitative real time RT-PCR for hTERT gene expression and found that p63TAα could repress endogenous hTERT mRNA expression in a dose-dependent manner (Fig. 3D). In addition, p63TAα inhibited endogenous telomerase activity as measured by TRAP assays (Fig. 3E). Finally, to confirm that p63TAα-mediated repression of endogenous hTERT expression involves SP1, we transfected HEK cells with sp1 siRNA. We found that p63TAα could no longer repress endogenous hTERT expression in sp1 knockdown cells, which was confirmed by Western blotting for SP1 expression (Fig. 3F). These results suggest that p63TAα requires SP1 for inhibition of the telomerase promoter and inhibition of hTERT mRNA expression.

p53-mediated Repression of hTERT Promoter Requires Distinct Transcriptional and Non-transcriptional Functions—We next investigated the role of other response elements present in the hTERT promoter, namely E-box and E2F. For these experiments, site-directed mutagenesis was used to eliminate individual response sites, and following transient transfection along with p53 or related factors, luciferase activity of mutated reporter vectors was compared with the parental non-mutated vector. Our results indicate that p53-mediated repression is significantly impaired by mutation of the proximal E-box site present in the hTERT promoter as shown by higher luciferase values for hTERT 24/25 1B and hTERT 24/25 1C constructs when co-expressed with p53 (Fig. 4, A–E). In contrast to the proximal E-box motif, disruption of the E2F site had negligible effects (Fig. 4D and E). In the presence of the wild type E-box motif, disruption of the E2F site had negligible effects on p53-mediated repression (Fig. 5, A and B). This was seen only when the proximal E-box site was simultaneously mutated (Fig. 4, D and E). These results suggest some type of cooperative effect between the distal E-box and
E2F signaling in p53-mediated regulation of the hTERT promoter expression. In contrast, our data clearly showed that mutation of the E2F site (hTERT-E2FM) efficiently prevented p63TAy-mediated repression, whereas it had negligible effects on p73α- or p73β-mediated repression (Fig. 5, A and B). These results demonstrate that p63TAy-mediated repression of the hTERT promoter is completely dependent on the E2F site. These results were further confirmed using human HEK cells (Fig. 5, C and D). Inhibition of endogenous hTERT expression and telomerase activity by p63TAy was also seen (Fig. 5, A and B). These results demonstrate that p63TAy-mediated repression of the hTERT promoter is completely dependent on the E2F site. These results were further confirmed using human HEK cells (Fig. 5C). Inhibition of endogenous hTERT expression and telomerase activity by p63TAy was also seen (Fig. 5, D and E). Surprisingly, although p63TAy-mediated repression mechanisms are similar in mouse and human cells, p53-mediated hTERT repression appears to use different pathways in MEF cells (Fig. 6, B and C). Although mutation of E-box and E2F sites abrogated p53-mediated repression in MEF cells, this had little effect on p53-mediated repression in human HEK cells. However, p53 was able to repress endogenous human hTERT expression (Fig. 6D) and telomerase activity (Fig. 6E). We then investigated whether p53 transcriptional activity may be required for its repressive effect. To this end, we used two transcriptionally inactive p53 mutants previously isolated from human T-cell lymphotrophic virus, type 1-transformed adult T-cell leukemia cells (M2 and M6; Fig. 7A) (44). When these constructs were tested, our results showed that in the absence of transcriptional functions p53 is still able to repress the hTERT promoter in MEF cells but is unable to repress the hTERT promoter in human HEK cells (Fig. 7, B and C). These data reveal the use of different signaling pathways for p53-mediated repression of hTERT expression in murine and human cells, transcription-independent for MEF cells and transcription-dependent for HEK cells. In agreement with these observations, wild type p53 was able to inhibit telomerase activity, whereas p53 mutants were unable to repress telomerase (Fig. 6D). These data raised the hypothesis that activation of effector genes might be required for p53-mediated repression in human cells. Strikingly, a previous study indicated that when the p53-inducible p21/CDKN1A (p21waf) gene was deleted p53 was no longer able to repress any of the 11 target genes that it otherwise down-regulated. Most of these genes were also repressed by ectopic expression of p21WAF in the absence of p53 (45). In addition, one report indicated that p53-mediated repression of hTERT occurred in a p21WAF-dependent man-

**FIGURE 5.** p63TAy represses hTERT promoter through E2F elements. A, schematic representation of hTERT promoter-reporter construct mutated for the E2F binding site (hTERT-E2FM). B and C, repression of hTERT-E2FM promoter-reporter construct by p53, p63TAy, p73α, or p73β was tested in transient transfections in MEF cells (B) and in human HEK cells (C), respectively. Results are representative of three independent transfections. Error bars represent S.D. Western blots indicate the level of each of the transfected plasmids in the assays. D and E, p63TAy represses hTERT expression and telomerase activity. HEK cells were transfected and assayed as described in Fig. 3 and under “Experimental Procedures.” dH2O, distilled H2O.
ner (46), whereas other studies suggested that p53-mediated repression of hTERT was SP1-dependent or E2F/Rb-dependent (47, 48). Based on these observations, we tested the need for p21WAF in p53-mediated repression of the hTERT promoter in human cells. Expression of p21waf was knocked down using siRNA, and the ability of p53 to repress the hTERT promoter was investigated by transient transfection and luciferase assays. Although p21WAF was effectively knocked down, p53 retained its ability to repress hTERT, suggesting that its effect is independent from p21WAF (Fig. 7E). Because p53-mediated repression of the hTERT promoter required transcription, we looked at c-myc, another gene target previously reported to be transcriptionally down-regulated at the mRNA level by p53 (49, 50). Transient transfection experiments confirmed that wild type p53 but not the transcriptionally inactive mutant p53 M6 effectively repressed c-myc gene expression (Fig. 7F). Thus, we hypothesized that the p53 effects on the hTERT promoter were dependent upon suppression of c-myc expression. Consistent with this idea, we found that p53 was no longer able to repress the hTERT promoter when ectopic c-myc expression was provided (Fig. 7G). The lack of p53 effect was not due to higher levels of the hTERT promoter activity because we showed above that p53 still retained repression activity when expressed along with SP1 despite a 6-fold induction in hTERT promoter activity in the presence of ectopic SP1 (Fig. 2, A and B). Finally, we knocked down c-Myc expression using c-myc-specific siRNA. Real time quantitative PCR demonstrated that loss of c-myc expression in HEK cells (as demonstrated by Western blot expression) resulted in inhibition of hTERT expression. Importantly, p53 lost its repressive effects on the hTERT promoter when c-myc was no longer expressed (Fig. 7H). All together, these data demonstrate that p53-mediated repression of hTERT promoter in human cells is dependent upon down-regulation of c-myc expression.

**p73α and p73β Transcriptional Repression of hTERT Promoter Involves NF-YB Factors**—The results shown above (Figs. 1–6) indicate that p73α- and p73β-mediated repression of the hTERT promoter is not affected by mutation of SP1, E-box, or E2F binding sites. We found that treatment with TSA, a selective inhibitor of histone deacetylases, significantly reduces p73-mediated repression of the hTERT promoter in both MEF and HEK cells (Fig. 8, A and B). A similar effect of TSA on p73

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**FIGURE 6.** p53-mediated repression of hTERT promoter occurs through E2F/E-box in MEF cells. A, schematic representation of hTERT promoter-reporter construct mutated for proximal and distal E-box and E2F binding sites (hTERT-E-box/E2FM). B and C, repression of hTERT-E-box/E2FM promoter-reporter construct by p53, p63TA, p73α, or p73β was tested in transient transfections in MEF cells and in human HEK cells, respectively. Results are representative of three independent transfections. Error bars represent S.D. Western blots indicate the level of plasmids transfected in each assay. C and D, p53 represses hTERT expression and telomerase activity. HEK cells were transfected with 4 μg of p53, and whole cell lysates were collected for RNA or TRAP assays to detect hTERT expression or telomerase activity, respectively. dH2O, distilled H2O.
repression of the platelet-derived growth factor receptor had been reported previously and shown to be mediated by the NF-YB factors (51). To further demonstrate involvement of NF-YB in p73-mediated hTERT promoter regulation, we used the characterized NF-YB1 and NF-YB2 shRNA vectors previously shown to abrogate expression of NF-YB factors in transient transfection experiments (Fig. 8C). Whereas exogenous overexpression of NF-YB1 and NF-YB2 shRNA partially rescued p73α, only inhibition of NF-YB2 expression completely abolished any p73α-mediated repression. Although the use of shRNA does not imply that p73 family members mediate all their repressive effects on the hTERT promoter through NF-YB factors, it does demonstrate that p73-mediated repression of the hTERT promoter involves NF-YB factors. Western blots demonstrate efficient knockdown of the p21WAF protein expression. F, real time RT-PCR for c-myc expression normalized to gapdh in HEK cells transfected with wild type p53 or the transcriptionally inactive p53 M6 mutant. G, ectopic expression of a c-myc vector (0.5–1.0 μg) prevents p53-mediated repression of the hTERT promoter in human HEK cells. Western blots demonstrate the level of transfected plasmids. H, p53 requires c-myc for transcription repression of endogenous hTERT. HEK cells were transfected with p53 and CD8 expression plasmids. Three hours later, cells were retransfected with 2 or 20 nm c-myc siRNA. Positively transfected cells were sorted as stated in Fig. 3D for hTERT expression. A Western blot indicates the knockdown of c-myc expression in HEK cells following transfection of 2 or 20 nm c-myc siRNA, dh2O, distilled H2O.

DISCUSSION

When human fibroblasts are transfected with SV40 large T antigen, which binds to and inactivates p53 and pRb, telomerase reactivation does not occur until additional genetic alterations are acquired (52). Similar results were observed with normal human breast epithelial cells in which a p53 construct mutated at codon 143, 175, 248, or 273 was overexpressed (53). Moreover, although mutations in p53 that lead to a transcriptionally inactive p53 in human cancers is frequent, mutations in p63 and p73 are rare possibly due to the existence of complex alternatively spliced variants. However, several reports have indicated epigenetic silencing of p73 and p63 promoters in human cancers. Our results demonstrate that p63 and p73 isoforms are strong repressors of the hTERT promoter and that several non-redundant pathways involving SP1, E-box/E2F, and NF-YB are implicated in the negative regulation of the hTERT promoter. This multifactorial control check presumably acts as a strong tumor suppressor mechanism, avoiding a situation in which the sole inactivation of p53 would result in higher telomerase expression, expanded lifespan, and the risk of immortalization. In fact, subsequent to p53 mutation in tumor cells, inactivation of p63 and/or p73 showed a correlation with tumor stage and grade. In addition, it is possible that the suppression of hTERT promoter activity by p53 and p53 family members plays a role in the cell cycle-dependent control of hTERT expression in normal cells. Cells transformed by human tumor viruses (hepatitis C virus; hepatitis B virus; human papillomavirus; human T-cell lymphotrophic virus, type I; Kaposi sarcoma-associated herpesvirus; and Epstein-
Barr virus) have reactivation of telomerase expression that coincides with inactivation of p53 and p73 (5).

The fact that mutations in all SP1 sites reduce hTERT promoter activation to near background levels suggests that SP1 sites are important for normal, basal activity of the hTERT promoter, but as shown by our results, SP1 is not directly involved in p53-, p63TA/H9253- or p73-mediated suppression of the telomerase promoter. Interestingly, TAp63/H9251 and TAp63/H9253 use distinct pathways to control hTERT expression, namely SP1 and E2F. This observation may be related to the fact that TAp63/H9251 lacks significant transcriptional ability, whereas TAp63/H9253 is a strong transcriptional activator. Further studies are needed.

Our results also showed that ectopic expression of c-Myc efficiently rescued p53-mediated repression of the hTERT promoter in human cells in part through down-regulation of c-Myc expression. Because only a partial rescue was observed using the E-box-mutated hTERT promoter construct, our results also suggest that c-Myc stimulates the hTERT promoter directly through E-box binding and indirectly through a yet to be identified mechanism.

![Figure 8](image1.png)

**FIGURE 8.** p73α and p73β repression of hTERT promoter involves NF-YB factors. A and B, p73α- and p73β-mediated repression of the hTERT promoter is rescued by treatment with histone deacetylase inhibitor (TSA) in MEF and HEK cells, respectively. Western blots indicate the level of transfected plasmids in each assay. C, p73α- and p73β-mediated repression of the hTERT promoter is rescued by co-expression of NF-YB1 or NF-YB2 shRNA. Results are representative of two independent transfections. Error bars represent S.D. D and E, p73α and p73β are strong transcriptional repressors of the endogenous hTERT promoter and telomerase activity. HEK cells were transfected with 2 or 4 μg of p73α or p73β, and total cell lysates were used in real time PCR for hTERT expression (D) or TRAP assays for telomerase activity (E). dH₂O, distilled H₂O.

![Figure 9](image2.png)

**FIGURE 9.** Telomerase promoter regulation by p53 family members. An overall summary of the regulation of the hTERT core promoter by p53, p63, and p73 family members in mouse and human cells is shown. p53 uses distinct mechanisms for repression of hTERT gene expression in mouse and human cells. Dashed lines indicate a mechanism specific to mouse cells. HDAC, histone deacetylase.

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In contrast, we found that both p73α and p73β repress the hTERT promoter to a similar extent, although only p73β has strong transcriptional activities when tested in transient assays. We also found that p73α-mediated repression of the hTERT promoter was partly alleviated by co-expression of shRNA directed against NF-YB1 or NF-YB2. However, p73β-mediated suppression of hTERT promoter was completely rescued by co-expression of NF-YB2 but not NF-YB1. These findings parallel those described for p73-mediated repression of platelet-derived growth factor β receptor (51). Our study also highlights differences in p53 family member-mediated repression of the hTERT promoter between mouse and human cells. Given the strong degree of homology between the human and mouse core hTERT promoters (used in this study) and similar homologies between human and mouse p53 family members, we feel that the differences between exogenously added human factors and endogenous factors in mouse cells are not solely responsible for the differences seen between human and mouse cells. In support of this conclusion, p63Taγ and p73 family members conserve the same mechanism to repress the hTERT promoter in mouse and human cells regardless of any interactions between exogenous and endogenous proteins. Overall, our results contribute to a better understanding of hTERT promoter regulation and the relationship between p53 and related family members and telomerase expression in normal and cancer cells (Fig. 9).

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