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ΔNp63α represses nuclear translocation of PTEN by inhibition of NEDD4-1 in keratinocytes

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Abstract

Np63 maintains the proliferative potential of keratinocytes by inhibiting the transcription and nuclear localization of the tumor suppressor PTEN as shown earlier by our laboratory. The goal of this study was to define the mechanisms by which Np63 mediates the nuclear exclusion of PTEN. We demonstrate here that Np63 reduces the ubiquitination of PTEN, a key signaling event in the nuclear translocation of PTEN. The decrease in ubiquitinated PTEN correlated with the ability of Np63 to bind to NEDD4 promoter and transcriptionally repress the E3 ubiquitin ligase NEDD4-1. Knockdown of NEDD4-1 in cultured keratinocytes was sufficient to attenuate the increase in nuclear PTEN observed upon silencing of Np63. *In vivo* examination of normal skin demonstrated that Np63 and NEDD4-1 were both expressed in the basal layer of the epidermis and this correlated with nuclear exclusion of PTEN. Altogether, these studies suggest that Np63-mediated suppression of nuclear PTEN in basal layer keratinocytes occurs through repression of NEDD4-1.

Keywords

Np63 ; Nuclear PTEN; and NEDD4-1

Introduction

The tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) is second only to p53 in the frequency of observed mutations or loss in human cancers. The discovery of nuclear PTEN has expanded the tumor suppressive functions of PTEN to include induction of cell cycle arrest, increased apoptotic signaling and maintenance of chromosome stability [21]. We previously demonstrated that the proto-oncogene Np63 not only transcriptionally represses PTEN, but also inhibits the nuclear localization of PTEN in epidermal keratinocytes [16]. The balance between Np63 and PTEN was shown to be critical in maintaining keratinocyte proliferation and that this balance is significantly perturbed in non-melanoma skin cancers [16].

Retention of PTEN within the nucleus is maintained by a balance between nuclear import and export. Addition of a single ubiquitin moiety to PTEN, termed mono-ubiquitination, serves as a nuclear translocation signal for PTEN [1,22,23]. NEDD4-1 (neuronal precursor developmentally down regulated 4-1) E3 ubiquitin ligase, known to target PTEN, also regulates Np63 expression during development [4,22].

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The ability of Np63 to regulate the nuclear translocation of PTEN in skin cells is of therapeutic interest as loss of nuclear PTEN has been associated with cancer progression[9,10,13,26]. In this study we demonstrate that Np63 mediates the nuclear exclusion of PTEN by transcriptionally down-regulating NEDD4-1, thereby preventing the ubiquitination of PTEN and its import into the nucleus.

Materials and Methods

Cell Lines, Reagents and Plasmids

Primary adult human keratinocytes, HaCaT cells and the squamous cell carcinoma line A431 were chosen to model normal, non-tumorigenic and cancerous keratinocytes, respectively. H1299 cells were chosen as they are p63 null, while PTEN and NEDD4-1 positive. Wild-type and p63^{-/-} mouse embryonic fibroblasts were provided by Dr. Elsa Flores (MD Anderson). All cells were maintained and transfected as previously described[16]. The 6x-His tagged ubiquitin (His-Ub) were obtained from Dr. Steven Berberich (Wright State University, Dayton, OH) and hemagglutinin tagged ubiquitin (HA-Ub) and human NEDD4-1 expression plasmids were graciously provided by Dr. Miguel Martins (University of Leicester, Leicester, UK). Two rounds of Oligofectamine (Life Technologies, Grand Island, NY) transfections with p63 specific siRNAs were conducted as previously described [16]. Allstars Non-Silencing Control (NSC) and NEDD4-1 specific siRNA, target sequence (5 CCGGAGAATTATGGGTGTCAA3), were purchased from Qiagen (Valencia, CA).

Real Time PCR—Taqman based qRT-PCR was performed as described previously [16]. AODs used were GAPDH (4325792), p63 (Hs_00978340_m1), NEDD4-1 (Hs_0040645_m1). Fold change in genes of interest, relative to endogenous loading controls, was calculated using the comparative CT method on SDS2.2.2 software [19]. Student's t-tests were used to determine significant difference.

Immunoblot and Immunoprecipitation analyses—Immunoblot analysis was performed as described previously [16]. Subcellular fractionations were carried out using the NE-PER kit as per manufacturer's instructions (Pierce, Rockford, IL). Proteins were detected with the following antibodies: anti-p63 (4A4), anti- α -actin (AC-15), anti- α -tubulin (B-7), (Santa Cruz Biotechnologies, Santa Cruz, CA); anti-PTEN #9559, anti-NEDD4 #3607, anti-PARP #9542 (Cell Signaling, Boston, MA). Anti-Lamin A/C (BD Biosciences #612162) was donated by Dr. Steven Berberich (Wright State University). For immunoprecipitation studies whole cell extracts from HaCaT and A431 were pre-cleared with Protein G Dynabeads (Life Technologies) followed by overnight incubation at 4°C with 1 μ g Nedd4-1 specific antibody (anti-Nedd4-1 #sc-25508, Santa Cruz) or non-specific IgG control. Immunoprecipitated protein complexes were captured with Protein G Dynabeads and subjected to immunoblot analysis.

Immunofluorescence—Paraffin embedded embryonic mouse sections, a kind gift from Dr. Satrajit Sinha (SUNY Buffalo), were stained as previously described [16]. All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo [15].

Ubiquitination Assay—H1299 cells were transfected with the indicated expression plasmids and either HA-Ub or His-Ub. For experiments using HA-Ub, H1299 cells were lysed in a high salt buffer (300 mM NaCl, 100 mM Tris pH=8, 0.2 mM EDTA, 0.1% NP40, 10% glycerol). Protein lysates were immunoprecipitated as described above with 1 μ g anti-

hemagglutinin 12CA5. Ubiquitination assays with the His-Ub expression plasmid were completed as described previously [14].

Chromatin Immunoprecipitation (ChIP) Assay—HaCaT cells were grown to 70–80% confluency and subjected to chromatin immunoprecipitation using p63 specific antibodies (4A4 and H-129) as described previously [16]. Primers used for PCR amplification of regions within Nedd4 promoter [6] are listed in table 1 (online resource 1).

Results

Previous studies have implicated multiple ways by which PTEN translocates into the nucleus [5,8,17,22]. One such mechanism is mono-ubiquitination of PTEN [22]. To test whether Np63 affected this process, the ubiquitination of endogenous PTEN was measured after transfection of p63 null H1299 cells with HA-Ub together with either empty vector or Np63. Overexpression of Np63 reduced the levels of ubiquitinated PTEN, detected as higher molecular weight ladders with a PTEN specific antibody following immunoprecipitation of HA-tagged ubiquitin complexes (Fig. 1a). Similar to the effects on endogenous PTEN, Np63 significantly reduced ubiquitination of exogenous PTEN (Fig. 1b). This confirms that the reduction in ubiquitination of PTEN was not a by-product of increased transcriptional repression from overexpression of Np63.

Examination of a gene expression microarray in HaCaT and A431 cells after knockdown of p63 suggested NEDD4-1 is negatively regulated by Np63 (data not shown). Interestingly, NEDD4-1 was the first ubiquitin ligase shown to control the subcellular distribution of PTEN [22]. To ascertain if Np63 mediates PTEN ubiquitination by transcriptionally inhibiting NEDD4-1, the expression of NEDD4-1 was first measured in mouse embryonic fibroblasts (MEFs) from wild type and p63^{-/-} animals. NEDD4-1 protein levels were dramatically higher in p63^{-/-} MEFs compared to wild type cells (Fig. 2a). Protein levels of p63 could not be detected in wild type or p63^{-/-} MEFs (data not shown). To confirm that Np63 negatively regulates Nedd4-1, we transfected two different p63-specific siRNAs in primary adult human keratinocytes and the HaCaT and A431 keratinocyte cell lines. As shown in Figure 2b, silencing Np63 led to a significant increase in both transcript and protein levels of NEDD4-1 levels when compared to control cells. Consistent with the knockdown studies, over expression of Np63 in the p63 null H1299 cell line led to decreases in both NEDD4-1 and PTEN protein, confirming that Np63 negatively regulates the expression of both NEDD4-1 and PTEN (Fig. 2c). We further demonstrated that NEDD4-1 is a direct target of Np63 by performing ChIP assays. We observed binding of endogenous Np63 to several putative p63 binding sites within the NEDD4 promoter (Figure 2d) in HaCaT cells. Taken together, these data confirm that NEDD4-1 is negatively regulated by Np63. To determine if Np63-mediated regulation of NEDD4-1 affected the ability of Np63 to inhibit PTEN expression, we tested whether knockdown of NEDD4-1 synergizes with silencing of Np63 to further enhance PTEN expression. Knockdown of either Np63 or NEDD4-1 increased PTEN levels, consistent with the ability of Np63 and Nedd4-1 to transcriptionally repress PTEN or lead to proteosomal degradation of PTEN, respectively (Fig. 2e) [22,25]. Concomitant knockdown of both Np63 and NEDD4-1, however, did not further increase total cellular levels of PTEN (Fig. 2e).

Since loss of NEDD4-1 did not synergize with knockdown of Np63 to increase total cellular levels of PTEN, we speculated that the interplay between Np63 and NEDD4-1 was controlling the subcellular distribution of PTEN instead. To test this possibility we first confirmed that endogenous NEDD4-1 interacts with PTEN in keratinocytes. Interestingly, immunoprecipitation of whole cell extracts from both HaCaT and A431 cells with a

NEDD4-1 specific antibody or control IgG demonstrated that both endogenous Np63 and PTEN interact with endogenous NEDD4-1 (Figure 3a).

To confirm that Np63 was inhibiting the nuclear import of PTEN by repressing NEDD4-1 mediated ubiquitination of PTEN, we attempted to rescue the ubiquitination of PTEN upon over expression of Np63. Similar to the results in Figure 1b, Np63 over expression significantly reduced the levels of PTEN detected after isolation of His-tagged ubiquitin (Fig. 3b). Co-expression of both NEDD4-1 and Np63 attenuated the effects of Np63 on ubiquitination of PTEN (Fig. 3b).

Having confirmed that Np63 regulates the ubiquitination of PTEN by repressing NEDD4-1 (Fig. 3b), we sought to determine if the relationship between Np63 and NEDD4-1 mediated nuclear levels of PTEN in keratinocytes. Knockdown of p63 in HaCaT cells increased NEDD4-1 levels and also trended toward increased nuclear and cytoplasmic PTEN (Fig. 3c), however these changes were not statistically significant. None the less, small changes in PTEN levels have been shown to have significant biological effects [3], thus we believe the modest changes observed in nuclear PTEN may be biologically significant. Conversely, loss of NEDD4-1 resulted in reduced nuclear PTEN levels, but increased cytoplasmic levels of PTEN (Fig. 3c). Concomitant knockdown of Np63 and NEDD4-1 attenuated the increase in nuclear PTEN as compared to knockdown of only

Np63, suggesting that inhibition of NEDD4-1 by Np63 is responsible for the suppression of nuclear PTEN (Fig. 3c). In epidermal tissue both Np63 and NEDD4-1 are highly expressed in the basal layer (Fig. 3d). The localization of Np63 and NEDD4-1 to the basal layer also correlates with the exclusion of PTEN from the nuclei of basal layer keratinocytes (Fig. 3d, lower panel and [16]), demonstrating that the ability of Np63 to inhibit NEDD4-1 mediated ubiquitination of PTEN inhibits the nuclear translocation of PTEN *in vivo* as well as *in vitro*.

Discussion

In the present study we have demonstrated that by negatively regulating the expression of NEDD4-1, p63 was able to indirectly control the ubiquitination, and thus the localization, of PTEN (Fig. 3e). The inhibition of NEDD4-1 by Np63 provides a counter balance to the negative transcriptional regulation of PTEN by Np63 [16]. Restriction of NEDD4-1 expression to the basal layer of the epidermis and the exclusion of PTEN from the nuclei of basal layer keratinocytes (Fig. 3d), further demonstrates that the inhibition of NEDD4-1 by

Np63 is physiologically relevant in maintaining homeostasis in normal epidermal tissue. Since nuclear PTEN is most frequently associated with arrested or differentiated cells, its absence is likely critical to preserving the proliferative capability of the basal layer of the epidermis [10,5,26]. The mechanisms by which nuclear PTEN inhibits cell proliferation are still being elucidated, but studies have demonstrated that PTEN targets a subset of proteins within the nucleus distinct from those associated with cytoplasmic PTEN. For instance, direct dephosphorylation of CREB by PTEN reduces expression of CREB target genes and subsequently decreases proliferation [11]. Additionally, nuclear PTEN has been implicated in controlling chromosome stability [20] and in Rad51-mediated DNA repair [12]. The unique roles of nuclear PTEN highlight the need to better understand how PTEN is transported throughout the cell.

Np63 could also regulate ubiquitination of PTEN by competing for binding to NEDD4-1. This is supported by previous studies showing that Np63 is ubiquitinated by NEDD4-1 [4]. The ability of NEDD4-1 to ubiquitinate PTEN and Np63, combined with the results from this study demonstrating that Np63 inhibits NEDD4-1 (Fig. 2), suggests that Np63 /NEDD4-1/PTEN form a natural negative feedback mechanism. This feedback loop

serves to keep p63 and NEDD4-1 levels in check so that nuclear PTEN levels are kept low, protecting the proliferative capacity of basal layer keratinocytes. This feedback loop may represent a key step in inducing keratinocyte differentiation and the development of a stratified epidermis, since high calcium has been demonstrated to activate NEDD4-1 while causing the degradation of p63 [24,7].

In addition to regulating the localization of PTEN, p63 also leads to the activation of the Akt survival pathway via repression of PTEN [16]. Activation of Akt has also been shown to lead to nuclear export of PTEN [18]. Interestingly, Akt signaling also reduces NEDD4-1 expression [2] suggesting there is likely a cross talk between the Akt-mediated nuclear export of PTEN and p63-mediated inhibition of nuclear import of PTEN via NEDD4-1. Repression of NEDD4-1 by p63 may thus serve to limit the ubiquitin mediated degradation of PTEN, and subsequent hyperactivation of Akt in skin. Further studies will be required to understand the complex interplay between p63 and NEDD4-1 in regulating the PTEN/Akt axis to control proliferation in normal and pathological skin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Np63 decreases ubiquitination of PTEN. **a** H1299 cells were transfected with HA tagged ubiquitin (HA-Ub) and either empty vector or Np63 expression plasmids. Endogenous ubiquitinated PTEN was immunoprecipitated with an anti-HA antibody and detected by anti-PTEN antibodies by immunoblot. **b** H1299 cells were transfected with the 6xhistidine tagged ubiquitin (His-Ub) and the indicated expression plasmids. Ubiquitinated proteins were isolated using Ni-NTA beads and ubiquitination of exogenous PTEN was detected by immunoblot with an anti-PTEN antibody.

A)

B)

C)

Fig. 2.

Np63 represses NEDD4-1. **a** Whole cell extracts from primary wild type and p63^{-/-} MEFs were subjected to immunoblot analysis for the indicated proteins. The changes in NEDD4-1 protein levels, relative to wildtype, are listed above each band. Note: p63 was undetectable at the protein level in wild type and p63^{-/-} MEFs. **b** Primary adult human epidermal keratinocytes, HaCaT and A431 cells were transfected with non-silencing control (NSC) siRNA or siRNAs targeting all isoforms of p63. Total RNA was extracted and transcript levels of p63 and NEDD4-1 were analyzed by qRT-PCR. Y-axis represents the change in NEDD4-1 and p63 transcript levels relative to NSC transfected cells. Error bars represent standard deviation from three independent experiments; * = $p < 0.05$. Representative immunoblots of p63 and Nedd4-1 are shown in the bottom panels. The change in protein levels, relative to NSC transfected cells, is listed above each band. **c** H1299 cells were transfected with empty vector or Np63 (1, 2 and 3 μ g) as indicated and whole cell extracts were subjected to immunoblot analysis for the indicated proteins. **d** Schematic of the *NEDD4-1* promoter showing locations of putative p63 binding sites (**top**

panel). ChIP assay was performed on HaCaT cells, wherein chromatin was immunoprecipitated with normal IgG control antibody or antibodies that recognize all p63 isoforms (4A4) or p63 isoforms (H129) as indicated. Eluted DNA was PCR-amplified with primers specific for multiple regions of the *NEDD4-1* promoter as shown in the top panel. **e** HaCaT cells were transfected with NSC or siRNA against p63 and/or NEDD4-1 and whole cell lysates were subject to immunoblot analysis for the indicated proteins. The change in protein levels, relative to NSC transfected cells, is listed below each band.

A)

B)

E)

Fig. 3.

Np63 and NEDD4-1 antagonistically regulate nuclear PTEN levels. **a** Endogenous NEDD4-1 was immunoprecipitated from whole cell extract of A431 and HaCaT cells. Co-precipitation of endogenous NEDD4-1, Np63 and/or PTEN was detected by immunoblot analysis. **b** H1299 cells were transfected with His-Ub and the indicated expression plasmids. Ubiquitinated proteins were isolated using Ni-NTA beads and ubiquitination of exogenous PTEN was by immunoblot analysis. **c** HaCaT were transfected with NSC or siRNA against p63 and/or NEDD4-1 and subjected to subcellular fractionation and immunoblot analysis one day post transfection. A representative experiment is shown in the top panel. Nuclear protein levels were normalized to the Lamin A/C, while α -tubulin served as a loading control for normalization of cytoplasmic proteins. The average change in nuclear and cytoplasmic PTEN from three independent experiments is plotted in the bottom panel. Y-axis represents the change PTEN levels relative to NSC transfected cells after normalization to the loading control. **d** Representative images showing the localization of p63, NEDD4-1, and PTEN skin of wild type E18.5 mouse embryos. **e** Schematic of the how Np63 regulates nuclear translocation of PTEN. Np63 is able to suppress the nuclear import of PTEN by decreasing mono-ubiquitination of PTEN via repression of the E3 ligase NEDD4-1.