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Pirh2 E3 Ubiquitin Ligase Modulates Keratinocyte Differentiation Through p63

Yong-Sam Jung, Yingjuan Qian, Wensheng Yan, and Xinbin Chen[§]

Comparative Oncology Laboratory, University of California, Davis, CA 95616, USA

Abstract

p63, a homologue of the tumor suppressor p53, is essential for the development of epidermis and limb. p63 is highly expressed in epithelial cell layer and acts as a molecular switch that initiates epithelial stratification. However, the mechanisms controlling p63 protein level is still far from fully understood. Here, we demonstrate a regulatory protein for the p63 activity. We found that Pirh2 E3 ubiquitin ligase physically interacts with p63 and targets p63 for polyubiquitination and subsequently proteasomal degradation. We also found that ectopic expression of Pirh2 in HaCaT cells suppresses cell proliferation. Consistent with this, we found that along with altered expression of Np63 protein, ectopic expression of Pirh2 promotes, whereas knockdown of Pirh2 inhibits, keratinocyte differentiation. Collectively, our data suggest that Pirh2 plays a physiologically relevant role in keratinocyte differentiation through posttranslational modification of p63 protein.

Keywords

p63; Pirh2; E3 ligase; Ubiquitination; keratinocyte differentiation

Introduction

p63, a p53 ancestor gene, is expressed as the N-terminal transactivation domain-containing (TA) and -deletion (ΔN) isoforms, the former of which is transcribed from the P1 promoter upstream of exon 1 and the latter of which from the P2 promoter in intron 3 (Yang *et al.*, 1998). Both TA and ΔNp63 give rise to at least five C-terminal alternative spliced isoforms (α, β, γ, δ, and ε) (Mangiulli *et al.*, 2009). Similar to p53, TAp63 is a transcription factor containing the N-terminal transactivation domain, the central DNA binding domain, and the tetramerization domain (Yang *et al.*, 1998). Unlike p53, TA/ΔNp63α contains a C-terminal sterile alpha motif (SAM) domain involved in protein-protein interaction and regulation of development (Ponting, 1995; Yang and McKeon, 2000). It has been shown that TAp63 exerts p53-like activities in cell cycle arrest and cell death via regulating expression of some p53 target genes, including p21, Mdm2, vitamin D receptor, and Bax (Dohn *et al.*, 2001;

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[§]Corresponding to Dr. Xinbin Chen, xbchen@ucdavis.edu.

Conflict of Interest

The authors declare no conflict of interest.

Guo *et al.*, 2009; Kommagani *et al.*, 2006; McKeon, 2004; Senoo *et al.*, 2007; Truong *et al.*, 2006). While Np63 lacks the N-terminal transactivation domain conserved in TAp63, it carries a unique transactivation domain, which consists of 13 unique amino acids for Np63 along with the proline-rich domain (Dohn *et al.*, 2001; Helton *et al.*, 2006; King *et al.*, 2003; Wu *et al.*, 2003). It is well known that Np63 plays a key role in the maintenance of proliferative potential and self-renewal of basal epidermal cells (King *et al.*, 2003; Koster *et al.*, 2004; Yang *et al.*, 1998). Interestingly, the p63 gene is rarely mutated and even amplified in some cancers (Chikh *et al.*, 2007; Flores *et al.*, 2005; Hagiwara *et al.*, 1999; Hibi *et al.*, 2000) although mutation of the p53 gene is a common event in the majority of human cancers. Nevertheless, human patients and mice deficient in p63 exhibit severe abnormalities of epidermis and limb formation, indicating that p63 plays a critical role in epidermal development (Mills *et al.*, 1999; Su *et al.*, 2009a; Su *et al.*, 2009b; Yang *et al.*, 1999). Therefore, p63 is likely to be regulated in a tissue-specific manner.

The p53 protein turnover is regulated by several E3 ubiquitin ligases, including Mdm2 (Mouse double minute 2) and Pirh2 (p53-induced RING-H2) (Brooks and Gu, 2006; Leng *et al.*, 2003; Wu *et al.*, 1993). Interestingly, both Mdm2 and Pirh2 are RING-finger E3 ligases directly regulated by p53 and thus form a negative regulatory feedback loop with p53 to promote p53 ubiquitination and degradation (Leng *et al.*, 2003; Wu *et al.*, 1993). However, there are differences between Mdm2 and Pirh2. For instance, Mdm2 primarily degrades p53 in unstressed cells, but Pirh2 is able to degrade p53 upon DNA damage (Maya *et al.*, 2001; Prives and Hall, 1999; Shieh *et al.*, 1997). In addition, the protein stability of p73, another p53 family member, is regulated by Pirh2, but not by Mdm2 (Balint *et al.*, 1999; Jung *et al.*, 2011b; Ongkeko *et al.*, 1999). Similarly, degradation of p63 protein occurs independent of Mdm2-mediated ubiquitination (Calabro *et al.*, 2002; Galli *et al.*, 2010). Therefore, it is likely that Mdm2 specifically polyubiquitinates and degrades p53, whereas Pirh2 is capable of regulating the protein stability of other p53 family members.

In this study, we found that p63 protein stability is regulated by Pirh2. We showed that knockdown of Pirh2 upregulates, whereas overexpression of Pirh2 downregulates, p63 expression. In addition, Pirh2 physically interacts with and polyubiquitinates p63, and consequently targets p63 for proteasome degradation. Finally, we showed that Pirh2 promotes keratinocyte differentiation via modulating Np63 expression. Taken together, our findings demonstrate that Pirh2 functions as an E3 ligase for the alpha isoforms of p63 and that Pirh2 plays a physiologically relevant role in keratinocyte differentiation via posttranslational modification of p63 protein.

Results

Pirh2 represses p63 expression

In addition to Mdm2, Pirh2 has been identified as an E3 ligase regulating p53 degradation (Leng *et al.*, 2003). Previously, we and others showed that Pirh2 polyubiquitinates and directs p73 for the proteasome-dependent degradation (Jung *et al.*, 2011b; Wu *et al.*, 2011). Since p63 shares a high degree of sequence identities with p53 and p73 (Joerger *et al.*, 2009), we hypothesize that Pirh2 regulates p63 stability. To test this, Pirh2 was transiently knocked down by Pirh2 siRNA in RKO and HCT116 cells. Indeed, we found that the level

of TAp63 α protein was increased in Pirh2-knockdown (KD) cells (Figure 1a, p63 panel, compare lanes 2 and 4 with 1 and 3, respectively). We would like to note that in both RKO and HCT116 cells, TAp63 α is the predominant form of p63 isoforms and can be reliably measured by Western blotting. As a positive control, p53 was also increased upon Pirh2-KD (Figure 1a, p53 panel, compare lanes 2 and 4 with 1 and 3, respectively). The level of actin was measured as a loading control. Consistent with this, we showed that the level of TAp63 α protein was gradually increased as Pirh2 was progressively knocked down over a 3-day period in HCT116 cells (Figure 1b). Next, we examined whether increased expression of Pirh2 leads to decreased expression of TAp63 α protein. To test this, FLAG-tagged Pirh2 was transiently overexpressed in HCT116 and HCT116 p53^{-/-} cells. We showed that upon ectopic expression of Pirh2, the level of TAp63 α protein was decreased in HCT116 and HCT116 p53^{-/-} cells (Figure 1c).

Next, we want to examine whether Pirh2 affects Np63 expression. To test this, Pirh2 was transiently knocked down (Figure 1d) or overexpressed (Figure 1e) in HaCaT cells, which express a high level of endogenous Np63 α (Yang *et al.*, 2002). Western blot analysis showed that Np63 α was increased by Pirh2-KD, but decreased by Pirh2 overexpression in HaCaT cells (Figure 1d and e, compare lane 2 with 1). To further examine the effect of Pirh2 on p63 degradation, Myc-TAp63 α was transiently expressed in MCF7 cells along with an increased dose of FLAG-Pirh2 (Figure 1f). We showed that the levels of ectopically expressed Myc-TAp63 α were decreased by FLAG-Pirh2 in a dose-dependent manner (Figure 1f, p63 panel). Similarly, the levels of endogenous Np63 α were decreased (Figure 1g, p63 panel) by FLAG-Pirh2 in a dose-dependent manner in HaCaT cells (Figure 1g, Pirh2 panel). Interestingly, we showed that the ability of Pirh2 to decrease the level of TAp63 α and Np63 α was blocked by treatment with MG132 proteasome inhibitor (Figure 1f and g, compare lane 6 with 5). These data suggest that Pirh2 targets p63 for proteasomal degradation.

Pirh2 physically associates with p63

Since an E3 ligase often physically interacts with its substrates, we want to examine whether Pirh2 physically associates with p63. To test this, endogenous p63 and Pirh2 in RKO and HaCaT were immunoprecipitated by anti-p63 and anti-Pirh2, respectively. Western blot analysis showed that endogenous Pirh2 was detected in the TAp63 α -immunocomplex (Figure 2a, top panel, lane 3). Conversely, endogenous TAp63 α was detected in the Pirh2-immunocomplex (Figure 2a, bottom panel, lane 3). Likewise, endogenous Pirh2 was also detected in Np63 α -immunocomplex (Figure 2b, top panel, lane 3). Conversely, endogenous Np63 α was detected in the Pirh2-immunocomplex (Figure 2b, bottom panel, lane 3). To rule out a potential cell-type specific effect, endogenous p63 and Pirh2 were immunoprecipitated by anti-p63 and anti-Pirh2, respectively, in MIA-PaCa-2, which expresses a high level of endogenous TAp63 α , and ME180, which expresses a high level of endogenous Np63 α . Again, we showed that Pirh2 associated with TAp63 α (Figure 2c) and Np63 α (Figure 2d). To confirm this protein-protein association between Pirh2 and TAp63 α / Np63 α , FLAG-tagged Pirh2 (FLAG-Pirh2) and Myc-tagged TAp63 α (Myc-p63 α) or Np63 α (Myc-Np63 α) were cotransfected into RKO cells, and then immunoprecipitated by anti-Myc and anti-FLAG, respectively. We showed that ectopically

expressed FLAG-Pirh2 was present in the anti-Myc (TAp63 α / Np63 α) immunocomplexes (Figure 2e and f, top panel). Conversely, ectopically expressed Myc-p63 α or Myc- Np63 α was detected in the anti-FLAG (Pirh2) immunocomplexes (Figure 2e and f, bottom panel). In addition, FLAG-Pirh2 was transfected into MIA-PaCa-2 and ME180 cells, and then immunoprecipitated by anti-p63 or anti-FLAG, respectively. We showed that ectopically expressed FLAG-Pirh2 was present in the anti-p63 (TAp63 α / Np63 α) immunocomplexes (Figure 2g and h, top panel). Conversely, endogenous TAp63 α or Np63 α was detected in the anti-FLAG (Pirh2) immunocomplexes (Figure 2g and h, bottom panel).

Pirh2-mediated degradation of p63 occurs through a ubiquitin-dependent pathway

To determine if the Pirh2-mediated degradation of p63 is ubiquitin-dependent, we measured the level of ectopically expressed p63 in ts20 cells at both the permissive (35°C) and nonpermissive (39°C) temperatures. The ts20 cells, a Chinese hamster lung cell line, has a thermo-inactive ubiquitin activating enzyme E1 (Chowdary *et al.*, 1994). We found that the level of TAp63 α was decreased by Pirh2 at the permissive temperature, but not much at the nonpermissive temperature (Figure 3a, compare lanes 1 and 3 with 2 and 4, respectively). As a control, we showed that endogenous p53 was destabilized at the permissive temperature but not at the nonpermissive temperature (Figure 3a, compare lane 2 and 4), consistent with previous reports that Pirh2-mediated p53 degradation is accomplished in a ubiquitin-dependent pathway (Chowdary *et al.*, 1994; Leng *et al.*, 2003). To confirm that reduction of TAp63 α in ts20 cells is due to proteasomal degradation, ts20 cells were treated with the proteasome inhibitor MG132. We found that the level of TAp63 α was not decreased upon treatment with MG132 (Figure 3a, lanes 5-8), demonstrating that the degradation of TAp63 α by Pirh2 requires proteasome function. Furthermore, we examine whether Pirh2 promotes Np63 α degradation in ts20 cells. We showed that like TAp63 α , Np63 α was targeted by Pirh2 for proteasomal degradation at the permissive temperature, which was blocked by treatment with MG132 (Figure 3b). Finally, we examined whether Pirh2 alters the protein half-life of TAp63 α in MIA-PaCa-2 and Np63 α in HaCaT cells. We showed that the protein half-life of TAp63 α was decreased from ~7 to 4 h (Figure 3c and d), and that of Np63 α was decreased from ~11 to 6 h by Pirh2 overexpression (Figure 3e and f).

Pirh2 promotes p63 polyubiquitination

To investigate whether Pirh2 can promote p63 degradation via ubiquitination, Myc-tagged TAp63 α , and FLAG-tagged ubiquitin were co-expressed in RKO cells with FLAG-tagged Pirh2, Pirh2- RING (lacks RING finger domain), or Pirh2-DN (contains two substitutive mutations from Cys to Ser at amino acids 145 and 148) (Figure 4a) (Jung *et al.*, 2010). We showed that Myc-TAp63 α was detected along with slower migrating bands by anti-Myc antibody in cells expressing Pirh2, but not Pirh2- RING and Pirh2-DN (Figure 4b, top p63 panel, compare lane 4 with lanes 5-6). We also showed that the slow migrating bands were recognized by anti-ubiquitin antibody (Figure 4b, Ub panel). This suggests that the slow migrating bands are polyubiquitinated TAp63 α . Similarly, Np63 α was polyubiquitinated by Pirh2 (Figure 4c).

Next, we investigated whether Pirh2 directly serves as an E3 ubiquitin ligase for p63. To test this, *in vitro* ubiquitination assay was performed with ³⁵S-labeled TAp63 α along with

recombinant GST-Pirh2 or GST-Pirh2-DN. We showed that TAp63 α was polyubiquitinated by Pirh2 but not by Pirh2-DN (Figure 4d, compare lanes 3-4). Similarly, we also showed that Np63 α was polyubiquitinated by Pirh2 but not by Pirh2-DN (Figure 4e, compare lanes 3-4). Together, these data demonstrate that Pirh2 is capable of polyubiquitinating TA/Np63 α and promoting TA/Np63 α degradation.

Pirh2 promotes keratinocyte differentiation via p63

Striking defects in skin and limb development observed in p63 knockout mice suggest that p63 is essential for epidermal homeostasis (Mills *et al.*, 1999; Yang *et al.*, 1999). It has been shown that Np63 is the dominant isoform expressed before epidermal stratification and throughout embryonic stages during epidermal, tooth, and hair development (Candi *et al.*, 2007; Koster and Roop, 2004; Laurikkala *et al.*, 2006). Indeed, abundant expression of Np63 in the basal layer of stratified epithelia maintains the proliferative potential and directs proper differentiation of progenitor cells (Koster *et al.*, 2004; Lee and Kimelman, 2002). Thus, we examined whether Pirh2 is implicated in keratinocyte differentiation. To test this, HaCaT cell line was chosen, since it has been used widely for *in vitro* keratinocyte differentiation (Deyrieux and Wilson, 2007). Since cell growth retardation is associated with differentiation, we first examined whether cell proliferation is affected by ectopic expression of Pirh2. Indeed, we found that HaCaT cell proliferation was inhibited by ectopic expression of Pirh2 (Figure 5a). Next, we examined whether Pirh2 has an impact on terminal keratinocyte differentiation, which can be measured by formation of cornified cell envelope (Pillai and Bikle, 1992). We found that knockdown of Pirh2 decreased, whereas ectopic expression of Pirh2 increased, cornified cell envelope formation induced by treatment of calcium (Figure 5b and c). In addition, we showed that knockdown of Pirh2 enhanced Np63 expression but decreased expression of involucrin and filaggrin (Figure 5d). Involucrin and filaggrin are markers commonly used to measure keratinocyte terminal differentiation (Candi *et al.*, 2005; Watt, 1983). Conversely, ectopic expression of Pirh2 decreased Np63 expression but increased expression of involucrin and filaggrin (Figure 5e).

Discussion

All proteins are continuously turning over, as they are being hydrolyzed and replaced by *de novo* synthesis. Many proteins are degraded by the ubiquitin-dependent proteasomal pathway (Rock *et al.*, 1994). In this study, we provide several lines of evidence to support that Pirh2 targets both TAp63 α and Np63 α proteins for ubiquitin-dependent proteasomal degradation. First, Pirh2 physically binds to p63. Second, Pirh2 promotes p63 ubiquitination through its E3 ligase activity. Third, Pirh2 promotes p63 degradation in a ubiquitin-dependent manner. Finally, Pirh2 promotes keratinocyte differentiation via degradation of Np63 α .

Since its discovery as homologs of the p53 tumor suppressor, p63 and p73 have gained tremendous attention and have been intensively investigated. However, although p63 and p73 perform p53-like functions, they are not classic Knudson-Type tumor suppressors since they are key regulators in development (Melino *et al.*, 2002). These indicate that p63 and

p73 are not just redundant family proteins for p53. Due to the functional diversity among p53 family members, it is not surprising that p53, p63, and p73 are differentially regulated. For example, p53 stability is primarily regulated by the ubiquitin-proteasome degradation pathway via E3 ubiquitin ligases, including RING-finger E3 ligases (Mdm2, Pirh2, and COP1) and HECT-domain E3 ligases (E6-AP and Arf-BP1) (Lee and Gu, 2010). Degradation of p63 protein is mediated by Dlx3 (Di Costanzo *et al.*, 2009), RACK1 (receptor for protein kinase C) (Li *et al.*, 2009), and HECT-domain containing E3 ligases, including ITCH (Rossi *et al.*, 2006) and WWP1 (Li *et al.*, 2008). p73 ubiquitination has been shown to be regulated by ITCH (Rossi *et al.*, 2005), FBXO45 (an F-box protein) (Peschiaroli *et al.*, 2009), and NEDL2 (a NEDD4-related HECT-domain containing E3 ligase) (Miyazaki *et al.*, 2003). To date, an E3 ligases that can target all the p53 family have not been reported. Here, we found that overexpression of Pirh2 decreases, whereas knockdown of Pirh2 increases, the level of TA/ Np63 α protein (Figure 1). In addition, we showed that Pirh2 physically associates with TA/ Np63 α (Figure 2) and promoting its polyubiquitination and degradation through proteasome (Figures 3 and 4). Together, we concluded that p63 is a substrate for Pirh2 E3 ubiquitin ligase. Previously, we and others showed that Pirh2 promotes p53/p73 protein turnover via polyubiquitinating. Thus, for the first time to our knowledge previously unreported, we showed that Pirh2 is a common E3 ubiquitin ligase for p53, p63, and p73.

The epidermis is the self-renewing stratified epithelium on the outermost layer of the skin. A variety of genes, such as involucrin, loricrin, and keratins, are involved in skin formation as a barrier against the environment (Candi *et al.*, 2005). Np63 α is not only the main isoform essential for epidermal development during embryogenesis, but also controls epithelial cell differentiation in the basal layer of epidermis in adult (King *et al.*, 2003; Koster and Roop, 2004). Np63 α is highly expressed in basal epithelial cells and downregulation of Np63 α initiates differentiation (King *et al.*, 2003; Koster *et al.*, 2004; Yang *et al.*, 1998). However, upstream regulator of Np63 α during differentiation is not clear. In this study, we found that overexpression of Pirh2 significantly inhibits cell proliferation in HaCaT cells (Figure 5a). Consistent with this, it was previously showed that depletion of Np63 reduces cell viability in head and neck squamous carcinoma epithelial cells (Rocco *et al.*, 2006). Moreover, we found that depletion of Pirh2 decreased, whereas overexpression of Pirh2 increased, the level of cornified cell envelope formation (Figure 5b and d). This suggests that Pirh2 is an important regulator in epithelial development. Since p63 is regulated by several E3 ligases, including as ITCH (Rossi *et al.*, 2006), Mdm2/Mdmx (Calabro *et al.*, 2002; Wang *et al.*, 2001), WWP1 (Li *et al.*, 2008), and SCF TrCP1 (Gallegos *et al.*, 2008), how a proper level of p63 isoforms is maintained during skin development and/or in response to DNA damage needs to be examined. In addition, what is the upstream signal that modulates Pirh2-mediated p63 degradation during epidermal differentiation is of great interest for future studies.

As a common regulator of p53 family members, it is reasonable to speculate that Pirh2 plays a role in tumorigenesis. Indeed, evidence showed that Pirh2 may act as an oncoprotein or tumor suppressor in different types of human cancers (Jung *et al.*, 2012). Downregulation of tumor suppressor proteins including p53, p73, and p27 may contribute to tumor progression

in cancers with high Pirh2 expression (Duan *et al.*, 2007; Duan *et al.*, 2004; Duan *et al.*, 2006). However, upregulation of oncogenic c-Myc leads to tumor formation in Pirh2 knockout mice and in cancers with low Pirh2 expression (Hakem *et al.*, 2011). Previously, we showed that Pirh2 inhibits DNA polymerase ϵ (PolH) activity via degrading PolH through 20S proteasome or promoting PolH monoubiquitination (Jung *et al.*, 2011a; Jung *et al.*, 2010). PolH is critical in translesion DNA synthesis, and loss of PolH results in early onset of skin tumor formation upon UV-induced replication stress in PolH-knockout mice (Ohkumo *et al.*, 2006). In addition, Pirh2 is associated with Keratin-8/18, which is abundantly expressed in epithelial cells, to regulate UV-induced cell death through modulating mitochondria distribution (Duan *et al.*, 2009). Here, we showed that Np63 α is targeted by Pirh2 for proteasomal degradation. Interestingly, except its essential role in skin development and in maintaining epidermal homeostasis in adults, elevated expression of Np63 α is correlated with multiple epithelial cancers (Candi *et al.*, 2007). Moreover, it has been shown that wild-type p53 mediates the tanning response (Cui *et al.*, 2007). Taken together, it is likely that Pirh2 plays a role in skin carcinogenesis through its targets and future studies deciphering how Pirh2 targets are involved in skin carcinogenesis will provide important clues for skin cancer treatment.

Materials and Methods

Cell Culture and Plasmids

RKO, MCF7, HaCaT, the temperature-sensitive ts20, HCT116, MIA-PaCa-2, ME180, and HCT116 p53^{-/-} were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Human full-length Pirh2, Pirh2-DN (an E3 ligase defective mutant), and Pirh2- RING (the RING finger domain deletion mutant) were used as previously described (Jung *et al.*, 2010). All Pirh2 proteins were N-terminally FLAG-tagged as previously described (Jung *et al.*, 2010). cDNAs encoding TAp63 α and Np63 α were N-terminally Myc-tagged as previously described (Gaiddon *et al.*, 2001). FLAG-tagged Ubiquitin expression vector in pcDNA3 was used as previously described (Jung *et al.*, 2010).

Antibodies

Antibodies used in this study were as follow: Rabbit polyclonal anti-Pirh2 (Bethyl Laboratories), mouse monoclonal anti-p63 (Santa Cruz Biotechnology), mouse monoclonal anti-Involucrin (IVL) (Santa Cruz Biotechnology), mouse monoclonal Filaggrin (Santa Cruz Biotechnology), mouse monoclonal anti-p53 antibodies (a mixture of DO-1, PAb1801, PAb240, and PAb421) derived from hybridoma's supernatant, mouse monoclonal anti-ubiquitin (Santa Cruz Biotechnology), rabbit polyclonal anti-Myc (Abcam), mouse monoclonal anti-FLAG (Sigma), and rabbit polyclonal anti-actin (Sigma).

Immunoprecipitation-Western Blot Analysis

The immunoprecipitation experiment was carried out as previously described (Jung *et al.*, 2008). Briefly, cells were washed with PBS, lysed in mammalian lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.4 mM PMSF), sonicated, and cleared by centrifugation. Cell lysates (300-500 μ g of total proteins) were incubated for 4

hours at 4°C with indicated antibodies coupled to the protein A/G-agarose beads (Sigma) and then washed with mammalian lysis buffer. Immunoprecipitated protein complexes and whole cell lysates (WCLs) were subjected to an SDS-PAGE. For each set, input controls were run (5% of WCL used for immunoprecipitation). IgG antibody was used as a negative control for each immunoprecipitation assay. Immuno-blots were visualized by SuperSignal West Femto Chemiluminiscent detection reagents (Pierce).

Small Interference RNA (siRNA)

Scramble and Pirh2 siRNA (sense 5'-CCAACAGACUUGUGA AGAA-dTdT-3' and antisense 5'-UUCUUCACAAGUCUGUUGG-dTdT-3') were purchased from Dharmacon (Junget al., 2011a). The siRNA duplex was introduced into cells using SilentFect (Bio-Rad) according to the manufacturer's protocol. Cells were harvested at indicated times after transfection for further experiments.

GST Fusion Protein Preparation

Glutathione S-transferase (GST)-tagged Pirh2 and Pirh2-DN were expressed by pGEX-4T-3 (Amersham Pharmacia Biotech). The recombinant GST-tagged proteins were purified as described previously (Jung et al., 2008). Briefly, the GST fusions of Pirh2 and Pirh2-DN were expressed in *E. coli* BL21 (DE3) (Novagene) upon induction with 0.5 mM IPTG for 4 h at 37°C. Bacterial cells were harvested and then resuspended in GST lysis buffer ((200 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 100 μM EDTA, 0.1% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride). Subsequently, cell lysates were sonicated and clarified by centrifugation. GST fusion proteins were purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Ubiquitination Assay

In vivo ubiquitination assay was performed as described (Leng et al., 2003). RKO cells were transfected with indicated plasmids and treated with 5 μM MG132 for 6 h prior to harvest. Cell lysates were immunoprecipitated with anti-Myc antibody followed by Western blot with anti-Myc and anti-Ubiquitin antibodies to detect p63 ubiquitination.

In vitro ubiquitination assay using recombinant GST fusion Pirh2 proteins was performed as described (Jung et al., 2011a). ³⁵S-labeled TAp63α and Np63α proteins were mixed with GST-Pirh2 or -Pirh2-DN and then incubated on ice for 1 h to form Pirh2:p63 protein complexes. The complexes were added to ubiquitination buffer containing E1, E2, and ubiquitin, and incubated at 30°C for 2 h. Finally, the reaction mixtures were separated on SDS-PAGE and analyzed by autoradiography. E1, E2, and ubiquitin were purchased from Boston Biochem. ³⁵S-labeled p63 was produced by the TNT T7-coupled reticulocyte lysates system (Promega).

Cell Proliferation Assay

To evaluate the calcium-induced differentiation effect on proliferation of HaCaT cells, cell proliferation assay was performed. HaCaT cells were transfected with control pcDNA3 vector or pcDNA3-2X-FLAG-Pirh2 for 24 h and then an appropriate number of cells were cultured in 6-well plates in triplicate. Cells were cultured in calcium-containing medium (1.5

mM) over a 7-day period. Cells were harvested and counted at indicated times. All values are given as mean \pm SD. Statistical comparisons were made by Student's t-test. A *P* value less than 0.05 was considered statistically significant.

Cornified Cell Envelope Assay

Cornified cell envelopes (CCEs) were analyzed as described previously (Qian *et al.*, 2011; Zhang *et al.*, 2010). Briefly, cells were harvested and resuspended in 1.1 ml of PBS containing 2 mM EDTA, and 100 μ l aliquotes were counted for the total number of cells. The remaining cells were centrifuged and resuspended in dissociation buffer (2% SDS, 20 mM dithiothreitol, 5 mM EDTA, and 0.1 M Tris-Cl, pH 8.5), and heating for 5 min. After cooling, insoluble CCEs were harvested and resuspended in 100 μ l of PBS, and counted with a hemacytometer. The data were measured as CCEs/total cells \times 100. The fold-change in relative CCE is a product of CCEs induced by Pirh2 overexpression or knockdown, divided by that induced by control.

Acknowledgments

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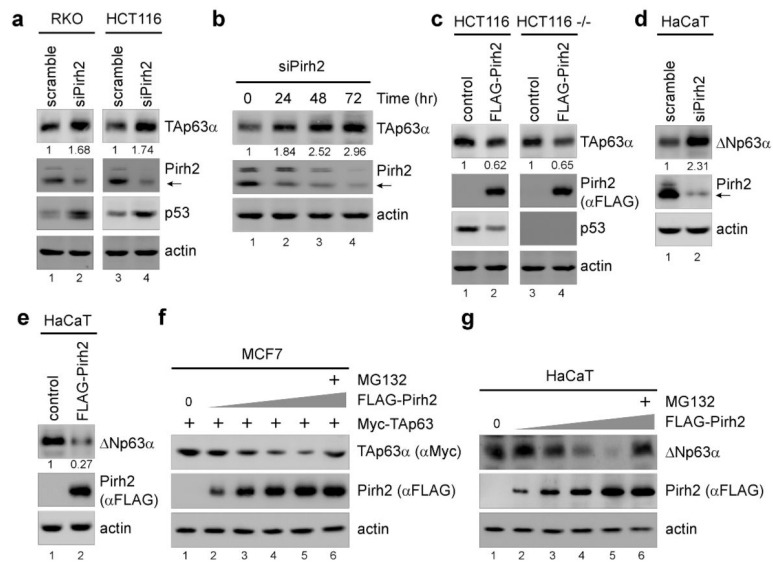


Figure 1. Knockdown of Pirh2 increases, whereas overexpression of Pirh2 decreases, p63 expression

(A) Cell lysates were prepared from RKO (left panel) and HCT116 (right panel) cells transfected with scramble or Pirh2 siRNA to knock down Pirh2 for 72 h. TAp63 α , Pirh2, p53, and actin were measured with their respective antibodies. The level of TAp63 α protein after normalization with actin was presented as fold change. (B) Cell lysates were prepared from HCT116 cells transfected with Pirh2 siRNA for 0, 24, 48, and 72 h. TAp63 α , Pirh2, and actin were measured with their respective antibodies. The level of TAp63 α protein after normalization with actin was presented as fold change. (C) Western blots were prepared with extracts from HCT116 (left panel) and HCT116 p53^{-/-} (right panel) cells that were mock-transfected or transfected with FLAG-Pirh2 for 36 h. The blots were analyzed as in (A) except anti-FLAG antibody was used to detect FLAG-Pirh2. The level of TAp63 α protein after normalization with actin was presented as fold change. (D) The experiment was performed as described for panel (A) except that HaCaT cells were used for transfection. The level of Δ Np63 α protein after normalization with actin was presented as fold change. (E) The experiment was performed as described for panel (C) except that HaCaT cells were used for transfection. The level of Δ Np63 α protein was evaluated through densitometry and results were presented as fold change. (F) Western blots were prepared with extracts from MCF7 cells transfected with Myc-TAp63 α along with an increasing dose of FLAG-Pirh2 for 36 h. The blots were analyzed as in (B) except anti-FLAG antibody was used to detect FLAG-Pirh2. (G) Western blots were prepared with extracts from HaCaT cells transfected with FLAG-Pirh2 for 36 h. Δ Np63 α and actin were measured with their respective antibodies. Anti-FLAG antibody was used to detect FLAG-Pirh2.

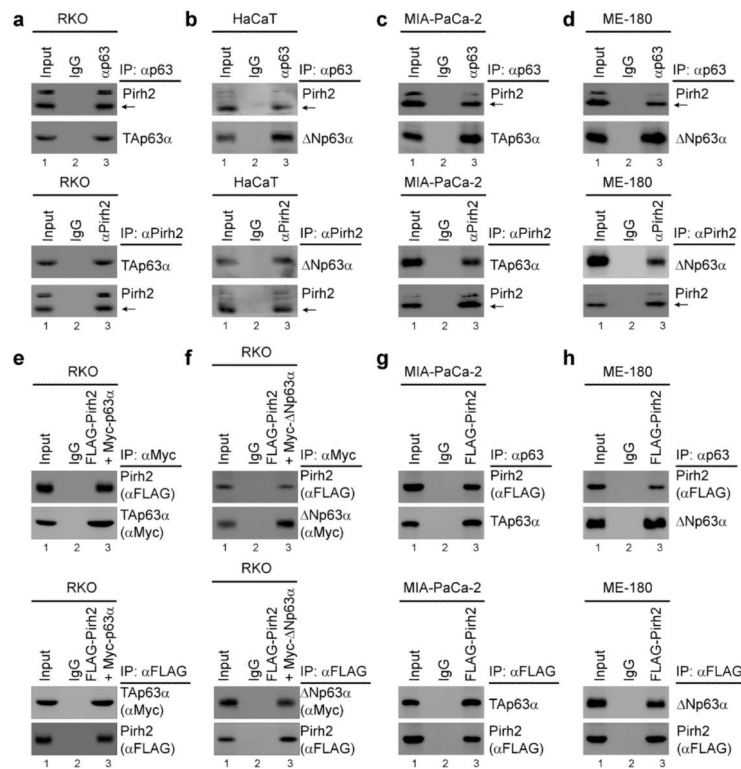


Figure 2. TAp63 α and Np63 α isoforms bind to Pirh2

(A) Top panel: cell extracts from RKO cells were immunoprecipitated with anti-p63 (4A4) or control IgG. The immunocomplexes were then used to detect TAp63 α and Pirh2 along with whole-cell lysates as input control. Bottom panel: the experiment was performed as described for top panel except that immunoprecipitation was performed with anti-Pirh2 antibody. (B) The experiments were performed as in panel (A) except HaCaT cells were used to immunoprecipitate Np63 α . (C) The experiments were performed as in panel (A) except MIA-PaCa-2 cells were used. (D) The experiments were performed as in panel (B) except that ME-180 cells were used. (E) The experiments were performed as in panel (A) except using anti-Myc to immunoprecipitate Myc-TAp63 α (top panel) and anti-FLAG to immunoprecipitate FLAG-Pirh2 (bottom panel) in RKO cells transiently transfected with Myc-TAp63 α and FLAG-Pirh2. (F) The experiments were performed as in panel (E) except RKO cells transiently transfected with Myc-Np63 α and FLAG-Pirh2. (G) The experiments were performed as in panel (A) except anti-FLAG was used to immunoprecipitate FLAG-Pirh2 in MIA-PaCa-2 cells transiently transfected with FLAG-Pirh2. (H) The experiments were performed as in panel (G) except MEF-180 cells were used.

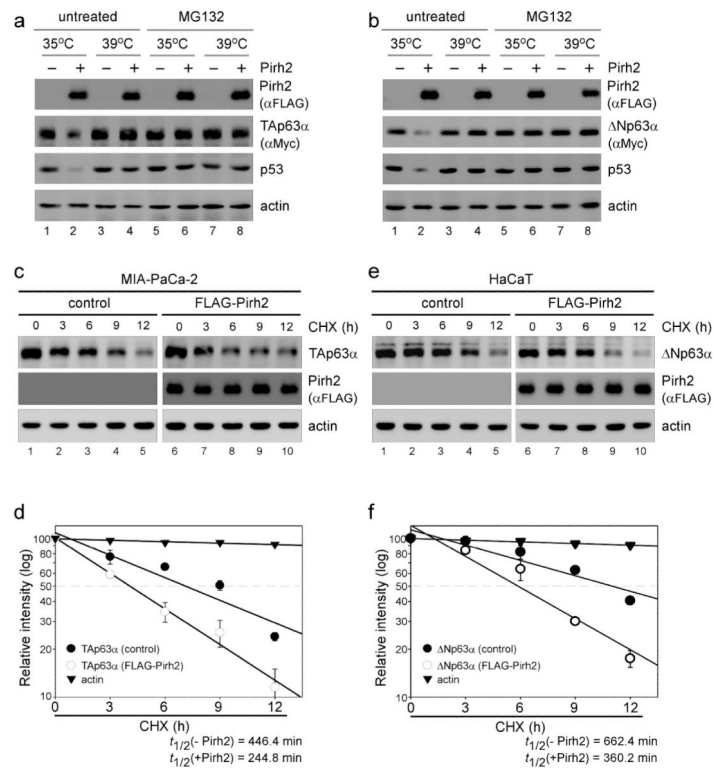


Figure 3. Pirh2-mediated degradation of p63 occurs through a ubiquitin-dependent proteasomal pathway

(A) ts20 cells were transiently transfected with Myc-TAp63 α along with FLAG-Pirh2 at 35°C for 24 h and then incubated at 35°C or 39°C for an additional 24 h in the presence or absence of MG132 (5 μ M). The levels of FLAG-Pirh2 and Myc-TAp63 α were measured by anti-FLAG and anti-Myc antibodies, respectively. Endogenous p53 and actin were measured by their respective antibodies. (B) The experiment was performed as in panel (A) except ts20 cells transiently transfected with Myc- Δ Np63 α and FLAG-Pirh2. (C) The levels of TAp63 α , FLAG-Pirh2, and actin were measured in extracts purified from MIA-PaCa-2 cells mock-transfected or transfected with FLAG-Pirh2 followed by treatment with CHX at the indicated times (0 to 12 h) were used to measure. (D) Calculated half-life of TAp63 α , using the data from panel (C). The percentage of intensity in log₁₀ was plotted versus time, and the $t_{1/2}$ was calculated from the log₁₀ of 50%. (E) The experiment was performed as in panel (C) except that HaCaT cells were used to measure the half-life of Δ Np63 α . (F) Calculated half-life of Δ Np63 α , using the data from panel (C).

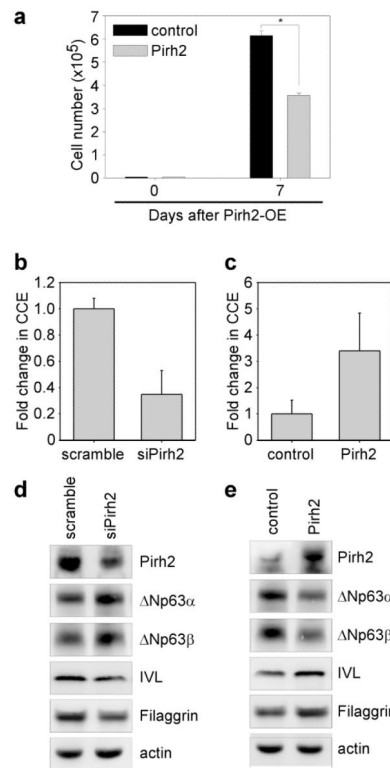


Figure 5. Pirh2 promotes keratinocyte differentiation

(A) HaCaT cells were transfected with a control vector or a vector expressing FLAG-Pirh2. 24 h after transfection, appropriate cells (5,000 cells) were seeded in 6-well plates in triplicate and then cultured for a 7-day period. The cell number was measured at the indicated time. Error bars represent the SD from three independent experiments (*, $P < 0.05$). (B) 70–80% subconfluent HaCaT cells were cultured with defined keratinocyte-SFM medium for 24 h, then transfected with scrambled siRNA or siRNA against Pirh2 for 3 days, followed by treatment of calcium (1.5 mM) for 12 days. CCEs were analyzed as described under “Materials and Methods”. (C) HaCaT cells were transfected with a control vector or a vector expressing FLAG-Pirh2 for 24 h, followed by treatment of calcium (1.5 mM) for 12 days. (D) The level of Pirh2, Np63 α , involucrin (IVL), filaggrin, and actin was measured in HaCaT cells used in (B). Pirh2, Np63 α , Np63 β , IVL, filaggrin, and actin were measured with their respective antibodies. (E) The experiment was performed as in panel (D) except that HaCaT cells were transfected with a control vector or a vector expressing FLAG-Pirh2, followed by treatment of calcium (1.5 mM) for 12 days.