STXBP4 regulates APC/C-mediated p63 turnover and drives squamous cell carcinogenesis

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Contributed by Carol Prives, April 3, 2018 (sent for review October 25, 2017; reviewed by Xinbin Chen, Peter K. Jackson, and Ute Moll)

Levels of the N-terminally truncated isoform of p63 (ΔN p63), well documented to play a pivotal role in basal epidermal gene expression and epithelial maintenance, need to be strictly regulated. We demonstrate here that the anaphase-promoting complex/cyclosome (APC/C) complex plays an essential role in the ubiquitin-mediated turnover of ΔNp63α through the M–G1 phase. In addition, syntaxin-binding protein 4 (Stxbp4), which we previously discovered to bind to ΔNp63, can suppress the APC/C-mediated proteolysis of ΔNp63. Supporting the physiological relevance, of these interactions, both Stxbp4 and an APC/C-resistant version of ΔNp63α (RL7-ΔNp63α) inhibit the terminal differentiation process in 3D organotypic cultures. In line with this, both the stable RL7-ΔNp63α variant and Stxbp4 have oncogenic activity in soft agar and xenograft tumor assays. Notably as well, higher levels of Stxbp4 expression are correlated with the accumulation of ΔNp63 in human squamous cell carcinoma (SCC). Our study reveals that Stxbp4 drives the oncogenic potential of ΔNp63α and may provide a relevant therapeutic target for SCC.

p63 | STXBP4 | p53 | APC/C | squamous cell carcinoma

P63, a member of the p53 family of transcription factors that is essential for the development of normal epidermal stratification, regulates the proliferative potential of epithelial stem cells (1–4). The N-terminally truncated isoform of p63 (ΔNp63) was initially shown to inhibit the activity of the transaction isoform of p63 (TAp63) and p53, thereby functioning in a dominant-negative manner (5, 6). Studies have also revealed that ΔNp63 is transcriptionally competent per se and therefore may be oncogenic if overexpressed in certain cellular contexts (7, 8), although the significance of the TA and ΔN isoforms in epithelial biology and oncology is not fully understood.

ΔNp63 protein expression is normally relegated to the basal layer of the epidermis, although ΔNp63 has been detected in both metaplasia and severe dysplasia and in more advanced tumor-progression stages (9). Accordingly, there is continued interest in how p63 protein expression is regulated. ΔNp63α is frequently overexpressed in a variety of squamous cell carcinomas (SCCs), including lung, head and neck, and cervix SCCs, suggesting that it has context-dependent pro-oncogenic roles in the early development of SCCs (10–12). Although the genomic region containing the *TP63* gene has been reported to be frequently amplified in SCCs, the levels of p63 protein are also regulated by ubiquitin-mediated proteolysis by E3 ubiquitin ligases such as Nedd4 (13), Itch (14), WWP1 (15), FBW7 (16), and Pirh2 (17).

Ubiquitin-mediated proteolysis not only governs p63 but also is broadly important in cellular processes, especially in cell-cycle progression (18). Two ubiquitin ligases in particular, SCF (Skip1/CUL1/F-box) and the APC/C (anaphase-promoting complex/cyclosome), control the ubiquitination and subsequent degradation of dozens of regulators of cell-cycle progression (19). Activation of the APC/C, a large multiprotein E3 ubiquitin ligase, is dependent on two WD40 domain proteins, Cdc20 and Cdh1/FZR1, which function as substrate adaptors that activate the APC/C and specifically recruit multiple substrates for ubiquitination (20, 21). Whereas Cdc20 activates the APC/C during early mitosis, Cdh1 plays an essential role starting in late anaphase that persists through the G1 phase (22). In addition, while Cdc20-APC/C primarily regulates mitotic progression, Cdh1-APC/C shows a broad spectrum of substrates in and beyond the cell cycle that play roles in genomic integrity, signal transduction, cell differentiation, and cancer formation (18).

We previously reported that syntaxin-binding protein 4 (Stxbp4) regulates ubiquitination and degradation of ΔNp63 by Rack1 and Itch (23) and that in the clinicopathological context Stxbp4 drives the oncogenic potential in a ΔNp63-dependent manner and is an independent prognostic factor in patients with lung SCC (24). However, the pathologic relevance of ΔNp63 and Stxbp4 in tumorigenesis is still far from fully understood. Here we describe a role for the APC/C complex in regulating ΔNp63α protein accumulation and provide evidence that Stxbp4 serves to regulate the APC/C-mediated proteolysis of ΔNp63α. Both Stxbp4 and an APC/C degradation-resistant mutant version of ΔNp63α endow keratinocyte cells with increased proliferative potential and decreased differentiation properties. Our data also suggest the need to degrade p63 to protect some cells from becoming oncogenic.

Results

APC/C Associates with ΔNp63α. Since ΔNp63α is essential for the control of stratified epithelial cells and SCCs, we sought to identify proteins that might interact with ΔNp63α and regulate

Significance

The N-terminally truncated isoform of p63 (ΔNp63) is overexpressed in some forms of squamous cell carcinoma (SCC). Here we show that the anaphase-promoting complex/cyclosome (APC/C) degradation machinery plays an essential role in regulating the proteolysis of ΔNp63. We report as well that syntaxin-binding protein 4 (Stxbp4) suppresses APC/C-mediated ubiquitination and proteolysis of ΔNp63 and thereby drives the oncogenic potential of SCC. Aberrancies in this newly defined mechanism could account for ΔNp63 overexpression in SCC. These findings suggest that Stxbp4 could be a relevant therapeutic target for SCC detection and treatment.

Author contributions: S.R. and C.P. designed research; S.R. and M.F. performed research; Y.L., D.M.O., A.M.C., and M.N. contributed materials; S.R. and Y.O. analyzed data; and S.R. and C.P. wrote the paper.

Reviewers: X.C., University of California, Davis; P.K.J., Stanford University; and U.M., Stony Brook University.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718546115/-/DCSupplemental.

Published online May 7, 2018.

its levels. We purified ΔNp63α-associated proteins from lysates of retrovirally infected human keratinocyte HaCaT cells expressing doubly tagged (FLAG- and HA-tagged) ΔNp63α at physiological levels using sequential immunoprecipitation with anti-FLAG followed by anti-HA antibodies. MALDI-TOF/MS analysis of the eluates from ΔNp63α-expressing keratinocytes revealed that three subunits of the APC/C, namely Cdc20, ANAPC6, and ANAPC2, were associated with p63 (*SI Appendix*, Fig. S1 *A* and *B*). We confirmed that these subunits as well as three other members of the APC/C (ANAPC11, Cdc27, and Cdh1/FZR1) were present in FLAG-HA-ΔNp63α immunoprecipitates (Fig. 1*A*). Consistent with these findings, endogenously expressed ΔNp63 and APC/C components were found to be associated in keratinocyte cells and were most readily detected when cells were treated with the proteasome inhibitor MG132 (Fig. 1*B*). Ectopically expressed ΔNp63 could also be shown to interact strongly with Cdc20 in mitotic cells (*SI Appendix*, Fig. S1*C*). A far-Western experiment showed that Cdc20 can directly bind to ΔNp63α in vitro, while negative controls (BSA and the other reticulocyte proteins) did not interact with ΔNp63α (Fig. 1*C*). Although the APC10 component of the APC/C was shown to be required for Cdc20's binding to its substrates (20), our data indicate that Cdc20 is a bona fide interacting partner of ΔNp63α.

The activation of the APC/C is dependent on two proteins, Cdc20 and Cdh1/FZR1, which function as substrate adaptors that activate the APC/C (Fig. 1*D*) (19, 20). As expected from the above-mentioned results, wild-type Cdc20 bound and targeted ΔNp63α for degradation (Fig. 1 *E* and *F*). Note as well that ΔN165-Cdc20 was unable to bind to the APC/C core subunits, thereby sequestering substrates instead of delivering them to the APC/C for degradation (25). On the other hand, ΔC471-Cdc20, which lacks the C-terminal 29 amino acids that are required for interaction with p21, did not show interaction with or degradation of ΔNp63 (Fig. 1 *E* and *F*), providing support for an association of ΔNp63 with the Cdc20 C terminus.

ΔNp63α Protein Is Regulated by the APC/C During the Cell Cycle and Is Degraded in Early Mitosis. To extend our findings, we monitored the impact of Cdc20 on three ΔNp63 isoforms (α, β, and γ) and found that ΔNp63α protein levels were dramatically decreased in the presence of either ectopic Cdc20 or Cdh1, while ΔNp63β and ΔNp63γ were only moderately affected by coexpression with either regulator of the APC/C (Fig. 2*A*). In the presence of Cdc20, the half-life of ΔNp63α was reduced from 6 h to 2 h, indicating that the Cdc20-induced decrease in ΔNp63α abundance was due to an increase in ΔNp63α protein degradation (Fig. 2*B*).

It is important to mention that while both Cdc20 and Cdh1 when overexpressed led to the degradation of ΔNp63α, siRNA ablation of Cdc20 substantially increased ΔNp63α, while siRNA-mediated depletion of Cdh1 had no such effect, and ΔNp63α levels were even modestly decreased (*SI Appendix*, Fig. S2). The reduced levels of ΔNp63α and the increased turnover in cells depleted of Cdh1 may be due to the fact that Cdh1 facilitates the degradation of Cdc20 in these cells, as previously reported (26). Supporting the observation that Cdc20 is more important than Cdh1 in ΔNp63 degradation in proliferating cells, in contrast to our observations with ΔNp63α, the levels of other well-characterized Cdh1-APC/C substrates such as Cdc20 itself (27) and cyclin B (28) are known to be substantially increased upon depletion of Cdh1. As will be shown later in this study, Cdh1 is indeed an important player in APC/C degradation of ΔNp63α, although in a different context.

Furthermore, treatment with the proteasome inhibitor MG132 suppressed the degradation of ΔNp63α coexpressed with Cdc20 or Cdh1, indicating that the APC/C complex indeed plays a role in ubiquitin proteasome-mediated turnover of ΔNp63α (Fig. 2*C*). Finally, in vitro degradation assays showed that the APC/C is required for the degradation of ΔNp63α (Fig. 2*D*). In brief, purified recombinant ΔNp63α was incubated with cell lysates that had been transfected with either control (siLUC) or Cdc20 siRNA. The lysates were supplemented with a degradation mixture, cycloheximide (CHX), and PK-ubiquitin, and then ΔNp63α protein levels were followed over 6 h. Under these conditions, degradation of recombinant ΔNp63α protein was markedly slower in extracts of

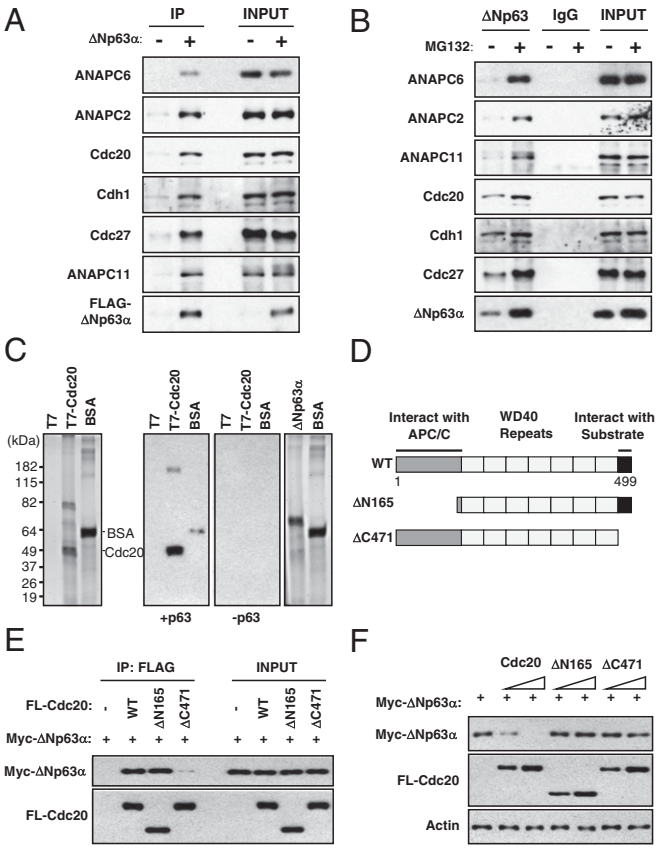

Fig. 1. The APC/C complex is a ΔNp63α interaction partner. (*A*) The APC/C interacts with ectopically expressed ΔNp63α. HaCaT cells were retrovirally infected with either MSCV empty vector or FLAG-HA doubly tagged ΔNp63α. Sequential immunoprecipitates (IP) with anti-FLAG and anti-HA of cell lysates were subjected to immunoblotting with the indicated antibodies. Input lanes contain 10% of total lysate. (*B*) The APC/C interacts with endogenous ΔNp63α. HaCaT cells were treated with or without 20 μM MG132 for 6 h. The cell lysates were immunoprecipitated with anti-ΔNp63 antibody or control IgG and were subjected to immunoblotting analysis with the indicated antibodies. (*C*) Cdc20 can directly interact with ΔNp63α in vitro. Purified ΔNp63α protein from sf9 cells (*Far Right*) and in vitro-translated Cdc20 proteins from reticulocyte lysate (*Far Left*) are shown. The immunoprecipitates were subjected to SDS/PAGE and stained by Coomassie Blue. Mock (T7) and T7-Cdc20 proteins were resolved on two parallel gels. The transferred membranes were then incubated with (+p63) or without (−p63) purified FLAG-ΔNp63α protein (*Middle Panels*) and were subjected to immunoblotting using FLAG antibody. (*D*) Schematic illustration of the modular structure of Cdc20 showing the location of the putative APC/C core and substrate-interacting regions along with constructs ΔN165 and ΔC471 that lack the regions that interact with the APC/C and substrate, respectively. (*E*) The C terminus of Cdc20 is indispensable for interaction with ΔNp63α. HEK293 cells were transfected with constructs encoding Myc-tagged ΔNp63α, FLAG-tagged wildtype, ΔN165, or ΔC471 Cdc20 polypeptides. Equivalent amounts of cell extracts were subjected to immunoprecipitation with anti-FLAG antibody and then were analyzed by immunoblotting with anti-Myc antibody (Myc-ΔNp63α). (*F*) Both the N and C termini of Cdc20 are important for ΔNp63α degradation. HEK293 cells were transfected with ΔNp63α in the presence of wild-type (WT), ΔN165, or ΔC471 Cdc20 polypeptides at a ratio of 1:5 or 1:10. The cells were harvested, and extracts were analyzed by immunoblotting with antibodies against Myc (Myc-ΔNp63α), FLAG (FL-Cdc20), or actin.

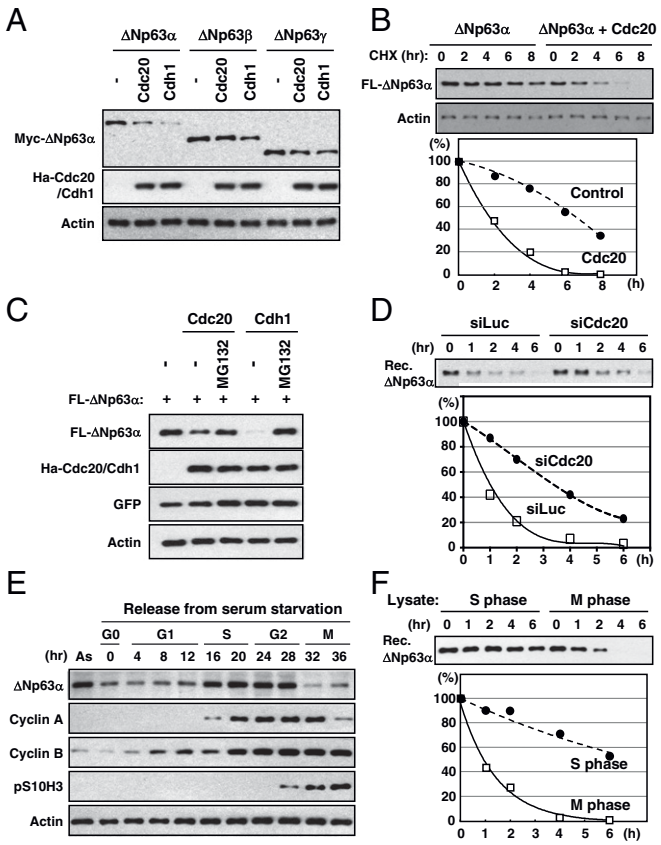

Fig. 2. Expression of Cdc20 or Cdh1 reduces ectopic p63 levels. (*A*) ΔNp63α is the preferred isoform degraded by the APC/C. HEK293 cells were cotransfected with Myc-tagged ΔNp63α, -β, or -γ in the presence of HA-tagged Cdc20/Cdh1 at a ratio of 1:10. The cells extracts were analyzed by immunoblotting using antibodies against Myc (Myc-ΔNp63α), HA (Ha-Cdc20/Cdh1), or actin. (*B*, *Upper*) Cdc20 expression increases the turnover of ΔNp63α. HEK293 cells were transfected with FLAG-tagged ΔNp63α in the presence or absence of HA-tagged Cdc20 at a ratio of 1:10. Twenty-four hours after transfection, cells were synchronized by nocodazole and incubated with 20 μg/mL CHX, harvested at the indicated time points, and analyzed for ΔNp63α levels. (*Lower*) Relative stability is shown. (*C*) ΔNp63α degradation is mediated by ubiquitin-mediated turnover. HEK293 cells were transfected with ΔNp63α in the presence of Cdc20 or Cdh1 at a ratio of 1:10. Eighteen hours after transfection, the cells were treated with 20 μM MG132 for 6 h. The cells were harvested, and extracts were analyzed by immunoblotting with anti-FLAG [FL-ΔNp63α–, anti-HA (Ha-Cdc20/Cdh1)], GFP (transfection control), or actin antibodies. (*D*, *Upper*) ΔNp63α is degraded in a Cdc20-dependent manner in vitro. HaCaT cells were transfected with control siRNA (siLuc) or siRNA for Cdc20 (siCdc20) and were synchronized by nocodazole. The cell extracts were supplemented with degradation mixture components, CHX, and PK-ubiquitin and were incubated with purified recombinant ΔNp63α (Rec. ΔNp63α) protein and harvested at the indicated times. (*Lower*) Relative stability is shown. (*E*) ΔNp63α protein varies with the cell-cycle stage and is preferentially degraded during early mitosis. HaCaT cells were synchronized in G0/G1 phase followed by release and were harvested at the indicated times. The cell lysates were immunoblotted with the indicated antibodies. Lane "As" contains extract of asynchronous cells. (*F*, *Upper*) Extracts prepared from HaCaT cells harvested in S phase and M phase (16 and 32 h after G0/G1 release, respectively) were supplemented with degradation mixture components, incubated with recombinant ΔNp63α protein (Rec. ΔNp63α), and harvested at the indicated times. (*Lower*) Relative stability is shown.

Cdc20-siRNA–containing cells. This provides further evidence that the accumulation of ΔNp63α is controlled specifically by the Cdc20–APC/C pathway in proliferating keratinocytes.

To determine whether ΔNp63α expression is regulated in a cell-cycle–dependent manner, HaCaT cells were arrested in G0/G1 phase by culturing in 0.5% serum and then were released into fresh medium. ΔNp63α levels were lowest at G0/G1 and remained low as cells progressed through the G1 phase (Fig. 2*E*). When cells reached the S/G2 transition (16–28 h after release from G0/G1), the levels of p63 peaked and then dropped markedly in M phase. To confirm that ΔNp63α protein is down-regulated in early mitosis, we showed that when HaCaT cells were synchronized in G2/M phase by a nocodazole block and then were released, the levels of ΔNp63α decreased rapidly as cells progressed through M phase (*SI Appendix*, Fig. S3 *A* and *B*). To further confirm that ΔNp63 protein levels in metaphase are regulated by the APC/C, we performed in vitro degradation assays where recombinant ΔNp63α protein was incubated with cell lysates in S or M phase (Fig. 2*F*). Indeed, ΔNp63α protein was degraded much more efficiently in M-phase lysates than in lysates from S-phase cells. Our results showing that ΔNp63α is a cell-cycle–regulated protein whose levels are highest during S and G2 phases and then are greatly decreased throughout mitosis are consistent with our finding that ΔNp63 is a substrate of the APC/C.

The Destruction Box Motifs in ΔNp63 Are Required for Its Ubiquitination and Degradation by the APC/C. APC/C substrates require a sequence known as a "D-box" (Destruction box: RXXLXXXXN) for target recognition (29). Intriguingly, although most substrates contain only a single minimal RXXL D-box motif, through sequence analysis we identified three D-box motifs in ΔNp63α. The first putative D-box (ΔD-box1: amino acids 225–228) is highly conserved in the DNA-binding domain of p63α, β, and γ. The second D-box (ΔD-box2: amino acids 393–396) is conserved in the oligomerization domain of p63α and β, and the third D-box (ΔD-box3: amino acids 549–552) is localized in the SAM motif (sterile α motif) within the C terminus only in p63α (Fig. 3*A* and *SI Appendix*, Fig. S4). No other potential APC/C recognition motifs, such as KEN (amino acids K-E-N) (26) or A-boxes (30), were identified in ΔNp63α.

To test the importance of these D-box motifs for degradation by the APC/C, we generated several mutated versions of ΔNp63α in which the Arg (R) and Leu (L) residues of ΔD-box1, D-box2, and ΔD-box3 were mutated to Ala (A) residues or combinations thereof. We then determined whether the protein levels of these ΔNp63α mutants were reduced in the presence of coexpressed Cdc20 or Cdh1 (Fig. 3*B*). While RL1 (ΔD-box1: lanes 4–6) and RL2 (ΔD-box2: lanes 7–9) mutant proteins were degraded similarly to wild-type ΔNp63α, the RL3 mutant (ΔD-box3: lanes 10–12) was relatively more stable. The double mutants RL5 (ΔD-boxes 1 + 3: lanes 19–21) and RL6 (ΔD-boxes 2 + 3: lanes 22–24) also showed resistance to degradation by Cdc20 and Cdh1. Mutation of three D-boxes (RL7; ΔD-boxes 1 + 2 + 3: mediated by coexpressed Cdc20 and Cdh1).

To further explore the importance of the ΔNp63α D-boxes, we performed an in vitro degradation assay where wild-type and different combinations of D-box–mutant ΔNp63α proteins were translated in reticulocyte lysates and the resulting recombinant proteins were incubated with lysates of HaCaT cells that harbored either control siRNA (siLuc) or siRNA vs. Cdc20. Consistent with the in vivo degradation assays, we confirmed that RL3 (ΔD-box3: lanes 7–8) was relatively more stable than RL1 (ΔD-box1: lanes 3–4) and RL2 (ΔD-box2: lanes 5–6) in control-treated lysates and that the triple D-box mutant RL7 (ΔD-box1+2+3: lanes 15–16) was incapable of being degraded by Cdc20 (Fig. 3*C*). We conclude that D-box3 is the dominant D-box in regulating ΔNp63α degradation by the APC/C complex. This may explain the relatively poor degradation of ΔNp63β and ΔNp63γ in the presence of Cdc20 and Cdh1 (Fig. 2*A*).

To show that ΔD-boxes are required for ubiquitin-mediated degradation by the APC/C, extracts of HEK293 cells that had been transfected with plasmids expressing HA-ubiquitin, Myc-Cdc20, and Flag-tagged wild-type or D-box–mutant variants of

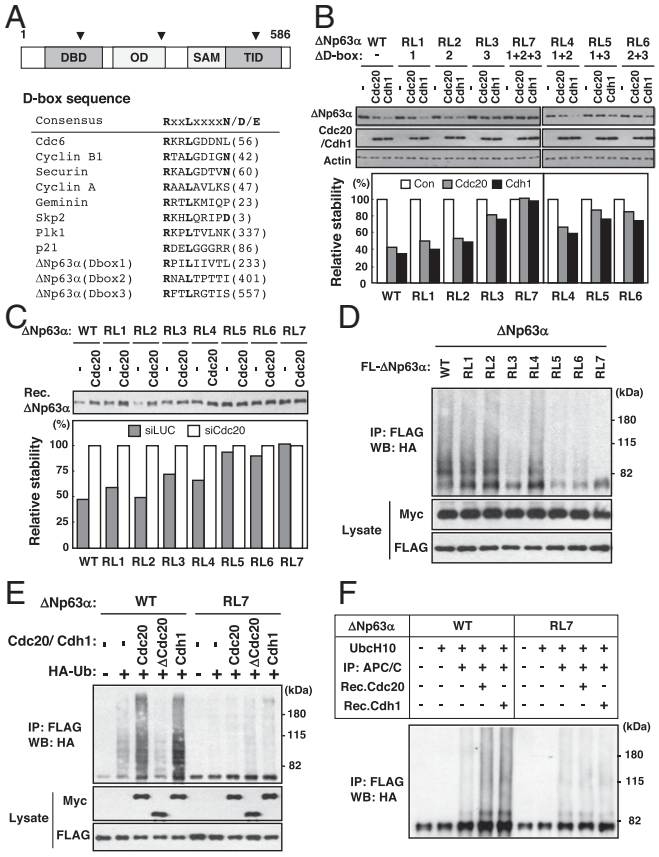

Fig. 3. The D-box motifs in Δp63 are required for its ubiquitination and degradation by the APC/C. (*A*, *Upper*) Schematic illustration of the modular structure of ΔNp63α. Arrowheads show the positions of putative D-box motifs (D-boxes 1, 2, and 3). (*Lower*) D-box sequences of several known APC/C substrates and the location of the three D-boxes within ΔNp63α. (*B*) The ΔNp63α triple D-box mutant (RL7-ΔNp63α) is resistant to degradation by APC/C. HEK293 cells were transfected with FLAG-tagged wild-type ΔNp63α (WT) or variants with mutated D-boxes: RL1 (ΔD-box1), RL2 (ΔD-box2), RL3 (ΔD-box3), RL4 (ΔD-box1+2), RL5 (ΔD-box1+3), RL6 (ΔD-boxes2+3), and RL7 (ΔD-box1+2+3) in the presence of Cdc20/Cdh1 at a ratio of 1:10. The cell extracts were analyzed by immunoblotting with anti-FLAG (ΔNp63α), anti-HA (Cdc20/Cdh1), or actin antibodies. (*C*, *Upper*) D-box3 in ΔNp63α is the most critical for Cdc20-APC/C degradation in vitro. HaCaT cells were transfected with control siRNA (siLuc) or siRNA for Cdc20. The cell extracts were supplemented with degradation mixture components and were incubated with wild-type (WT), RL1, RL2, RL3, RL4, RL5, RL6, or RL7 variant ΔNp63α recombinant proteins. The cell extracts were analyzed by immunoblotting with anti–FLAG-ΔNp63α. (*Lower*) The relative stability of the ΔNp63α variants is shown. (*D*) APC/C-mediated ΔNp63α ubiquitination requires ΔNp63α D-boxes. HEK293 cells were transfected with wild-type or ΔD-box variants of ΔNp63α in the presence of Cdc20 and ubiquitin. At 18 h after transfection, cells were synchronized by nocodazole and treated with 20 μM MG132 for 6 h and harvested. (*Upper*) The cell extracts were subjected to immunoprecipitation with FLAG antibody and immunoblotting with HA antibody. (*Lower*) Whole-cell lysates were also subjected to immunoblotting using anti-Myc (Cdc20) and anti-FLAG (FL-ΔNp63α) antibodies. (*E*) ΔNp63α ubiquitination is dependent on the APC/C complex. HEK293 cells were transfected with wild-type (WT) or degradation-resistant (RL7) ΔNp63α, Myc-tagged wild-type or ΔN165 Cdc20, or Cdh1 in the presence of HA-ubiquitin. At 18 h after transfection, the cells were treated with 20 μM of MG132 for 6 h and were harvested. (*Upper*) The cell extracts were subjected to immunoprecipitation with FLAG antibody and immunoblotting with HA antibody. (*Lower*) Whole-cell lysates were also subjected to immunoblotting assay with anti-Myc (Cdc20/Cdh1) and anti-FLAG antibodies. (*F*) The ΔNp63α D-boxes are required for its ubiquitination in vitro. FLAG-tagged wild-type (WT) or degradation-resistant (RL7) ΔNp63α proteins were incubated with immunopurified APC/C components from synchronized cells and recombinant Cdc20, and an in vitro ubiquitination assay was performed as described in *SI Appendix*.

ΔNp63α were subjected to immunoprecipitation with anti-FLAG antibody and were immunoblotted with an anti-HA antibody (Fig. 3*D*). Ubiquitination of RL3 (ΔD-box3) was significantly suppressed compared with wild-type, RL1 (ΔD-box1), and RL2 (ΔD-box2) ΔNp63α, while the triple D-box mutant RL7-ΔNp63α (ΔD-box1+2+3) completely suppressed ubiquitination in the presence of ectopic Cdc20. We speculate that hetero-oligomers of endogenous ΔNp63 with RL7-ΔNp63 can inhibit APC/C-mediated degradation of endogenous ΔNp63, although we cannot exclude other possibilities. To demonstrate that the appearance of ubiquitinated ΔNp63 requires Cdc20-APC/C, we used a previously described ΔN165 mutant of Cdc20 which lacks interaction with the APC/C (Fig. 1*D*). As shown in Fig. 3*E*, the ΔN165-Cdc20 variant which lacks the ability to bind to the APC/C lost the ability to promote ΔNp63α ubiquitination. This strongly indicates that Cdc20-mediated ΔNp63 ubiquitination requires the APC/C core complex.

We next investigated whether ΔNp63α can serve as a substrate for the E3 ligase activity of the APC/C complex. Recombinant His-tagged Cdc20 (His^6-Cdc20) protein was added to a reconstituted in vitro ubiquitination system (described in *SI Appendix*, *SI Materials and Methods*) containing biotinylated ubiquitin, recombinant human E1 and E2 (UbcH10) proteins, along with immunoprecipitated APC/C from G2/M-arrested HaCaT cells, Mg-ATP, and baculovirus-expressed and purified ΔNp63α protein. In the presence of purified Cdc20, the ΔNp63α protein was ubiquitinated, as shown by the appearance of discrete higher molecular weight ΔNp63α species (Fig. 3*F*). These in vitro experiments confirm that ΔNp63 is a bona fide substrate of the APC/C E3 ubiquitin ligase. In subsequent experiments described later in this study, the highly stable RL7 mutant form of ΔNp63α proved to be valuable for revealing that the APC/C regulates ΔNp63α roles in terminal differentiation and tumorigenesis.

Stxbp4 Inhibits APC/C-Mediated ΔNp63α Degradation in the Basal Epidermal Layer. We next investigated whether Stxbp4, which we previously discovered can stabilize ΔNp63α and thereby contribute to lung SCC (23, 24), might interfere with its degradation by the APC/C. Coexpressed Stxbp4 prevented the loss of ΔNp63α in the presence of Cdc20 and extended the half-life of ΔNp63α to longer than 8 h (Fig. 4*A*). To identify the ΔNp63α-interacting domain in Stxbp4, the interactions between various deletion mutants of Stxbp4 and ΔNp63α (Fig. 4*B*) were examined using immunoprecipitation-immunoblotting (Fig. 4*C*). Coimmunoprecipitation experiments identified the coiled-coil (CC) domain in Stxbp4 (spanning amino acids 291–405) as having a major role in interacting with ΔNp63α (Fig. 4*C*). In line with this, the ΔCC-Stxbp4 variant that lacks the CC domain was not able to suppress ΔNp63 degradation mediated by Cdc20-APC/C (Fig. 4*D*).

ΔNp63α is predominantly expressed in the proliferative basal layer of stratified epithelium, where it contributes to the maintenance of regenerative potential, epithelial integrity, and stemness (4). Levels of ΔNp63α protein decrease abruptly when cells undergo differentiation (31). Consequently, we examined Stxbp4 and ΔNp63 expression within samples of human epidermis (Fig. 4*E* and *SI Appendix*, Fig. S5). Consistent with previous reports, ΔNp63 was predominantly expressed in the basal cell layer alongside the basal layer marker cytokeratin 14 (32). Interestingly, Stxbp4 expression was also most prominent within the proliferative basal layer and colocalized with ΔNp63. These data suggesting that nuclear-localized Stxbp4 contributes to ΔNp63 accumulation in the basal epidermal layer were supported by subsequent experiments.

ΔNp63α Suppresses Terminal Differentiation in the Epidermis. To determine whether APC/C-mediated degradation of ΔNp63α contributes to the differentiation of keratinocytes, we introduced the degradation-resistant stable RL7-ΔNp63α variant into

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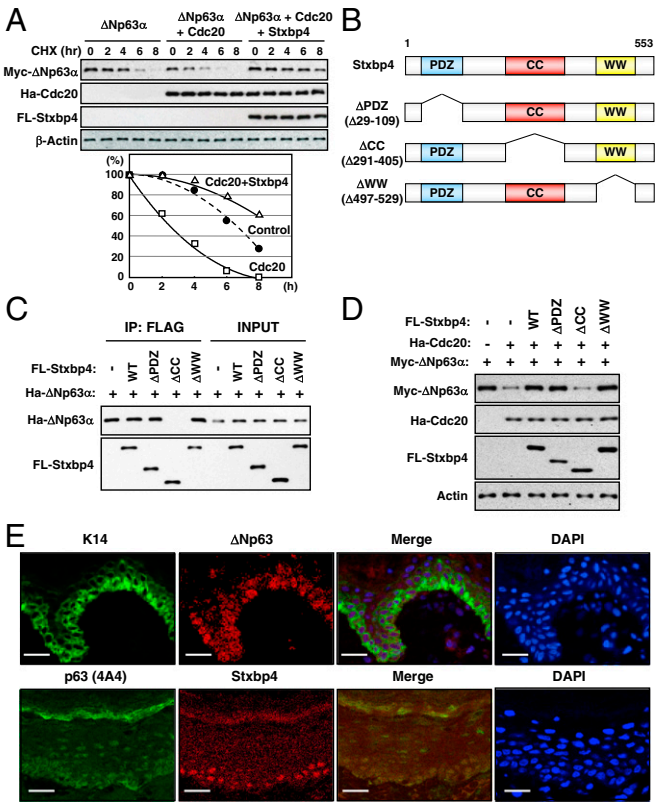

Fig. 4. Stxbp4 inhibits APC/C-mediated degradation of ΔNp63α. (*A*) Stxbp4 contributes to ΔNp63α stability by preventing Cdc20-APC/C–mediated degradation. (*Upper*) HEK293 cells were transfected with ΔNp63α in the presence of HA-Cdc20 alone (1:10) or both HA-Cdc20 and FLAG-Stxbp4 (1:10:10). At 36 h after transfection, the cells were synchronized by nocodazole, were treated with 20 μg/mL of CHX, and were collected at the indicated time points. Extracts were analyzed by immunoblotting with anti-Myc (Myc-ΔNp63α), anti-HA (Ha-Cdc20), anti-FLAG (FL-Stxbp4), or anti-actin antibodies. (*Lower*) Relative stability is shown. (*B*) Schematic illustration of the modular structure of Stxbp4. The PDZ (postsynaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens 1), CC (coiled-coil), and the WW (proline-rich) domains are indicated. (*C*) The CC domain of Stxbp4 is necessary for interaction with ΔNp63α. HEK293 cells were transfected with constructs encoding HA-tagged ΔNp63α or FLAG-tagged wild-type Stxbp4 or Stxbp4 proteins lacking the PDZ (ΔPDZ), CC (ΔCC), or WW (ΔWW) domains. At 18 h after transfection, cells were treated with 20 μM MG132 for 6 h and then were harvested. Equal amounts of protein were immunoprecipitated with anti-FLAG antibody and were analyzed by immunoblotting with anti-HA antibody. Ten percent of the lysates were used for the input lanes. (*D*) The CC domain of Stxbp4 is essential for the protection of ΔNp63α from APC/C degradation. HEK293 cells were cotransfected with ΔNp63α and Cdc20 in the presence of wild-type (WT), ΔPDZ, ΔCC, or ΔWW Stxbp4 polypeptides. At 36 h after transfection, cells were harvested and extracts were analyzed by immunoblotting with antibodies against Myc (Myc-ΔNp63α), HA (Ha-Cdc20), FLAG (FL-Stxbp4), or actin. (*E*) Stxbp4 is expressed and colocalizes with p63 in the basal layer of human epidermis. Immunohistochemistry analysis of a sample of human epidermis using anti-Stxbp4, anti-ΔNp63, anti-p63 (4A4), and anti-K14 antibodies as indicated. (Scale bars: 40 μm.)

primary human keratinocytes (pHKCs) by retrovirus infection and monitored their morphology upon differentiation caused by high-calcium conditions. After 96 h, the cells showed compacted morphology with tight cell junctions in control pHKCs. By contrast, pHKCs infected with the stable RL7-ΔNp63α variant or Stxbp4 showed less compacted morphology with fewer tight junctions (Fig. 5*A*). Further, while involucrin, one of the key proteins that facilitate terminal differentiation of the epidermis, became detectable in high-calcium–cultured pHKCs infected with an empty vector retrovirus (Mock), involucrin was greatly reduced in cells expressing RL7-ΔNp63α or Stxbp4 (Fig. 5*B*). We also found that expression of RL7-ΔNp63α as well as Stxbp4 significantly reduced the levels of involucrin mRNA after culturing cells for 3–4 d in high-calcium conditions (Fig. 5*C*). Importantly, the ΔNp63α interaction-deficient Stxbp4 (ΔCC-Stxbp4) did not show any changes in involucrin induction or ΔNp63α accumulation (compare Fig. 5*D* with Fig. 5*B*). Together these findings indicate that expression of Stxbp4, similar to that of RL7-ΔNp63α, negatively regulates differentiation both morphologically and biochemically, and this likely involves its interaction with ΔNp63α.

We next infected pHKCs with control (Mock), RL7-ΔNp63α–, or Stxbp4-expressing retroviruses and subjected them to the 3D organotypic raft culture protocol, a procedure that rather accurately replicates the formation of stratified epithelia. In human skin epidermis sections, the cornified differentiated layers of the

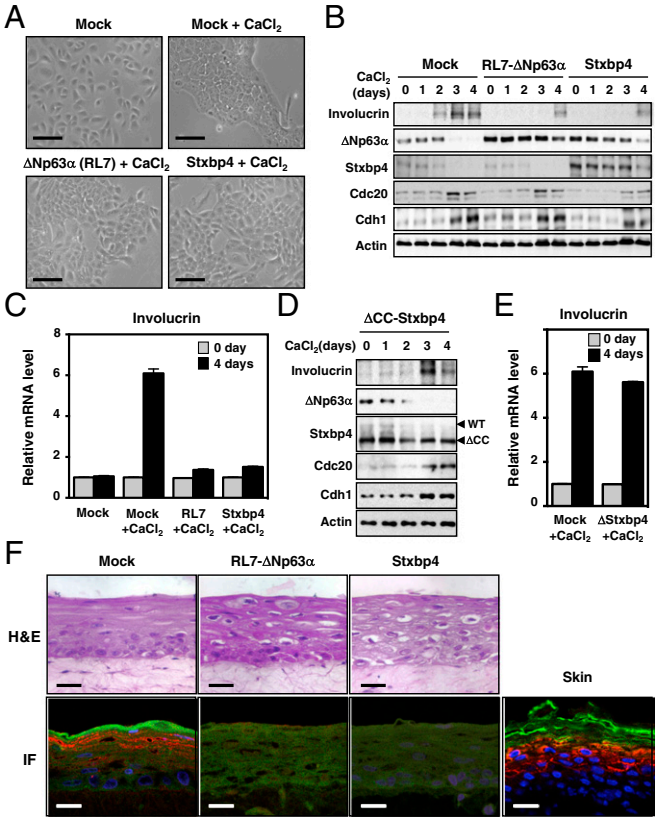

Fig. 5. ΔNp63α promotes proliferation and suppresses terminal differentiation in primary human keratinocytes. (*A*) APC/C degradation-resistant ΔNp63α and Stxbp4 suppress terminal differentiation. Empty vector (Mock), degradation-resistant ΔNp63α (RL7-ΔNp63α), and Stxbp4 retrovirally infected pHKCs were cultured in 1.5 mM $CaCl_2$ to induce differentiation and were imaged by an LSM 700 laser-scanning confocal microscope (Zeiss). (Scale bars: 50 μm.) (*B*) Retrovirus-infected pHKCs cultured in high-calcium conditions were harvested at the indicated times, and cell lysates were subjected to immunoblotting with the indicated antibodies. (*C*) Involucrin mRNA level as in *B* was determined by RT-qPCR. (*D*) Stxbp4 (ΔCC-Stxbp4) retrovirally infected pHKCs cultured in high-calcium conditions were harvested at the indicated times. The cell lysates were subjected to immunoblotting with the indicated antibodies. Full-length Stxbp4 is shown in *B*. (*E*) Involucrin mRNA levels as in *D* were determined by RT-qPCR. Stxbp4 mRNA is shown in *C*. (*F*) Stxbp4 and APC/C-resistant ΔNp63α-infected pHKCs do not undergo terminal differentiation in 3D organotypic raft cultures. 3D skin equivalents were generated using human pHKCs infected with empty vector (Mock), stable RL7-ΔNp63α–, and Stxbp4-expressing retroviruses. Involucrin (Alexa 594: red) and Loricrin (Alexa 488: green) were detected in pLPCX-infected keratinocytes (*Left*), and human skin epidermis was used for comparison (*Lower Right*). (Scale bars: 20 μm.) IF, immunofluorescence.

epidermis contain markers of terminal differentiation, namely loricrin (green) in the top layer and involucrin (red) in the layer beneath it (Fig. 5*F*, *Lower Right*). Mock-infected pHKCs exhibited a basal multilayered epidermis, and the cornified layer at the surface of the epidermis was readily detectable (Fig. 5*F*, *Upper*). Moreover, involucrin and loricrin were observed in the upper (suprabasal) layers similar to a control sample of human epidermis (Fig. 5*F*, *Lower Left* and *SI Appendix*, Fig. S6). In striking contrast, RL7-ΔNp63α– or Stxbp4-infected pHKCs failed to express involucrin and loricrin in these 3D skin equivalents, indicating that keratinocytes that cannot degrade ΔNp63α do not mature and terminally differentiate in these assay conditions (Fig. 5*F*, *Lower Middle* and *SI Appendix*, Fig. S6).

Cdh1 Knockdown Leads to the Accumulation of ΔNp63 and Prevents Epidermal Differentiation. We next examined the role of p63 turnover in regulating keratinocyte differentiation using a loss-of-function approach. shRNAs against ΔNp63, Cdc20, Cdh1, and Stxbp4 were lentivirally transduced into pHKCs (Fig. 6 and *SI Appendix*, Fig. S7). We first monitored the differentiation progress of these shRNA-infected pHKCs when cells were induced to differentiate using high-calcium conditions. Knockdown of ΔNp63 resulted in a modest up-regulation of involucrin mRNA (Fig. 6*A*), while keratinocytes infected with shRNA-Cdc20 and, even more dramatically, with shRNA-Cdh1 showed less involucrin mRNA (Fig. 6*A*) and protein induction (Fig. 6*B*). Relatedly, ΔNp63α protein levels were inversely proportional to the expression of involucrin in these high-calcium conditions (Fig. 6*B*).

3D models of skin equivalents revealed that ΔNp63α and Stxbp4 ablation in pHKCs exhibited differentiated morphology with involucrin expression that was more extensively detected in the multilayers, while Cdh1 knockdown failed to express detectable involucrin (Fig. 6*C*). Thus, by maintaining high ΔNp63α levels, the expression of involucrin and differentiation of keratinocytes is blocked in response to a differentiation stimulus. To further address the functional importance of the p63–APC/C axis, we showed that ΔNp63 silencing also significantly rescued the ability of Cdh1-knockdown pHKCs to differentiate, suggesting that the differentiation capacity induced by Cdh1 deficiency is in part attributable to ΔNp63 accumulation (Fig. 6 *D* and *E*). It is notable that shRNA-Cdh1 contributed to the accumulation of ΔNp63α in differentiating pHKCs, while ΔNp63α was reduced following siRNA-Cdh1 knockdown in proliferating keratinocytes (*SI Appendix*, Fig. S2). Taking these findings together with our data in *SI Appendix*, Fig. S2, we propose that ΔNp63α degradation is predominantly dependent on Cdc20-APC/C in proliferating keratinocytes, while Cdh1-APC/C plays a more prominent role in ΔNp63 degradation in differentiating cells. In summary, we conclude that the APC/C plays a complex but important role in regulating ΔNp63 and epithelial development in human keratinocytes.

A Role for Stxbp4 and ΔNp63α Stabilization in SCC. To evaluate the functional relevance of Stxbp4 in oncogenesis, we investigated colony formation in NIH/3T3 cells that were retrovirally transduced with vectors expressing wild-type or APC/C-resistant stable RL7-ΔNp63α or Stxbp4 (Fig. 7*A*). The results showed enhanced anchorage-independent colony formation in soft agar in cells ectopically expressing either RL7-ΔNp63α or Stxbp4, while wild-type ΔNp63α formed fewer and relatively smaller colonies. Correspondingly, s.c. transplantation of these clones into immune-deficient mice resulted in the formation of larger tumors arising from either stable RL7-ΔNp63α– or Stxbp4-expressing cells compared with either mock (control) or wild-type ΔNp63α-expressing cells (Fig. 7*B*). Further support for an oncogenic role for stable ΔNp63α was derived from experiments in which lentivirally expressed shRNAs were used to deplete

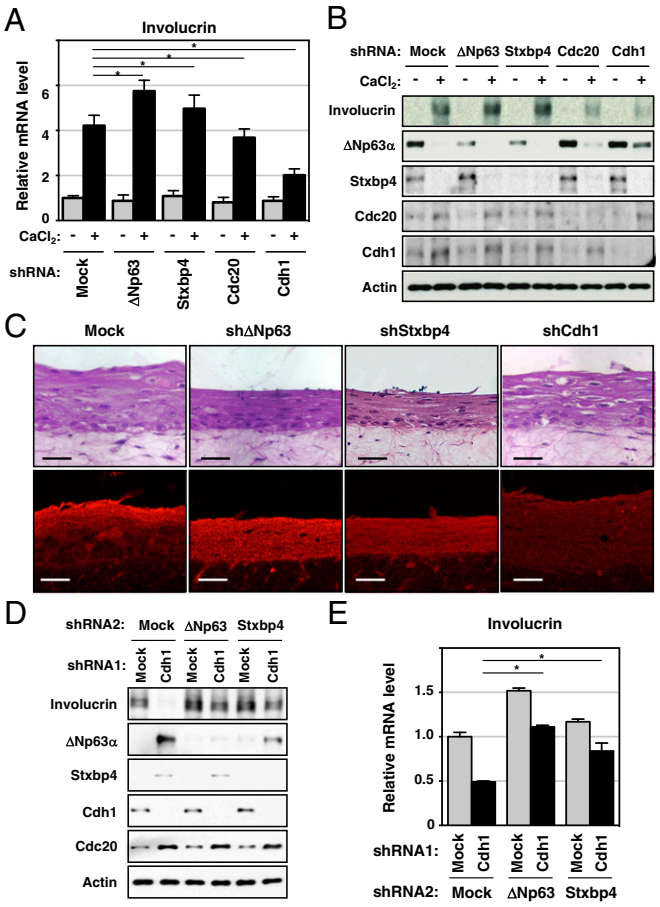

Fig. 6. Cdh1 is important for maintaining low levels of ΔNp63 and allowing terminal differentiation of keratinocytes. (*A*) Decreased expression of the terminal differentiation marker involucrin in shCdc20- and shCdh1-infected keratinocytes. pHKCs with lentivirally expressed shRNAs directed against Luc (Mock), ΔNp63α, Stxbp4, Cdc20, or Cdh1 were cultured in 1.5 mM $CaCl_2$ to induce differentiation for 4 d. The involucrin RNA level was determined by qRT-PCR. *$P < 0.05$. (*B*) shRNA-infected keratinocytes as in *A* were harvested, and cell lysates were analyzed by immunoblotting with the indicated antibodies. (*C*) 3D organotypic raft cultures were prepared using shRNA-infected keratinocytes as described in *A*. (*Upper*) 3D-cultured keratinocyte cells were stained by H&E. (*Lower*) Involucrin was detected by Alexa 594 staining (red) in these 3D skin equivalents. The experiment shows that shLUC-infected human keratinocytes can be terminally differentiated and that shRNAs directed against ΔNp63 or Stxbp4 enhance the expression of involucrin. (Scale bars: 20 μm.) (*D*) pHKCs were infected with lentiviruses containing empty vector (Mock) or Cdh1 shRNAs along with either ΔNp63α-shRNA or Stxbp4-shRNA vectors. These shRNA-infected keratinocytes were cultured in 1.5 mM $CaCl_2$ for 4 d to induce differentiation. The cells were harvested, and cell lysates were analyzed by immunoblotting with the indicated antibodies. (*E*) Involucrin mRNA from parallel samples in *D* was quantified by qRT-PCR. *$P < 0.05$.

Stxbp4 and ΔNp63 from a lung SCC cell line (RERF-LC-Sq1). When the resulting clones were s.c. transplanted into immunodeficient mice, the average tumor volume of cells harboring either shRNA-Stxbp4#1 or shRNA-Stxbp4#2 was significantly decreased and was similar to that seen with shRNA-ΔNp63 as compared with cells with a control shRNA (Fig. 7*C*).

Whole-transcriptome analysis (RNA-seq) revealed that Stxbp4-depleted SCC cells had different gene-expression profiles (Fig. 7*D*). Among the differentially expressed genes (DEGs), we identified 512 genes that were either significantly up-regulated (279 genes) or down-regulated (233 genes) (Fig. 7*D*). These candidate DEGs potentially represent a network involved in STXBP4-mediated biology. More than 30% of the affected

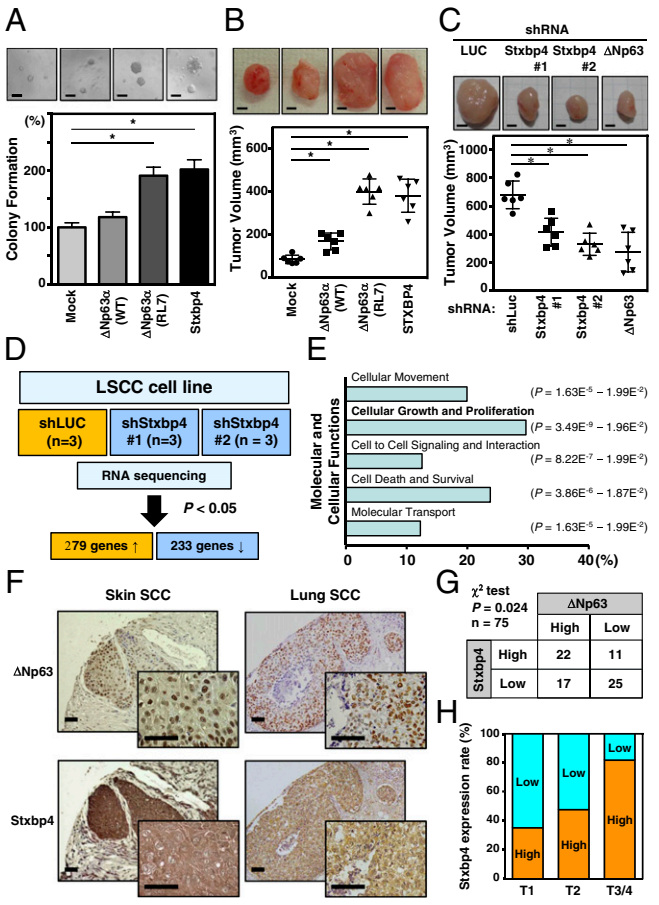

Fig. 7. Stxbp4 promotes oncogenesis in NIH 3T3 cells, and Stxbp4 expression correlates with ΔNp63α levels in SCC. (*A*, *Upper*) The growth of NIH/3T3 cells after being retrovirally transduced with empty vector control (Mock), ΔNp63α (wild type), ΔNp63α (RL7), or Stxbp4 was monitored by soft agar colony formation assays. (*Lower*) SDs are plotted. (Scale bars: 100 μm.) (*B*, *Upper*) Representative images of xenograft mouse tumors arising from 5×10^6 retrovirally transduced NIH/3T3 expressing wild-type ΔNp63α (WT), RL7-ΔNp63α, or Stxbp4 that were s.c. transplanted. (*Lower*) Tumor volumes resulting from six independent injections for each transfectant are shown. Tumor formation was measured after 30 d. (Scale bars: 2 mm.) (*C*, *Upper*) Representative images of xenograft mouse tumors arising from 1×10^7 transduced RERF-LC-Sq1 cells lentivirally depleted of Stxbp4 or ΔNp63α (WT) that were s.c. transplanted. (*Lower*) Tumor volumes resulting from six independent injections for each transfectant are shown. Tumor formation was measured after 20 d. *$P < 0.05$ in *A*–*C*. (Scale bars: 2 mm.) (*D*) A total of 512 genes that were either significantly up-regulated (279 genes) or down-regulated (233 genes) were identified by whole-transcriptome analysis (RNA-seq) of RERF-LC-Sq1 cells compared with cells depleted by shRNA for Stxbp4 (shStxbp4#1 and shStxbp4#2). (*E*) Functional analysis of DEGs as described in *D* was performed by Ingenuity Pathway Analysis (IPA) software (Ingenuity, Qiagen), and five of the most significant pathways are listed. (*F*) Representative immunohistochemical staining of ΔNp63 (*Upper*) and Stxbp4 (*Lower*) in skin SCC (*Left*) and lung SCC (*Right*). Immunostaining of Stxbp4 demonstrates the nucleus and cytoplasmic immunostaining pattern. (Scale bars: 20 μm.) (*G*) Stratification of human skin samples from SCC patients ($n = 75$) based on the expression of Stxbp4 and ΔNp63. The χ^2 test was performed ($P = 0.024$). (*H*) Stxbp4 expression rate on human skin samples from SCC patients stratified according to the indicated T factor (primary tumor size) showing relative proportions of low vs. high Stxbp4 levels as outlined in *SI Appendix*.

genes were in the functional class of Cellular Growth and Proliferation (Fig. 7*E*). Forty-five common genes identified in Stxbp4-depleted SCC cells and lung SCC are listed in descending order in *SI Appendix*, Fig. S8. These results indicate that Stxbp4 has oncogenic activity both in vitro and in vivo (as does stable RL7-ΔNp63α) and further suggest that Stxbp4 could be a critical driver of tumor propagation through regulating the ΔNp63α pathway.

While ΔNp63 is reported to be a diagnostic marker highly specific for lung SCCs (3, 33), ΔNp63 in particular has context-dependent pro-oncogenic roles in the early development of SCCs (10, 11). Our results showing Stxbp4 regulates ΔNp63 in basal layers of the epidermis support a potential role for Stxbp4 in regulating ΔNp63 signaling in SCC. To investigate the clinical significance of Stxbp4 expression, we analyzed a panel of tissue microarrays from skin SCC and lung SCC patients (Fig. 7*F*). Significantly higher levels of Stxbp4 were present in skin SCC that showed an accumulation of ΔNp63 ($P = 0.024$; χ^2 test) (Fig. 7*G*). Notably, Stxbp4-positive tumors correlated with disease stage (Fig. 7*H*). In line with our previous report using lung SCC specimens (24), higher levels of Stxbp4 expression were significantly correlated with the accumulation of ΔNp63 (23, 34) and with prognosis according to the combined lung SCC dataset from The Cancer Gene Atlas (*SI Appendix*, Fig. S9) (35). Our results showing that STXBP4 has oncogenic activity both in vitro and in vivo suggest that it could be a critical driver of tumor propagation in SCC, most likely in a ΔNp63-dependent manner.

Discussion

p63 has added a new dimension to the role of the p53 family in cancer biology due to its involvement in the DNA damage response and in the regulation of cell-cycle arrest (6, 7), apoptosis (36, 37), stemness (31), and tumorigenesis (38, 39). ΔNp63α is the predominant p63 isoform expressed in the basal layer of stem cells within stratified epithelia (40, 41). Given the essential roles of ΔNp63 in basal–epidermal gene expression and stratification maintenance of epithelial progenitor proliferative potential (although its expression is not limited to stem cells), it is important to elucidate how ΔNp63 protein expression is controlled. The correct balance between the tumor-suppressive role of the TAp63 isotype and the oncogenic role of the ΔNp63 isotype, along with the tissue-specific environment, may be critical for proliferation and differentiation of both epithelial stem cells and tumor cells. Further studies are needed to determine whether there are qualitative or quantitative differences in the expression of p63 and which isoforms are responsible for discrete functions such as stemness, cell-cycle arrest, and tumorigenesis (11). It follows that elucidation of how p63 protein expression is regulated is highly relevant to its roles in the development of certain tumors.

Our data show that depletion of Cdc20 but not Cdh1/FZR1 induces ΔNp63 accumulation in proliferating cells (Fig. 6*B* and *SI Appendix*, Fig. S2). Note as well that Cdh1 levels and, to a lesser extent, Cdc20 levels increase while ΔNp63 levels decrease as the differentiation process progresses (Fig. 5*B*). It was established that Cdc20-APC/C primarily regulates mitotic progression, while Cdh1-APC/C shows a broad spectrum in its targets in and beyond the cell cycle, playing roles in signal transduction, differentiation, and tumorigenesis. Nevertheless, we cannot exclude the possibility that Cdh1 and Cdc20 are modified differently by calcium and differentiation signaling (21, 22). Our data demonstrate that Cdh1 and, less effectively, Cdc20 participate in ΔNp63 down-regulation and the stimulation of terminal differentiation in the context of differentiation (Fig. 6 *A* and *B*). Our results have considerable relevance to tumorigenesis, as follows: First, the ability of Cdh1 to recruit its substrates to the APC/C core complex is attenuated in transformed cancer cells (21). Second, mutations within and down-regulation of Cdh1 are observed in several cancers (42). Third, many substrates of Cdh1, including M/S-phase cyclins and kinases, and DNA replication factors are frequently overexpressed in some tumors (43). Finally, in experiments using mouse models, Cdh1 heterozygosity results in the development of epithelial tumors, suggesting that Cdh1 may be a haploinsufficient tumor suppressor (44). We

speculate that one outcome of the down-regulation of Cdh1 in cells derived from epithelia might be the accumulation of ΔNp63, with profound consequences for tumorigenesis.

In the present study, we found that ΔNp63 protein stability can also be tightly regulated by an interacting partner, Stxbp4, and we showed that Stxbp4 works in opposition to the APC/C in primary and immortalized keratinocytes. Increasing expression of Stxbp4 led to oncogenic outcomes in NIH/3T3 cells that were similar to the expression of degradation-resistant RL7-ΔNp63α (Fig. 7*B*). These results are consistent with our previous study showing that Stxbp4 suppresses ubiquitination and degradation of ΔNp63 by Rack1 and Itch as well as the APC/C (23). Here we demonstrate that ΔNp63 protein stability can be tightly regulated by two of its interacting partners, Stxbp4 and the APC/C, and show that they work in opposite manners.

Using degradation-resistant ΔNp63α and Stxbp4 (Fig. 5*F*) in in vitro differentiation culture models and 3D organotypic pHKC raft cultures, we found that ΔNp63α degradation is necessary for terminal differentiation in these experimental set-ups. This is consistent with previous studies with normal keratinocytes and squamous carcinomas, in which the expression of ΔNp63 is localized in the undifferentiated cells and is absent from areas of terminal differentiation (36, 45). Since elevated expression of ΔNp63 is observed throughout the depth of the epithelium from metaplasia to severe dysplasia and in the early steps in SCC tumorigenesis (9, 32), ΔNp63 has been identified as a highly specific diagnostic marker for SCCs from adenocarcinoma (AC) in lung cancer (3, 33). This indicates that p63 is required for the maintenance and/or differentiation of progenitor cell populations necessary for epithelial development, while it needs to be lost in the upper layers. Thus, it is likely that ΔNp63 in particular has context-dependent pro-oncogenic roles in the early development of SCCs (10, 11). Relatedly, in a mouse model where p63 is expressed in a single-layered lung epithelium, there is a shift to stratified squamous epithelium (8, 10), suggesting that the accumulation of ΔNp63 keeps cells in the terminal differentiation-resistant stem cell-like state and induces squamous cell carcinoma. These findings support the hypothesis of the oncogenic role of these isoforms in various epithelial cancers (44).

In summary, given the critical role of ΔNp63 in epithelial stem cells and SCCs, our detailed mechanistic study of the regulation of p63 protein stability has not only basic but also translational significance. For example, manipulating protein–protein interactions between Stxbp4 and ΔNp63 might be of some therapeutic value in treating cancers, especially SCC, or pathogenesis of psoriasis (46, 47). Since knockdown of ΔNp63 in head and neck SCC can induce p73-dependent apoptosis (11, 39), efforts to disrupt the interaction between Stxbp4 and p63 using peptidomimetics or small molecules might destabilize ΔNp63 in those cells and therefore eventually be useful as a new therapeutic approach.

Materials and Methods

Plasmids expressing FLAG-HA doubly tagged ΔNp63α, FLAG-tagged wild-type and ubiquitination-resistant (RL7) ΔNp63α, and FLAG-tagged Stxbp4 were cloned into the MSCV or pLPCX retrovirus expression vector (Clontech, Takara Bio). FLAG-tagged Cdc20 and Cdh1 were kindly provided by M. Pagano, New York University School of Medicine, New York. 3×FLAG-tagged Stxbp4 pCMV7 has been previously described (23). Purification of the doubly tagged ΔNp63α complex and MALDI-TOF mass analysis and immunohistochemical analysis were as previously described (48, 49). Immunohistochemistry and scoring methods were as previously described (24, 34). Details of extended methods used in this paper including cell culture, antisense oligonucleotides, baculovirus protein purification, cell synchronization, FACS analysis, far-Western immunoblotting, in vitro ubiquitination assay, in vitro degradation assay, immunofluorescence, immunohistochemistry, real-time qPCR, 3D organotypic raft cultures, anchorage-independent growth assay, and s.c. transplantation in mice are described in *SI Appendix, SI Materials and Methods*. All animal procedures were approved by the Animal Ethics Committee of Gunma University.

ACKNOWLEDGMENTS. We thank Dr. Scott W. Lowe for providing the lentiviral shRNA pMLP vector and Ella Freulich for expert technical assistance. This work was supported by NIH Grants CA87497 and CA58316 and also in part by the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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