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Cell density-dependent acetylation of ΔNp63α is associated with p53-dependent cell cycle arrest

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ABSTRACT

ΔNp63α is a p63 isoform that is predominantly expressed in the epidermal stem cells and in cancer. To find the regulatory pathways of ΔNp63α, we assessed whether ΔNp63α is acetylated and determined the functional implications of acetylation. First, the hinge region of p63 was shown to be acetylated by PCAF, similarly to other p53 family members. Second, acetylation synergistically induced cytoplasmic localization of ΔNp63α. Finally, acetyl-ΔNp63α was induced during high-density culture, suggesting that acetylation of ΔNp63α may reinforce cell cycle arrest upon cell contact. Altogether, these findings suggest that acetylation of ΔNp63α contributes to the epidermal homeostasis.

Structured summary of protein interactions:
PCAF acetylates **ΔNp63α** by acetylation assay (View Interaction: 1, 2, 3)
ΔNp63α physically interacts with **ΔNp63α** by anti tag coimmunoprecipitation (View interaction)
ΔNp63α physically interacts with **p53** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

p63, an ancient homolog of p53, is indispensable for the maintenance of stratified epithelia [1–3]. Recent findings suggest that p63 is involved in protection against metastasis [4,5] and in maintenance of the genomic integrity of female germ cells [6]. The pleiotropic functions of p63 are performed by distinct forms of p63. ΔNp63α is an isoform predominantly expressed in epidermal stem cells and cancer. ΔNp63α antagonizes p53 and maintains proliferative capacity of epidermal stem cells and progenitors [7–9]. On the other hand, TAp63α is the only form expressed in the female germ cells and activates death program upon genotoxic insults [6].

Although the developmental role of p63 is well defined, its involvement in cancer has remained elusive. Unlike p53, mice heterozygous for the gene encoding p63 do not show overt tumor-susceptibility, and p63 mutations are rarely found in human cancer [10,11]. In contrary, ΔNp63α is frequently over-expressed in squamous cell carcinomas and other cancers [12–15]. Recent whole exome sequencing identified a p63 mutation in head and neck squamous cell carcinoma as well [16]. In addition, p63 deficiency activated cellular senescence [17], and the expression of ΔNp63α inhibited oncogene-induced senescence and cooperated with the oncogenic Ras in tumor promotion [18]. These findings suggest that ΔNp63α might contribute to tumorigenesis at an early stage. Furthermore, depletion of ΔNp63α sensitized cancer cells to genotoxic drugs [19,20], suggesting that regulation of ΔNp63α expression may likely be a matter of life and death for normal as well as cancer cells.

The epidermal stem cell-specific expression of ΔNp63α is first induced at the transcription level, and the stem cell-specific transcriptional elements are being partially characterized [21,22]. P63 protein also undergoes extensive post-translational modifications, which regulate its stability and function. Phosphorylation and sumoylation of p63 have been linked to both its activation and degradation [23–25]. The concept of post-translational modifications is not unfamiliar in this protein family. For instance, p53, upon genotoxic stress, becomes stabilized and subjected to multiple post-translational modifications including phosphorylation and acetylation. The acetylation event is absolutely required for p53 to exert its transcription activation [26,27]. It is conceivable that p63 may also be acetylated, given that p53 and p63 share many regulatory pathways.

In this study, we assessed whether p63 is acetylated and identified two acetylation sites in the hinge region. The biochemical properties of acetylation mimic form of ΔNp63α revealed that acetylations promote cytoplasmic localization of ΔNp63α. In addition, by generating acetyl-p63 specific antibodies, we found that acetylations occur when cells were grown to high density for both

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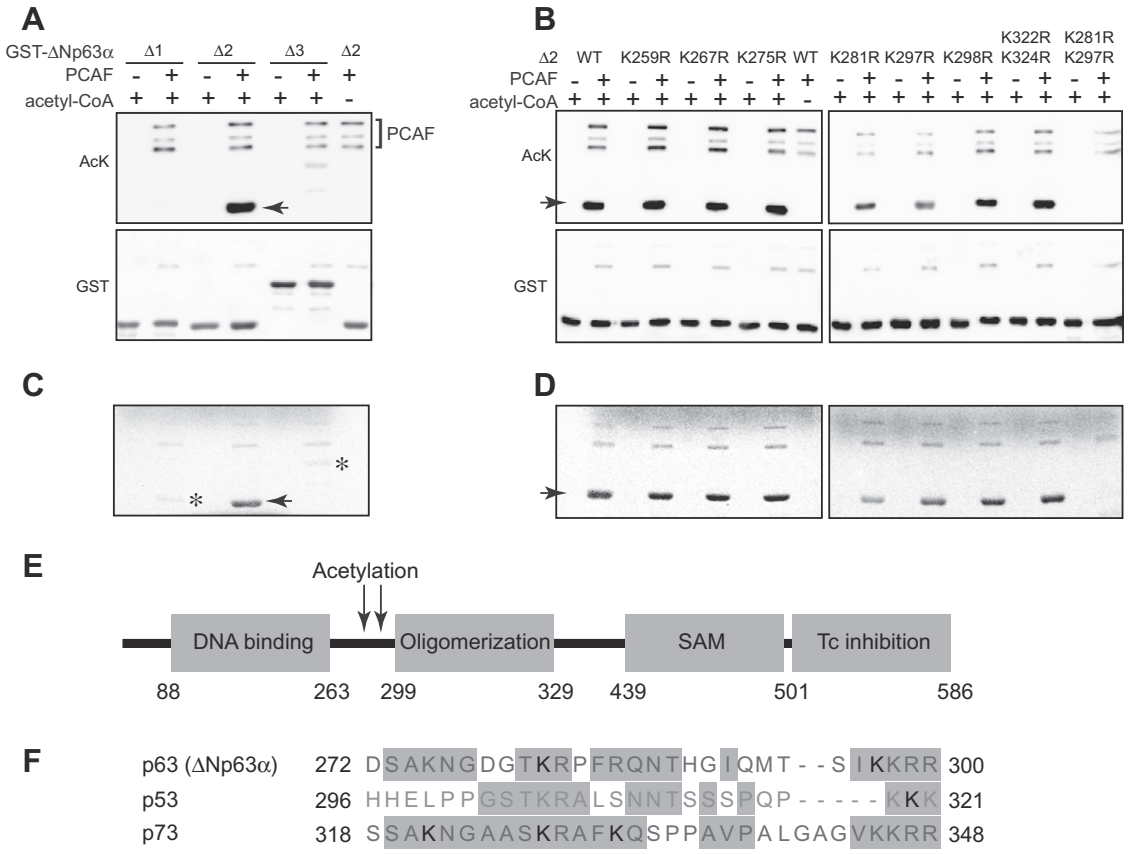

Fig. 1. Hinge region of p63 is acetylated by PCAF. (A) In vitro acetylation was performed on ΔNp63α fragments; Δ1 (aa. 88–184), Δ2 (aa. 256–348), and Δ3 (aa.343–586). Western blotting (WB) by anti-acetyl Lys (AcK) Ab shows Δ2 fragment, located between the DNA binding and oligomerization domain, is robustly acetylated (arrow). Weak acetylation was also detected on the Δ1 and C-terminal Δ3 fragments (asterisks). (B) In vitro acetylation was performed on ΔNp63α fragments with [1-^{14}C] acetyl-CoA and detected by autoradiogram. (C) Lysines in the Δ2 fragment were substituted with arginine as indicated and in vitro acetylations were carried out. Western blotting with anti-AcK Ab shows acetylation was decreased in the K281R and K297R single substitution mutants and completely absent in the double substitution K281RK297R mutant. (D) In vitro acetylation was performed on Δ2 mutants with [1-^{14}C] acetyl-CoA and detected by autoradiogram. (E) A diagram showing acetylation sites of ΔNp63α on the hinge region, K281 and K297. (F) Alignment of human p53, p63, and p73 sequences around K281 and K297 by Vector NTI software (Invitrogen). P63 sequences are numbered as in ΔNp63α. Lysines marked by bold **K** characters are known acetylation sites. K320 in p53 could be aligned to K297 of ΔNp63α.

cancer cells as well as normal keratinocytes. Taken together, our results suggest that acetylation of ΔNp63α is a regulatory mechanism that modulates cell growth and maintains epidermal homeostasis.

2. Materials and methods

2.1. Cell culture

ME180 cells (ATCC, Manassas, VA) were maintained in RPMI-1640 supplemented with 10% v/v fetal bovine serum and antibiotics (Hyclone, Logan, UT). Saos-2 (ATCC) and 293T cells (Invitrogen, Carlsbad, CA) were cultured in DMEM supplemented with 10% v/v fetal bovine serum and antibiotics. Human epidermal keratinocytes were cultured in KGM-gold media (Lonza, Switzerland). Cells were split at 1×10^6 per 10 cm culture dish unless stated otherwise.

2.2. DNA constructs and antibodies

pcDNA 3.myc-ΔNp63α and pcDNA 3.myc-p53 were kind gifts from F. Mckeon (Harvard Medical School, MA). GFP-ΔNp63α was subcloned from pcDNA3.myc-ΔNp63α into pEGFP C1 (Invitrogen) and p63 deletion constructs were subcloned into pGEX 4T-2 (GE healthcare, Piscataway, NJ). To generate arginine or glutamine substitution mutants, site-directed mutagenesis was performed using npfu forte (Biostream, Korea).

Ac-p63 specific antibodies were obtained by immunization of rabbits with chemically acetylated p63 peptide Ac-K281[KNGDGT(AcK)RPFR] or Ac-K297 [QMTSI(AcK)KRRS-C] (AbFrontier, Korea). The collected sera were affinity purified on immobilized acetylated peptides after removal of antibodies recognizing unacetylated p63. Monoclonal anti-p63 (4A4) and anti-Myc (9E10) Abs were from F. Mckeon. The following commercial antibodies were utilized: anti-acetyl lysine (4G12 mouse monoclonal, Millipore, Billerica, MA); anti-acetyl lysine (Rabbit polyclonal), anti-p53 pSer15, anti-αβ tubulin, and anti-Lamin A/C Abs from Cell Signaling (Danvers, MA); anti-p53 (clone DO-1) from Oncogene Science (Cambridge, MA); anti-p21, anti-β-actin, and anti-GFP (mouse monoclonal) Abs from Santa Cruz (Santa Cruz, CA).

2.3. siRNA and DNA transfection

siRNA and DNA plasmids were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. siRNA (Bioneer, Korea) target sequences are as follows. Luciferase, CGUACGCGGAAUACUUCGGAdTdT; p63, CCUAGUCAUUUGAUUCGAGUAdTdT; p53, AAGACUCCAGUGGUAAUCU ACdTdT.

2.4. Protein purification and in vitro acetylation assay

GST-fused ΔNp63α deletion fragments and GST-PCAF HAT domain (amino acids 352–832) were purified from *Escherichia coli* by glutathione 4 Sepharose fast flow beads (GE healthcare). In vitro

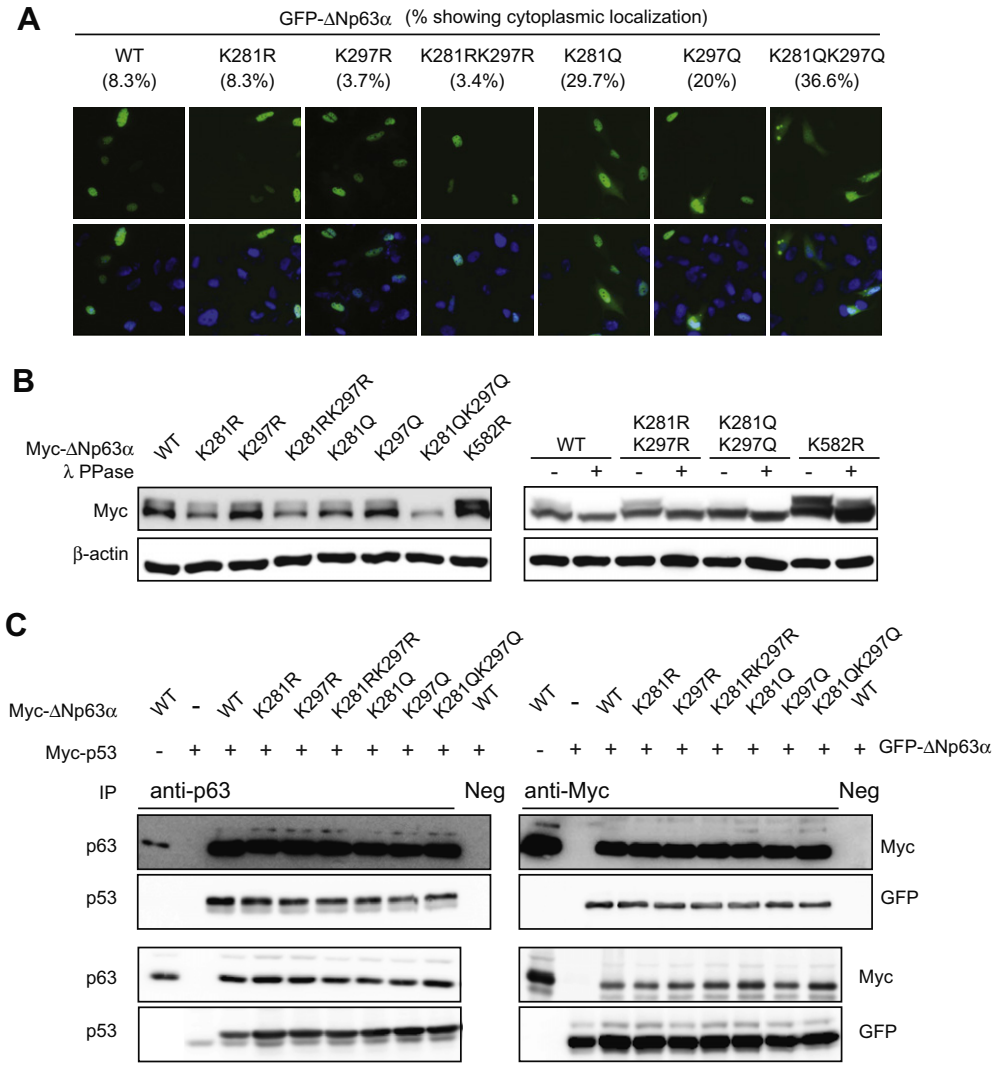

Fig. 2. ΔNp63α acetylation mimic mutants are preferentially localized in the cytoplasm. (A) Saos-2 cells were transfected with GFP-tagged wild type, acetylation-defective, and acetylation-mimic ΔNp63α. Wild type and acetylation-deficient mutants are mainly in the nucleus whereas acetylation-mimic mutants show both nuclear and cytoplasmic localization. GFP signals were analyzed in 60–80 cells per each group and the percent of cells showing cytoplasmic ΔNp63α is indicated in the parentheses. Experiments were repeated three times. (B) Phosphorylation of WT and mutant ΔNp63α were visualized by slow migration in the polyacrylamide gel and treatment with lambda phosphatase. Sumoylation defective mutant K582R was used as a heavily phosphorylated control. (C) p63–p63 or p63–p53 interaction was assessed by transfection in 293T cells followed by co-immunoprecipitation.

acetylation assay was performed on the purified GST-ΔNp63α fragments using active PCAF for 1 h at 30 °C in HAT buffer (250 mM Tris–HCl [pH 8.0], 50% glycerol, 0.5 mM EDTA, 5 mM DTT, and 1 mM unlabeled acetyl-coA). Proteins were separated by SDS–PAGE and analyzed by Western blot using anti-acetyl lysine antibodies. For detection by autoradiogram, in vitro acetylation assay was performed using 0.05 μCi of [1-^{14}C] acetyl-CoA (GE healthcare) and signals detected by BAS2500 phosphor Imager (Fujifilm, Japan).

2.5. Cytoplasmic fractionation

We followed the protocols by Wysocka et al. [28]. Briefly, cells were harvested in cold PBS and lysed in (10 mM HEPES [pH7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M Sucrose, 10% glycerol, 1 mM DTT, and 0.1% Triton X-100). The supernatant was collected as the cytoplasmic fraction and pellets were further lysed in 3 mM EDTA, 0.2 mM EGTA, and 1 mM DTT. The supernatant was collected as the nuclear soluble fraction and pellets were boiled in SDS sample buffer as the chromatin/nuclear matrix associated fraction. Protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 2 μg/ml Na_3VO_4, 1 μg/ml leupeptin, 10 mM *N*-ethylmaleimide) were added to all the lysis solutions. Chemicals were from Sigma (St. Louis, MO).

2.6. Western blot and Immunoprecipitation

Cells were lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% NP-40) supplemented with protease inhibitors. Immunoprecipitation was carried out with 2–4 mg of cell lysates using anti-p63 or anti-Myc antibody and protein A/G beads (Sigma). For direct Western blot analysis, 50–100 μg of cell lysates were loaded and probed with appropriate antibodies.

2.7. RT-PCR

RNA was isolated by Trizol reagent (Invitrogen), and 5 μg RNA was reverse transcribed by Superscript II (Invitrogen) in a 20 μl reaction. For each PCR, 1 μl cDNA was utilized using following primers. p21 forward, AAGACCATGTGGACCTGT; p21 reverse, GGTAGAAATCTGTCATGCTG; p27 forward, GTGCGAGA GAGGCGGTCGTG; p27 reverse, TCCACCGGGCCGAAGAGGTT; 14-3-3σ

forward, AGAGCTGAGGCCACCTGGGG; 14-3-3σ reverse, CGGTCTTGCACTGGCGCGC; Bax forward, TGACATGTTTTCTGACGGCAAC; Bax reverse, GGAGGCTTGAGGAGTCTCACC; Noxa forward, GAGATGCCTGGGAAGAAGG; Noxa reverse, ACGTGCACCTCCTGAGAAAA; GAPDH forward, GAAGGTGAAGGTCGGAGTC; GAPDH reverse, GAAGATGGTGATGGGATTT.

For real time detection, PCR reactions were ran on the One Step system (Applied Biosystems, Foster City, CA) using p21 or GAPDH primers in combination with SyBr (Applied Biosystems).

2.8. Flowcytometry

For the assessment of cell cycle profile, single-cell suspensions were fixed in 70% EtOH and stained with Propidium Iodide. Cells were analyzed by FACSCanto (Beckton Dickinson) using FACSDiva software.

2.9. Image analysis

For the detection of GFP-ΔNp63α, Saos-2 cells were transfected with WT or mutant construct, and fixed in 4% paraformaldehyde 48 h later. Slides were mounted in Vectashield mounting medium with DAPI (Vector Lab, Burlingame, CA) and analyzed on the Axiovert inverted microscope with a 40× oil lens (Carl Zeiss, Germany). Phase contrast images were photographed on the Axiovert microscope.

3. Results

3.1. Identification of acetylation sites for p63

In order to assess the acetylation of ΔNp63α, we performed an in vitro acetylation assay with purified proteins. We generated GST-tagged deletion constructs of ΔNp63α and set up acetylation reactions using PCAF as the acetyl transferase (Fig. 1A). We detected a strong signal for acetyl-lysine on the Δ2 fragment, containing 3′ regions post DNA-binding domain and the whole oligomerization domain. By comparison, there was only a weak signal for acetyl-lysine on the Δ1 and Δ3 fragments. We also performed an acetylation assay using [1-^{14}C]-labeled acetyl-CoA and confirmed our results by autoradiogram (Fig. 1C). The robustly-acetylated Δ2 fragment contained 8 lysine residues, in which we mutated a single lysine (K) to arginine (R) and performed an in vitro acetylation assay. We found that K281R and K297R mutants were compromised in their acetylation, and double acetylation mutant K281R297R was not acetylated at all (Fig. 1B). Arginine mutants for other lysines (K259R, K267R, K275R, K298R, K322RK324R) showed similar level of acetylation to the wild type (WT). ^{14}C autoradiography confirmed our results (Fig. 1D). Together, these results identified two acetylation sites of ΔNp63α localized in the hinge region between the DNA binding and oligomerization domain (Fig. 1E). Both lysines are conserved in other p53 family members (Fig. 1F).

3.2. Analysis of acetylation-defective or mimic mutant ΔNp63α

To gain insights on the function of acetylation, we first characterized the acetylation-defective arginine or acetylation-mimic glutamine (Q) substitution mutants of ΔNp63α. We expressed the WT or mutant ΔNp63α and analyzed their expression pattern in Saos2 cells. As shown in Fig. 2A, GFP-tagged WTΔNp63α is mainly localized in the nucleus, as are the acetylation-defective mutants. By comparison, acetylation-mimic mutants showed both nuclear and diffuse cytoplasmic localization. In particular, a larger proportion of K281QK297Q double acetylation-mimic mutant was cytoplasmic. These data suggest that dual-acetylation on the two sites may cooperate for the nuclear export of ΔNp63α. Interestingly, the K281QK297Q mutant showed a low degree of phosphorylation compared to the WT and other mutants (Fig. 2B). As the phosphorylation could affect multimerization for p63 [29], we determined whether acetylation mutants possessed different ability to form a homotypic or heterotypic complex with p53 (Fig. 2C). However, WT and all the mutant ΔNp63α's were able to form p63–p63 and p63–p53 complexes, indicating that acetylation on the hinge region did not affect oligomerization between the family members.

3.3. ΔNp63α acetylation in high-density cell culture

Next, to determine whether acetylations on K281 and K297 sites occur in vivo and if so, to define the physiologic role, we generated acetyl-p63 specific antibodies on these sites. The rabbit polyclonal antibodies exclusively detected acetylated K281 (Ac-K281) or K297 (Ac-K297) for in vitro acetylated p63 fragments (Fig. 3A). Using these antibodies, we determined the presence of acetylated p63 in ME180 cervical cancer cells and detected low levels of K281 and K297 acetylation, verified by siRNA for p63 (Fig. 3B). Then in search of conditions where ΔNp63α acetylation might be stimulated, we found that acetylations increase when cells are grown to higher confluency by increased plating cell number (Fig. 4A and B) or by increased culture time (Fig. 4C and D). Acetylation was more profound for the K297 site, and the timing of acetylation on K281 preceded that on K297 site (Fig. 4C). This suggests that p63 acetylations might occur sequentially in response to cell contact. Acetylation on K281 and K297 sites did not increase in response to DNA damage inflicted by gamma irradiation or high-dose UV irradiation (data not shown). The overall increase of ΔNp63α acetylation in confluent culture was confirmed by detection of acetyl lysine after p63 immunoprecipitation as well (Fig. 4E). In this condition, p53 was modestly phosphorylated and stabilized (Fig. 4A and C), indicating transient activation of the

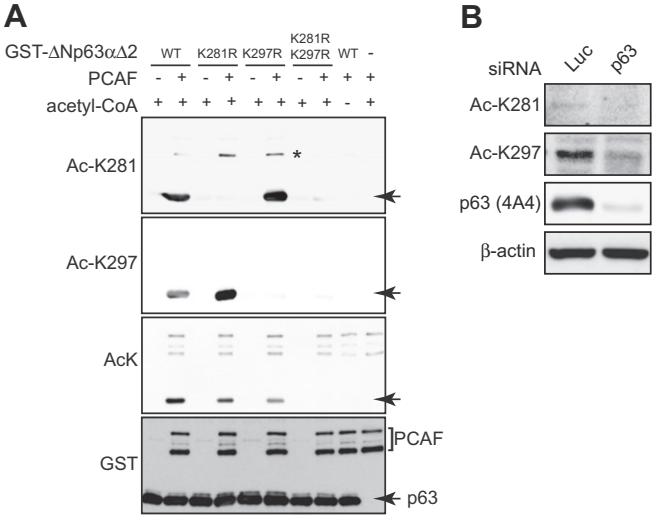

Fig. 3. Generation of acetyl-p63 antibodies to the K281 and K297 sites. Rabbit polyclonal antibodies were generated to the acetylated p63 peptides spanning Ac-K281 or Ac-K297, and affinity purified. (A) Anti-AcK281 Ab detects only acetylated Δ2 fragments, not unacetylated fragment or K281R mutant. Anti-AcK297 Ab detects only acetylated Δ2 fragments, not unacetylated fragment or K297R mutant. Anti-AcK281 Ab shows slight cross reactivity to PCAF. Blots were also detected by anti-AcK Ab to show acetylation status and anti-GST Ab for the protein loading control. (B) ME180 cells were transfected with siRNA against p63 and WB performed with anti-AcK281 or AcK297 Abs. Signals for the Ac-K281 and Ac-K297 disappear after p63 silencing, verifying the Ab specificity.

p53 pathway. Concomitantly, the cell cycle inhibitor p21 was upregulated. In the confluent culture condition, the upregulation of p21 could be ascribed to p53 activity, as silencing p53 blocked p21 induction (Fig. 4F). On the other hand, silencing p63 further upregulated p21 levels, suggesting that repression of ΔNp63α contributed to the induction of p21.

To further investigate the factors responsible for the density-dependent growth inhibition, we assessed mRNA levels of p21 and other cell cycle inhibitory genes (Fig. 5). Among these, induction of p21 was substantial, although we failed to detect upregulation of others including p27 and 14-3-3σ. We also checked mRNA levels for pro-apoptotic p53 target genes but did not find any differences. The fold-induction of p21 mRNA was assessed by real time PCR to be 4–6 fold when cells reached to >90% confluency compared to the low density culture (30% confluent) condition (Fig. 5A and B).

3.4. Correlation of acetylations and cytoplasmic localization of ΔNp63α

The density-dependent induction of p21 can be ascribed to the activation and stabilization of p53 (Fig. 4F). It is also possible that ΔNp63α is modified in such a way to lose the ability to repress p21. In Fig. 2A, we demonstrated that acetylation-mimic mutants of ΔNp63α are more preferentially localized to the cytoplasm. Therefore, we assessed whether ΔNp63α, upon high cell density, can be acetylated and localized in the cytoplasm. To do this, we harvested cells at low or high density, and separated them into cytoplasmic, nuclear soluble, and chromatin/nuclear matrix- associated fractions. As shown in Fig. 6A, a high proportion of ΔNp63α was associated with chromatin or nuclear matrix for low density cells. By comparison, the chromatin/nuclear matrix-bound fraction

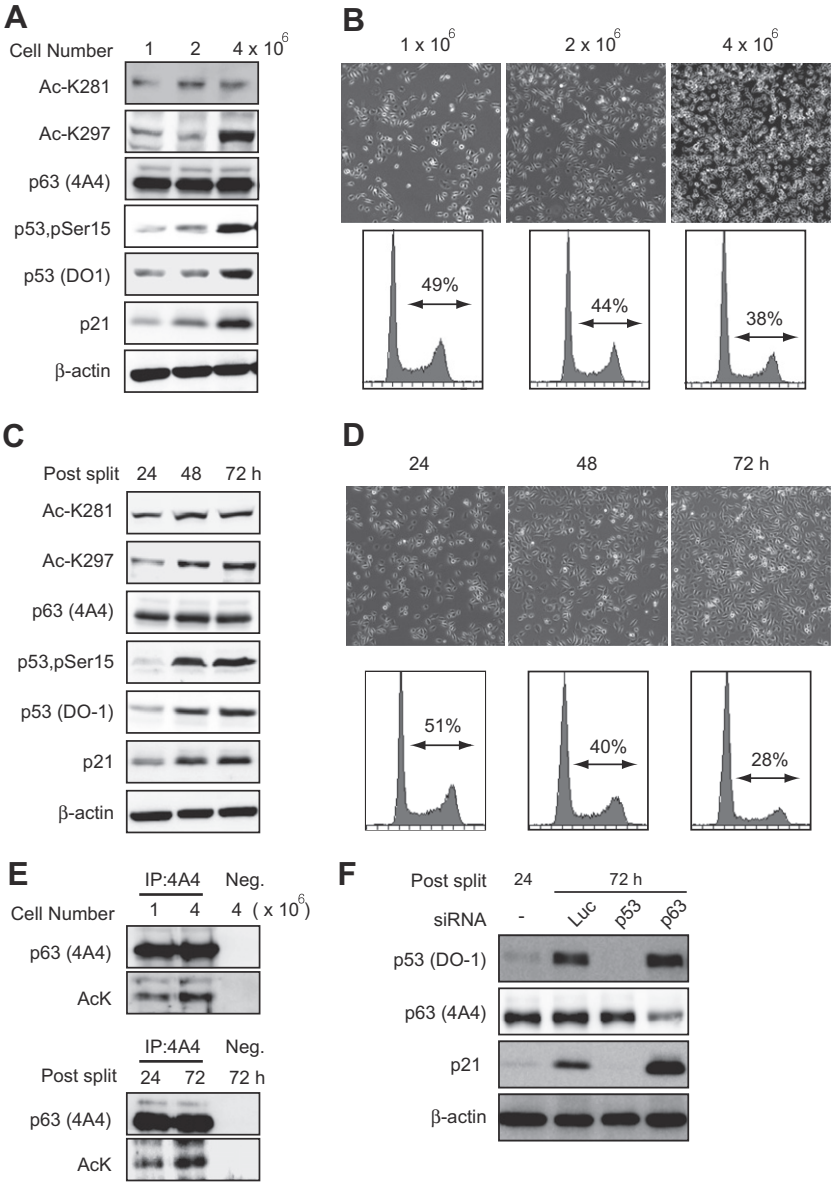

Fig. 4. High cell density induces acetylation on K281 and K297 sites of ΔNp63α. (A) ME180 cells were split at 1×10^6, 2×10^6, and 4×10^6 cells per 10 cm dish and harvested 24 h later. At higher cell density, ΔNp63α acetylation on K281 and K297 is induced. Levels of p53, phosphor-p53 (pSer15), and p21 are also increased. (B) Phase contrast images (upper panel) and Propidium Iodide staining (lower panel) of ME180 cells at 1×10^6, 2×10^6, and 4×10^6 cells per 10 cm dish. (C) ME180 cells were split at 1×10^6 cells per 10 cm dish and harvested 24, 48, and 72 h later. With increased culture time, ΔNp63α acetylations on K281 and K297 are increased. Levels of p53, phosphor-p53 (pSer15), and p21 are also increased. (D) Phase contrast images (upper panel) and Propidium Iodide staining (lower panel) of ME180 cells 24, 48, and 72 h post split. (E) Acetylation of ΔNp63α was assessed by immunoprecipitation with 4A4 Ab and WB with anti-AcK Ab. (F) ME180 cells were transfected by siRNA against p53 or p63 and the level of p21 protein was determined.

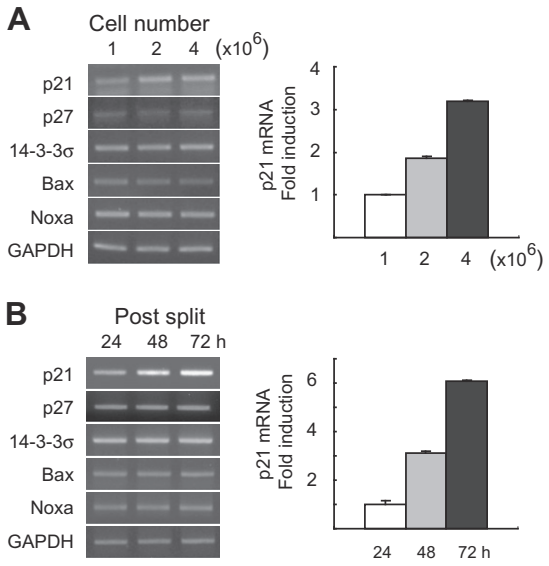

Fig. 5. High cell density induces upregulation of p21 mRNA. RNA was isolated from ME180 cells plated at different numbers (A) or cultured for 24, 48, or 72 h (B). mRNA levels determined for cell cycle inhibitors (p21, p27, and 14-3-3σ) and proapoptotic p53 target genes (Bax and Noxa). p21 mRNA levels were also assessed by real time RT-PCR. GAPDH was used as a normalization control.

significantly decreased in confluent cells, whereas the cytoplasmic fraction increased. Interestingly, Ac-K281-ΔNp63α was predominantly found in the nuclear soluble fraction, whereas Ac-K297-ΔNp63α was mostly cytoplasmic. These data link K281 and K297 acetylations to the nuclear export signal and also suggest that acetylations on K281 and K297 sites may occur in a sequential manner. Importantly, K281 and K297 acetylations were also induced in normal keratinocytes in high-density culture conditions (Fig. 6B), suggesting that normal epidermal compartment might utilize p63 acetylation to modulate cell growth. Taken together, acetylation of ΔNp63α could be a homeostatic mechanism by which cells regulate their proliferative rate without permanent commitment.

4. Discussion

Regulation of cell growth is fundamental in the development and homeostasis of multicellular organisms, and any alteration may lead to serious diseases including cancer. P63 is a p53 homolog whose expression as a ΔNp63α form is restricted to the epidermal stem cell compartment and essential for the proliferative potential [9]. Therefore, the absence of ΔNp63α results in developmental defects in the epidermis and all the derivatives [3]. The lack of ΔNp63α also causes cellular senescence and premature aging [17], and ΔNp63α has been recently implicated in the bypass mechanism of cellular senescence in oncogene-induced transformation [18]. In this study, we provide a new set of evidence linking acetylation of ΔNp63α to density-dependent inhibition of cellular proliferation as a homeostatic mechanism.

ΔNp63α in the stratified epithelia controls the proliferative capacity of stem cells and progenitors [9]. This function is well-supported by the finding that p63-deficient mice exhibit a malformation of stratified epithelia attributed to the failure in the maintenance of stem and progenitor cell populations [3]. In addition, overexpression of ΔNp63α induces epidermal hyperplasia [30], further strengthening the association between ΔNp63α expression and proliferative capacity. The association is also evident for the studies by Pellegrini on the isolated corneal epithelium [31]. Holoclones isolated from the basal cells of the limbal epithelium express high levels of ΔNp63α and retain high proliferative capacity as well as the potential to form complete epithelial layers in culture. By comparison, paraclones no longer express ΔNp63α and terminally differentiate after a few rounds of cell division. Meroclones express low to moderate levels of ΔNp63α and possess higher levels of proliferative capacity than paraclones. These characteristics of corneal epithelial cells indicate that the level of ΔNp63α determines the proliferative potential of cells at a distinct differentiation stage. In comparison, acetylation of ΔNp63α seems to be a reversible mechanism to control the rate of cell proliferation within a compartment. In order to maintain homeostasis, proliferating cells need to accommodate environmental stimuli and adjust cell cycle progression accordingly. High cell density would transcend an environmental cue to induce ΔNp63α acetylation and p53 activation. These changes stimulate the expression of the cell cycle arrest gene p21 and hence slow down cell proliferation. The transient and reversible growth inhibition will eventually help cell survival.

In the confluent cell culture condition, p21 was the only abundantly induced p53 target. The basis for this has been suggested by others that low levels of p53 might induce genes with a high affinity for p53 binding, which are genes related to the cell cycle arrest but not apoptosis [32]. ME180 cells are known to harbor HPV sequences [33], and therefore their p53 was thought to be non-functional. However, in our study we were able to show that the p53 function is somewhat preserved, and that it could induce transcription of p21. It may be true that the available p53 in these cells are low, that the cell cycle arrest genes are preferentially induced in these cells. Additionally, we found that HaCaT cells, an

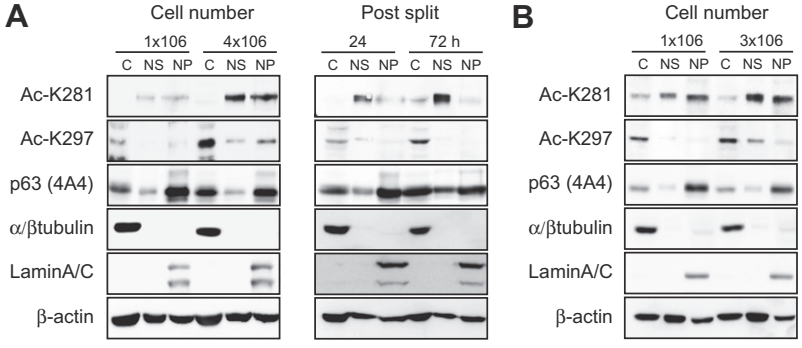

Fig. 6. Acetyl-ΔNp63α is accumulated in the cytoplasmic fraction in high density culture. (A) ME180 cells plated at different numbers or harvested 24 and 72 h post plating were fractionated into cytoplasmic [C], nuclear soluble [NS], and nuclear matrix/chromatin bound [nuclear pellet, NP] portions. Nuclear soluble Ac-K281- and cytoplasmic Ac-K297-ΔNp63α are increased at high cell density. Cell fractionation was monitored by cytoplasmic αβ tubulin and nuclear Lamins. (B) Normal HEKs were cultured at 1×10^6 or 3×10^6 cells per 10 cm dish for 24 h and fractionated as in (A).

immortalized keratinocyte cell line harboring the mutant p53 [34], did not upregulate p21 in the confluent culture condition (Lee, unpublished data). By comparison, normal keratinocytes moderately upregulated p21 (Lee, unpublished data), suggesting that p53 and acetyl-ΔNp63α inter-dependently regulate cell cycle progression for normal epidermal cells. Therefore, acetylation of ΔNp63α is a mechanism that leads to skewing cellular responses towards cell cycle arrest instead of permanent cell death.

In our study, we did not pursue the acetylation of C-terminal fragment of p63 because our assay demonstrated much weaker acetylation on the C-terminus compared to the mid-region. However, this could have been due to the limitations of our assay and further investigation is necessary to completely delineate the acetylation map of ΔNp63α. For p53, acetylation on the hinge region (K320, may be equivalent to K297 of ΔNp63α) by PCAF, and heavy acetylation on the C-terminal region as well as the DNA binding region by CBP have been reported [27,35,36]. Acetylation-defective p53 mutant on the DNA binding and C-terminal region failed to activate the p21 gene [27]. By comparison, acetylation on the hinge region negatively regulated the pro-apoptotic activity of p53 [37]. For p73, acetylation on the hinge region was mediated by p300 and was responsible for the DNA damage-dependent induction of apoptotic targets [38]. For p63, p300 acetylated and induced the transcription targets of TAp63γ isoform, yet the acetylation sites were not defined [39]. In the present study, we identified two acetylation sites in ΔNp63α and related them to the cell density-dependent growth inhibition. We also found that acetylation of ΔNp63α can be enhanced by CBP or less by p300 in cultured cell lines (Lee, unpublished data). The CBP/p300-derived acetylation was not affected by arginine substitution of K281 or K297, indicating that additional acetylation sites may exist. These sites may reside in the N-or C-terminal p63 and correspond to the p300-mediated acetylation sites described by MacPartlin et al. [39]. Therefore, in line with our hypothesis, acetylation of p63 appears to be tightly linked to its function, similar to p53, and levels of complexity exist. Further investigation is warranted.

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References

[1] Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R. and Bradley, A. (1999) P63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398, 708–713.
[2] Yang, A. et al. (1998) P63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol. Cell 2, 305–316.
[3] Yang, A. et al. (1999) P63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398, 714–718.
[4] Lindsay, J., McDade, S.S., Pickard, A., McCloskey, K.D. and McCance, D.J. (2011) Role of DeltaNp63gamma in epithelial to mesenchymal transition. J. Biol. Chem. 286, 3915–3924.
[5] Adorno, M. et al. (2009) A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell 137, 87–98.
[6] Suh, E.K. et al. (2006) P63 protects the female germ line during meiotic arrest. Nature 444, 624–628.
[7] Parsa, R., Yang, A., McKeon, F. and Green, H. (1999) Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. J. Invest. Dermatol. 113, 1099–1105.
[8] Lee, H. and Kimelman, D. (2002) A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. Dev. Cell 2, 607–616.
[9] McKeon, F. (2004) P63 and the epithelial stem cell: more than status quo? Genes Dev. 18, 465–469.
[10] Flores, E.R. et al. (2005) Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer Cell 7, 363–373.
[11] Keyes, W.M. et al. (2006) P63 heterozygous mutant mice are not prone to spontaneous or chemically induced tumors. Proc. Natl. Acad. Sci. U S A 103, 8435–8440.
[12] Nishi, H. et al. (1999) Mutation and transcription analyses of the p63 gene in cervical carcinoma. Int. J. Oncol. 15, 1149–1153.
[13] Park, B.J., Lee, S.J., Kim, J.I., Lee, C.H., Chang, S.G., Park, J.H. and Chi, S.G. (2000) Frequent alteration of p63 expression in human primary bladder carcinomas. Cancer Res. 60, 3370–3374.
[14] Wang, T.Y. et al. (2001) Histologic and immunophenotypic classification of cervical carcinomas by expression of the p53 homologue p63: a study of 250 cases. Hum. Pathol. 32, 479–486.
[15] Yamaguchi, K. et al. (2000) Frequent gain of the p40/p51/p63 gene locus in primary head and neck squamous cell carcinoma. Int. J. Cancer 86, 684–689.
[16] Stransky, N. et al. (2011) The mutational landscape of head and neck squamous cell carcinoma. Science 333, 1157–1160.
[17] Keyes, W.M., Wu, Y., Vogel, H., Guo, X., Lowe, S.W. and Mills, A.A. (2005) P63 deficiency activates a program of cellular senescence and leads to accelerated aging. Genes Dev. 19, 1986–1999.
[18] Keyes, W.M. and Mills, A.A. (2006) P63: a new link between senescence and aging. Cell Cycle 5, 260–265.
[19] Liefer, K.M., Koster, M.I., Wang, X.J., Yang, A., McKeon, F. and Roop, D.R. (2000) Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. Cancer Res. 60, 4016–4020.
[20] Lee, H.O., Lee, J.H., Choi, E., Seol, J.Y., Yun, Y. and Lee, H. (2006) A dominant negative form of p63 inhibits apoptosis in a p53-independent manner. Biochem. Biophys. Res. Commun. 344, 166–172.
[21] Antonini, D. et al. (2006) An autoregulatory loop directs the tissue-specific expression of p63 through a long-range evolutionarily conserved enhancer. Mol. Cell Biol. 26, 3308–3318.
[22] Harmes, D.C. et al. (2003) Positive and negative regulation of deltaN-p63 promoter activity by p53 and deltaN-p63-alpha contributes to differential regulation of p53 target genes. Oncogene 22, 7607–7616.
[23] Westfall, M.D., Joyner, A.S., Barbieri, C.E., Livingstone, M. and Pietenpol, J.A. (2005) Ultraviolet radiation induces phosphorylation and ubiquitin-mediated degradation of DeltaNp63alpha. Cell Cycle 4, 710–716.
[24] Huang, Y.P. et al. (2004) Altered sumoylation of p63alpha contributes to the split-hand/foot malformation phenotype. Cell Cycle 3, 1587–1596.
[25] Ghioni, P., D'Alessandra, Y., Mansueto, G., Jaffray, E., Hay, R.T., La Mantia, G. and Guerrini, L. (2005) The protein stability and transcriptional activity of p63alpha are regulated by SUMO-1 conjugation. Cell Cycle 4, 183–190.
[26] Dai, C. and Gu, W. (2010) P53 post-translational modification: deregulated in tumorigenesis. Trends Mol. Med. 16, 528–536.
[27] Tang, Y., Zhao, W., Chen, Y., Zhao, Y. and Gu, W. (2008) Acetylation is indispensable for p53 activation. Cell 133, 612–626.
[28] Wysocka, J., Reilly, P.T. and Herr, W. (2001) Loss of HCF-1-chromatin association precedes temperature-induced growth arrest of tsBN67 cells. Mol. Cell Biol. 21, 3820–3829.
[29] Deutsch, G.B. et al. (2011) DNA damage in oocytes induces a switch of the quality control factor TAp63alpha from dimer to tetramer. Cell 144, 566–576.
[30] Yang, X. et al. (2011) DeltaNp63 versatilely regulates a Broad NF-kappaB gene program and promotes squamous epithelial proliferation, migration, and inflammation. Cancer Res. 71, 3688–3700.
[31] Pellegrini, G. et al. (2001) P63 identifies keratinocyte stem cells. Proc. Natl. Acad. Sci. U S A 98, 3156–3161.
[32] Jackson, J.G. and Pereira-Smith, O.M. (2006) P53 is preferentially recruited to the promoters of growth arrest genes p21 and GADD45 during replicative senescence of normal human fibroblasts. Cancer Res. 66, 8356–8360.
[33] Reuter, S., Delius, H., Kahn, T., Hofmann, B., zur Hausen, H. and Schwarz, E. (1991) Characterization of a novel human papillomavirus DNA in the cervical carcinoma cell line ME180. J. Virol. 65, 5564–5568.
[34] Lehman, T.A. et al. (1993) P53 mutations in human immortalized epithelial cell lines. Carcinogenesis 14, 833–839.
[35] Gu, W. and Roeder, R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 90, 595–606.
[36] Sakaguchi, K., Herrera, J.E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C.W. and Appella, E. (1998) DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev. 12, 2831–2841.
[37] Chao, C. et al. (2006) Acetylation of mouse p53 at lysine 317 negatively regulates p53 apoptotic activities after DNA damage. Mol. Cell Biol. 26, 6859–6869.
[38] Costanzo, A. et al. (2002) DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes. Mol. Cell 9, 175–186.
[39] MacPartlin, M., Zeng, S., Lee, H., Stauffer, D., Jin, Y., Thayer, M. and Lu, H. (2005) P300 regulates p63 transcriptional activity. J. Biol. Chem. 280, 30604–30610.