

## C-terminal $\alpha$ Domain of p63 Binds to p300 to Coactivate $\beta$ -Catenin



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### Abstract

*TP63* (p63), a member of the tumor suppressor *TP53* (p53) gene family, is essential for ectodermal tissue development and suppresses malignant progression of carcinomas. The most abundant isoform,  $\Delta Np63\alpha$  (referred to as p63), lacks the N-terminal transactivation (TA) domain, and was originally characterized as a dominant-negative type suppressor against p53 family proteins. It also binds to TCF/LEF to inhibit  $\beta$ -catenin. Nevertheless, transcriptional activation by p63 has also been observed in varied systems. To understand the puzzling results, we analyzed the structure–function relationship of p63 in the control of  $\beta$ -catenin-dependent transcription. p63 acted as a suppressor of moderately induced  $\beta$ -catenin. However, when nuclear targeted S33Y  $\beta$ -catenin was applied to cause the maximum enhancer activation, p63 displayed a  $\beta$ -catenin-coactivating function. The DNA-binding domain of p63 and the target sequence facilitated it. Importantly, we newly found that, despite the absence of TA domain, p63 was associated with p300, a general adaptor protein and chromatin modifier causing transcriptional activation. C-terminal  $\alpha$  domain of p63 was essential for p300-binding and for the coactivator function. These results were related to endogenous p63-p300 complex formation and Wnt/ $\beta$ -catenin-responsive gene regulation by p63 in squamous cell carcinoma lines. The novel p63-p300 interaction may be involved in positive regulation of gene expression in tissue development and carcinogenesis.

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### Introduction

p63 (TP63), a member of the tumor suppressor p53 (TP53) gene family [1–3], is essential for ectodermal tissue development and controls carcinogenesis and malignant progression [4–6]. This gene encodes transcriptional activation domain-containing isoforms (TA-p63) that resemble p53 and N-terminally truncated isoforms ( $\Delta N$ -p63). Both TA and  $\Delta N$  transcripts undergo alternative splicing to form  $\alpha$ ,  $\beta$ , and  $\gamma$  variants [1].

The TA domain of p63 has a single p300-binding site, whose sequences are conserved among p53 family proteins, to transactivate target genes [7]. In contrast,  $\Delta N$ -p63 proteins lack the entire TA domain and instead have  $\Delta N$ -specific sequences of only 14 amino acid residues. Based on the structural aspect and inhibition of p53 and TA-p63,  $\Delta N$ -p63 proteins were originally defined as dominant-negative type suppressors against p53 family [1].

Well-differentiated squamous cell carcinomas of head-and-neck, as well as carcinomas of lung, urothelia, breast, skin etc., exhibit high-level expression of p63 [3,8]. Importantly, the loss of p63

Abbreviations: TA, transactivation; NF-Y, nuclear transcription factor Y; WRE, Wnt response element.

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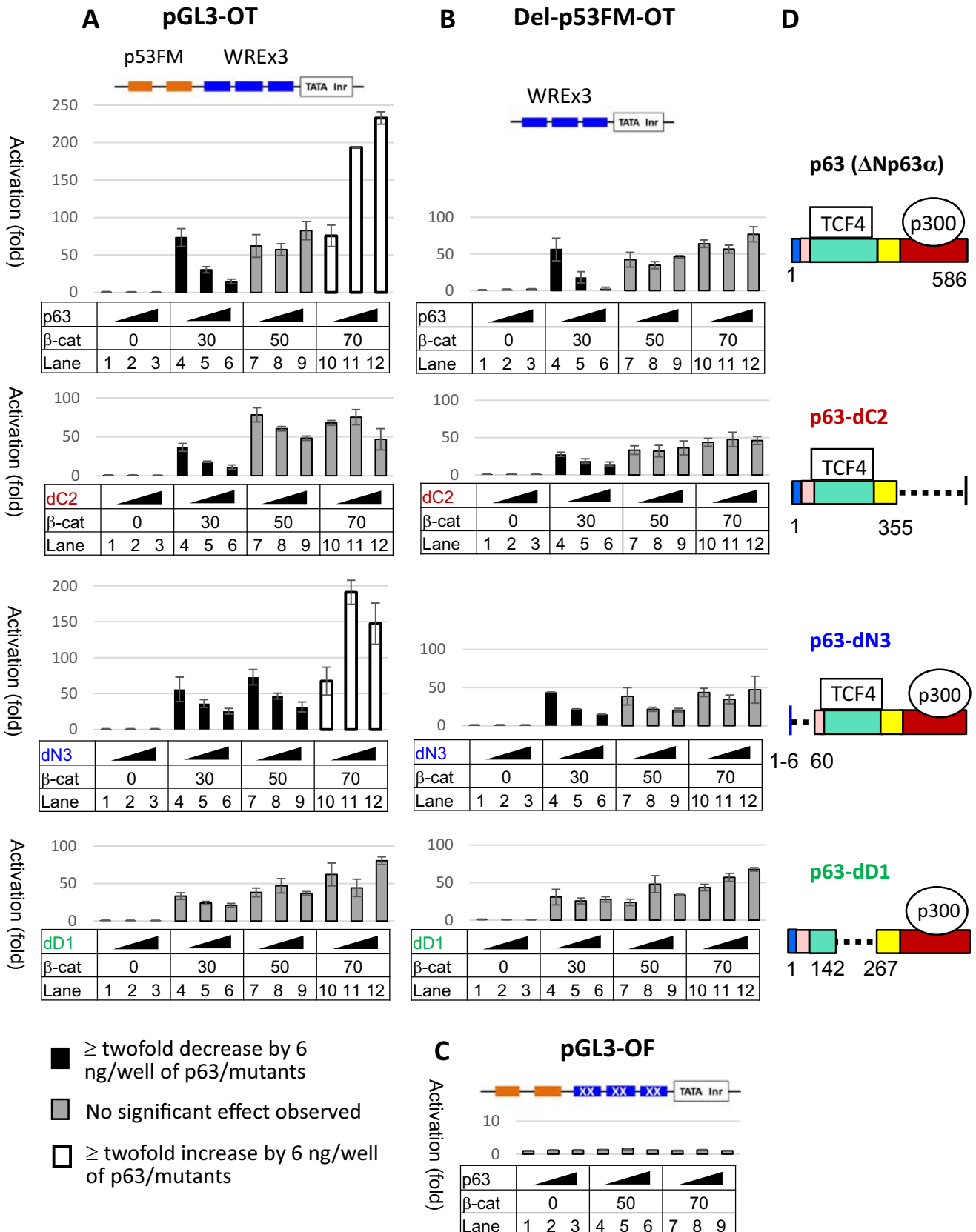
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expression was associated with malignant progression [9–12]. As the ΔN-p63-α isoform is the most abundant p63 protein in these carcinomas and keratinocyte stem cells [1,13–15], ΔNp63α is hereafter referred to as p63 unless otherwise specified.

A genome-wide search for p63-binding sites resulted in the identification of direct p63-binding sequences corresponding to the p53-binding motif as well as the motifs of transcription factors essential for tissue development [16]. Unlike the original characterization, p63



lacking the TA domain appeared to upregulate transcription in some cases [16,17]. For instance, p63 enhances *PERP* expression, where the molecular mechanism remains unidentified [18]. p63 directly activates *HSP70* by interacting with nuclear transcription factor Y (NF-Y) [19], although cell cycle G2/M promoters were repressed by the p63-(NF-Y) interaction [20].

Others and we reported that p63 binds to HMG domain of lymphoid enhancer factor/T-cell factor (TLE/TCF) proteins to antagonize  $\beta$ -catenin [21,22]. However, some results were not absolutely consistent with this conclusion. In a p63-expressing squamous cell carcinoma line, Wnt/ $\beta$ -catenin target genes such as *MMP7* and *AXIN2* were indeed upregulated by p63 knockdown, whereas expression of *SNAI2*, *CCND2* and *MYC* decreased as p63 diminished. When analyzed with pGL3-OT, a widely used Wnt/ $\beta$ -catenin-responsive reporter plasmid, p63 suppressed  $\beta$ -catenin-dependent transcription in Saos-2 and Huh7. In contrast, a  $\beta$ -catenin-enhancing effect of p63 was observed in HEK293 cells with the same plasmid [21–23].

We considered these disputable results as manifestation of as yet unknown mechanisms with p63. This study explored molecular interactions underlying the positive and negative regulation of Wnt/ $\beta$ -catenin-dependent gene expression by p63.

## Materials and Methods

### Plasmids

The p63-coding sequences (NM\_001114980, variant-4 of TP63) from the codon-optimized clone (Origene Technologies, RC225987) were recloned into pCMV6-Ac-HA vector to express p63 with C-terminal hemagglutinin (HA) tag (pCMV6-p63HA). The p300 expression vector, pCMVb-p300, was a gift from William Sellers, Dana Farber Cancer Institute (Addgene plasmid # 10717). Plasmids for the expression of nuclear-targeted S33Y- $\beta$ -catenin and Myc-tagged-TCF4 as well as pGL3-OT, pGL3-OF, and pGL3-OT-del-p53FM were previously described [22]. Deletion mutants, p63-dN3 and p63-dD1, were generated from pCMV6-p63HA using the PrimeSTAR Mutagenesis Basal Kit (Clontech-Takara Bio, R046A). PCR primers used were as follows: p63-dN3 (p63-dN3-F, 5'-TGGAAA ACTCCACCTTCG ATGCTCT-3'; p63-dN3-R, 5'-AAGGTGGAGTTTTCCAGG TACA ACAT-3') and p63-dD1 (p63-dD1-F, 5'-CTGAGCA CAAGCAGCAAGTTTCGGACAC-3'; p63-dD1-R 5'-TTGCTGCTTGTGCTCAGCTTTTTTGTAG-3'). pCMV6-p63HA was digested with *MunI* and *NotI* to remove the C-terminal domain sequences and circularized with a inker (dC2HA-FOR, 5'-AATTACGCGTACGC-3'; dC2HA-REV, 5'-GGCCGCGTACGCGT-3') to form p63-dC2. As the codon-optimized pCMV6-Entry clone caused an eight-fold increase in p63 protein synthesis as compared with the previously used pRc/CMV clones [22], we proportionally decreased the amount of p63-expression vectors. Codon-optimized adenovirus type-5 Flag (KDD)-tagged E1A (Accession number AP\_000197) and E1b-55 K

(AP\_000199) expression vectors were from Origene Technologies (VC100110 and VC100112).

### Luc Reporter Assay

Cells were seeded ( $8 \times 10^4$  cells/well) in 24-well plates 24 h before transfection. In addition to the *luc* vector (25 ng/well) and Myc-TCF4 expression vector (25 ng/well), those of nuclear-targeted S33Y- $\beta$ -catenin (0, 30, 50, and 70 ng/well) and p63 (0, 3, and 6 ng/well) were introduced. A total of 130 ng of DNA was transfected into each well with Effectene (Promega). Cells were lysed with Glo-lysis buffer (Promega) at 48 h. *Luc* enzyme assay was carried out as previously described [22]. All transfection experiments were performed in triplicate, and results shown as mean  $\pm$  S.D. Each transfection was repeated at least two times with similar results.

### Immunoprecipitation and Western Blotting

Saos-2 cells were seeded ( $10^6$  cells/well) in 6-cm plates 24 h prior to transfection. DNA (2  $\mu$ g) was transfected in each plate with Turbofectin 8.0 (Origene). At 48 h, plates were chilled on ice, washed, and lysed with IP Lysis Buffer (Thermo Scientific, 87,787) supplemented with protease inhibitor cocktail (Sigma-Aldrich, S8820). After centrifugation, cell lysates were incubated with agarose conjugates with anti-HA (MBL, 561–8, rabbit IgG), anti-Myc (Abcam, Ab1253, goat IgG), or anti-p300 (Santa Cruz, sc-48,343, mouse IgG) for 2 h at 4 °C. Protein complexes were washed four times, and dissociated in SDS-PAGE sample buffer. We used 7.5% polyacrylamide gel (Mini-PROTEAN TGX, Bio-Rad) for SDS-PAGE. Anti-Myc (MBL, 562), anti-p63 (Abcam, Ab735), anti-p300 (Santa Cruz, sc-585), anti-NFYC (Santa Cruz, sc-390,861), anti-Flag (Sigma, F3165, M2), anti-E1A (Santa Cruz, sc-58,658) and anti-HDAC1 (Abcam, ab19845) antibodies were used for western blotting. Alkaline phosphatase-conjugated secondary antibodies (Cell Signaling Technology, #7056S and #7054S) were used in combination with Immune-Star AP Substrate (Bio-Rad, 1,705,018).

### Cell Lines

Saos-2 cells were obtained from RIKEN BRC Cell Bank (RCB0428), and cultured in RPMI1640 medium (substituting McCoy's 5A) supplemented with 10% fetal bovine serum. We also used FaDu (ATCC HTB-43) and A431 (ATCC CRL-1555) from American Type Culture Collection, and HSC-1 from JCRB (JCRB1015).

### p63 Knockdown

p63 RNA-silencing was carried out by transfection of siRNA targeting p63 (p63i) and control siRNA (Ci) with siLentFect (Bio-Rad) as described [14,22].

## Results

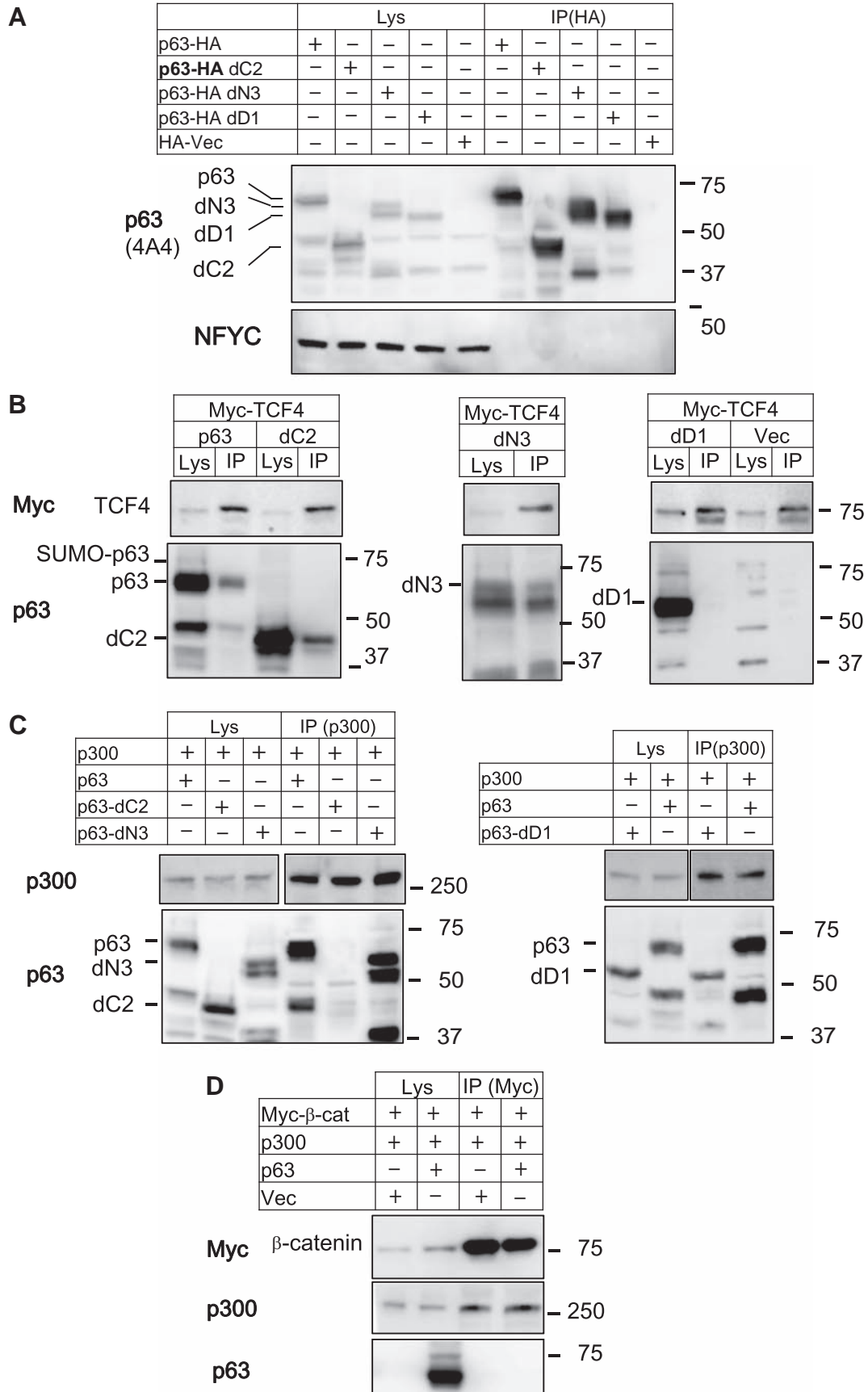
### Positive and Negative Control of $\beta$ -Catenin by p63

*Luc* reporter gene expression assay was performed in p53-null, p63-untranscribed Saos-2 cells. To focus on the nuclear events, we used the constitutively active form of  $\beta$ -catenin (S33Y  $\beta$ -catenin) in

**Figure 1.** Suppression and coactivation of  $\beta$ -catenin by p63. *Luc* reporter assay was performed with pGL3-OT (A), del-p53FM-OT (B), and pGL3-OF (C). Expression vector of nuclear-targeted S33Y- $\beta$ -catenin was used at indicated amounts (ng/well). Increasing amounts of p63 expression plasmids (0, 3, and 6 ng/well) were also introduced (dark right triangle). Dark columns indicate that *luc* activity decreased to one-half or less by p63/mutants ( $\geq$  twofold decrease by 6 ng/well of p63/mutants), while white columns indicate an increase of twofold or more by p63/mutants ( $\geq$  twofold increase by 6 ng/well). Gray columns show that the alteration was not significant. Domain structures of p63 and mutants are outlined (D). The N-terminal  $\Delta$ N-specific region (blue), proline-rich region (peach), DNA-binding domain (right green), oligomerization domain (yellow), and C-terminal  $\alpha$ -domain (red) are depicted. Protein interactions detected in Figure 2 are also summarized.

this study. In addition to the wild-type (WT) p63, the following deletion mutants were examined: p63-dC2, p63-dN3, and p63-dD1 (Figure 1D). p63-dC2 had a deletion of the entire α-domain at the

C-terminus (residues 356–586). p63-dN3 lacked the N-terminal structure (residues 7–59), while p63-dD1 lacked a large part of the DNA-binding domain (143–266) but retained the presumed



nuclear localization signal and oligomerization domain as well as the  $\alpha$ -domain. Different amounts of p63 and mutants (0, 3, and 6 ng for one well with  $8 \times 10^4$  cells) (Figure 1A, dark right triangle) were transfected in combination with S33Y  $\beta$ -catenin expression plasmid (0, 30, 50, and 70 ng/well). Constant amounts of the Myc-TCF4 expression vector and pGL3-OT *luc* plasmid having three copies of Wnt/ $\beta$ -catenin response element (WRE) and a p53 family protein binding motif (p53FM) [22] (Figure 1A) were contained in the transfection mixture.

When S33Y  $\beta$ -catenin was increased from 30 to 50 and 70 ng/well, in the absence of p63, we observed no further increase in *luc* expression (Figure 1A, top panel, lanes 4, 7, and 10, respectively), implying that  $\beta$ -catenin-dependent transcription reached the maximum level by saturation of myc-TCF4 with S33Y  $\beta$ -catenin. Under the assay conditions, we examined how p63 influenced  $\beta$ -catenin-driven transcription. Consistent with our previously described results with WT  $\beta$ -catenin [22], p63 exerted an inhibitory effect against S33Y  $\beta$ -catenin at  $\leq 30$  ng/well (Figure 1A, top, compare lane 4 with lanes 5 and 6). Interestingly, p63 failed to suppress S33Y  $\beta$ -catenin at 50 ng/well (compare lane 7 with lanes 8 and 9), but enhanced S33Y  $\beta$ -catenin at 70 ng/well (lanes 10–12). p63 was unable to activate *luc* expression without  $\beta$ -catenin transfection (lanes 1–3).

p63-dC2 retained the S33Y  $\beta$ -catenin (30 ng) suppressive activity (Figure 1A, second panel, lanes 4–6), which was less prominent with S33Y  $\beta$ -catenin at 50 ng/well. In contrast to WT p63, dC2 mutant failed to enhance S33Y  $\beta$ -catenin at 70 ng/well. Thus, the  $\alpha$  domain seemed responsible for the  $\beta$ -catenin-enhancing function of WT p63.

p63-dN3 showed both  $\beta$ -catenin-suppressing (Figure 1A, the third panel, lanes 4–6) and coactivating (lane 10–12) effects as p63 did, although a minor alteration in kinetics was observed: This mutant suppressed not only 30 ng/well but also 50 ng/well of S33Y  $\beta$ -catenin (lanes 7–9). Thus, the N-terminal structure of p63 was unnecessary for suppression and coactivation of  $\beta$ -catenin. p63-dD1 neither down-regulated nor up-regulated S33Y  $\beta$ -catenin at 30–70 ng/well (Figure 1A, bottom). The DNA binding domain may contribute to the control of  $\beta$ -catenin in both directions.

### p53FM Facilitates Coactivation of $\beta$ -Catenin by p63

We performed *luc* assays with the pGL3-OT-del-p53FM plasmid (here referred to as Del-p53F-OT), in which the p53 family protein-binding motif (p53FM) was deleted (Figure 1B) [22]. As observed with pGL3-OT (Figure 1A, top), S33Y  $\beta$ -catenin at 30 ng/well hit the plateau of transcriptional activation, allowing no further activation with 50 or 70 ng/well of S33Y  $\beta$ -catenin (Figure 1B, top, lanes 4, 7, and 10, without p63). WT p63 suppressed S33Y  $\beta$ -catenin at 30 ng/well (Figure 1B, top, lanes 4–6) but failed to decrease or

increase *luc* expression at 50 ng/well of S33Y  $\beta$ -catenin (lanes 7–9). Thus, p63 did not enhance 70 ng/well of S33Y  $\beta$ -catenin (lanes 10–12) in the absence of p53FM.

p63-dC2 and p63-dN3 also suppressed the lower amount of S33Y  $\beta$ -catenin when tested with del-p53FM-OT (Figure 1B, second and third panels, lanes 4–6). Neither of the p63 mutants enhanced *luc* expression induced by S33Y  $\beta$ -catenin at 70 ng/well (lanes 10–12). As observed with pGL3-OT (Figure 1A, bottom), p63-dD1 showed neither positive nor negative influence on *luc* expression by 0–70 ng/well of S33Y  $\beta$ -catenin (Figure 1B, bottom, lanes 1–12). Thus, the p53FM sequence in pGL3-OT was found to facilitate the p63- and p63-dN3-mediated coactivation of S33Y  $\beta$ -catenin when *luc* expression reached plateau.

We also used pGL3-OF, a widely used control plasmid, wherein each WRE was destroyed by two nucleotide mutations preserving the p53FM site (Figure 1C). p63 had no inhibitory or stimulatory effect on *luc* expression in the presence (50 and 70 ng/well, lanes 4–9) or absence (lanes 1–3) of S33Y  $\beta$ -catenin. Thus, p63 (in the isoform of  $\Delta Np63\alpha$ ) was evidently unable to transactivate p53FM. These experiments with del-p53FM-OT and pGL3-OF indicated that p53FM facilitated  $\beta$ -catenin coactivation by p63. However, this coactivation required higher amounts of S33Y  $\beta$ -catenin and functional WREs, and, therefore, was distinguished from transactivation of the target site by TA-p63.

### p63 Domain-Specific Interactions With Proteins

To understand the mechanisms underlying the positive and negative effects of p63 on  $\beta$ -catenin-dependent transcription, we analyzed protein–protein interactions. HA-tagged p63 and the deletion mutants (p63-dC2, p63-dN3, and p63-dD1) were transfected into Saos-2 cells, followed by immunoprecipitation with an anti-HA antibody (Figure 2A). Ectopically expressed WT and mutant p63 often appeared with lower molecular weight proteins, indicating intracellular degradation. Particularly, p63-dN3 was observed as a doublet, of which the upper band corresponded to the full-length protein. The dN3 deletion may have exposed an N-terminal peptide sensitive to cellular enzymes [24].

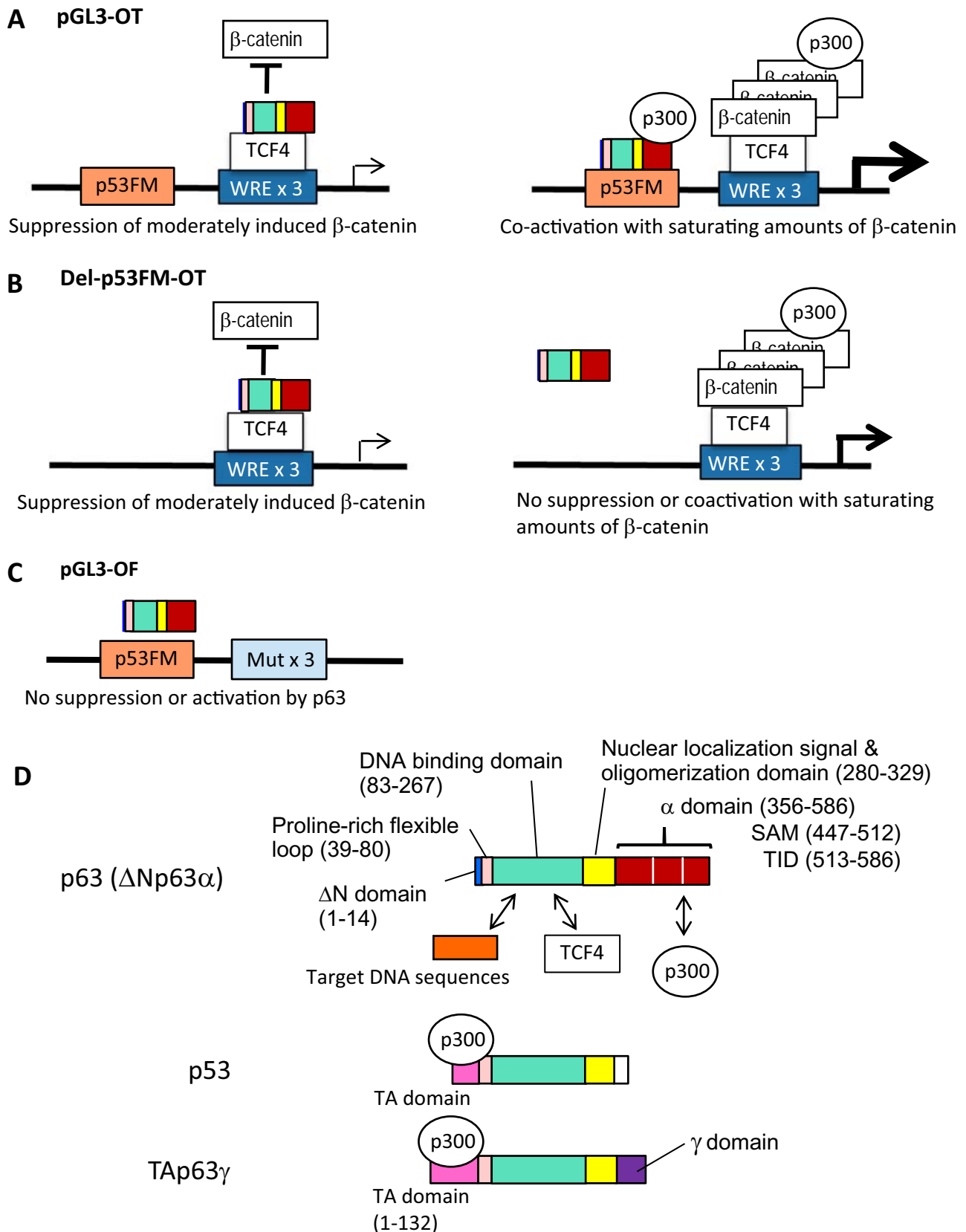
While p63 and its mutant proteins were efficiently precipitated, the abundantly expressed C subunit of NF-Y (NF-YC), was absent in p63 precipitates. Unlike the observation of cell cycle  $G_2/M$  gene repression by p63 [20], the p63-(NF-YC) interaction may be irrelevant to this experimental system.

After transfection of Myc-TCF4 in combination with p63 or its mutants, proteins were precipitated with an anti-Myc antibody (Figure 2B). Consistent with our previous results, p63 was coprecipitated with Myc-TCF4 [21,22]. p63-dC2 and p63-dN3 were also associated with Myc-TCF4 with varied efficiencies. In

**Figure 2.** Interactions of p63 with coactivator proteins of  $\beta$ -catenin. (A) HA-tagged p63 ( $\Delta Np63\alpha$ ) and its truncated forms were transfected into Saos-2 cells and precipitated with an anti-HA antibody. Proteins in the cell lysates (Lys) and immunoprecipitates (IPs) were analyzed by western blotting with antibodies against p63 and NF-YC. Positions of the full-length WT and mutant p63 bands are indicated. Positions and molecular masses (in kilodaltons) of the standards are also indicated. (B) Myc-TCF4 was co-transfected with HA-p63, its deletion mutant, or the empty vector (Vec), and precipitated with an anti-Myc antibody. Western blots were probed for Myc-TCF4 and p63. The weak band of WT p63 at 80 kD is reactive with anti-p63 and anti-SUMO-1 antibodies, and is indicated as SUMOylated p63 (SUMO-p63) [42]. (C) After transfection of p300 with HA-p63 or its deletion mutants, IPs with an anti-p300 antibody were analyzed. Western blots were probed for p300 and p63. Due to the poor detection of p300 in lysates, results with an increased sample amount (2-fold) are shown. (D) Myc-tagged  $\beta$ -catenin and p300 was transfected with or without HA-p63. IPs by the anti-Myc antibody were probed for Myc- $\beta$ -catenin, p300, and p63.

contrast, p63-dD1 was undetectable in the Myc-TCF precipitate. These observations were supported by the result that the amino acids 81–290 of p63 are essential for the interaction with the HMG domain of LEF/TCF [21].

Considering that p63 was able to enhance β-catenin (Figure 1A), we hypothesized involvement of p300 (EP300), a transcriptional coactivator/adaptor protein with histone acetyltransferase activity [25]. In fact, p300 is known as a coactivator of β-catenin [26]. After transfection



of p300 in combination with p63, p63-dC2, p63-dN3, or p63-dD1, proteins were precipitated with an anti-p300 antibody (Figure 2C). As the result, p63 appeared as a dense band on the western blot, suggesting a strong interaction between p63 and p300. Both p63-dN3 and p63-dD1 were also coprecipitated with p300, but p63-dC2 was not. Thus, we found that p300 is a partner of p63, for which the C-terminal domain of p63 is essential. The protein interactions observed in Figure 2, B and C are schematically shown in Figure 1D.

To test whether p63 impairs the interaction between  $\beta$ -catenin and p300, Myc-tagged  $\beta$ -catenin and p300 were transfected with or without p63. The anti-Myc antibody precipitated p300 with Myc- $\beta$ -catenin, and the interaction was unaffected by p63 (Figure 2D). Thus, p63 neither inhibited nor facilitated the  $\beta$ -catenin-p300 association. Furthermore, p63 was absent in the immune complex, indicating that the tripartite complex of " $\beta$ -catenin-p300-p63" was improbable. The p63-p300 complex (Figure 2C) may exist apart from the  $\beta$ -catenin-p300 complex.

### Hypothetical Model of the Positive and Negative Regulation of $\beta$ -Catenin by p63

Based on the results of reporter assays (Figure 1) and immunoprecipitation (Figure 2), we hypothesized mechanisms underlying the suppression and coactivation of  $\beta$ -catenin by p63 (Figure 3). p63 antagonizes  $\beta$ -catenin by binding to LEF/TCF (Figure 3A, left) [21,22]. The HMG-binding region of p63 (residues 81–290) overlaps with the DNA-binding domain (83–267) (Figure 3D). When the TCF4 molecules are saturated with increased amounts of  $\beta$ -catenin to achieve the maximum *luc* expression, p63 seems to act with p53FM to coactivate  $\beta$ -catenin (Figure 3A, right). Because p63-dC2 was able to suppress but not coactivate  $\beta$ -catenin with pGL3-OT (Figure 1A), the interaction of the C-terminus with p300 may be essential for the coactivator function of p63.

In the absence of p53FM in the enhancer region, p63 inhibited lower concentrations of  $\beta$ -catenin (Figures 1B, 3B, left). During the maximum transcriptional activation by  $\beta$ -catenin, however, p63 neither inhibits nor enhances  $\beta$ -catenin (Figure 3B, right). p63 may be "competed-out" by  $\beta$ -catenin occupying TCF4, but lack any alternative element to interact. In addition, p63, in the form of  $\Delta$ Np63 $\alpha$ , does not directly transactivate p53FM in the absence of WRE sequences (Figures 1C, 3C).

### Analyses of Endogenous p63 in Squamous Cell Carcinomas

We next assessed relevance of the p63-p300 interaction and the model of  $\beta$ -catenin coactivation in p63-expressing squamous cell carcinoma line, FaDu [14]. Since p300 (*EP300*) was initially identified as an adenovirus E1A-binding protein [27], we transfected Flag-tagged E1A (Flag-E1A) (Figure 4A, left). In addition, Flag-E1B-55 K was used as the control protein without an affinity to p300 [28]. When

precipitated with an anti-Flag antibody, Flag-E1A was co-precipitated with endogenous p300. In contrast, the Flag-E1B-55 K precipitate did not contain p300. Neither of the precipitates contained endogenous p63. We next carried out immunoprecipitation with an anti-p300 antibody after Flag-E1A transfection (Figure 4A, right). Endogenous p63 was coprecipitated with p300, as efficiently as Flag-E1A was. p63 may be one of the numerous transcription factors interacting with p300 [25]. In contrast, HDAC1 (histone deacetylase 1) was little detected in the anti-p300 precipitate. Thus, our experimental system revealed specific p300-protein interactions. Furthermore, endogenous p63-p300 complex formation occurred physiologically in FaDu cells.

As shown previously,  $\beta$ -catenin-responsive genes, MMP7 (matrix metalloproteinase-7), and CCND2 (cyclin D2), were sensitively responded to p63-knockdown, in which the former was upregulated by p63-knockdown, while the latter was down-regulated [22]. As analyzed for gene expression with a whole genome microarray, p63-knockdown efficiencies (fold change in p63) differed depending on cell lines, FaDu, HSC-1, and A431, probably reflecting the siRNA transfection efficiencies (Figure 4B). Nevertheless, we constantly observed that MMP7 expression was increased by p63 silencing, while CCND2 expression declined as p63 diminished.

To assess the possibility of  $\beta$ -catenin coactivation by p63 (Figure 3A, right) with the chromosomal DNA sequences, we tested the regulatory sequences, MMP7-WRE1 (found in Enhancer ID GH11J102530, Genome Locator), and CCND2-WREb (GH12J004268), by the reporter assay (Figure 4C). When activated by WT  $\beta$ -catenin approximately 4-fold, these enhancers were evidently repressed by p63 as reported [22]. With S33Y  $\beta$ -catenin (30 ng/well) causing 7–9-fold activation, p63 was still able to repress these enhancers (Figure 4C, a, b, lanes 4–6). When activated 10-fold by S33Y  $\beta$ -catenin (50–70 ng/well, a, lanes 7–12), p63 neither enhanced nor repressed MMP7-WRE1-rep. In contrast, upregulation of CCND2-WREb-rep by p63 became detectable under the full activation by S33Y  $\beta$ -catenin (Figure 4C, b, lanes 7–12).

## Discussion

We observed that p63 influences  $\beta$ -catenin-driven transcription in various modes, as examined with pGL3-OT, its truncated forms, and chromosomal MMP7 and CCND2 enhancer sequences. p63 can act in the  $\beta$ -catenin-suppressing and coactivating modes, depending on the enhancer structure and the magnitudes of  $\beta$ -catenin activation. We also analyzed interactions between p63 and  $\beta$ -catenin-related essential nuclear proteins in p63-transfected and endogenously expressing carcinoma cells. Our results newly showed that p300 is a partner of p63. The C-terminal domain of p63 interacts with p300, which is correlated to the  $\beta$ -catenin coactivating function of p63.

**Figure 3.** Speculative mechanisms of positive and negative control of  $\beta$ -catenin by p63. Based on the previous [21,22] and present study results, possible  $\beta$ -catenin controlling mechanisms by p63 are graphically presented. Blue and orange boxes indicate WRE and half-sites of p53 family protein binding motif (p53FM), respectively. (A) Experiments with pGL3-OT. Suppressor (left) and coactivator (right) modes of p63 in relation to the amounts of S33Y  $\beta$ -catenin transfection (Figure 1A). Bent arrows indicate relative transcription activities. (B) Experiments with del-p53FM-OT. Suppression of the low concentrations of  $\beta$ -catenin (left) and loss of the inhibitory effect with an increase in the amount of  $\beta$ -catenin (Figure 1B). (C) Experiments with pGL3-OF having two-nucleotide mutations in each WRE (Mut). p63 ( $\Delta$ Np63 $\alpha$ ) did not upregulate transcription through p53FM juxtaposed to the Mut sites (Figure 1C). (D) Domain structure of p63 ( $\Delta$ Np63 $\alpha$ ) in comparison with p53 and TAp63 $\gamma$ , the most potent transactivator produced from TP63 [1,2,43]. Amino acid residue numbers are indicated for each domain (colored box). We referred to NM\_001114980.1 for TP63 variant 4 ( $\Delta$ Np63 $\alpha$ ), NM\_000546.5 for TP63 variant 1, and NM\_001114979.1 for TP63 variant 3 (TAp63 $\gamma$ ).





autonomously express both Wnt ligands and receptors under the normal culture conditions [29].

The HMG domain of TCF/LEF binds to the target DNA sequences, or WRE. In addition to p63, other factors of distinct signaling pathways are known to physically interact with HMG. For instance, Smad3 binds to HMG of LEF1/TCF to synergistically activates *Xtwn* gene with  $\beta$ -catenin, wherein Smad-binding and TCF/LEF-binding elements are tandemly aligned [30]. The HMG-binding region of p63 (residues 81–290) determined by Drewelus et al. [21] overlaps with the DNA-binding domain (83–267), which contains an ‘aggregation-prone peptide’, PILTIVTLE (226–233), identified in p53 [31]. The lack of TCF4-binding activity in p63-dD1 may imply contribution of the hydrophobic peptide to the p63-TCF4 interaction.

The coactivation mode of p63 ( $\Delta$ Np63 $\alpha$ ) was distinguished from the well-described transactivation mode of p53 and TA-p63, because the coactivation required stronger induction of  $\beta$ -catenin and functional WREs to which TCF/LEF proteins bind. The TA domain of TA-p63 isoforms interacts with p300 with a stretch conserved among p53 family proteins, (Figure 3D) for transcriptional activation [7]. In the case of TAp63 $\alpha$ , interestingly, the ‘transcription inhibitory’ domain (TID) at the extremely C-terminus of the  $\alpha$ -domain directly binds to the TA domain to block the transactivating function [32–34]. In the scheme, The TID protects the TA domain from p300-binding in the inactive dimer complex, but does not seem to compete against the TA domain for p300 either in the dimer or in the active tetramer. In the central part of  $\alpha$  domain lies a sterile alpha motif (SAM) with which various protein interactions are expected [35]. Although the possibility of TID-p300 interaction is not ruled out, the SAM domain may be able to serve as the p300-binding site in the  $\Delta$ Np63 $\alpha$ -p300 complex.

The TID was also found to interact with HDAC1 and HDAC2 histone deacetylases to suppress target genes of p53, such as p21, 14–3-3 $\sigma$ , and p16/INK4a in epidermal progenitor cells, and *PUMA* in squamous cell carcinomas [36,37]. As p63 can regulate transcription both positively and negatively depending on the enhancers [16,22], these results are not necessarily opposed to our finding. In addition, the negative regulation may result from the dominant negative effect, interaction with cofactors, or cooperation with HDACs.

p300 is regarded as an epigenetic factor that accelerates transcription by relaxing the chromatin structure and assembling various enhancer-binding proteins and the initiation complex [25]. A recent study explained that histone H3 acetylation at Lys 27 (H3K27ac) by p300 or its close relative CBP is essential for enhancer activation [38]. A comprehensive epigenetic study suggested that p63 binding sites at active enhancers with H3K27ac predict p63 target genes in human epidermal differentiation [39]. Furthermore,

inherited p63 mutants that manifest epidermal defects poorly bind to the enhancers involved in keratinocyte differentiation, where H3K27ac signal is also decreased [40]. Thus, our finding may offer a molecular basis for the proposed ‘bookmarking’ function of p63 [41].

### Conclusion

We newly detected complex formation between the C-terminal  $\alpha$  domain of p63 and p300, proposing a model of  $\beta$ -catenin coactivation by p63. In addition to the dominant-negative type suppressor function against p53 family transactivators, p63 (in the form of  $\Delta$ Np63 $\alpha$ ) may be able to play as a positive regulator of gene expression through the functions of p300.

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**Figure 4.** Functions of endogenous p63 in squamous cell carcinomas. (A) Specific immunoprecipitation of endogenous p300. (Left panel) Interaction of endogenous (end) p300 with transfected E1A. FaDu cells were transfected with Flag-E1A and Flag-E1B expression vectors, the lysates (lys) were subjected to immunoprecipitation (IP) with an anti-Flag antibody. Proteins detected in western blotting, as well as the positions of size markers (in kilodaltons) are indicated. SUMO- $\Delta$ Np63 $\alpha$  is also marked. (Right panel) Detection of endogenous p300-p63 complex formation. FaDu cells were transfected with Flag-E1A, followed by lysis and immunoprecipitation with an anti-p300 antibody. (B) Alteration of *CCND2* and *MMP7* expression by p63 knockdown. DNA microarray analysis was performed with Whole Human Genome Microarray kit (Agilent). Alteration of *CCND2* and *MMP7* expression (fold change) were plotted in relation to p63 (p63si versus Csi). Results were obtained with A431, HSC-1, and FaDu (three experiments) cells. (C)  $\beta$ -catenin suppressing and coactivating reactions of p63. *MMP7*-WRE1-rep-luc (a) and *CCND2*-WREb-rep-luc (b) [22] were tested for *luc* expression with different amounts of S33Y  $\beta$ -catenin and p63 in Saos-2 cells.

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