Transactivation-dependent and -independent Regulation of p73 Stability*

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The tumor suppressor p53 regulates its own stability by transcriptionally activating Mdm2, Pirh2, and COP1, which target p53 for degradation. However, whether such a negative feedback mechanism exists to regulate the stability of p73, the structural and functional homologue of p53, is unclear. Unlike p53, p73 is not mutated in cancers, but its expression is significantly elevated. Thus, we have investigated the regulation of p73 turnover. Our data suggest the existence of a negative feedback mechanism for p73 degradation. p73 mutants with compromised transactivation activity are generally more stable than the full-length TAp73 form. TAp73 appears to promote its own turnover as well as that of other p73 forms, including the ΔNp73 that lacks the amino-terminal transactivation domain, in a transactivation-dependent manner. This degradation-inducing property of TAp73 was inhibited only by p73 mutants that also inhibit the transactivation activity TAp73 but not by mutant p53, highlighting the specificity in the regulation of p73 stability. Moreover, regions in the amino and carboxyl termini of p73 confer both stabilizing and destabilizing effects on the protein, independent of its transactivation ability. Finally, we have identified the regions between amino acids 56 and 248 of p73 as being the region of a negative feedback mechanism for p73 degradation. Taken together, the data suggest that p73 turnover is tightly regulated in a transactivation-dependent and -independent manner, resulting in the controlled expression of the various p73 forms.

p73 belongs to a family of tumor suppressors, including p53 and p63, that are structurally and functionally similar (1), and has homology with p53 in the transactivation, tetramerization, and DNA binding domains (2). However, unlike p53, p73 is present in multiple isoforms because of alternate splicing at the carboxyl terminus, giving rise to at least six isoforms for p73 (p73α–β), which are known as the TA forms (1–3). In addition, the presence of promoter sequences in the intron of the gene results in the expression of an amino-terminal truncated version of p73 that lacks the transactivation domain, known as the ΔN form, which acts as a dominant negative inhibitor of the full-length protein (1, 2).

Functionally, TAp73 forms are similar to p53, and they are able to induce apoptosis when overexpressed (4). Moreover, both TAp73α and TAp73β have been shown to transactivate many p53-responsive promoters, although the relative efficiencies vary on different promoters (5). Cell death induced by overexpression of TAp73 occurs in the absence of p53, and both TAp73α and TAp73β have been shown to suppress foci formation in p53 null cells (3). Moreover, the absence of p73 was shown to result in resistance to apoptosis in response to chemotherapeutic drugs and other stress signals (6, 7). Nonetheless, p73 null mice do not develop spontaneous tumors like their p53 null counterparts, indicating that although p73 is structurally similar to p53, it might not be as potent as p53 in its tumor-suppressive activities (8). In support of this, it was shown that TAp73 has reduced ability to activate some of the common p53 target genes, suggesting that p73 might be the weaker of the two (5, 9). This also raises the possibility that p73 may be differentially regulated compared with p53, thereby leading to differential effects on target gene activation and cellular physiology.

p73 also differs from p53 at the biochemical level. p53 is a short-lived protein whose stability is regulated by an autoregulatory negative feedback loop (10). This feedback loop consists of several targets that are induced by p53, such as Mdm2, Pirh2, and COP1, which possess ubiquitin-protein isopeptide ligase activities and have been shown to degrade p53 (11–14). By contrast, Mdm2 was shown not to degrade p73, although it was able to bind to it (15, 16), suggesting that the protein turnover mechanism might be different for these two different but homologous proteins. Moreover, the role of Pirh2 and COP1 in p73 turnover is not well understood. On the other hand, several proteins have been shown to affect p73 stability, including the mismatch repair protein PMS2, tyrosine kinase c-Abl, AP-1 transcription factor c-Jun, Hect ubiquitin-protein ligase Itch, p300, PML, NEDD2, and cyclin G (17–24). Of these, p300, cyclin G, PML, and c-Abl have been shown to also cause an increase in p53 levels when co-expressed (22, 25–27). However, expression of Itch or PMS2 was not found to cause an increase in p53 stability (17, 20), and cells lacking c-Jun were found to have increased p53 (28). Thus, distinct mechanisms exist in differentially regulating p53 and p73 stability.

Despite the functional similarities between p53 and p73, the latter gene is rarely mutated in human cancers (29). However, both TAp73 and ΔNp73 were shown to be overexpressed in many cancers (30, 31). This intriguing observation has led to the suggestion that ΔNp73 might be inhibiting both TAp73 and p53 functions, thereby negating the functional consequence of an activated TAp73 (31). Nonetheless, regulatory mechanisms controlling p73 stability in cancer cells have not been ad-
dressed. By contrast, many studies show that p53 overexpressed in tumors is generally mutated (32). Mutated p53 is unable to activate Mdm2, leading to the breaking of the auto-regulatory negative feedback loop, resulting in accumulation of the mutant p53 (33). However, whether such auto-regulatory negative feedback loops exist that regulate p73 stability is not clear. A recent report (34) suggested that the transactivation activity of TAP73 was associated with its turnover. Consistently, we and others (19, 35–37) have noted that the ΔN forms of p73 are generally more stable than the TAP73 forms, suggesting the existence of a p73 transactivation-dependent self-degradation mechanism in operation. We have thus investigated the structural requirements for p73 turnover, and we show the existence of a transactivation-dependent auto-regulatory negative feedback loop. Moreover, p73 stability is also regulated in a transactivation-independent manner. Detailed results are presented.

MATERIALS AND METHODS

Cells and Transfections—The p53 null human lung cancer cell line H1299 (ATCC), human osteosarcoma cell line Saos2 with the tetracycline-inducible p73β, and mdm2−/−/p53−/− mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine fetal serum, as described (19). p73β was induced in the Saos2-p73β cells by addition of tetracycline as described (38). H1299 cells stably expressing the various p53 hot spot mutants were generated as described (39).

2 × 105 cells (in 6-well dishes) were used in transfection experiments using Lipofectamine PLUS reagent, as per the manufacturer’s protocols (Stratagene). Cells were transiently transfected with the various plasmids as indicated in the figure legends. Generally, 100 and 500 ng of total amounts of transfected plasmids were used in the transfection experiments. Generally, 100 and 500 ng of total amounts of transfected plasmids were used in many previous studies analyzing p73 stability and transactivation properties. As shown in Fig. 1A, compare with Esgfp levels. Similar results were obtained with untagged proteins (data not shown).

RESULTS

Both DNA Binding and Amino-terminal Transactivation Domains Contribute to p73 Stability—We and others (19, 35–37) have noted previously that the steady-state levels of ΔNp73 were higher than TAP73. In order to confirm these observations, we performed transient transfection experiments to determine the steady-state levels of the various p73 forms. All experiments described here were performed in the presence of the Esgfp plasmid, which was used to monitor transfection efficiency, as described (19). Transfection of equal amounts of FLAG-tagged, pCDNA3-based expression plasmids in p53 null H1299 cells resulted in the ΔNp73α or ΔNp73β forms being more abundantly expressed than the TAP73α or TAP73 forms, respectively (Fig. 1A). We thus evaluated if the combined loss of both DNA binding and transactivation properties would have a synergistic effect on p73 steady-state levels. As shown in Fig. 1C, 292p73α appeared to be slightly less abundant than the ΔNp73β form. However, the ΔN292p73β mutant was the most abundant, and its levels were higher than that of ΔNp73β, suggesting that both the DNA binding and transactivation properties and/or domains contribute to the regulation of p73 levels.

We assessed the half-lives of the variants in the presence of the protein synthesis inhibitor cycloheximide, which has been used in many previous studies analyzing p73 stability and half-life (20, 23, 34, 44, 45), to determine whether the differences in steady-state levels of the various p73 variants were because of altered protein stability. TAP73β had the shortest half-life, followed by 292p73β, ΔNp73β, and ΔN292p73β (Fig. 1D). 50% of TAP73β remained at around 1.5 h post-cycloheximide treatment compared with >80% of 292p73β (Fig. 1E). However, more than 90% of ΔNp73β and ΔN292p73β remained at this time point. Extended analysis indicated that at 8 h after cycloheximide treatment, 80% of ΔNp73β remained, whereas about 95% of ΔN292p73β was still present (Fig. 1E). Taken together, the results suggest that both the DNA binding and amino-terminal transactivation domains of p73 contribute to the regulation of p73 stability.

Inhibition of p73 Transactivation Activity Results in Extended p73 Half-life—We next sought to investigate the role of transactivation activity in regulating p73 stability. Co-transfection of mdm2Luc (luciferase gene driven by the p53-responsive mdm2 promoter) in H1299 cells with the various p73 expression plasmids indicated that all the p73 mutants had no significant transactivation activity (Fig. 2, A and B, p73β and p73α, respectively). Co-expression of TAP73α or TAP73β with the various p73 mutants resulted in the abrogation of transactivation activity for TAP73α or TAP73β, indicat-

1 The abbreviations used are: HA, hemagglutinin; Esgfp, enhanced green fluorescent protein.
ing all of the p73 variants tested were capable of inhibiting TAp73 transactivation activity (Fig. 2, A and B).

Co-expression of ∆Np73β with low levels of FLAG-tagged TAp73β (100 ng) resulted in an increase of TAp73β steady-state levels, as determined by immunoblotting with anti-FLAG or anti-p73β (GC15) antibodies (Fig. 2C, compare lanes 1 and 6). Moreover, co-expression of 292p73β, ∆N292p73β, and p73DD (which contains p73 tetramerization domain) also led to an increase in TAp73β levels (Fig. 2C, compare lane 1 with lanes 7–9). Furthermore, analysis of TAp73β stability indicated that co-expression of the ∆Np73β, 292p73β, and p73DD led to an increase in the half-life of TAp73β (Fig. 2, D and E). The half-life of TAp73β that was about 1.5 h was extended to about 3.5 and 3–4 h in the presence of ∆Np73β and 292p73β, respectively, and it was almost not degraded up to 6 h in the presence of p73DD (Fig. 2, D and E, and Fig. 1D). Similar results were obtained with TAp73α (data not shown). Taken together, the results demonstrate that the inhibition of TAp73 transcriptional activity by the various p73 mutants resulted in a concomitant stabilization of the TAp73 protein and hence suggest

![Diagram](image_url)
FIG. 2. Inhibition of p73 transactivation activity by p73 mutants stabilizes p73. A and B, p73 mutants inhibit p73-mediated transactivation activity. H1299 cells were transfected with the reporter plasmid expressing the firefly luciferase gene under the transcriptional control of the mdm2 promoter together with the indicated plasmids (100 ng each). In each case, total amounts of transfected DNA were equalized with pCDNA3 vector. Luciferase activity was analyzed as described 48 h post-transfection. Transfections were carried out in triplicate, and the standard errors are indicated.

C, steady-state levels of TAp73β in the presence of various p73 mutants. H1299 cells were transfected with 100 ng of TAp73β in the absence or presence of the indicated p73 mutants as described, and the p73 levels were determined by immunoblotting. * indicates FLAG-TAp73β that migrates slower, in lanes showing both FLAG-TAp73β and 292p73β.

D and E, TAp73β half-life in the presence of the indicated p73 mutants were determined as described in Fig. 1, D and E. CHX, cycloheximide.
that the transactivation activity of TAp73 contributes to the regulation of its own stability.

Role of the Amino-terminal Domain in Regulating p73 Stability—We next explored if the amino-terminal region of p73 has any other transactivation-independent role in the regulation of p73 stability. To this end, we generated several amino-terminal deletion mutants of TAp73/H9252, as indicated in Fig. 3A. Analysis of transactivation activity of these mutants indicated that/H9004\2-9p73/H9252 had significant transactivation activity, whereas the other mutants (i.e. /H9004\2-23, /H9004\2-40, and /H9004\2-56) were compromised in their activity, similar to /H9004\Np73/H9252 (Fig. 3B).

Analysis of the steady-state levels indicated that regions between amino acids 2 and 9 and 23 and 40 had a stabilizing effect on p73, as deletion mutants /H9004\2-9 and /H9004\2-40 were less abundant than TAp73/H9252, whereas deletion mutants /H9004\2-23 and /H9004\2-56 were more abundant than TAp73/H9252 (Fig. 3C, compare lane 2 with lanes 4–6). Moreover, /H9004\2-56p73/H9252 was as abundant as /H9004\Np73/H9252, together suggesting that regions between amino acids 9 and 23 and 40 and 56 probably conferred a destabilizing effect on the protein (Fig. 3C, compare lanes 2–5 and lane 7). The half-lives of these mutants were consistently variable. /H9004\2-9p73/H9252 was the most stable, similar to /H9004\Np73/H9252 (Fig. 3D and E). The data together suggest that the amino terminus of p73 contains regions that would affect p73 stability. Moreover, these regions also appear to regulate p73 stability in a manner independent of its transactivation activity, as the transcriptionally active /H9004\2-9p73/H9252 and conversely transcriptionally inactive /H9004\2-40p73/H9252 were less stable than TAp73/H9252.

Role of the Carboxyl-terminal Domain in Regulating p73 Stability—Similar experiments were performed to evaluate the
role of the carboxyl terminus in regulating p73 stability. Deletion mutants were generated, which lacked clusters of lysine residues that are thought to be essential for ubiquitination and other modifications that regulate protein stability (41), as shown in Fig. 4A. 1–425 lacked 4 lysine residues, and 1–358 and 1–248 lacked a total of 10 and 18 lysine residues, respectively, compared with TAp73β (Fig. 4A) (40). Analysis of transcriptional activity revealed that 1–425 was competent in ac-

Fig. 4. Role of the carboxyl terminus in regulating p73 stability. A, schematic representation of the various p73 carboxyl-terminal mutants used. B, transactivation activity of the various p73 carboxyl-terminal mutants. C, immunoblot analysis of the carboxyl-terminal mutants used in this study. D and E, half-life of the p73 carboxyl-terminal mutants, as described above. CHX, cycloheximide.
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Activating the Mdm2 promoter activity, whereas 1–358 had compromised activity (Fig. 4B). By contrast, 1–248 was completely transcriptionally inert (Fig. 4B).

Analysis of steady-state levels indicated that 1–425 and 1–358p73 mutants were expressed abundantly, whereas the expression of the 1–248p73 mutant was dramatically reduced (Fig. 4C, compare lanes 2 and 3, immunoblotted with anti-FLAG antibody, and lanes 4 and 5, which were detected with anti-HA antibody). Half-life analysis confirmed these findings. Although 1–425 was more stable than TAp73β (t1/2 = 6 h), 1–358p73 was very stable and 1–248 was highly unstable, with a half-life of about less than an hour (Fig. 4A, D and E). These results suggest that the region between amino acids 358 and 499 may contain motifs that confer destability, whereas the region between amino acids 248 and 358 contain motifs conferring stability to p73. Together, the findings indicate the regions in the carboxyl terminus of p73 also contribute to p73 stability in a manner separate from its transactivation ability, as the transcriptionally active 1–425p73 was more stable and the transcriptionally inactive 1–248p73 was less stable than TAp73β.

**Domain Requirements for TAp73-mediated Degradation**—As the results suggested that TAp73 contributes to the regulation of its own turnover, we next investigated the domain requirements for degradation induced by TAp73. To this end, we evaluated the effect of TAp73β or TAp73α on various p73 mutants. Co-expression of TAp73β or TAp73α with ΔNp73β resulted in the reduction in the stability of the latter protein (Fig. 5A, compare lane 4 with lanes 5 and 6), indicating the entire amino-terminal domain in ΔNp73β is not required for degradation induced by both TAp73α and TAp73β. Further analysis using other p73 mutants indicated that both the amino and carboxyl termini of p73β from amino acids 2–56 and 258–499, respectively, were not required for efficient degradation induced by TAp73β, as the steady-state levels of all the mutants tested were reduced in the presence of TAp73β (Fig. 5, B, compare lanes 3–7 with lanes 8–12, and C, compare lanes 3–5 with lanes 6–8).

Subsequently, the regions in TAp73 that are required to induce degradation were determined, using ΔNp73β as a degradation target. As noted earlier, TAp73β was more efficient than TAp73α in inducing degradation (Fig. 5A, compare lanes 4–6). Co-expression of the various mutants with FLAG-ΔNp73β indicated that the lack of amino acids 2–9 had minimal effect on the ability of the mutants to induce degradation of ΔNp73β (Fig. 5D, compare lane 2 with lane 8). However, lack of up to the first 56 amino acids gradually and partially compromised the ability to induce efficiently the degradation of ΔNp73β (Fig. 5D, compare lane 2 with lanes 9–11). By contrast, ΔNp73β was unable to mediate its own degradation, as determined by immunoblotting with anti-FLAG antibody (Fig. 5D, compare lanes 2–12). Analysis of the carboxyl-terminal mutants indicated that 1–425p73 was able to cause the degradation of untagged-ΔNp73β, similar to that observed with TAp73β (Fig. 5E, compare lane 1 with lanes 5–6, top panel probed with anti-p73β(GC15) antibody). However, 1–248 and 1–358p73 mutants were unable to significantly affect the steady-state levels of ΔNp73β (Fig. 5E, compare lane 1 with lanes 7–8). These data suggest that both the amino terminus (amino acids between 9 and 56) and the carboxyl terminus (amino acids between 248 and 425) of TAp73 are required for the induction of efficient degradation of ΔNp73β.

**Inhibition of Transactivation Activity Alone Is Not Sufficient to Stabilize p73**—Next, we examined if inhibition of the transactivation property of TAp73 alone was sufficient to stabilize it. To this end, two sets of experiments were carried out. First, we examined if there was a correlation between the degree of inhibition of transactivation activity of TAp73 and its stability. Co-expression of TAp73β with the various mutants indicated that although Δ2-9p73β did not affect the transactivation activity of TAp73β, the remaining amino-terminal mutants reduced its activity to a similar extent as co-expressing ΔNp73β (Fig. 5F). However, none of the carboxyl-terminal p73 mutants had any inhibitory effect on the transactivation activity of TAp73 (Fig. 5F). Steady-state analysis indicated that only Δ2-56p73β was capable of causing an increase in FLAG-TAp73 levels, similar to ΔNp73β (Fig. 5B, compare lane 2 with lanes 11 and 12, both anti-FLAG and anti-p73β(GC15)). By contrast, none of the other amino-terminal mutants were able to increase TAp73 steady-state levels, although they were all capable of inhibiting p73 transcriptional activity to various extents (Fig. 5B, compare lane 2 with lanes 9 and 10). However, consistent with the lack of any effect on the transactivation property of TAp73β, none of the carboxyl-terminal mutants affected TAp73β steady-state levels (Fig. 5C, compare lane 2 with lanes 6–8). The data thus suggest that transactivation inhibition alone is not sufficient for stabilization of p73.

Second, we evaluated if ΔNp73β-mediated inhibition of degradation occurs with all the p73 mutants. Co-expression of ΔNp73β resulted in the increase in steady-state levels of all amino-terminal p73 mutants (Fig. 5D, compare lanes 3–6 with lanes 8–11, anti-p73β(GC15)), consistent with the earlier findings that the amino-terminal region is required for the induction of p73 degradation, and inhibition of this property leads to increased steady-state levels of the mutants. These results were also supported by the effect of ΔNp73β on the extension of Δ2-9 and Δ2-23p73 half-lives (Fig. 5, G and H). By contrast, steady-state levels of all the carboxyl-terminal mutants were unaffected by co-expression with ΔNp73β expression (Fig. 5E, compare lanes 2–4 with lanes 6–8, anti-HA and anti-FLAG antibodies). Although ΔNp73β was able to stabilize the various amino-terminal p73 mutants with degradative potential, it had hardly any effect on 1–425p73, which was also capable of inducing degradation (Fig. 5E, compare lane 2 with lane 6). Therefore, ΔNp73β-mediated increase in p73 levels occurs not only by inhibition of transcription but also via other mechanisms involving the carboxyl terminal regions. Taken together, the data suggest that stabilization of TAp73 occurs through the inhibition of its transactivation activity and probably by other transactivation-independent mechanisms.

**Mutant p53 Is Incapable of Stabilizing p73**—Mutant p53 has been demonstrated to bind and inhibit TAp73 activity (42, 43). We thus explored if mutant p53-mediated inhibition of TAp73 transcriptional activity would also lead to increased TAp73 stability. Reporter assays indicated that two p53 mutants, 175p53 and 248p53, both in the T2P or 72R forms (polymorphic forms) (39), were equally capable of inhibiting TAp73β-mediated transactivation in H1299 cells (Fig. 6A). Examination of the steady-state levels of TAp73β in the presence of co-expressed p53 mutant plasmids indicated no significant increase of TAp73β steady-state levels, although ΔNp73β was able to cause an increase of TAp73β levels in the same experiments (Fig. 6B, compare lane 1 with lanes 8–13). In order to further confirm if mutant p53 would not affect the TAp73 steady-state levels, we utilized two other cellular systems. First, we used a TAp73-inducible Saos2 cell system in which TAp73β expression was induced by addition of tetracycline (38). Examination of steady-state levels again indicated that expression of p53 mutants did not cause an elevation of TAp73β levels (Fig. 6C, compare lanes 2 with lanes 3–6 and 7). Second, we transfected TAp73β expression plasmids into H1299 cells stably expressing the various p53 mutants, and we examined the TAp73β steady-state levels (Fig. 6D, compare lanes 1–4 with lanes 5–8). The results indicate that neither TAp73β nor ΔNp73β expression was affected in a manner separate from its transactivation ability.
Consistent with the earlier findings, expression of TAp73/H9252 in the presence of mutant p53 did not affect its steady-state levels (Fig. 6D, compare lane 1 with lanes 2–5). These results thus indicate that although the various mutant p53 are capable of inhibiting TAp73 transactivation activity, their expression does not alter TAp73 steady-state levels.

FIG. 5. Domain requirements for TAp73-mediated degradation and effect of p73 mutants on p73 stability. A, effect of TAp73 on ΔNp73. ΔNp73β was transfected in the presence or absence of TAp73α or TAp73β, and the steady-state levels were determined by immunoblotting. All plasmids used here are FLAG-tagged. B and C, steady-state levels of TAp73β in the presence of various p73 mutants were determined by immunoblotting. * indicates position of the p73 mutants, which are hardly visible. D and E, effect of p73 mutants on ΔNp73. Steady-state levels FLAG-ΔNp73β (D) or untagged ΔNp73β (E) were analyzed in the presence or absence of various p73 mutants. F, the indicated p73 mutants were co-transfected with TAp73β and the Mdm2-promoter-reporter construct. Luciferase activity was analyzed as described 48 h post-transfection. G and H, half-life of p73 mutants in the presence of ΔNp73β. CHX, cycloheximide.
Finally, we investigated if TAp73 could degrade p53. Earlier experiments indicated that co-expression of TAp73/H9252 in p53 null H1299 cells led to a decrease in p53 steady-state levels (Fig. 6B, compare lanes 3–7 with lanes 9–13). Because TAp73 is able to induce Mdm2, we examined the effect of co-expression of TAp73β on p53 in p53<sup>−/−</sup>mdm2<sup>−/−</sup> mouse fibroblasts. Expression of wild-type and mutant p53 was also reduced in the presence of TAp73β in p53<sup>−/−</sup>mdm2<sup>−/−</sup> cells (Fig. 6E, compare lanes 3–7 with lanes 9–13), suggesting that p53 may be degraded in a p73-mediated but Mdm2-independent manner.

**FIG. 6.** Inhibition of p73 transactivation activity by p53 mutants does not result in stabilization of p73. A, p53 mutants inhibit p73-mediated transactivation activity. H1299 cells were transfected with the mdm2 promoter-luciferase construct together with the indicated plasmids, and luciferase activity was analyzed. B, H1299 cells were transfected with TAp73β and in the absence or presence of the indicated p53 mutants, and p73 steady-state levels were determined. DO1 antibody was used to determine p53 levels. C, Saos2 cells with the tetracycline-inducible TAp73β were transfected with the indicated p53 mutants. Cells were then treated without or with tetracycline 24 h after transfection to induce p73 activity, and cells were harvested a further 24 h later for immunoblot analysis. D, H1299 cells stably expressing empty vector (pCDNA) or the indicated p53 mutants in either the proline (72P) or the arginine (72R) form were transiently transfected with TAp73β expression plasmid, and the steady-state levels of TAp73β were determined 24 h later. E, effect of TAp73β expression on p53 steady-state levels was analyzed in p53<sup>−/−</sup>mdm2<sup>−/−</sup> cells as described in B.
Ubiquitination and p73 Degradation—Because the region between amino acids 56 and 248 of p73 was sufficient for p73-mediated auto-degradation, we next examined if this region was also sufficient for ubiquitin-mediated degradation. Co-expression of increasing amounts of FLAG-tagged ubiquitin expression plasmid with the various p73 mutants led to a dose-dependent decrease in the steady-state levels of all the amino- and carboxyl-terminal mutants (Fig. 7, A and B), suggesting that the region between amino acids 56 and 248 of p73 is sufficient for ubiquitin-mediated p73 degradation.

**DISCUSSION**

We and many other investigators (19, 35–37) have noted that the ΔNp73 forms are generally more stable than the TAp73 forms. Recently, Wu et al. (34) have suggested that there could be a link between the p73 transcriptional activity and its degradation. We have now extended these findings and shown that both the DNA binding and transactivation domains are necessary for TAp73 to regulate its turnover. By using co-expression experiments, we noted that expression of TAp73 led to a decrease in the levels of ΔNp73 and other mutants, highlighting that TAp73 had the capacity to induce the degradation of p73 proteins. This degradation-inducing property of TAp73 correlated with its transactivation potential, as the various amino-terminal mutants with compromised transcriptional activity (including delatNp73) were less able to induce ΔNp73 degradation. Moreover, inhibition of transactivation potential of TAp73 by various p73 mutants such as ΔNp73, 292p73, and p73DD correlated with increased TAp73 half-lives, suggesting that the transactivation activity of TAp73 was involved in regulating its own turnover. These data are consistent with that reported by Wu et al. (34). On the other hand, carboxyl-terminal mutants of p73, such as 1–358 and 1–248 that were unable to inhibit TAp73-mediated transactivation activity, were also unable to cause an increase in TAp73 steady-state levels. Together these data strongly suggest the existence of a

![Image of Figure 7](http://www.jbc.org/)
mechanism that regulates p73’s own abundance in a transcription-dependent manner.

Although TAp73 was the only natural p73 variant tested that appeared to possess the degradation-inducing property, all p73 forms tested in this report were capable of being degraded by this activity. The domain between amino acids 56 and 248 of p73 was found to be the minimal region required for p73-mediated degradation, suggesting that TAp73-mediated degradation would be able to target all natural variants of p73 in the cell (including all isoforms and the ΔN forms). Moreover, ubiquitin-mediated p73 degradation was also found to require this same region, indicating that p73-mediated protein degradation could occur via ubiquitination. However, we cannot exclude the possibility that several mechanisms may be involved in the isoform-specific p73 degradation.

Besides p73 being its own substrate, we have also evaluated if p53, the related protein, could also be degraded by TAp73. Co-expression of TAp73 with p53 (both wild-type and mutants) resulted in the reduction of p53 steady-state levels in several cellular systems. The results suggest that p53 might be also a substrate of the p73-mediated degradation pathway. In this respect, it is noteworthy that there is a very high degree of homology between p53 and the amino acids 56–248 of p73 (2). Although Pirh2, the p53-activated ubiquitin-protein isopeptide ligase that binds to p53 in the regions between amino acids 82–292 and degrades it, was also shown to bind to p73 (13), Wu et al. (34) suggested that Pirh2 might not be the target of p73 that regulates its stability. Moreover, we have ruled out Mdm2 as an intermediate in this process because p53 levels were also reduced in the presence of TAp73 in p53/−/− mdm2/−/− cells. Thus, it remains to be elucidated if other ubiquitin-protein isopeptide ligases such as COP1 have any role in p73-mediated auto-regulation of its stability.

The ability of TAp73 to induce degradation of all the p73 forms was inhibited by the naturally occurring ΔNp73, suggesting the existence of a network regulating p73 stability. This inhibitory effect correlated with the inhibition of the transactivation activity of TAp73. Although p53 mutants were able to inhibit the transcriptional activity of TAp73, our data indicate that inhibition of the transcriptional activity of TAp73 by various p53 mutants was not sufficient to cause an increase in TAp73 levels. However, inhibition of TAp73 activity by the truncated p73DD mutant consisting of the p73 oligomerization domain was able to stabilize TAp73, consistent with a recent report (44) that indicated that the oligomerization potential of p73DD was necessary for the stabilization of TAp73. These data together suggest that the mode of inhibition of the transcriptional activity of TAp73 is critical in the inhibition of the TAp73-mediated degradation. Although both mutant p53 and ΔNp73 are able to bind TAp73, the mechanisms are different. ΔNp73 and p73DD are thought to bind TAp73 through the oligomerization domain, whereas mutant p53 requires the core domain to interact with p73 (42, 43), and this difference might affect the manner in which the degradation potential of p73 is inhibited. Consistently, some of the amino-terminal deletion mutants that were able to inhibit the transcriptional activity of TAp73 were not able to inhibit TAp73-mediated degradation, indicating that other motifs in p73 might influence this process. Moreover, the various amino- and carboxyl-terminal mutants had different rates of degradation, which was not always congruent with the transcriptional activity. These data together suggest that there may be other regions in p73 that affect its stability in a transcription-independent manner. The data thus indicate that although inhibition of transactivation activity of p73 is necessary, it alone is not sufficient to prevent its degradation-inducing function.

One feature that has emerged from this work is that independent regions in the amino and carboxyl termini of p73 affect its stability in a manner independent of the transactivation activity. There are independent regions between amino acids 2 and 9, 23 and 40, and 248 and 358 conferring stability, whereas regions between amino acids 9 and 23, 40 and 56, and 358 and 499 confer a destabilizing effect to TAp73β. Of these, the stability-conferring region between amino acids 248 and 358 contains the residues that are acetylated by p300, resulting in stabilization of p73 (40). However, within the stability-conferring regions between amino acids 358 and 499 is the region where Pin-1 was shown to bind and stabilize p73 (amino acids 412, 442, and 482) (45). Moreover, NEDL2 was shown to stabilize p73 by binding proline-rich motifs in the region between amino acids 401 and 487 (24). Thus, it seems that the combination of factors might influence the stability of p73 and that the net effect of stabilizing versus destabilizing factors might dictate the state of the p73 stability. Consistently, the amino-terminal regions between amino acids 2 and 56 contain at least two stability-conferring and two destabilizing regions. Thus, the net effect of these regions on the regulation of p73 stability would presumably be dependent on the spatial and temporal effect of factors that are bound to these regions. Further detailed investigations are required to identify proteins that may bind to these regions and affect p73 stability.

Another striking feature that emerges from these findings is that although ΔNp73 could stabilize TAp73, it itself is degraded. At the first instance, this appears to be a conundrum, as the inhibition of the transactivation activity of TAp73 would be expected to result in the prevention of the degradation-inducing potential for TAp73, and hence, one would not expect degradation of either TAp73 or ΔNp73. At least two possible scenarios could be envisaged to explain these results. First, ΔNp73 might be preferentially degraded in a TAp73-dependent manner, especially because it does not possess the amino-terminal motifs that regulate stability. On the other hand, it is also possible that the stoichiometric balance of TAp73:ΔNp73 would determine which protein is degraded. Thus, one could envisage a dynamic reaction ongoing in the cell to regulate both TAp73 and ΔNp73 levels. Further experiments are required to test these hypotheses.

In summary, we have demonstrated the existence of an auto-regulatory negative feedback mechanism that regulates p73 stability and the domain requirements that govern this degradation process. Physiologically, one could envisage a situation where the levels of p73 protein in the cells are kept at low basal levels through the auto-regulatory negative feedback loop, similar to the p53-Mdm2 loop. In pathological conditions such as cancer, both TAp73 and ΔNp73 have been shown to be up-regulated (29–31). This could be due to increased activation of ΔNp73 that could lead to stabilization of TAp73. Thus, expression of TAp73 in cancers might be a consequence of the inhibition of the TAp73 self-regulating machinery. On the other, it is also plausible that TAp73 is modified in cancer cells such that the destruction machinery is unable to degrade it. Further investigations are required to test these hypotheses.

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