

Differential effects of p63 mutants on transactivation of p53 and/or p63 responsive genes

Shama K Khokhar^{1,*}, Ramakrishna Kommagani^{1,*}, Madhavi P Kadakia^{1,2}

¹Department of Biochemistry and Molecular Biology, Boonshoft School of Medicine, Wright State University, 122 Diggs Bldg, 3640 Colonel Glenn Highway, Dayton, OH 45435, USA; ²Center for Genomics Research, Boonshoft School of Medicine, Wright State University, 3640 Col Glenn Highway, Dayton, OH 45435, USA

p63, known to play a role in development, has more recently also been implicated in cancer progression. Mutations in p63 have been shown to be responsible for several human developmental diseases. Differential splicing of the p63 gene gives rise to p63 isoforms, which can act either as tumor suppressors or as oncogene. In this report, we studied the effects of naturally occurring TAp63 γ mutants on the regulation of p53/p63 and p63 specific target genes. We observed significant differences among p63 mutants to regulate the p53/p63 and p63 specific target genes. Additionally, we observed a differential effect of p63 mutants on wildtype-p63-mediated induction of p53/p63 and p63 specific target genes. We also demonstrated that these mutants differentially regulate the binding of wildtype p63 to the promoter of target genes. Furthermore, the effects of these mutants on cell death and survival were consistent with their ability to regulate the downstream targets when compared to wildtype TAp63 γ . In summary, our data demonstrate that p63 mutants exhibit differential effects on p63 and p53/p63 specific target genes and on the induction of apoptosis, and provide further insight into the function of p63.

Keywords: p63 mutants, Hdm2, p21, VDR, Shh

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Introduction

p63 is a member of the p53 tumor suppressor gene family. Its high sequence homology to p53 led to the speculation that p63 functions as tumor suppressor similar to p53. Unlike p53 knockout mice which develop spontaneous tumors, p63 knockout mice, however, exhibit several developmental defects [1-3]. Similar to p53, p63 also serves as a sequence-specific DNA binding transcription factor that activates target genes, leading to an inhibition of cell cycle progression and induction of apoptosis [4, 5]. The p63 gene, with alternate promoter usage and differential C-terminal splicing, gives rise to six isoforms with remarkably diverse activities as transcription factors; TAp63 γ , TAp63 α and TAp63 β contain the N-terminal transactivation domain (TA), whereas the Δ Np63 γ , Δ Np63 α and Δ Np63 β are transcribed from an

internal promoter and lack the full-length transactivation domain.

Mutations in p63 are documented in several different human developmental disorders [6]. Although little is known about the precise mechanism that underlies the phenotypic specificity that is observed in different mutational classes, it is possible that each of these p63 mutations causes a specific perturbation of normal p63 function. Even though p63 mutations are rarely detected in human cancers, several studies implicate p63 in cancer progression and loss of p63 and p73 has been associated with aggressive tumor progression and poor prognosis [7-10]. Heterozygous mice carrying mutations in p63 display a higher tumor predisposition than p63 knockout mice [11]. In contradiction, another report indicated that p63 heterozygous mutant mice were not prone to spontaneous or chemically induced tumors [12]. However, loss of p63 co-operates with a loss of p53 in tumor development, but the exact mechanism of this action is still unclear. p63 isoforms containing the transactivation (TA) domain limit tumor progression by inducing expression of insulin growth factor binding protein-3 (IGFBP-3), an apoptotic gene and Maspin, as well as inhibiting the expression of

*These authors contributed equally to this work

Correspondence: Madhavi Kadakia

Tel: +1-937-775-2339; Fax: +1-937-775-3730

E-mail: madhavi.kadakia@wright.edu

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vascular endothelial growth factor (VEGF), known to play an important role in angiogenesis and neovascularization, and heat shock protein 70 (HSP70), an anti-apoptotic gene [13-17]. In contrast, ΔNp63 isoforms, which lack the TA domain, repress the expression of IGFBP-3 and induce the expression of HSP70 and VEGF; this induction is due to the presence of its own small but functional transactivation domain [18, 19]. Thus, it seems that, while ΔNp63 isoforms act as an oncogene, p63 isoforms containing the TA domain perform tumor-suppressive activities. Finally, p63, like p53, activates major apoptotic pathways by activating signaling via death receptors and mitochondria, which sensitizes the cancer cells towards chemotherapy [20]. Endogenous p63 is induced by many chemotherapeutic agents, and blocking this function with p63 mutants might confer chemoresistance [21]. Together, all these studies point toward the relevance of studying the biological effects of p63 mutants. Our goal is to assess whether heterozygous mutants observed in human developmental syndromes affect the normal function of p63. Specifically we studied the effects of these p63 mutants on wildtype-mediated regulation of target genes specific for both p53 and p63, as well p63 specific target genes, because several laboratories, including ours, have demonstrated that, while p63 can bind to promoters of genes regulated by p53, p63 also has unique DNA-binding sites that are not bound by p53 and therefore these target genes are uniquely regulated by p63 [22-24]. Therefore, studying both of these sets of genes would enable us to assess the potential effects of these mutants on both p53 and p63.

A series of naturally occurring mutants of p63 shown to occur in human developmental syndromes (Figure 1A) were constructed using PCR-based site-directed mutagenesis. Since these germline mutations are in the DNA-binding domain, which is conserved in all the p63 isoforms, these mutations can affect the functions of all the p63 isoforms. We chose to carry out this study with TAp63γ, reported to be the most potent transactivator amongst p63 isoforms [25]. Additionally, several studies have reported that TAp63 isoforms regulate genes involved in cancer and development [16, 17, 26-32]. The TAp63γ mutants tested in this study included those observed in Ectrodactyly, Ecotodermal dysplasia, Clefting-EEC (R280C, R204W, R279H, C306R and R227Q), Split Hand/Foot Malformation-SHFM (K194E and R280C) and Acro-Dermato-Ungual-Lacrimal-Tooth-ADULT (R298Q) syndromes. Our data demonstrate that while p63 mutants observed in SHFM (K194E, R280C), EEC (R227Q) and ADULT (R298Q) syndromes up-regulate the expression of all p63/p53 target genes, only mutations observed in EEC (R227Q) and ADULT (R298Q) retain the wildtype TAp63γ transactivation potential necessary to regulate

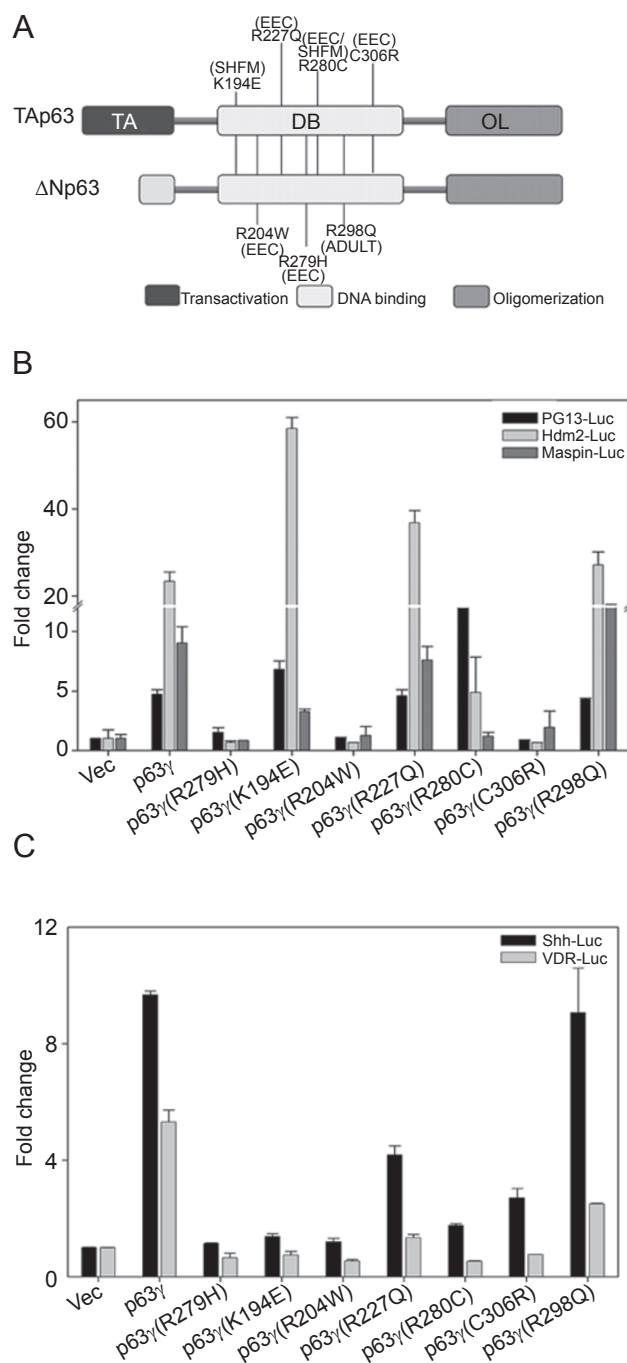


Figure 1 Differential transactivation potential of p63 mutants on p53/p63 and p63 specific reporters. **(A)** A schematic representation of p63 gene architecture and location of p63 missense mutations tested and the syndrome associated with each of the mutations in this study. **(B and C)** H1299 cells were transfected with **(B)** PG13-Luc, Maspin-Luc and Hdm2-Luc reporters or **(C)** Shh-Luc and VDR-Luc reporters alone or along with wildtype TAp63γ or TAp63γ mutants and CMV-R-Luc plasmids using lipofectamine 2000 as indicated. The ratio of firefly luciferase units to Renilla luciferase units was calculated for normalizing luciferase units to transfection efficiency. The Y-axis represents the fold change in relative luciferase units compared with empty vector.

p63 specific target genes tested in this study. Although the TAp63 γ mutants tested in this study interact with wildtype p63, they do not affect the localization of wildtype TAp63 γ . Interestingly, these mutants can regulate the ability of the wildtype p63 to bind to the promoter regions of the target genes. Finally, TAp63 γ (R227Q), TAp63 γ (R298Q) and TAp63 γ (K194E) mutants also retain the ability of wildtype TAp63 γ to induce apoptosis.

Results

TAp63 γ (R227Q) and TAp63 γ (R298Q) mimic wildtype TAp63 γ in regulation of p63 specific targets

We examined the effect of naturally occurring TAp63 γ mutants and wildtype TAp63 γ on target gene reporters which were specific for both p53 and p63, such as PG13-Luc, Hdm2-Luc and Maspin-Luc (Figure 1B), as well as on p63 specific target gene reporters such as Shh-Luc and VDR-Luc (Figure 1C) [23, 24]. These studies were performed in H1299 cells, since these cells lack p53 and therefore we can attribute the induction of genes to p63 (wild type or mutant). As expected, wildtype TAp63 γ used as a positive control significantly up-regulated the activity of all the reporters tested. TAp63 γ (R227Q) and TAp63 γ (R298Q) mutants exerted similar effects as wildtype TAp63 γ showing significant up-regulation of all the tested reporters (Figure 1B and 1C). Interestingly, the TAp63 γ (K194E) mutant, which is observed only in SHFM syndrome, significantly increased the activity of p53 and p63 specific reporters but had no effect on p63 specific reporters. Similarly, the TAp63 γ (R280C) mutant observed in both SHFM and EEC also up-regulated PG-13 Luc and Hdm2-Luc reporter activity, but did not significantly affect the activity of Maspin-Luc, VDR-Luc and Shh-Luc. Finally, mutants TAp63 γ (R279H), TAp63 γ (R204W) and TAp63 γ (C306R), all observed only in EEC syndrome, had little or no effect on the activity of all the reporters tested (Figure 1B and 1C).

We next examined the effect of these mutants on endogenous expression levels of Hdm2, p21, Shh and VDR (Figure 2). H1299 cells were transfected with TAp63 γ and TAp63 γ mutants or an empty vector backbone, after which the relative expression of these genes was assessed at the transcript levels and protein levels (Figure 2). Consistent with our results from the transactivation data (Figure 1), cells transfected with TAp63 γ , TAp63 γ (R227Q), TAp63 γ (R298Q), TAp63 γ (K194E) and TAp63 γ (R280C) showed a significant increase at both transcript and protein levels of Hdm2 and p21, also shown to be targets of p63 [23]. Like wildtype p63, TAp63 γ (R227Q) and TAp63 γ (R298Q) mutants were able to induce Shh and VDR expression, but both TAp63 γ (K194E) and TAp63 γ (R280C) were unable

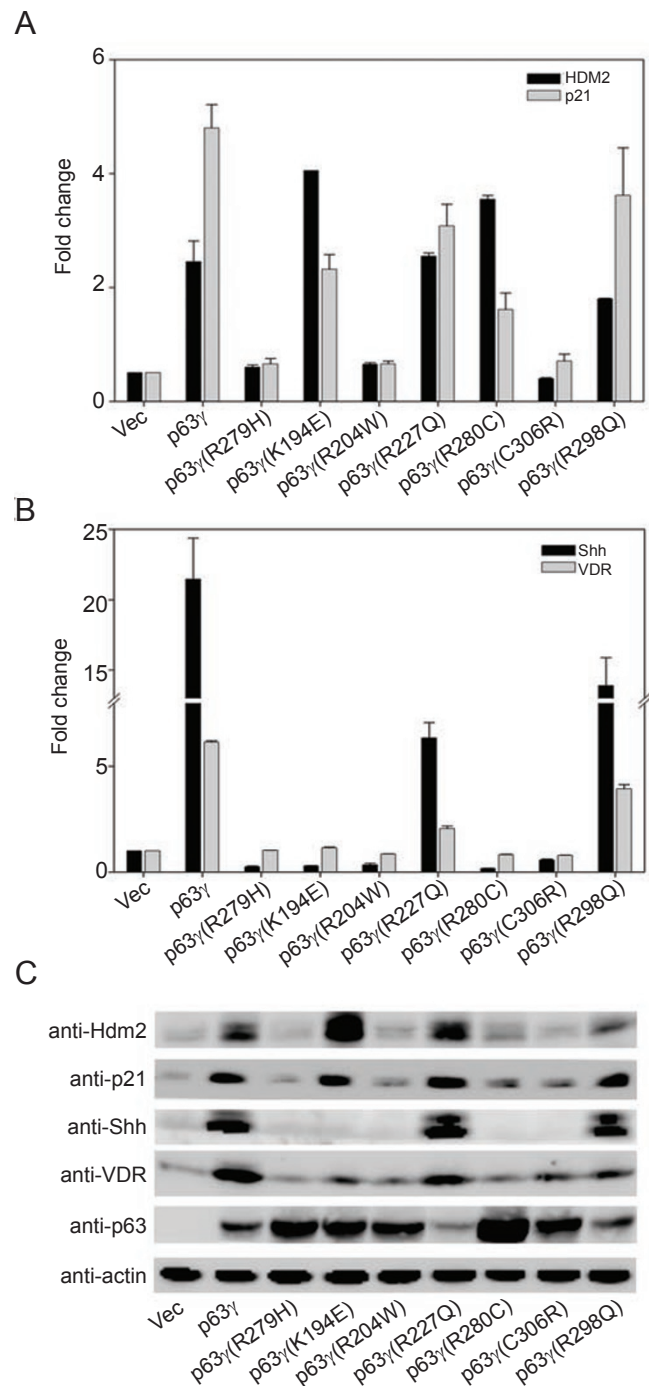


Figure 2 Differential activation of p53/p63 and p63 specific target genes by p63 mutants. **(A)** H1299 cells were transfected with TAp63 γ , TAp63 γ mutants or empty vector alone as indicated. At 24 h post-transfection, total RNA was extracted and **(A)** Hdm2 and p21; **(B)** Shh and VDR transcript levels were detected using TaqMan-based real-time PCR. The Y-axis represents the fold change in transcript levels relative to vector-transfected cells. **(C)** Immunoblot analysis was performed to confirm the overexpression of p63 and endogenous expression of Hdm2, p21, Shh and VDR using gene specific antibodies. Immunoblotting for β -actin served as the loading control.

to induce tested p63 specific targets Shh and VDR. Finally, mutants TAp63γ (R279H), TAp63γ (R204W) and TAp63γ (C306R) showed little or no effect on the transcript and protein levels of Hdm2, p21, Shh or VDR (Figure 2). In addition, we observed differences in the levels of overexpressed mutants, which indicated variability in the stability of these mutants. For example, similar to wildtype TAp63γ, TAp63γ (R227Q) and TAp63γ (R298Q) mutants were less stable when compared to the other mutants tested in this study. Taken together, these results demonstrate that while TAp63γ (R227Q) and TAp63γ (R298Q) mutants behave in a manner similar to wildtype p63, the mutants TAp63γ (K194E) and TAp63γ (R280C) observed in SHFM syndrome can

only induce targets specific for both p53/p63. The mutants TAp63γ (R279H), TAp63γ (R204W) and TAp63γ (C306R), all observed only in EEC syndrome, had no effect on any of the tested p53/p63 or p63 target genes.

TAp63γ mutants do not affect TAp63γ-mediated Hdm2 induction, but EEC syndrome mutants inhibit TAp63γ-mediated Shh induction

In order to assess the effects of TAp63γ mutants on wildtype TAp63γ-mediated transactivation of genes responsive to p53 and/or p63, H1299 cells were transfected with Hdm2-Luc or Shh-Luc reporter along with TAp63γ alone or with increasing dose of TAp63γ mutants as indi-

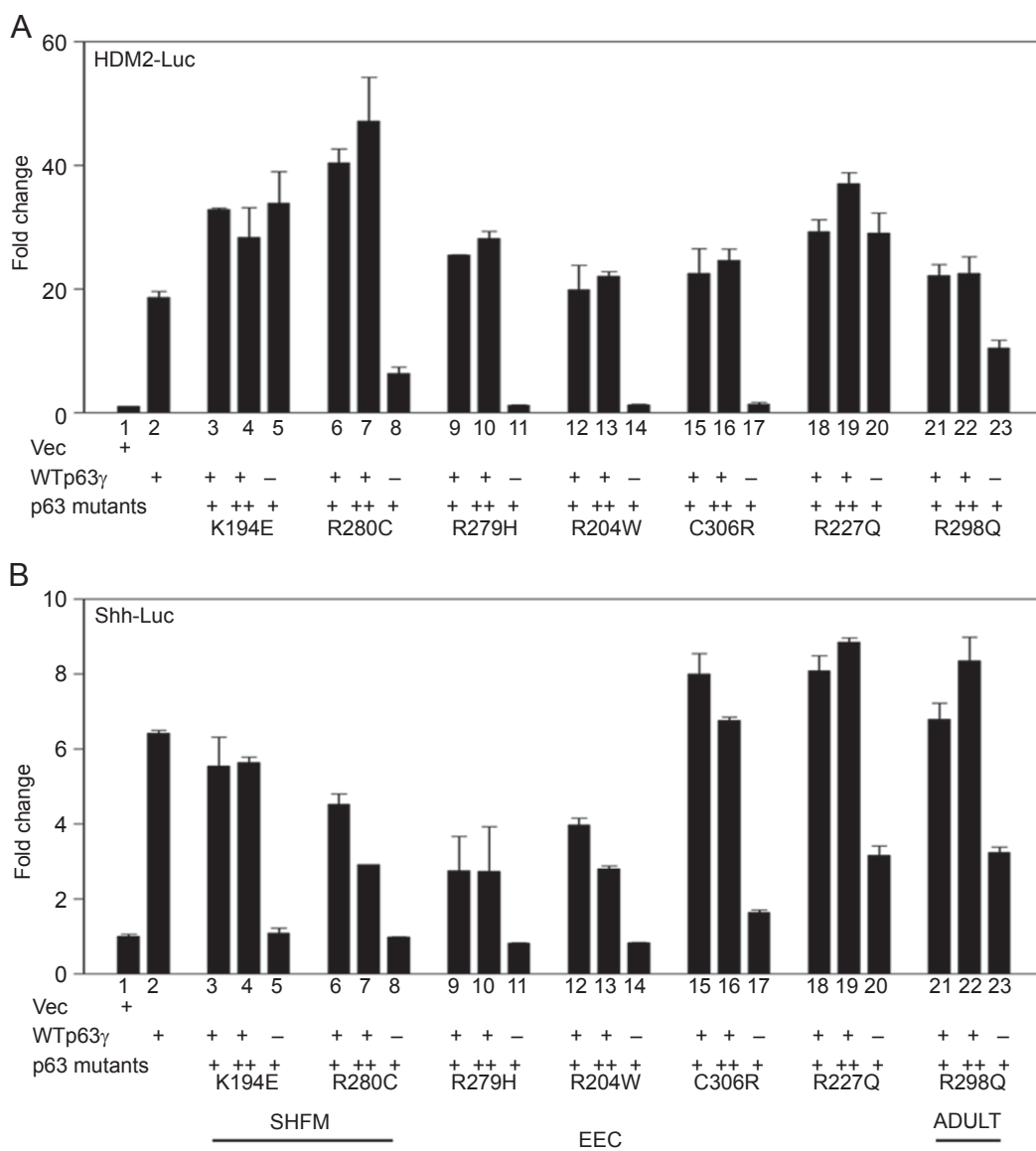


Figure 3 TAp63γ mutants differentially affect the wildtype-TAp63γ-mediated transactivation. H1299 cells were transfected with the Hdm2-Luc (A) or Shh-Luc reporter (B) alone or co-transfected with wildtype TAp63γ alone or along with TAp63γ mutants as indicated using lipofectamine 2000. The Y-axis represents the fold change in relative luciferase units compared with empty vector.

cated (Figure 3). As expected, TAp63 γ by itself led to a significant increase in the Hdm2 reporter activity (Figure 3A). Co-transfection of wildtype TAp63 γ with TAp63 γ (R280C), TAp63 γ (K194E) and TAp63 γ (R227Q), which can themselves induce Hdm2, led to a significant increase in wildtype-mediated Hdm2-Luc reporter activity. However, co-transfection of wildtype TAp63 γ with TAp63 γ (R298Q), which can also induce Hdm2, only led to a modest increase in Hdm2 reporter activity. Interestingly, the TAp63 γ (R279H), TAp63 γ (R204W) and TAp63 γ (C306R) mutants, which were unable to transactivate Hdm2 by themselves, did not inhibit wildtype-TAp63 γ -mediated transactivation of Hdm2-Luc reporter activity (Figure 3A). The effects of these p63 mutants on TAp63 γ -mediated Hdm2 induction was also monitored at the transcript and protein levels (Figure 4A and 4B). Interestingly, TAp63 γ -mediated Hdm2 induction was not significantly affected by TAp63 γ (K194E), TAp63 γ (R280C), TAp63 γ (R227Q) and TAp63 γ (R298Q) mutants. Consistent with the transactivation data, TAp63 γ (R279H), TAp63 γ (R204W) and TAp63 γ (C306R) mutants did not inhibit wildtype-TAp63 γ -mediated transactivation of Hdm2.

Next we monitored the effects of TAp63 γ mutants on wildtype-TAp63 γ -mediated transactivation of a p63 specific target, Shh [24]. We observed that TAp63 γ -induced transactivation of Shh reporter was significantly inhibited by TAp63 γ (R280C), TAp63 γ (R279H) and TAp63 γ (R204W) mutants (Figure 3B). Interestingly, the TAp63 γ (R227Q) and TAp63 γ (R298Q) mutants (Figure 3B) showed a modest increase in wildtype-TAp63 γ -mediated transactivation of Shh reporter. Although, the TAp63 γ (C306R) and TAp63 γ (K194E) mutants do not induce Shh reporter activity, they did not affect the ability of wildtype TAp63 γ to induce Shh reporter activity (Figure 3B). The effects of these p63 mutants on TAp63 γ -mediated induction of Shh were also studied at the transcript and protein levels. TAp63 γ (R227Q) and TAp63 γ (R298Q) mutants did not affect TAp63 γ -mediated induction of Shh (Figure 4A and 4B). While TAp63 γ (R279H) mutant significantly inhibited wildtype-TAp63 γ -mediated Shh induction, TAp63 γ (R204W) mutants only led to a modest decrease in the TAp63 γ -mediated Shh induction (Figure 4A and 4B). Once again, TAp63 γ (C306R) and TAp63 γ (K194E) mutants did not affect wildtype-TAp63 γ -mediated Shh induction. Together, the co-transfection studies clearly demonstrate that, while none of the TAp63 γ mutants affect the ability of TAp63 γ to induce Hdm2, a target regulated by both p63 and p53; some of the EEC mutants tested in this study inhibit the ability of TAp63 γ to induce Shh.

TAp63 mutants do not affect the localization of wildtype TAp63

To assess whether p63 mutants affect the localization

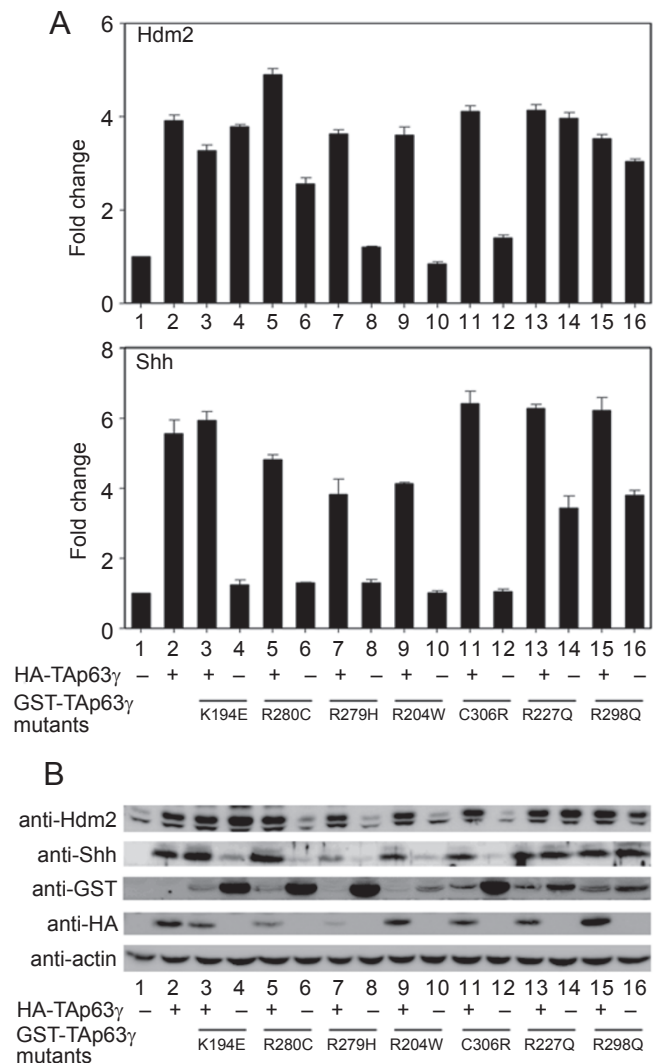


Figure 4 Effect of TAp63 γ mutants on TAp63 γ -mediated induction of Hdm2 and Shh expression. H1299 cells were transfected with wildtype TAp63 γ alone or co-transfected with TAp63 γ mutants as indicated. **(A)** Transcript levels of Hdm2 (upper panel) and Shh (lower panel) were detected using TaqMan-based real-time PCR. The Y-axis represents fold change in transcript levels relative to vector-transfected cells. **(B)** Immunoblot analysis was performed to detect the endogenous protein expression of Hdm2 and Shh and to confirm the overexpression of HA-tagged TAp63 γ and GST-tagged TAp63 γ mutants. Immunoblotting for β -actin served as the loading control.

of wildtype TAp63 γ immunofluorescence assays were performed by transfecting H1299 cells with HA-tagged wildtype TAp63 in the presence or absence of GST-tagged TAp63 γ mutants (Figure 5). We have shown the data for three representative mutants – TAp63 γ (R227Q), which mimics wildtype; TAp63 γ (K194E), which is the only mutant that showed partial cytoplasmic localization and also upregulated target genes specific for p63 and p53; and TAp63 γ (R279H), which leads to a dose-dependent

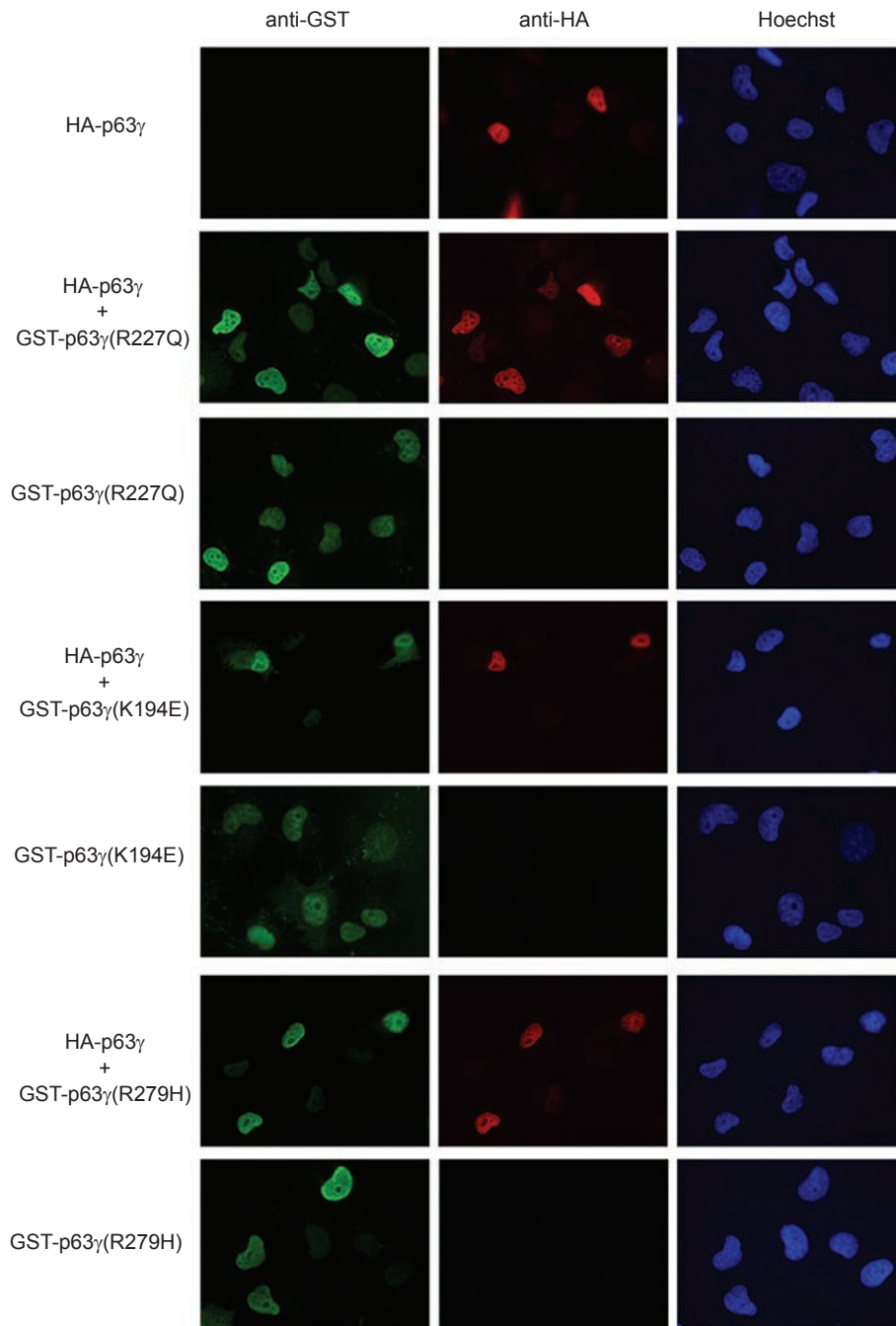


Figure 5 TAp63 γ mutants do not affect the localization of wildtype TAp63 γ . H1299 cells were transfected with HA-tagged TAp63 γ alone or along with GST-tagged TAp63 γ mutants as indicated. At 24 h post-transfection immunofluorescence assay was performed. Wildtype TAp63 γ and TAp63 γ mutant expression was detected using mouse anti-HA and rabbit anti-GST primary antibodies, respectively, and subsequently with the corresponding secondary antibodies. The nucleus was stained with Hoechst dye and the cells were examined using a fluorescence microscope.

decrease in TAp63 γ -mediated induction of Shh, a p63 specific target [24]. As shown in Figure 5, TAp63 γ , when expressed alone or together with the mutants, was localized to the nucleus. Similarly, TAp63 γ (R227Q), TAp63 γ (K194E) and TAp63 γ (R279H) mutants primarily localized to the

nucleus. All the other TAp63 γ mutants (R204W, R280C, C306R and R298Q) were also localized to the nucleus and did not affect the localization of wildtype TAp63 γ (data not shown). Examined together, we show that all the TAp63 γ mutants are localized to the nucleus. Furthermore, when

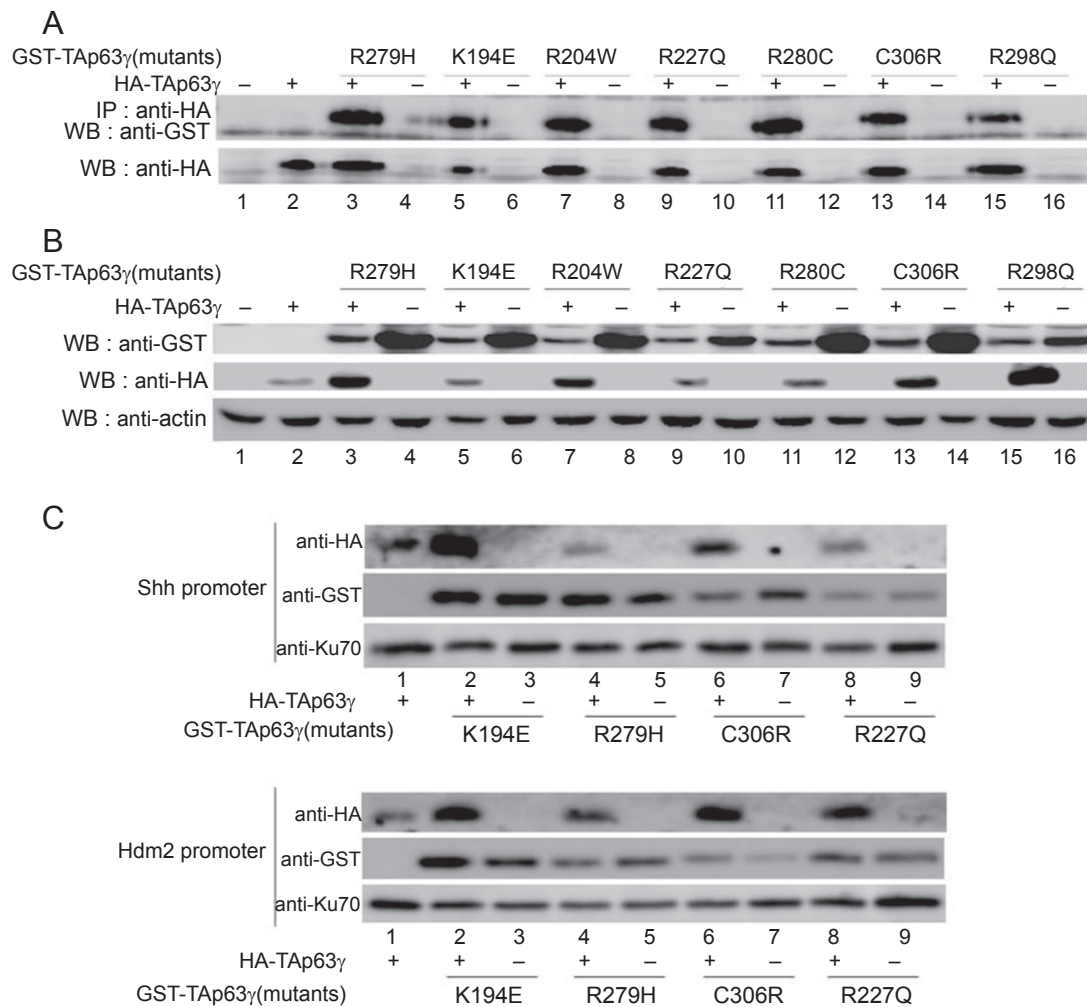


Figure 6 TAp63 γ mutants interact with wildtype TAp63 γ and affect its DNA-binding activity. H1299 cells were transfected with HA-tagged TAp63 γ alone or along with GST-tagged TAp63 γ mutants as indicated. At 24 h post-transfection, whole cell lysates were prepared. **(A)** In total 300 μ g of protein from each sample was subjected to immunoprecipitation using anti-HA mouse antibody. Immunoprecipitated protein samples were resolved on SDS-PAGE gel and immunoblotted with anti-GST rabbit antibody. **(B)** To confirm the overexpression, of wildtype and mutant proteins, equivalent amounts of protein from each condition were fractionated onto a SDS-PAGE and immunoblotted with anti-HA mouse and anti-GST rabbit antibodies. **(C)** H1299 cells were transfected with HA-tagged wildtype and GST-tagged mutant TAp63 γ plasmids. Nuclear extracts prepared from these cells representing the wildtype p63 and/or GST-tagged p63 mutant proteins were incubated with equivalent amounts of streptavidin-coated biotinylated Shh or Hdm2 promoters as indicated. HA-tagged wildtype p63 and GST-tagged mutant p63 binding were assessed by immunoblotting with anti-HA and anti-GST antibodies. Immunoblotting with Ku-70 antibody was performed to demonstrate equal amounts of DNA in each condition.

these mutants were co-transfected with wildtype TAp63 γ , cells still retained the wildtype TAp63 γ in the nucleus.

TAp63 γ mutants interact with wildtype TAp63 γ and affect the DNA-binding activity of wildtype

We next examined whether the mutant p63 can associate with wildtype p63. H1299 cells were transfected with expression vectors encoding GST-TAp63 γ mutants in the presence or absence of wildtype HA-TAp63 γ as shown in

Figure 6. Whole cell extracts were subjected to immunoprecipitation experiments using anti-HA antibody against wildtype TAp63 γ (Figure 6A) and subsequently immunoblotted with anti-GST to detect GST-tagged TAp63 γ mutants. Our results clearly indicate that all the p63 mutants associated with wildtype TAp63 γ . Overexpression of the wildtype and mutant p63 in whole cell extracts was confirmed by immunoblotting with anti-HA and anti-GST antibodies (Figure 6B). Given the differential effects of the p63 mutants on

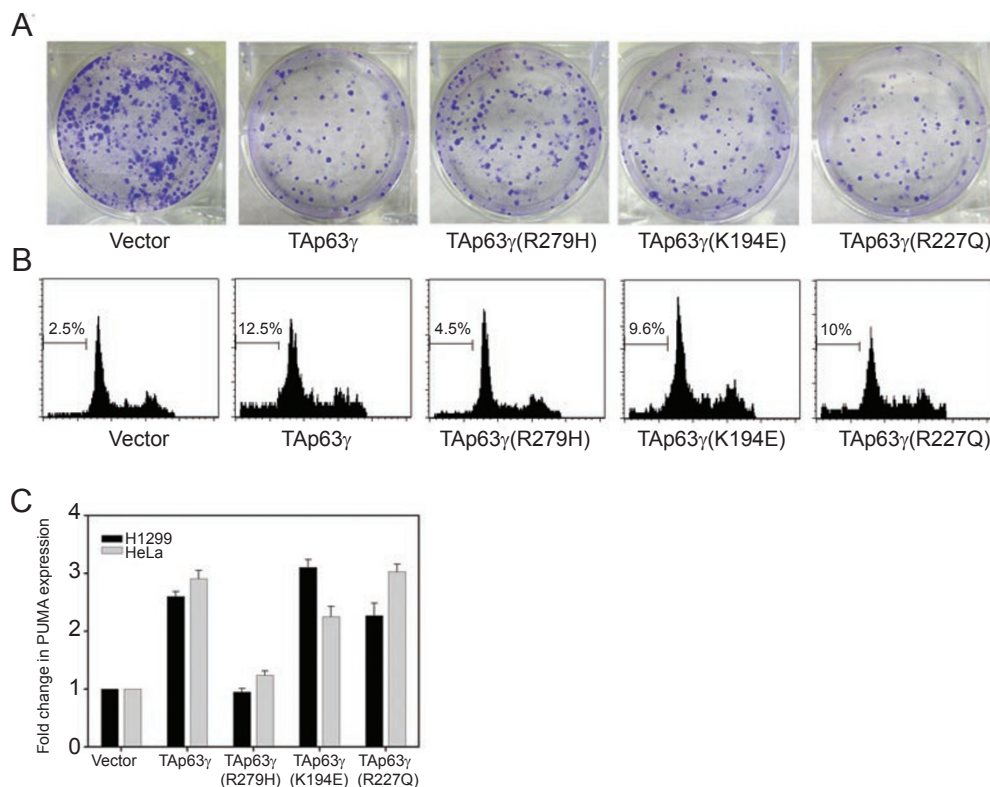


Figure 7 Effect of TAp63 γ mutants on cell growth. H1299 cells were transfected with either TAp63 γ or representative TAp63 γ mutants or empty vector control as indicated. **(A)** The colonies stained with crystal violet from a representative experiment are shown. **(B)** H1299 cells were co-transfected with either TAp63 γ mutants or wild TAp63 γ and membrane-bound hybrid-US9GFP (PAB35). Cells were harvested at 48 h post-transfection, fixed in 70% ethanol and DNA stained with propidium iodide solution, as described in Materials and Methods. DNA distribution was analyzed using flow cytometry using CellQuest Program. The histogram represents the percentage of sub-G1 cells positive for PI and membrane-bound hybrid GFP. **(C)** Total RNA was extracted and TaqMan-based real-time PCR analysis was performed to detect the transcript levels of PUMA. The Y-axis represents the fold change in PUMA transcript levels relative to vector-transfected cells.

the transactivation activity of wildtype TAp63 γ , we next performed DNA pull-down assay to examine the effect of representative p63 mutants on the binding of wildtype p63 to the Shh and Hdm2 promoters (Figure 6C). As expected, wildtype TAp63 γ binds to both Shh and Hdm2 promoters, although binding of wildtype p63 on Shh promoter was higher than on Hdm2 promoter. The higher binding of TAp63 γ to the Shh promoter can be due to the presence of p63 specific DNA-binding sequences in the Shh promoter region as shown earlier [24]. A significant increase in binding of wildtype TAp63 γ on Shh and Hdm2 promoters was observed in the presence of TAp63 γ (K194E) and TAp63 γ (C306R) mutants. This is consistent with our results in Figures 3 and 4 which show that co-transfection of these mutants with wildtype p63 led to an increase in wildtype-p63-mediated Shh induction. Interestingly, TAp63 γ (R279H) mutant significantly lowered the binding of wildtype TAp63 γ on Shh promoter, however, modestly increased the binding of wildtype TAp63 γ on Hdm2 pro-

motor. Once again this result is consistent with our observation that TAp63 γ (R279H) mutant led to a dose-dependent decrease in wildtype-p63-mediated Shh induction but had no effect on wildtype-p63-mediated induction of Hdm2. In contrast, TAp63 γ (R227Q) mutant significantly increased the binding of wildtype TAp63 γ on Hdm2 promoter, but had a modest effect on the binding of wildtype TAp63 γ on Shh promoter. Interestingly, we observed that the p63 mutants alone were also able to bind to the Shh and Hdm2 promoter regions as shown in the lanes with mutant alone, which further supports our data that some of these DNA-binding mutants by themselves can regulate certain target genes as shown in Figures 1-3. This is consistent with previous findings that DNA-binding domain mutants can retain the ability to bind to promoter regions of target genes [33]. Altogether, our results demonstrate a promoter-specific differential effect of TAp63 γ mutants on the DNA-binding activity of wildtype TAp63 γ .

Differential effects of TAp63 γ mutants on cell growth

TAp63 isoforms have been reported to be involved in promoting apoptosis during development and cancer progression. In particular, p63 has been shown to be required for p53-mediated apoptosis in mouse embryonic fibroblasts [34, 35]. Towards understanding the biological function of the mutants, we studied the effects of TAp63 γ mutants on cell survival and apoptosis using colony formation assay and flow cytometry, respectively. Once again we focused on representative mutants TAp63 γ (K194E), TAp63 γ (R279H) and TAp63 γ (R227Q). As expected based on the transactivation data, TAp63 γ (R227Q) and TAp63 γ (K194E) led to reduced cell growth similar to wildtype TAp63 γ when compared to control vector (Figure 7A and 7B). In contrast, TAp63 γ (R279H), a dominant-negative mutant, showed an increase in cell growth when compared to wildtype TAp63 γ (Figure 7A). Consistent with colony formation assay results, cells transfected with TAp63 γ (K194E), TAp63 γ (R227Q) and wildtype showed 9.6%, 10% and 12.5% sub-G1 population, respectively, and therefore increased apoptosis relative to vector (2.5%) (Figure 7B). In contrast, cells with the TAp63 γ (R279H) mutant showed significantly reduced levels of apoptosis (4.5%), consistent with the colony formation assay results (Figure 7A). We also measured the effects of TAp63 γ mutants on the transcript levels of PUMA, a well-known player in DNA-damage-induced apoptosis shown to be induced by TAp63 γ (Figure 7C) [36]. We observed that in two different cell lines, H1299 and HeLa, wildtype TAp63 γ , TAp63 γ (K194E) and TAp63 γ (R227Q) showed a significant induction of PUMA, whereas TAp63 γ (R279H) had little or no effect on PUMA expression. These results correlated well with the sub-G1 fraction quantitation indicative of apoptosis in Figure 7B. These results demonstrate that TAp63 γ mutants exert differential effects on cell survival and growth inhibition, which might explain the phenotypic variations observed within p63-associated diseases.

Discussion

p63 plays an indispensable role in epithelial morphogenesis and cancer progression [2]. Pathogenic mutations of p63 are shown to be responsible for several human syndromes which exhibit developmental defects. Although distinct p63 mutational patterns are observed with each developmental syndrome, the impact of these p63 mutations on gene expression and physiology of cells during development and cancer progression is still not clear. It was previously reported that arginine codons at positions 204, 227, 279 and 280 of p63 are important for specific and nonspecific interactions with DNA target sequences; mutations within these residues are highly detrimental to

DNA binding and transactivation activity of p63 [37]. Interestingly, many of these p63 mutations observed in EEC syndrome correspond exactly to the hotspot mutations in the p53 gene: p63 R204, R279 and R280 are analogous to p53 R175, R248 and R249, respectively, an exception being R227 mutation, which is only observed with p63 [38]. Our studies demonstrate that arginine mutants TAp63 γ (R279H and R204W) and cysteine mutant TAp63 γ (C306R) lack transactivation activity based on their inability to induce the expression of p21, Hdm2, Shh, VDR and PUMA (Figures 1 and 7). Interestingly, p63 heterozygous mutant mice are predisposed to tumor formation [11, 39]. It is therefore possible that the inability of TAp63 γ (R279H), TAp63 γ (R204W) and TAp63 γ (C306R) mutants to induce cell cycle arrest or apoptotic genes might predispose the patients harboring these mutations to a greater incidence of tumor formation. Both TAp63 γ (R227Q) and TAp63 γ (R298Q) mutants mimic their wildtype counterpart in their ability to transactivate both p63/p53 and p63 specific targets, and therefore such mutations are less likely to increase the risk of these patients towards cancer development. Together these results demonstrate that TAp63 γ (R227Q) and TAp63 γ (R298Q) mutants are similar to wildtype p63 in transactivation potential as well as stability (Figures 1 and 2). Unlike other EEC mutants studied, the fact that TAp63 γ (R227Q) acts like wildtype p63 can be attributed to the fact that TAp63 γ (R227Q) is a rare EEC mutation, of which the phenotype lacks the characteristic orofacial clefting and there are fewer limb defects than typically observed in EEC syndrome [40]. Additionally, a distinct phenotypic overlap between ADULT and EEC syndromes has been reported [41], which might reflect the ability of the mutants to retain the transcriptional potential of the wildtype TAp63 γ .

Interestingly, all p63 mutants tested were very stable, with the exception of mutants TAp63 γ (R227Q) and TAp63 γ (R298Q), which were as unstable as the wildtype TAp63 γ (Figure 2C). Although TAp63 γ (K194E) mutant is very stable when compared to wild type, it can significantly transactivate Hdm2 but not Shh. On the contrary, low amounts of TAp63 γ (R227Q) and TAp63 γ (R298Q) can lead to induction of both Hdm2 and Shh. Therefore, we believe that the specificity of targets is not merely dictated by the protein levels or stability.

Differential regulation of p53/p63 and p63 specific target genes by mutants associated with EEC syndrome suggests that the molecular basis of phenotypic variation observed within the EEC syndrome could be a result of the perturbation of the different signaling pathways normally regulated by p63. Our results show that, while TAp63 γ (R279H) mutant was unable to affect the TAp63 γ -mediated induction of p53/p63 target genes, it significantly inhibited the wildtype-

p63-mediated induction of p63 specific genes (Figures 3 and 4). This indicates that the EEC mutant TAp63 γ (R279H) may not always act in a dominant-negative fashion towards all target genes. Adding to this complexity, our results show that, while TAp63 γ (R280C) and TAp63 γ (K194E) mutants significantly induced the p53/p63 responsive genes, these mutants did not induce the p63 specific target genes (Figures 1 and 2). Lack of Maspin induction by TAp63 γ (R280C) alone and not by TAp63 γ (K194E) might be attributed to the fact that the TAp63 γ (R280C) mutation has been observed in both EEC and SFHM syndrome patients, unlike the TAp63 γ (K194E) mutation, which is observed only in SFHM syndrome patients. Our results are consistent with the observation that Arg 280 and Lys 194 residues, although involved in the maintenance of the overall structure of the DNA-binding domain, when mutated, only have subtle effects on the DNA-binding capacity of p63 [33]. It is plausible that subtle differences in the transactivation ability of these mutants might be critical not only for the clinical variability observed in the same syndrome, but also for other pathogenic conditions associated with alterations in p53 and p63.

Our results demonstrate that TAp63 γ (R279H), TAp63 γ (R204W) and TAp63 γ (R280C) mutants act in a dominant-negative manner to inhibit the wildtype-TAp63 γ -mediated transactivation (Figure 3). In contrast, TAp63 γ (R227Q) and TAp63 γ (R298Q) enhance the wildtype-TAp63 γ -mediated transactivation (Figure 3). Despite the fact that TAp63 γ (K194E) and TAp63 γ (C306R) mutants by themselves were unable to induce Shh, co-transfection of these mutants with wildtype TAp63 γ did not affect wildtype-TAp63 γ -mediated transactivation of Shh. Interestingly, wildtype p63 interacts with all the mutants tested and localization of wildtype p63 was not affected by any of the mutant (Figures 5 and 6), suggesting that the ability of mutants to inhibit wildtype activity might not be simply due to formation of heterotetramers, but could be due to the ability of dominant-negative mutants to compete with wildtype p63 for binding to p63 specific responsive elements. We clearly demonstrated that TAp63 γ (R279H) inhibits the wildtype TAp63 binding to p63 specific target gene promoter (Shh), but modestly enhances the wildtype TAp63 γ binding to p53/p63 specific target gene promoter (Hdm2). This is consistent with the ability of the TAp63 γ (R279H) mutant to inhibit wildtype-TAp63 γ -mediated transactivation of p63 specific genes but not of p53/p63 specific genes (Figures 3 and 4). The inability of TAp63 γ (R279H) to induce genes is likely due to its lack of transactivation potential, since TAp63 γ (R279H) binds to both Shh and Hdm2 promoters (Figure 6). Although TAp63 γ (K194E) and TAp63 γ (C306R) mutants were able to enhance the ability of wildtype TAp63 to bind to the Hdm2 and Shh

promoter regions (Figure 6C), these mutants were unable to enhance the wildtype-TAp63 γ -mediated transactivation of target genes. This suggests that enhancing the wildtype p63 binding on target promoters is not enough to enhance the wildtype-mediated induction of target genes.

Previously published reports indicated that exogenous TAp63 can activate genes involved in cell cycle arrest and apoptosis [42, 43]. Consistent with our transactivation results, we demonstrated the differential ability of p63 mutants in promoting apoptosis. While the TAp63 γ (R227Q) and TAp63 γ (K194E) mutants significantly promote cell death, TAp63 γ (R279H) mutant was unable to promote significant cell death. The differential effect of mutants on cell survival may also dictate the complex phenotypic variations in p63-associated diseases. In conclusion, our data demonstrate that the different TAp63 γ mutants vary in their ability to transactivate p53/p63 and p63 target genes. The phenotypic variations observed within p63-related syndromes could in part be due to the differential effects of these mutants on canonical and non-canonical downstream signaling pathways of p63. Although several p63 mutants were able to transactivate targets specifically regulated by p63 and p53, only TAp63 γ (R227Q) and TAp63 γ (R298Q) mutants were able to induce Shh and VDR, two p63 specific target genes. Since both these genes are known to play a critical role in epithelial development, specifically, limb development and keratinocyte differentiation shown to be defective in p63 knockout mice, regulation of these two genes by p63 might play a crucial role in the biological function of p63. Undoubtedly, a better understanding of the effects exerted by these mutants may improve our comprehension of cancer biology and aid in better therapeutic strategies at least in cancers involving dysfunctions of p63.

Materials and Methods

Cell lines and plasmids

H1299, a human non-small lung carcinoma cell line (obtained from ATCC), and HCT 116 p53 $^{-/-}$, a colon epithelial cell line (a generous gift from Dr Steve Berberich, Wright State University), which are devoid of p53, were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine calf serum (FBS) and 1% PS (penicillin and streptomycin) at 37 °C in humidified 5% CO₂. Expression plasmids encoding GST-TAp63 and HA-TAp63 γ were constructed as described earlier [44]. GST-tagged TAp63 mutants were created using PCR-based site-directed mutagenesis method using sense and antisense primers. The primer sets for the mutants included in this study are (1) K194E sense(5'-CAT GCC TGT CTA CAA AGA AGC TGA GCA CGT CAC-3') and antisense(5'-GTG ACG TGC TCA GCT TCT TTG TAG ACA GGC ATG-3'); (2) R204W sense(5'-GGA GGT GGT GAA GTG GTG CCC CAA CCA TG-3') and antisense(5'-CAT GGT TGG GGC ACC ACT TCA CCA CCT CC-3'); (3) R227Q sense(5'-CTC CTA GTC ATT TGA TTC AAG TAG AGG GGA ACA GC-3') and antisense(5'-GCT

GTT CCC CTC TAC TTG AAT CAA ATG ACT AGG AG-3'); (4) R280C sense (5'-GGA GGG ATG AAC CGC TGT CCA ATT TTA ATC ATT GTT ACT-3') and antisense(5'-AGT AAC AAT GAT TAA AAT TGG ACA GCG GTT CAT CCC TCC-3'); (5) C306R sense (5'-GGC CCG GAT CCG TGC TTG CCC AG-3') and antisense(5'-CTG GGC AAG CAC GGA TCC GGG CC-3'); and (6) R298Q sense (5'-GCA AGT CCT GGG CCA ACG CTG CTT TGA GG-3') and antisense(5'-CCT CAA AGC AGC GTT GGC CCA GGA CTT GC-3'). The primer sets for the R279H mutant has been described earlier [24]. PG13-Luc reporter plasmid containing 13 copies of p53-binding DNA consensus sequence was obtained from Dr Steven Berberich. Other reporters, Maspin-Luc and Hdm2-Luc, were a kind gift from Dr Lindsey Mayo. Shh and VDR full-length promoter constructs were constructed as reported earlier. Membrane-bound hybrid GFP plasmid, PAB35, was a kind gift from Dr Lynn W Enquist.

Transactivation studies

To measure the PG13-Luc, Hdm2-Luc, Maspin-Luc, Shh-Luc and VDR-Luc reporter activities, cells were seeded in 24-well plates and transfected with 100 ng of reporter constructs along with desired plasmids and a renilla luciferase plasmid to normalize for transfection efficiency using lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 24 h post-transfection, whole cell extracts were made in Passive Lysis buffer and dual luciferase assay was performed to detect both firefly and Renilla luciferase activity as per the manufacturer's protocol (Promega, Madison, WI). The relative luciferase activity was measured by calculating the ratio of firefly luciferase activity to Renilla luciferase activity.

Protein isolation and immunoblotting studies

H1299 cells were seeded onto six-well plates and transiently transfected with desired plasmids. At 24 h post-transfection, cells were harvested in RIPA buffer (0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, phosphate buffered saline, pH 7.4). Equivalent protein extracts were subjected to immunoblotting using anti-VDR D-6, anti-p21 C-19, anti-Shh H160, anti-p63 4A4 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mdm2 (Calbiochem, San Diego, CA) and anti- β -actin (Sigma, St Louis, MO) antibodies to detect VDR, p21, Shh, p63, Hdm2 and β -actin expression, respectively. HA-tagged wildtype TAp63 γ and GST-tagged mutants were also detected using 12CA5 anti-HA (Roche Molecular Biochemicals, CA) and Z5 anti-GST (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, respectively.

RNA isolation and TaqMan-based real-time PCR

For RNA studies, cells were transfected with the desired expression plasmid. At 24 h post-transfection, cells were lysed directly on the culture plate using the RNAeasy method as per the manufacturer's protocol (Qiagen, Valencia, CA). Quantitative real-time PCR analysis was performed as described earlier using an assay on Demand reagents specific for p21 (Hs_00355782_m1), Hdm2(Hs_00242813_m1), Shh((Hs_00179843_m1), VDR (Hs_0017213_m1) and PUMA (Hs_00248075_m1) (PE Applied Biosystems, Foster City, CA) [24].

Immunoprecipitation assay

H1299 cells were transiently transfected with expression plasmids encoding HA-tagged wildtype TAp63 γ and GST-tagged TAp63 γ mutants alone or in combination, as indicated. Cells were harvested

for total protein using RIPA buffer. Equivalent amount of protein was precleared with 20 μ l of rec-protein G-sepharose beads (Invitrogen, Carlsbad, CA) for 1 h at 4 $^{\circ}$ C, followed by O/N incubation with 1 μ g of monoclonal anti-HA 12CA5 antibody (Roche Diagnostics, Indianapolis, IN). The next day, immunoprecipitated samples were incubated with rec-protein G-sepharose beads for an hour, followed by 4 \times washes with RIPA buffer to remove the unbound proteins. Immunoprecipitated samples with beads were run on 10% SDS gel and immunoblotted with rabbit polyclonal anti-GST Z5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence studies

H1299 cells were plated on sterilized coverslips at a density of 1.5×10^5 cells/six-well plate. At 24 h after seeding, expression plasmids encoding HA-tagged wildtype TAp63 or GST-tagged TAp63 γ mutants were transiently transfected either alone or in combination. For immunofluorescence staining, after washing with $1 \times$ DPBS, cells were fixed for 8 min with 3% paraformaldehyde and permeabilized for 20 min with 1.0% Triton X-100. Cells were blocked with 0.5% normal goat serum (NGS) and incubated with primary antibodies for 1 h at room temperature. Primary antibodies used to detect HA-TAp63 γ , and GST-tagged mutants were mouse monoclonal anti-HA 12CA5 (Roche Diagnostics, Indianapolis, IN) at a dilution of 1:100 and rabbit polyclonal anti-GST Z5 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200. After three washes with 0.5% NGS, cells were incubated with secondary goat anti-rabbit, fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:250, and secondary donkey anti-mouse, Texas red dye-conjugated IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:275 for 1 h at room temperature. Hoechst dye 33342 (Sigma, St Louis, MO) was used for nuclear staining. Preparations were examined using fluorescence microscopy.

DNA-binding assays

H1299 cells were transfected with desired plasmids and nuclear extracts were harvested using NucBuster Protein Extraction Kit as per the manufacturer's protocol (Novagen, Madison, WI). Biotinylated Shh and Hdm2 promoters were generated by amplifying the full-length Shh and Hdm2 promoters from Shh-Luc and Hdm2-Luc reporters, respectively, using biotinylated primers (IDT, Coralville, IA). Primers used are as follows: for Shh, 5'-5-biotinylated GAG CTC TCT GTG CTT GAT GAC TGA AGC-3' and 5'-CTC GAG CTC GCC CAT GGA ACT GAT GAC-3'; for Hdm2, 5'-5-biotinylated- TAC TGG CCC GGC AGC GAG CGG TCA CTT TTG-3' and 5'-CTG GGA AAA TGC ATG GTT TAA ATA GCC CCA-3'. Equivalent amounts of biotinylated Shh and Hdm2 promoters preincubated with streptavidin beads were resuspended in EMSA buffer (5% glycerol, 10 mM HEPES pH 7.9, 75 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl₂, and 1 mM EDTA) and were incubated with nuclear extracts prepared from cells transfected with wildtype p63 alone and/or along with GST-tagged p63 mutants. A total of 60 μ g of nuclear extract was added to the biotinylated promoter and streptavidin beads complexes and incubated at 4 $^{\circ}$ C for 1 h. After incubation the DNA:protein complexes were washed with EMSA buffer and wildtype p63, GST-tagged p63 mutant proteins bound to the biotinylated promoters were run on SDS gel, and binding of wildtype and mutant p63 was assessed by immunoblot analysis using anti-HA and anti-GST antibodies, respectively.

Flow cytometry

H1299 cells were plated at a density of 2.25×10^5 cells/six-well plate and co-transfected with expression plasmids encoding membrane-bound hybrid-US9GFP (PAB35) with either TAp63γ or TAp63γ mutants or empty vector using lipofectamine 2000. Membrane-bound GFP plasmid (PAB35) was co-transfected along with either TAp63γ or TAp63γ mutants, to distinguish the transfected cells from the non-transfected cells. At 48 h post-transfection, cells were harvested for flow cytometry. Cells were collected by trypsinization, pelleted and resuspended in phosphate-buffered saline ($1 \times$ PBS) and fixed in 70% ethanol at -20°C . The cells were stained in PBS containing 50 μg/ml propidium iodide and 100 μg/ml RNase A (Sigma, St Louis, MO) for 30 min and analyzed by flow cytometry using CellQuest software (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA). For each analysis 10 000 cells positive for GFP and propidium iodide fluorescence were collected.

Colony formation assay

H1299 cells were seeded at a density of 2.5×10^5 cells/six-well plate and transfected with expression plasmids encoding TAp63γ, TAp63γ mutants or vector using lipofectamine 2000 as indicated. At 24 h post-transfection, cells were trypsinized, pelleted and resuspended in fresh media for counting. From each condition 1 000 cells were plated in a six-well plate and media was changed after every 2 days. After 15 days of seeding and monitoring cell growth, the media was aspirated and cells were washed with $1 \times$ DPBS and 1 ml of crystal violet dye (0.1% crystal violet in 10% ethanol) was added to each well for 5 min. The plates were subsequently washed with water twice and left to dry at room temperature for 2 days and pictures taken.

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