FEBS Letters 581 (2007) 5640–5644

SuPr-1-mediated desumoylation regulates the repressor activity of ΔNp63α

Hae-ock Lee[a], Mioh Cho[a], Jung-Hwa Lee[a], Hyo-Sil Kim[b], Yungdae Yun[b], Hyunsook Lee[a,*]

[a] *Department of Biological Sciences and Research Center for Functional Cellulomics, Seoul National University, San 56-1 Shillim-dong, Gwanak-ku, Seoul 151-742, Republic of Korea*

[b] *Division of Molecular Life Sciences, Ewha University, 11-1 Daehyun-dong, Seodaemoon-ku, Seoul 120-750, Republic of Korea*

Received 19 August 2007; revised 5 November 2007; accepted 5 November 2007

Available online 20 November 2007

Edited by Ivan Sadowski

Abstract ΔNp63α is exclusively expressed in stem cells and progenitor cells of the stratified epithelia. It promotes cell proliferation by antagonizing p53 and related TAp63/TAp73. Here, we report that specific desumoylation by SUMO protease SuPr-1 provides a fine-tuning mechanism for ΔNp63α repressor activity. We found that disrupting the sumoylation site compromised ΔNp63α repressor activity profoundly against TAp63γ and TAp73β-mediated transcription activation, but not to p53-mediated transcription. We further found that SuPr-1 specifically bound to sumoylated ΔNp63α and hydrolyzed SUMO. Consequently, SuPr-1 expression reduced ΔNp63α repressor activity to TAp63γ and TAp73β, whereas p53-mediated transactivation was unaffected. Collectively, these data suggest that SuPr-1-mediated ΔNp63α desumoylation elaborately regulates epithelial growth.

Keywords: ΔNp63α; SuPr-1; Sumoylation; Desumoylation; Transcription control

1. Introduction

The *p63*gene*(AIS/KET/CUSP/p40/p51/p73L)* encodes two different transcripts. One transcript encodes TAp63, which is structurally and functionally similar to p53. ΔNp63, which lacks most of the transcription-activating domain of TAp63, is directed by an internal intronic promoter [1]. In addition, alternative splicing at the C-terminus generates at least three splice variants—α, β, and γ; the α form is the longest and contains the sterile alpha motif and transcription inhibition domain [2,3]. Among the different isotypes, ΔNp63α is exclusively expressed in the epidermal stem cells and progenitor cells, and required for epidermal development and for the maintenance and regeneration of epidermal stem cells and progenitor cells [4–6].

Although first thought that ΔNp63α mainly acts as a dominant-negative form for p53 and TAp63 in vitro and in vivo, recent findings also suggest that ΔNp63α retains transcription activation capacity as well and activates key target genes required for epidermal morphogenesis [4]. However, ΔNp63α mainly functions as a transcription repressor in epidermal cell proliferation during zebrafish embryogenesis [5,6]: scheduled overexpression in zebrafish embryos suggested that ΔNp63α antagonizes p53 for epidermal outgrowth. Thus, it appears that the repressor function of ΔNp63α is essential, and therefore may be conserved, for maintaining epidermal cell proliferation in vertebrates [6].

ΔNp63α is a highly modified protein whose ubiquitination, phosphorylation, sumoylation, have been reported: poly-ubiquitination of ΔNp63α induces proteasome-mediated degradation [7] and SUMO modification of K637 residue of TAp63α (K582 for ΔNp63α) was reported to decrease transcription activation function [8–10]. In the case of ΔNp63α, sumoylation, as well as neddylation, induced the degradation of the protein during zebrafish embryogenesis [8].

We were specifically interested in understanding the effect of sumoylation–desumoylation in regulating ΔNp63α's repressor activity, since it is conserved from fish to humans and is crucial in the proliferation and survival of epithelial cell [5,6,11,12]. Here, we report that SuPr-1 functions as a specific SUMO protease for ΔNp63α, and regulates the repressor activity towards TAp63γ- and TAp73β-mediated transactivation. Our study suggests that the sumoylation–desumoylation switch provides a fine-tuning regulatory mechanism for ΔNp63α-mediated transcription control.

2. Results

2.1. Confirmation of *ΔNp63α* sumoylation at lysine 582

In order to assess the effect of sumoylation that affects ΔNp63α repressor function, we substituted five potential sumoylation consensus motifs with high probability (K494 and K582) and low probability (K139, K259, and K275) lysine residues with arginine residues using in vitro mutagenesis and constructed mutant Δ*Np63α* expression vectors.

Next, various mutant Δ*Np63α* expression constructs tagged with Myc epitope at the N-termini were cotransfected into 293T cells with *GFP-SUMO* expression plasmids, then subjected to immunoprecipitation followed by Western analysis. Wild-type ΔNp63α and the other mutants were efficiently sumoylated, whereas K582R mutant or the double mutant (K582 and K494 substituted to arginine) were not (Fig. 1A). This confirmed that K582 (K647 for TAp63α) is the sumoylation site [8–10].

Sumoylation of ΔNp63α at the K582 site was further confirmed by immunoprecipitation and Western blotting with a

*Corresponding author. Fax: +82 2 886 4335.
E-mail address: HL212@snu.ac.kr (H. Lee).

0014-5793/$32.00
doi:10.1016/j.febslet.2007.11.013

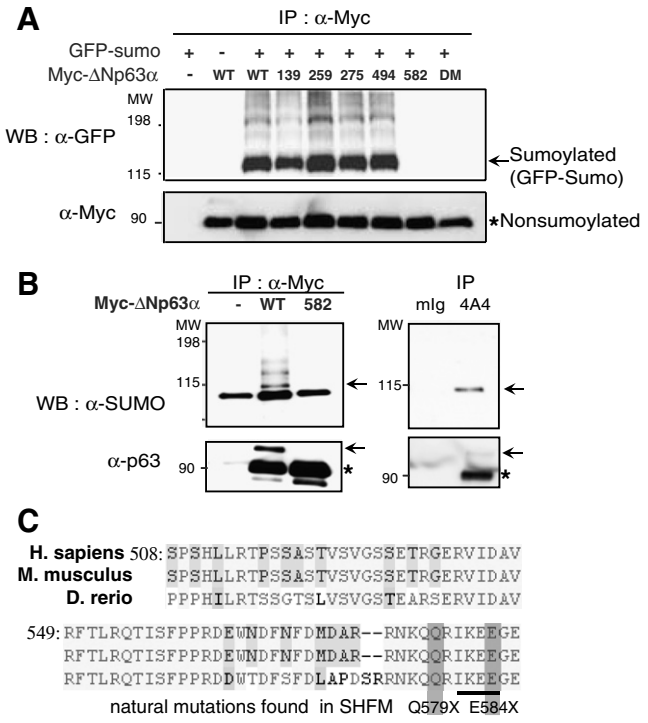

Fig. 1. ΔNp63α is sumoylated at K582. (A) 293T cells transfected with various *Myc-ΔNp63α* expression plasmids with or without *GFP-SUMO-1* were subjected to immunoprecipitation (IP) with anti-Myc mAb (9E10) and Western blotting (WB) with anti-GFP antibody. Mono-sumoylation bands are indicated with an arrow. Slow-migrating forms above the mono-sumoylated band, presumably the result of polysumoylation, are also detected. The same blot was reprobed with 9E10 for normalization. (B) 293T cells transfected with *Myc-ΔNp63α* (left panel) or ME180 cells without transfection (right panel) were immunoprecipitated with 4A4 anti-p63 mAb and immunoblotted with anti-SUMO-1 (GMP-1) mAb. Arrows indicate sumoylated ΔNp63α and non-sumoylated forms are marked with asterisks. The same blot was reprobed with 4A4 for immunoprecipitation control. Immunoprecipitation with mouse IgG (mIg) was included as a negative control (right panel). (C) Transcription inhibition domains of zebrafish, mouse, and human ΔNp63α are aligned. K582 sumoylation motif (underlined) and mutations found in human patients with split-hand–split-foot malformation are also highlighted.

SUMO-specific antibody. Wild-type ΔNp63α was efficiently detected by anti-SUMO antibodies, whereas the K582R mutant remained undetected (Fig. 1B, left panel). We then asked whether endogenous ΔNp63α was also sumoylated by employing ME180 cervical carcinoma cell line that expresses ΔNp63α. From immunoprecipitation followed by immunoblotting with anti-SUMO antibody, we confirmed that endogenous ΔNp63α undergoes sumoylation (Fig. 1B, right panel). In addition, a slower migrating band corresponding to the sumoylated ΔNp63α was readily detected for wild-type ΔNp63α but not for the K582R mutant (Fig. 1B, bottom panels). These combined results confirmed that ΔNp63α is sumoylated at K582 in vivo. Notably, K582 resides in the transcription inhibition domain at the extreme C-terminus [3], which is conserved from fish to humans (Fig. 1C). Interestingly, mutations associated with the epidermal syndrome split-hand–split-foot malformation, Q579X (634 for TAp63) [13] and E584X (639 for TAp63), lie adjacent to K582 or directly affect K582 sumoylation [10]. Since ΔNp63α is responsible for epidermal development among different p63 isotypes [4–6], these results imply that K582 sumoylation in ΔNp63α may have an essential function in modulating ΔNp63α activity in epidermal homeostasis [10].

2.2. Sumoylation controls transcription repression of ΔNp63α

In order to explore the outcome of sumoylation on the transcriptional repressor function of ΔNp63α, we adopted a luciferase reporter assay. In order to assess the transcription activation by all three p53 family members, we employed a luciferase reporter construct linked to a putative p53-responsive element (PG13-Luc). All three constructs activated PG13, with TAp63γ to the highest level (70-fold compared to 10-fold for p53). ΔNp63α acted as a strong repressor in a dose-dependent manner, with the effect most profound for p53, then to TAp73β-mediated transactivation. For similar repression levels, greater amounts of ΔNp63α were required for TAp63γ-mediated transcription (Fig. 2A). In comparison, the repressor activity of sumoylation-defective mutant K582R was compromised; greatly affecting TAp73β and TAp63γ, but less so to p53. When we compared the repressor activity of WT or K582R mutant on p53/TAp63/TAp73 target genes, we confirmed that the endogenous mRNA levels of *p21* and *Bax* were repressed by wild-type but less so by K582R mutant (Fig. 2B), consistent with the reporter analyses (Fig. 2A).

The decreased repressor activity of K582R mutant can result from changes in protein stability or cellular localization. In order to test whether sumoylation/desumoylation affects nuclear localization, we performed Western analysis after biochemical fractionation in U2OS cells transfected with *wild-type ΔNp63α* or *K582R* encoding plasmids. The result showed that there was little difference in protein levels or nuclear localization between wild-type and sumoylation-defective mutant (Fig. 2C).

Next, we examined the ΔNp63α's interaction affinity to p53, TAp63γ, and TAp73β. Wild-type and K582R were bound to all three p53 members. However, there was 2-fold decrease in binding of K582R to TAp63γ, and a slight decrease for TAp73β, compared to that of the wild-type (Fig. 2D), whereas the binding ability to p53 between the wild-type and K582R was not different. These results may account for the difference between wild-type and K582R in the repressor activity towards TAp63γ-mediated transactivation. As ΔNp63α interacts with number of transcription factors [14] (our unpublished data), sumoylation may provide differential binding platform for ΔNp63α.

2.3. SUMO-1 protease SuPr-1 selectively reduces ΔNp63α transcriptional repression

Because ΔNp63α sumoylation is readily detected under normal culture conditions, we speculated that desumoylation, may be a controlling step in regulating repressor activity exerted by ΔNp63α. Therefore, we asked if the SUMO-1-specific protease SuPr-1 [15] or SENP-1 [16] would associate with ΔNp63α. As shown in Fig. 3A (upper panel), there was a robust interaction of slow-migrating ΔNp63α with catalytically inactive SuPr-1 (C466S) [15] but with a weak interaction to catalytically active SuPr-1. These data suggest that SuPr-1 interacted with sumoylated ΔNp63α and rapidly hydrolyzed SUMO-1. Desumoylation was confirmed in that SuPr-1 hydrolyzed SUMO-1 from ΔNp63α in a dose-dependent manner (Fig. 3B). In contrast, the C466S mutant was unable to desumoylate ΔNp63α

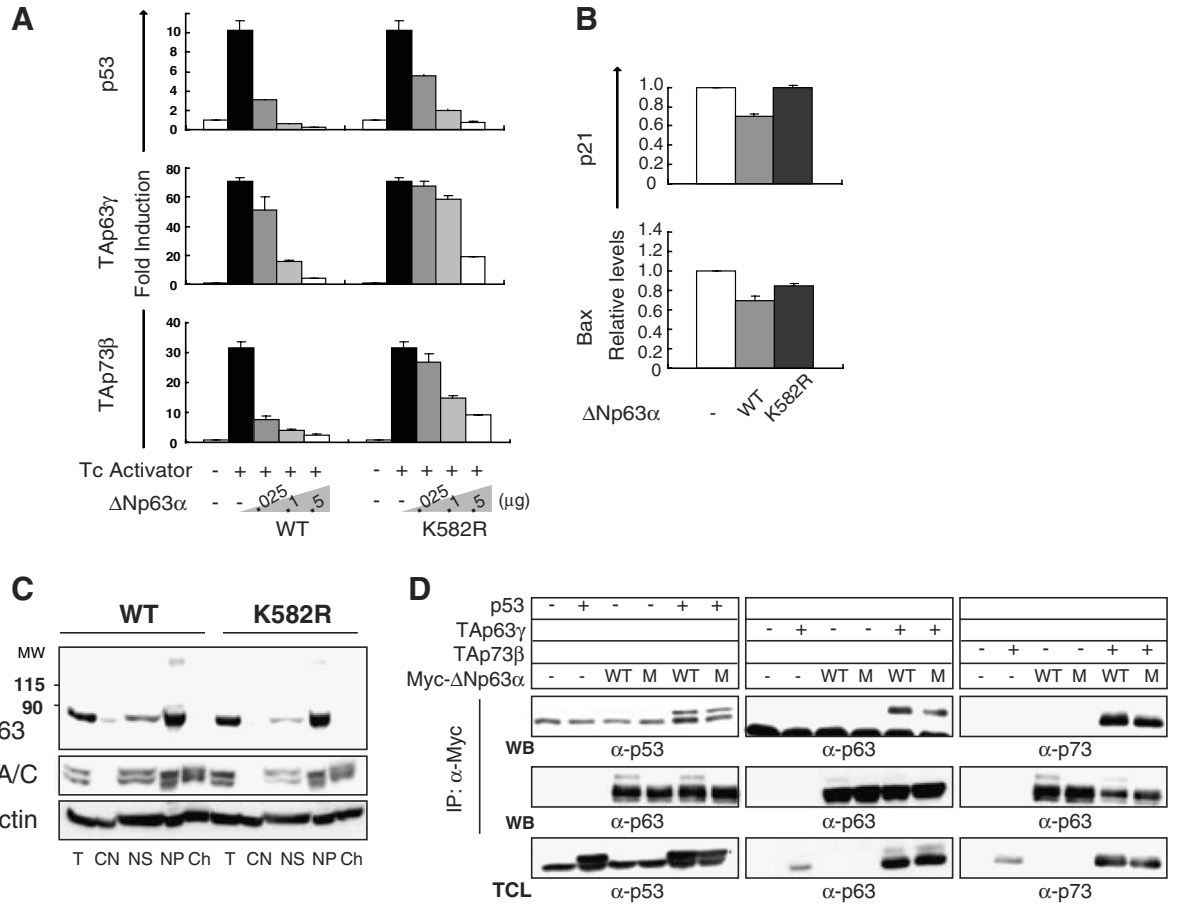

Fig. 2. Sumoylation modulates transcription repressor activity of ΔNp63α. (A) BHK21 cells were co-transfected with *PG13-luc* and *pRL-TK* to assess transcription repressor function of ΔNp63α on p53-, TAp63γ-, or TAp73β-mediated transcription. To compare the repressor activities of wild-type (WT) vs. K582R ΔNp63α, varying amounts of repressor were employed and examined for their effects. The ratios for transcription activator (p53, TAp63γ, or TAp73β) vs. repressor (ΔNp63α) were 20:1, 5:1, and 1:1, respectively. The y-axis shows the fold induction of firefly luciferase activity normalized with renilla luciferase activity from cells transfected with reporter alone. Note that the values of fold induction in y axis for TAp63γ-mediated transcription are higher. Values are averages of duplicate transfections and represent six independent experiments. (B) U2OS cells were transfected with WT or K582R mutant ΔNp63α, and real time RT-PCR was performed to check for the transcript levels of *p21* and *Bax*. Levels were normalized with *GAPDH* expression and control vector-transfected group. (C) U2OS cells were transfected with wild-type (WT) ΔNp63α or K582R. Forty-eight hours post-transfection, cells were harvested and fractionated [23]. T, total cell lysates; CN, cytoplasmic and nuclear soluble fraction; NS, nuclear soluble fraction; NP, nuclear pellet; Ch, nuclear pellet subjected to DNase digestion. (D) 293T cells cotransfected with *Myc*-ΔNp63α (WT or K582R) and *p53*, *TAp63γ*, or *TAp73β* expression plasmids were subjected to immunoprecipitation with anti-Myc mAb and Western blotting with anti-p53, anti-p63, or anti-p73 mAbs (upper panels). The same blot was reprobed with anti-p63 antibody (4A4) as an immunoprecipitation control (middle panels). Western blotting with anti-p53, anti-p63, or anti-p73 mAb in total cell lysates (TCL) indicates that similar amounts of expression plasmids were expressed (lower panels).

(Fig. 3B). When we tested for SENP1, another SUMO protease, the result showed that ΔNp63α did not interact with SENP1 (Fig. 3A, lower panel). These data suggest that SuPr-1 serves as a specific SUMO protease for ΔNp63α and may regulate its function.

The association of ΔNp63α and SuPr-1 was corroborated by indirect immunofluorescence microscopy. ΔNp63α colocalized with ectopically expressed SuPr-1; wild-type ΔNp63α co-localized with the enzymatically inactive SuPr-1, but less so with the wild-type SuPr-1. Co-localization of sumoylation-defective mutant K582R with catalytically inactive mutant SuPr-1 was reduced compared to wild-type ΔNp63α (Fig. 4).

Does desumoylation, mediated by SuPr-1, influence the repressor activity of wild-type ΔNp63α? Indeed, coexpression of SuPr-1 weakened the repressor activity of wild-type ΔNp63α to levels comparable to that of the K582R mutant to TAp63γ- and TAp73β-mediated transactivation (Fig. 5). Consistent with the result in Fig. 2A, repression of p53-mediated transcriptional activation was not influenced by SuPr-1 coexpression (Fig. 5, upper panel). Therefore, SuPr-1 is likely to be involved in selectively regulating ΔNp63α repressor activity to TAp63γ or TAp73β. Taken together, these observations suggest that ΔNp63α interacts with SuPr-1, and the ensuing desumoylation regulates the repressor activity of ΔNp63α.

3. Discussion

In this study, we found that SuPr-1 specifically desumoylates ΔNp63α. Unlike numerous substrate-specific E3 ubiquitylation ligases, only three types of SUMO ligases have been

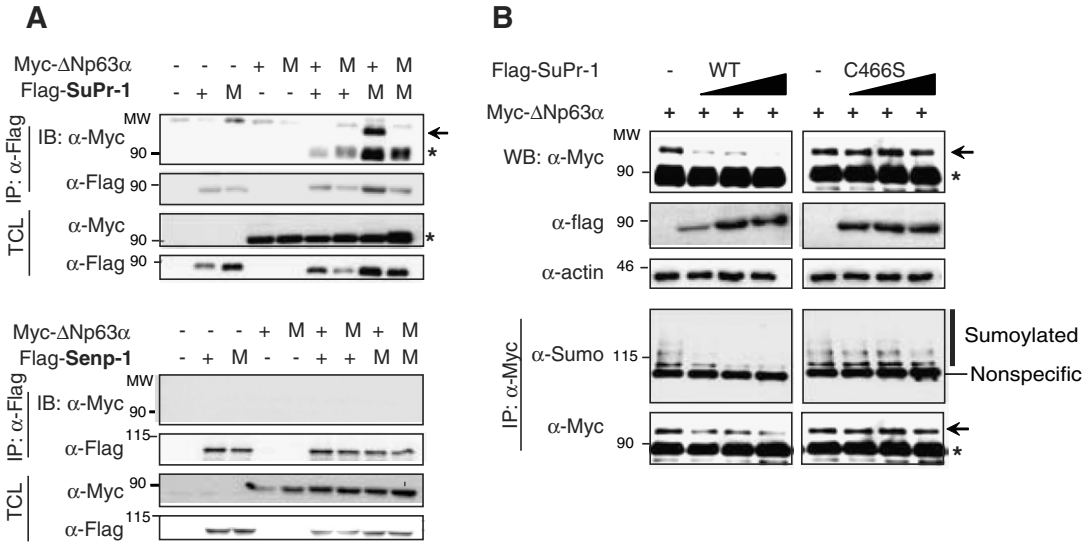

Fig. 3. SuPr-1 associates with ΔNp63α and hydrolyzes SUMO. (A) 293T cells were cotransfected with *Myc-ΔNp63α*, wild-type (WT) or K582R mutant, *flag*-SuPr-1-encoding plasmids (WT or C466S mutant) or *flag-Senp1* (WT or C603S) as indicated. Forty-eight-hours post-transfection, lysates were subjected to immunoprecipitation with anti-Flag mAb and Western blotting with anti-Myc mAb to determine binding between ΔNp63α and SuPr-1 or SENP1. The arrow indicates sumoylated ΔNp63α bound to SuPr-1; the star designates the non-sumoylated form. ΔNp63α did not bind to SENP1. The same blot was reprobed with anti-Flag mAb to normalize for immunoprecipitation. Western blotting for total cell lysates (TCL) is also shown for transfection control. (B) 293T cells were cotransfected with *Myc-ΔNp63α* and *flag-SuPr-1*, wild type (WT) or C466S expression plasmids to test whether SuPr-1 is capable of desumoylating ΔNp63α. Forty-eight-hours post-transfection, cells were subjected to Western blotting (upper panels) or immunoprecipitation with anti-Myc mAb and Western blotting with anti-SUMO mAb (lower panels). The arrow indicates sumoylated ΔNp63α, which diminishes with increasing amounts of *Flag-SuPr-1* expression.

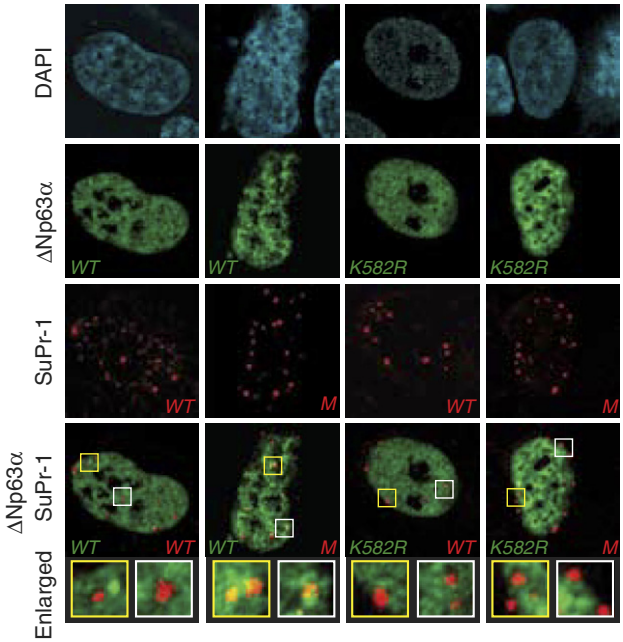

Fig. 4. Colocalization of SuPr-1 and ΔNp63α. U2OS cells were cotransfected with GFP-tagged WT – or K582R – Δ*Np63α* – expression plasmids with *flag*-SuPr-1 encoding plasmid. Cells were stained with anti-Flag mAb (Alexa 568, red), and DAPI (pale blue). Merged images are shown in yellow. Wild-type SuPr-1 (WT, Red) and catalytically inactive SuPr-1, C466S, (M, Red) immunostainings are compared with respect to wild-type ΔNp63α (WT, green) and K582 (K582R, green) localization, respectively. Representative co-staining of ΔNp63α and SuPr-1 are boxed, and the corresponding enlarged images are shown at the bottom.

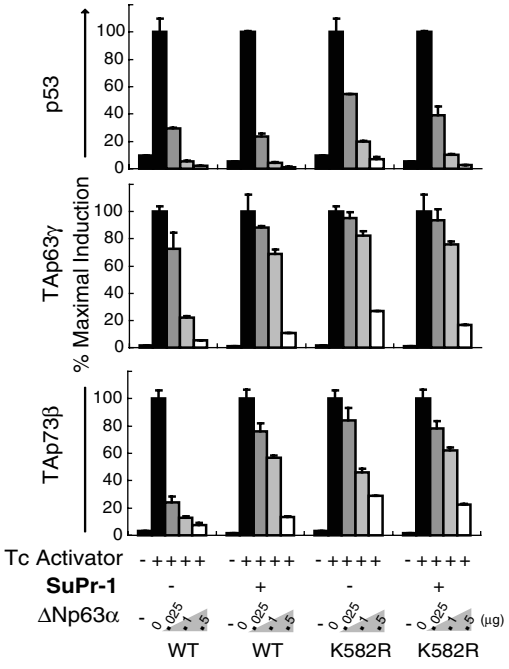

Fig. 5. SuPr-1 overexpression down-regulates the repressor activity of ΔNp63α towards TAp63γ- and TAp73β-mediated transactivation. BHK21 cells transfected with *PG13-luc*, and various effectors (p53, TAp63γ, TAp73β, respectively) were assayed with luciferase with or without *flag-SuPr-1*. The *y*-axis shows maximal percent readings from firefly luciferase activity normalized with renilla luciferase activity. Values are averages of duplicate transfections and represent three independent experiments.

described in mammals: PIAS, RanBP2, and Pc2 [17]. Desumoylation, on the other hand, is mediated by SUMO-specific proteases called SENPs (sentrin-specific protease 1–7 in mammals) [18]. We found SuPr-1 (the N-terminally truncated SENP2 isoform), but not SENP1, interacted with ΔNp63α and reduced repressor activity toward TAp63γ and TAp73β. Interestingly, repression of p53-mediated transcription activation was not influenced by SuPr-1. These findings suggest that SuPr-1-mediated desumoylation provides a means to differentially regulate ΔNp63α repressor activity toward TAp63γ/TAp73β versus p53.

The crosstalk between p53 family members plays a critical role in making decisions in cell fate. The balance between the dominant-negative form of p53 family members and transactivating members also controls tumor cell survival [19,20]. Moreover, recent discovery of ΔN forms of p53 [21] and interplay between p63 and p73 isoforms [22] add more complexity to the p53 network to guarantee the existence of versatile regulatory mechanisms. Our study suggests that SuPr-1-mediated desumoylation provides a fine-tuning mechanism that favors transcription repressor activity of ΔNp63α towards different p53 family members.

4. Materials and methods

4.1. Immunoprecipitation and Western blotting

Cells were lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% NP-40) supplied with protease inhibitors (1 mM PMSF, 1 μg/ml Aprotinin, 2 μg/ml Leupeptin, 1 μg/ml Pepstatin A, 1 μg/ml NaOVa4; Roche, 125 μM NEM; Sigma). Chemiluminescence of Western blots was processed under LAS-3000 (Fuji Film, Japan).

4.2. Reporter assay

All transfections were equalized with the pcDNA3-Myc vector for an equal DNA quantity and normalized with luciferase activity with pRL-TK. Luciferase activity was measured 48-h later using the Dual Luciferase Assay System (Promega, Madison, WI).

4.3. Real-time PCR

The relative levels of BAX and p21 mRNAs were determined by real-time quantitative PCR with SYBR (Applied Biosystems, Foster City, CA) and normalized to GAPDH products. Primer sequences were – p21 forward: AAGACCATGTGGACCTGT, p21 reverse: GGTAGAAATCTGTCATGCTG, Bax forward: TGACATGTTTTCTGACGGCAAC, Bax reverse: GGAGGCTTGAGGAGTCTCACC, GAPDH forward: GAAGGTGAAGGTCGGAGTC, GAPDH reverse: GAAGATGGTGATGGGATTTC.

Acknowledgements: We are grateful to Drs. Leonard Zon (Children's Hospital, Boston), and F. McKeon for SuPr-1 expression constructs, and p63 antibodies, respectively. This work was supported by Grants from KOSEF (M02-2004-000-20560-0), National Cancer Control Program (0320250-3 and 0620070), and 21C Frontier Functional Human Genome Project (M106KB010018-07K0201) in Korea.

References

[1] 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.
[2] Chi, S.W., Ayed, A. and Arrowsmith, C.H. (1999) Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain. EMBO J. 18, 4438–4445.
[3] Serber, Z. et al. (2002) A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. Mol. Cell Biol. 22, 8601–8611.
[4] Koster, M.I., Dai, D., Marinari, B., Sano, Y., Costanzo, A., Karin, M. and Roop, D.R. (2007) p63 induces key target genes required for epidermal morphogenesis. Proc. Natl. Acad. Sci. USA 104, 3255–3260.
[5] Bakkers, J., Hild, M., Kramer, C., Furutani-Seiki, M. and Hammerschmidt, M. (2002) Zebrafish DeltaNp63 is a direct target of Bmp signaling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. Dev. Cell 2, 617–627.
[6] Lee, H. and Kimelman, D. (2002) A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. Dev. Cell 2, 607–616.
[7] 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.
[8] Bakkers, J., Camacho-Carvajal, M., Nowak, M., Kramer, C., Danger, B. and Hammerschmidt, M. (2005) Destabilization of DeltaNp63alpha by Nedd4-mediated ubiquitination and Ubc9-mediated sumoylation, and its implications on dorsoventral patterning of the zebrafish embryo. Cell Cycle 4, 790–800.
[9] 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.
[10] Huang, Y.P. et al. (2004) Altered sumoylation of p63alpha contributes to the split-hand/foot malformation phenotype. Cell Cycle 3, 1587–1596.
[11] Yang, A. et al. (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398, 714–718.
[12] 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.
[13] van Bokhoven, H. et al. (2001) p63 Gene mutations in eec syndrome, limb–mammary syndrome, and isolated split hand-split foot malformation suggest a genotype–phenotype correlation. Am. J. Hum. Genet. 69, 481–492.
[14] 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.
[15] Ross, S., Best, J.L., Zon, L.I. and Gill, G. (2002) SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. Mol. Cell 10, 831–842.
[16] Gong, L., Millas, S., Maul, G.G. and Yeh, E.T. (2000) Differential regulation of sentrinized proteins by a novel sentrin-specific protease. J. Biol. Chem. 275, 3355–3359.
[17] Melchior, F., Schergaut, M. and Pichler, A. (2003) SUMO: ligases, isopeptidases and nuclear pores. Trends Biochem. Sci. 28, 612–618.
[18] Gill, G. (2004) SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? Genes Dev. 18, 2046–2059.
[19] 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.
[20] Rocco, J.W., Leong, C.O., Kuperwasser, N., DeYoung, M.P. and Ellisen, L.W. (2006) p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. Cancer Cell 9, 45–56.
[21] Bourdon, J.C., Fernandes, K., Murray-Zmijewski, F., Liu, G., Diot, A., Xirodimas, D.P., Saville, M.K. and Lane, D.P. (2005) p53 isoforms can regulate p53 transcriptional activity. Genes Dev. 19, 2122–2137.
[22] Jacobs, W.B. et al. (2005) p63 is an essential proapoptotic protein during neural development. Neuron 48, 743–756.
[23] 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.