SCF\textsuperscript{βTrCP1} Activates and Ubiquitylates TAp63γ\textsuperscript{**}

Jayme R. Gallegos\textsuperscript{1,1}, Joel Litersky\textsuperscript{1}, Hunjoo Lee\textsuperscript{2}, Yi Sun\textsuperscript{4}, Keiichi Nakayama\textsuperscript{3}, Keiko Nakayama\textsuperscript{**}, and Hua Lu\textsuperscript{***, + + + 2}

From the \textsuperscript{1}Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon 97239, the \textsuperscript{2}Department of Biochemistry and Molecular Biology, University of Indiana, Indianapolis, Indiana 46202, the \textsuperscript{3}Department of Radiation Oncology, University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan 48109, the \textsuperscript{4}Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan, \textsuperscript{5}CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan, and the \textsuperscript{6}Department of Developmental Biology, Center for Translational and Advanced Animal Research, Graduate School of Medicine, Tohoku University, 2-1 Seiryo, Aoba-ku, Sendai 980-8575, Japan

\textsuperscript{*} This work is supported in part by NCI, National Institutes of Health Grants CA 93614, CA 095441, and CA 079721 (to H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{†} The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

\textsuperscript{‡} Recipient of funding from an Oregon Health and Science University Department of Dermatology predoctoral fellowship (National Institutes of Health) and an Oregon Health and Science University Ophthalmology and Immunology predoctoral research fellowship (National Institutes of Health).

\textsuperscript{§} To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Indiana, 635 Barnhill Dr., MS 4053, Indianapolis, IN 46202. Tel.: 317-278-0920; E-mail: hualu@iuu.edu.

\textsuperscript{1} The abbreviations used are: TA, transactivation domain; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; GFP, green fluorescent protein; EGFP, enhanced GFP; HA, hemagglutinin; sIRNA, smaller interfering RNA; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; WB, Western blot; RT, reverse transcription; qPCR, quantitative PCR; MEF, mouse embryo fibroblast.

p63 is a member of the p53 tumor suppressor family that is critical for epithelial differentiation and also has an important role in cancer progression. Currently, the molecular mechanisms governing regulation of p63 function remain largely unclear. This study identifies a unique E3 ubiquitin ligase for p63, SCF\textsuperscript{βTrCP1}. SCF\textsuperscript{βTrCP1} is able to bind p63γ isoforms, with a higher affinity for the TAp63γ isoform. Strikingly, co-expression of TAp63γ and βTrCP1 leads to the stabilization of TAp63γ. This stabilization of TAp63γ leads to up-regulation of p21 at the mRNA and protein level by increased binding of TAp63γ at the p21 promoter. The up-regulation of p21 causes a subsequent increase in G\textsubscript{1} phase cell cycle arrest. Last, SCF\textsuperscript{βTrCP1} is able to ubiquitylate TAp63γ, and this ubiquitylation, as well as the increased activity of TAp63γ, is ablated with the expression of a ubiquitin-deficient mutant of βTrCP1 (ΔFβTrCP1). Therefore, our study reveals that SCF\textsuperscript{βTrCP1} is an E3 ligase that activates p63 through ubiquitylation.

p63 is a member of the p53 tumor suppressor family. The six major isoforms of p63 have several conserved regions common to the p53 family members, such as the transactivation domain (TA),\textsuperscript{3} the DNA binding domain, and the oligomerization domain (1–3). The TA isoforms have a full-length TA, whereas the N\textsubscript{p}63 isoforms are the shortest and the B and allow subsequent up-regulation of NF\textsubscript{κ}B (10). βTrCP1 (β-transducin repeats-containing protein 1, Fwd1, etc.) is one further, the C-terminal regions of these isoforms are alternatively spliced. These variants are termed α, β, and γ (1, 3). The p63α isoforms are the longest and most commonly expressed in adult tissues (2, 3), whereas the p63γ isoforms are the shortest and the most potent transcriptional activators of the six isoforms (3, 4). Because of its strong transactivation and its role in the stress response, TAp63γ is considered the p63 isoform “most like” p53.

The biological role of p63 is more complex than that of a classical tumor suppressor. p63 knock-out mice have a severe defect in epithelial stratification and fail to form associated tissues, such as teeth, hair, and mammary glands (2, 5). Also, it is debated whether p63 might act as a tumor suppressor or an oncogene (1). Recent data suggest that the N\textsubscript{p}63 isoforms, when overexpressed, may be tumorigenic, whereas the TA isoforms may play a more important role in tumor suppression (1). Several papers have suggested a reciprocal relationship between N\textsubscript{p}63α and TAp63γ, since, under a variety of cellular stimuli, the levels of one are often high when those of the other are low, and vice versa (1). What is clear is that a tightly controlled balance of p63 isoform levels in the cell is critical to homeostasis.

This tight balance is maintained through both direct binding of p53 family member transcription sites and heterodimerization among the p53 family members. Although commanding a unique set of target genes, p63 also shares several target genes with the other p53 family members, such as p21 (6–8). In a p53-deficient background, TAp63γ may rescue the growth-inhibitory function of p53 by activating some of these common target genes and causing growth arrest in response to various forms of stress (6–8). The elucidation of both common and isoform-specific signaling pathways will be critical to our understanding of the role of p63 in development and oncogenesis.

In an effort to understand one of the biochemical mechanisms that regulate p63, we searched for a p63-specific regulator that could modulate p63 ubiquitylation and, therefore, its activity. A search of the literature revealed that the inhibitor of IκB kinase α knock-out mouse displayed an epidermal phenotype very similar to that of the p63 knock-out mouse (9). The inhibitor of IκB kinase kinase complex works in conjunction with the E3 ubiquitin ligase complex SCF\textsuperscript{βTrCP1} to degrade the IκB and allow subsequent up-regulation of NF\textsubscript{κ}B (10). βTrCP1 (β-transducin repeats-containing protein 1, Fwd1, etc.) is one.
of the many substrate recognition components of the SCF complex (10). Humans have two βTrCP proteins, which act in homo- or heterodimers as part of the SCF complex: βTrCP1 and βTrCP2. When exogenously expressed, βTrCP1 is nuclear, whereas βTrCP2 is cytoplasmic (10). βTrCP has two major protein motifs: an F-box motif that connects βTrCP to the rest of the SCF complex through its interaction with Skp1 and a WD domain, which binds the many substrates of βTrCP (10). βTrCP proteins can recognize a canonical DXXS motif, where the Ser residues are usually phosphorylated (10). However, several noncanonical sequences and regions have also been found. These regions usually depend on a cluster of highly charged or phosphorylated residues and are found in proteins, such as Emi1 and Cdc25a (10–12).

Therefore, it is very plausible that SCFβTrCP1 may also regulate the p63 pathway. Our study, as described here, tests this idea. We have chosen to focus on the p63γ isoforms, because they are the most potent regulators of transcription and may be important in modulating the activity of the other family members during cell growth. Our data demonstrate that SCFβTrCP1 binds TAp63γ and, to a lesser degree, ΔNp63γ. This interaction takes place at sites on both the N and the C termini of TAp63γ. Further, this binding leads to subsequent stabilization of TAp63γ, leading to increased activation of p21, because of increased binding of TAp63γ to the p21 promoter. In turn, the up-regulation of p21 leads to an increase in G1 phase cells. Further, βTrCP1 is able to ubiquitylate TAp63γ in vitro and in cells, demonstrating that although it regulates the activity of TAp63γ, βTrCP1 also has a dual role as an E3 ligase to TAp63γ. This E3 ligase activity is critical to the increased activation of TAp63γ by βTrCP1, since a βTrCP1 mutant that can bind but not ubiquitylate TAp63γ (ΔβTrCP1) is unable to mediate this activity. Thus, this study provides insight into a novel ubiquitin-dependent activation mechanism for TAp63γ.

EXPERIMENTAL PROCEDURES

Cell Culture—Hi-5 insect cells were maintained in HFO SFX insect cell medium (Perbio) with 1× penicillin/streptomycin (Cellgro) at 25 °C. Human embryonic kidney epithelial cells (HEK293) and human non-small cell lung carcinoma cells (H1299) were cultered as described previously (7). Embryonic mouse embryonic fibroblasts (MEFs) were maintained according to the previous protocol (13). HaCaT and HEK-neuronal E6/E7 cells were obtained from Dr. Mihail Iordanov (Oregon Health and Science University) and cultured as described previously (14, 15).

Antibodies and Vectors—p63 was detected using Pab4A4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or p63-NTA antibody (generated by our laboratory). Polyclonal α-GFP antibody (Santa Cruz Biotechnology) was used for exogenous GFP-p63 IPs, and α-His antibody (Qiagen) was used to detect the His-tagged p63, as indicated. βTrCP1 was detected using α-FLAGM2 antibody (Sigma) or endogenous βTrCP1 antibodies, N-15 or H-300 (Santa Cruz Biotechnology). Tubulin was detected using antibody from Sigma. p21 was detected using p21 polyclonal sc-H164 (Santa Cruz Biotechnology). Bax was detected using Bax Ab5 (Neomarkers). pCDNA3.1 empty vector, pEGFP-TAp63γ (and related mutants F1–F4), pEGFP-ΔNp63γ, and pCDNA3His-ubiquitin were generated as described previously (7, 16). pCDNA3FLAG-βTrCP1, pCDNA3FLAG-ΔβTrCP1, and baculoviral expression constructs of Roc-1, HA-Cul1, His-Skp1, and FLAG-βTrCP1 were kindly provided by Dr. Yi Sun (University of Michigan) (17).

Transient Transfection/siRNA Transfection—Transient transfection used Transfextin (Bio-Rad) reagent according to the manufacturer’s directions at ~70% confluence for all cell lines. For each experiment, a 1:2 μg ratio of pEGFP-TAp63γ to pCDNA3-FLAGβTrCP1 vector or a 1:2.5 μg ratio of pEGFP-TAp63γ to pCDNA3-FLAGΔβTrCP1 vector was used and scaled to the appropriate size of the plate (for ChlP, 0.2 μg of pEGFP-TAp63γ; 2 μg of pCDNA3-FLAGβTrCP1 or 2.5 μg of pCDNA3-FLAGΔβTrCP1 was used per 10-cm plate). siRNA oligonucleotide infection was performed using cells at ~50% confluence. For each experiment, a βTrCP1/2 siRNA oligonucleotide pool (Santa Cruz Biotechnology) or scramble siRNA oligonucleotide mix was added to each plate at a final concentration of 100 nm. The cells were harvested 48 h post-transfection.

Preparation of Purified His-TAp63γ, GST-FLAG-βTrCP1, and SCFβTrCP1 Complex—All of the His-TAp63γ, GST-FLAG-βTrCP1, and His-TAp63γ fragments (F1–F4) were purified as described previously (7, 16). His-TAp63γ was purified using Ni2+-nitrilotriacetic acid purification, followed by purification on a Poros HQ affinity column (PerSeptive Biosystems). The SCFβTrCP1 complex was purified as described previously (17). The fractions were run on SDS-PAGE and checked by Western blot using polyclonal α-Roc1 (Neomarkers), monoclonal 12CA5 anti-HA (generated in the Lu laboratory), monoclonal α-FLAGM2, and monoclonal α-His antibodies to detect separate complex components.

Western Blot Analysis—Clariﬁed whole cell lysates were loaded directly onto an SDS gel and probed with antibodies, as noted in the ﬁgure legends, according to the previous protocol (7, 16). Band density was calculated using Adobe Photoshop or Optiquant software and normalized to tubulin. For Western blot analysis (WB) indicating protein levels after co-expression, cells were harvested 48 h post-transfection.

In Vitro Binding—Protein-protein association assays were conducted as reported (7, 16).
**RESULTS**

**βTrCP1 Expression Stabilizes TAp63γ**—To determine if βTrCP1 could act as a regulator for TAp63γ, we wished to determine if βTrCP1 would have an effect on the stability of TAp63γ, since βTrCP1 is a well-known regulator of protein stability. To answer this question, we used primary MEFs at passage 2 derived from βTrCP1+/− and βTrCP1−/− littermates (13) and examined the endogenous levels of TAp63γ mRNA and protein. Strikingly, there was a significantly lower amount of TAp63γ protein in the βTrCP1−/− MEFs compared with the βTrCP1+/− MEFs (30%), although the TAp63β protein level is not affected (Fig. 1A, top). Since the p63 antibody is pan-specific, O3C keratinocytes were used as positive control for the p63 isoforms (23, 24). Further, when the mRNA levels were examined using semiquantitative RT-PCR with TA-specific p63 primers, little change was observed (less than 0.5-fold) in TAp63β between the βTrCP1+/− and the βTrCP1−/− MEFs (Fig. 1A, bottom). Since the protein level of TAp63β is minor compared with the level of TAp63γ, by WB, this RT-PCR measurement is representative of the TAp63γ RNA levels. Next, to determine if this observation was specific to βTrCP1 expression, we repeated this experiment in H1299 cells, a cell line that is p53 null and has undetectable endogenous expression of p63 and p73 by WB. In fact, in the presence of increased βTrCP1, the protein level of TAp63γ increased significantly above the level of TAp63γ alone (Fig. 1B, lane 4 versus lane 2). Therefore, TAp63γ protein levels are also increased by a subsequent increase of βTrCP1. These results suggest that βTrCP1 must mediate the endogenous protein stability of TAp63γ through a post-translational mechanism.

In order to verify these intriguing observations, we measured the half-life of exogenous TAp63γ in the presence of βTrCP1. Again, to our surprise, although βTrCP1 has been shown to be involved in the degradation of its substrates, our data demonstrate that it stabilizes TAp63γ. As shown in Fig. 1C, although the exogenous GFP-TAp63γ alone has a half-life of 2–3 h, comparable with others’ published data (25), the GFP-TAp63γ co-expressed with the FLAG-βTrCP1 was highly stabilized over 6 h. We also confirmed this result using a labeled pulse-chase (data not shown).

Further, to examine the effect of βTrCP on endogenous p63 stability, we used a human keratinocyte line with several p63 isoforms and βTrCP1 (HaCaT). The cells were treated with siRNA oligonucleotides against βTrCP1/2 (since βTrCP2 often rescues βTrCP1 if knocked down alone). An initial experiment was performed to determine the efficiency of knockdown at both 24 and 48 h post-transfection to find the optimal time to start the degradation assay. Knockdown was confirmed, at 0.47-fold versus the scramble control at 48 h (Fig. 1D). This degree of reduction is biologically significant, since several groups have shown that βTrCP1 is affected by both haploinsufficiency and dominant-negative mutants in vivo (10), compared with the control, by WB (Fig. 1D). Therefore, for our half-life, time 0 for the cycloheximide exposure in Fig. 1E is 48 h after oligonucleo-
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**FIGURE 1. βTrCP1 expression stabilizes TAp63γ. A. βTrCP1 knock-out mice have lower endogenous protein levels of TAp63γ. βTrCP1+/− or βTrCP1−/− primary MEFs were harvested for either WB (top) or RT-PCR (bottom). In the WB, 150 μg of whole cell lysate for MEFs was loaded beside 30 μg of whole cell lysate from O3C mouse keratinocytes. B. βTrCP1 expression stabilizes raises steady-state protein levels of exogenous TAp63γ. H1299 cells were transfected for 48 h and harvested for WB. 50 μg of whole cell lysis was used. Loading control denotes nonspecific band. C. βTrCP1 expression stabilizes TAp63γ post-cycloheximide treatment. HaCaT cells were transfected with GFP-TAp63γ and FLAG-βTrCP1. 48 h later, they were treated with cycloheximide (CHX) and harvested over the indicated time course. Shown is representative WB with quantification of p63 signal intensity/tubulin signal intensity of three experiments graphed below.**, \*p < 0.03. D, βTrCP1 levels are reduced in βTrCP1/2-depleted cells. HaCaT cells were treated with control scramble siRNA oligonucleotides or βTrCP1/2 siRNA oligonucleotides and harvested after 24 or 48 h. 80 μg of whole cell lysate was used for WB analysis. The protein levels were quantified and normalized to tubulin. The numbers above are calculated fold-reduction shown. The lane numbers are denoted below. E, βTrCP1/2 knockdown destabilizes endogenous p63 post-cycloheximide treatment. HaCaT cells were transfected with βTrCP1/2 siRNA oligonucleotides or scramble control oligonucleotides. 48 h later, they were treated with cycloheximide and harvested over the indicated time course. Quantification of p63 signal intensity/tubulin signal intensity of representative blot graphed below.

TAp63γ binds βTrCP1—In order to determine how βTrCP1 regulates TAp63γ stability, we next asked whether βTrCP1 could bind TAp63γ in cells. HEK293 cells, which express an undetectable endogenous level of p63, were transfected with GFP-TAp63γ, GFP-ΔNp63γ, or FLAG-βTrCP1, followed by co-IP and WB. As shown in Fig. 2A, both TAp63γ and ΔNp63γ immunoprecipitated using FLAG antibody for βTrCP1 (lanes 1...
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**FIGURE 2. p63γ binds βTrCP1.** A, TAp63γ and ΔNp63γ bind βTrCP1 in cells. GFP-TAp63γ, GFP-ΔNp63γ, and FLAG-βTrCP1 were expressed in HEK293 cells. 448 μg of clarified whole cell lysate co-immunoprecipitated per sample, with 15.6% of the prebound lysate loaded as input. WB was performed using the indicated antibodies. B, TAp63γ binds βTrCP1 in vitro. GST-βTrCP1 was bound to GST-agarose bead. 60 ng of purified His-TAp63γ was bound to bead, 10% input, and binding samples were analyzed by WB. C, TAp63γ and TAp63α bind βTrCP1 endogenously. HEK293 cells were harvested, and 850 μg of total lysate was immunoprecipitated using the noted antibodies (left). The panel on the right shows the IgG control taken from the same gel, same exposure.

**FIGURE 3. βTrCP1 binds TAp63γ N and C termini.** A, a schematic of wild-type TAp63γ and the TAp63γ fragments. The transactivation (TA), DNA binding domain (DBD; black pattern), and oligomerization domain (OD; white pattern) are indicated. The fragments (F1–F4) and the encompassing amino acid residues are noted. B, in vitro mapping of βTrCP1 binding to TAp63γ. GST-βTrCP1 was incubated with 40 ng of His-TAp63γ F1–F4, as indicated, 100% input was loaded as a control. The upper band in lanes 3 and 10 is nonspecific. Below is a summary of the binding of βTrCP1 to TAp63γ. TAp63γ is shown as in A, with the stronger and weaker interaction with βTrCP1 shown as the solid and dashed lines, respectively.

βTrCP1 (supplemental Fig. 1). Further, we tested whether this interaction existed endogenously using co-IP in HEKn-E6/E7 cells, a human keratinocyte line that has high levels of p63 and βTrCP1 (supplemental Fig. 2). By co-IP, βTrCP1 immunoprecipitated with ΔNp63α and TAp63γ but not with TAp63β endogenously (Fig. 2C, lane 2). Also, p63 immunoprecipitated with βTrCP1 endogenously (lane 1). These results were also repeated using HaCaT and HeLa cell lysates. Therefore, βTrCP1 is able to interact with several isoforms of p63 both exogenously and endogenously. Of note, endogenous βTrCP1 appeared to associate with ΔNp63α more efficiently than with TAp63γ (Fig. 2C). This result, although intriguing, was not surprising, since βTrCP1 bound to both the N and C termini of p63 as shown below. Also, we observed that TAp63γ was more efficiently ubiquitylated than was ΔNp63α (data not shown) (see Fig. 7), and this could explain why most of the βTrCP1-bound p63 molecules were ΔNp63α, but not TAp63γ, since most of the latter became polyubiquitin-TAp63γ.

**βTrCP1 Binds the TAp63γ N and C Termini**—In order to further characterize the interaction of TAp63γ with βTrCP1, we mapped the regions where TAp63γ contacts βTrCP1 in vitro. We generated four His-tagged deletion mutants of TAp63γ, termed F1–F4 (Fig. 3A). TAp63γF1, the TA-containing deletion mutant (lane 6), bound strongly to GST-βTrCP1, whereas F3 and F4, the C terminus-containing mutants, also bound, but less efficiently than F1 (lanes 10 and 12) but not the DNA binding domain-containing mutant, F2 (Fig. 3B). These results suggest that the N terminus and, to a lesser extent, a region in the last 59 residues of the protein were important in βTrCP1 binding. These results are also consistent with the
However, when TAp63 was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression at the mRNA level, RT-PCR for p21, bax, βTrCP1, p63, and β-actin was performed in H1299 cells as shown (right). qPCR was also performed, and p21 and p63 were normalized to GAPDH (GAPDH) Representative data are shown. *, p < 0.0002. B, TAp63γ activation of p21, but not Bax, is increased by βTrCP1 expression. A, TAp63γ activation of p21, but not Bax, is increased by βTrCP1 expression at the mRNA level. RT-PCR for p21, bax, βTrCP1, p63, and β-actin was performed in H1299 cells as shown (right). qPCR was also performed, and p21 was normalized to GAPDH (GAPDH). Representative data are shown. *, p < 0.0002. B, TAp63γ activation of p21, but not Bax, is increased by βTrCP1 expression at the protein level. 70 µg of whole cell lysate was analyzed by WB.

FIGURE 4. TAp63γ activation of p21, but not Bax, is increased by βTrCP1 expression. A, TAp63γ activation of p21, but not Bax, is increased by βTrCP1 expression at the mRNA level. RT-PCR for p21, bax, βTrCP1, p63, and β-actin was performed in H1299 cells as shown (right). qPCR was also performed, and p21 was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Representative data are shown. *, p < 0.0002. B, TAp63γ activation of p21, but not Bax, is increased by βTrCP1 expression. A, TAp63γ activation of p21, but not Bax, is increased by βTrCP1 expression at the mRNA level. RT-PCR for p21, bax, βTrCP1, p63, and β-actin was performed in H1299 cells as shown (right). qPCR was also performed, and p21 was normalized to GAPDH (GAPDH) Representative data are shown. *, p < 0.0002. B, TAp63γ activation of p21, but not Bax, is increased by βTrCP1 expression at the protein level. 70 µg of whole cell lysate was analyzed by WB.

FIGURE 5. TAp63γ binding at the p21 promoter increases in the presence of βTrCP1 in cells. A, schematic of the p21 promoter. The primer regions used for ChIP at the p21 promoter are shown by gray boxes with labels according to primer set. +, a CATG core sequence; *, a CGTG core sequence. B, TAp63γ binding to the p21 promoter increases in the presence of βTrCP1 in cells. H1299 cells were transfected and subjected to ChIP using the indicated ChIP antibody/qPCR primer combinations. Representative data are shown. *, p < 0.002.

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A.

B.

Weaker interaction shown between ΔNP63γ and βTrCP1 in Fig. 2A.

TAp63γ Activation of p21, but Not Bax, Is Increased by βTrCP1 Expression—Since TAp63γ was stabilized by βTrCP1, and they interacted directly, we next asked whether βTrCP1 could affect TAp63γ function as a transcription factor. Two target genes of TAp63γ are p21 and bax (1, 4, 8), so we examined the effect of TAp63γ and βTrCP1 co-expression on the p63 transcriptional targets p21 and bax. Consistent with our previous reports (7), TAp63γ alone activated p21 at both the mRNA and protein levels significantly over the vector alone control and also had some effect on Bax (Fig. 4, A and B, lane 2). However, when TAp63γ was co-expressed with βTrCP1, p21 mRNA levels increased 55% (Fig. 4A, lane 4 and graph), and p21 protein levels increased ~50% over the TAp63γ control (Fig. 4B, lane 4). In contrast, Bax expression did not increase over the TAp63γ control at either the mRNA or protein level (Fig. 4, A and B, lane 4). Additionally, the TAp63γ mRNA expression levels do not change in the presence of βTrCP1, again suggesting that the up-regulation of the protein level is occurring through a post-translational mechanism, supporting our endogenous result in Fig. 1A. Therefore, the co-expression of TAp63γ with βTrCP1 causes apparent up-regulation of p21, but not bax, over the level of either TAp63γ or βTrCP1 alone.

TAp63γ Binding at the p21 Promoter Increases in the Presence of βTrCP1—Since βTrCP1 stabilized TAp63γ, and βTrCP1 also augmented TAp63γ activation of p21 levels, we next asked if βTrCP1 could enhance the presence of TAp63γ at the p21 promoter. The regions on the promoter that were used for analysis began at amino acids −2283, −20, and +7878 and spanned 50 bp downstream in each case. These regions are called P1, P2, and P3, respectively (Fig. 5A). The P1 primers include a high affinity site for the p53 family members, whereas the P3 primers are a negative control. Last, sequence analysis of the p21 promoter revealed both CATG sequences and CGTG sequences that may be favorable for p63 binding near the P2 primer set (4). Thus, we also tested this region for TAp63γ binding. Consistent with our earlier results, TAp63γ alone resulted in a 20-fold increase of promoter binding over the vector alone control, whereas co-expression of TAp63γ with βTrCP1 resulted in an increase of over 50-fold of TAp63γ bound to the p21 promoter (Fig. 5B). The P3 primers in the gene were negative. Interestingly, P2 primers also could detect
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![Image 1](image1.png)

![Image 2](image2.png)

**FIGURE 6.** TAp63γ activation of growth arrest is increased by βTrCP1 expression. Representative curves of H1299 cells treated with nocodazole, subjected to fluorescence-activated cell sorting, and gated for GFP-positive signal are shown below as generated by FlowJo software and plotted to scale on the same axis, as indicated. G1, 2n peak by PI staining; G2, 4n by PI staining. The percentage of GFP-positive cells in each phase of the cell cycle was determined, and data for three experiments were plotted (top). *, p = 0.02.

**FIGURE 7.** SCFβTrCP1 ubiquitylates TAp63γ. A, TAp63 isoforms are more ubiquitylated in cells. H1299 cells were analyzed by WB with 50 μg of whole cell lysis (bottom) or by Ni2+-nitrilotriacetic acid precipitation (top). B, SCFβTrCP1 ubiquitylates TAp63γ in vitro. Shown is 100 ng of purified His-TAp63γ and ATP reaction buffer with noted substrates, analyzed with α-p63 antibody. *, degradation of purified TAp63γ. C, SCFβTrCP1 ubiquitylates TAp63γ in cells. Ubiquitylation was analyzed as in A.

an active site of TAp63γ recruitment on the p21 promoter (Fig. 5B), demonstrating the existence of a novel site for p63 binding at the p21 promoter. Therefore, the increased stability of TAp63γ in the presence of βTrCP1 leads to increased TAp63γ bound to the promoter, using both a canonical p53 family binding region and a novel p63 binding region, accounting for the activation of p21 noted above.

TAp63γ Activation of Growth Arrest Is Increased by βTrCP1 Expression—Since our data clearly pointed to a role of βTrCP1 in TAp63γ expression of p21, and p21 is an established regulator of cell cycle arrest, we asked whether the up-regulation of p21 protein would translate to a functional role in G1 cell cycle arrest. Consistent with our previous data, although TAp63γ alone increases the proportion of cells in G1 (26%), the cells co-expressing TAp63γ and βTrCP1 showed a significant increase (42%) (Fig. 6). Therefore, the increased activity of p21 upon TAp63γ co-expression with βTrCP1 translates to an equally striking up-regulation of G1 cell cycle arrest.

p63γ Is Ubiquitylated by SCFβTrCP1—Our data above identify βTrCP1 as a regulator of TAp63γ, which acts by increasing TAp63γ through a post-translational mechanism. Since SCFβTrCP1 is an active member of the SCF complex, we next examined whether SCFβTrCP1 could ubiquitylate TAp63γ. First, we wished to examine the general ubiquitylation of p63 in H1299 cells. Using a Ni2+-nitrilotriacetic acid binding assay, GFP-TAp63γ, Myc-TAp63α, and Myc-ΔNp63γ showed a shift to higher weight moieties in the presence of ubiquitin (Fig. 7A, lanes 4, 6, and 8), but not when transfected alone, demonstrating that all of the isoforms in this case are ubiquitylated. Interestingly, the ΔN isoform, although expressed at equivalent levels, has less ubiquitylation than the TA isoforms, as shown by the much lighter laddering. Therefore, several isoforms of p63 are ubiquitylated, with the TA isoforms ubiquitylated to a greater degree, suggesting that ubiquitin may be one way to account for the differences in p63 isoform activity in the cell. Next, to ask if the ubiquitylation we observed was βTrCP1-dependent, we performed an in vitro ubiquitylation assay using a purified SCF complex, p63, E1, E2, and ubiquitin. In fact, in the presence of all of the ubiquitylation components, TAp63γ shifted to several ubiquitylated forms of the protein, which
were absent in the control lanes (Fig. 7B, lane 7). We then tested whether FLAG-βTrCP1 expression would increase the ubiquitylation of TAp63γ in H1299 cells. In this assay, we used FLAG-βTrCP1 or the F-box mutant of βTrCP1, FLAG-ΔFβTrCP1. ΔFβTrCP1 is a dominant negative truncation mutant of βTrCP1 that lacks the F-box. Therefore, the ΔFβTrCP1 protein can bind the substrates of βTrCP1, but those substrates are not presented to the SCF complex for ubiquitylation (data not shown) (10, 26). Consequently, the level of generally ubiquitylated TAp63γ precipitated in cells (Fig. 7C, lane 3) increased specifically in the presence of FLAG-βTrCP1 (lane 5) and decreased in the presence of the FLAG-ΔFβTrCP1 (lane 7). Further, as an additional control to ensure that our result was not due to the function of another SCF complex, we also performed experiments co-expressing TAp63γ with another SCF substrate recognition component, such as Skp2, and these experiments were negative (data not shown). Thus, this finding demonstrates that SCFβTrCP1 could act as a specific E3 ubiquitin ligase for TAp63γ and that the ubiquitylation of TAp63γ requires the presence of the rest of the SCF complex. Therefore, while also acting functionally as a regulator of TAp63γ, βTrCP1 acts as an E3 ubiquitin ligase for TAp63γ.

p63γ Activation by SCFβTrCP1 Is Mediated by Its Ubiquitylation—Since we found that βTrCP1 could both activate and ubiquitylate TAp63γ, we next wished to determine if the activation was related to the ubiquitin modification. Therefore, we repeated some of the activation assays using the FLAG-ΔFβTrCP1 mutant. First, we examined TAp63γ half-life in H1299 cells in a similar manner to Fig. 1C, with the addition of the ΔFβTrCP1 mutant. Where co-expression of GFP-TAp63γ and FLAG-βTrCP1 stabilized the TAp63γ (Fig. 8A, lanes 7–12), co-expression of GFP-TAp63γ with FLAG-ΔFβTrCP1 at the same expression ratio as in Fig. 7C did not stabilize the TAp63γ when compared with the GFP-TAp63γ alone (lanes 13–18). In order to observe if this loss of TAp63γ stabilization also correlated to a functional decrease, we continued by examining the level of p21 expression by qPCR. Again, when GFP-TAp63γ and the FLAG-ΔFβTrCP1 were co-expressed, there was no significantly increased expression of p21 compared with the p21 induction of GFP-TAp63γ alone, whereas p21 expression is significantly increased again in the presence of both GFP-TAp63γ and FLAG-βTrCP1 (Fig. 8B). Therefore, we wanted to see if this loss of activation was also true at the level of promoter binding. Again, we used H1299 cells and performed ChIP. As shown in Fig. 8C, although the GFP-TAp63γ has the expected increase of binding to the p21 P1 and P2 promoter regions (top and bottom, respectively), and GFP-TAp63γ and FLAG-βTrCP1 co-expression leads to a significant increase in promoter binding, the level of GFP-TAp63γ bound to the p21 promoter in the presence of FLAG-ΔFβTrCP1 significantly decreases compared with GFP-TAp63γ alone on both the P1 and P2 regions. The P3 results were similarly negative, as in Fig. 5 (data not shown). Concomitantly, these data demonstrate that the increased stabilization and activation of TAp63γ by βTrCP1 are direct results of the ubiquitylation itself, since the ΔFβTrCP1 mutant is still able to bind TAp63γ but not ubiquitylate it.

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DISCUSSION

In this study, we provide evidence for a novel activation pathway between TAp63γ and βTrCP1, beginning with the observation that endogenous and exogenous steady-state levels and the half-life of TAp63γ increase dramatically in the presence of βTrCP1. βTrCP1 binds to TAp63γ directly and also to ΔNp63γ to a lesser degree, consistent with our data mapping βTrCP1 binding to the N and C termini of TAp63γ. This interaction is also present endogenously. The result of this interaction is the subsequent stabilization of TAp63γ and increased activation of p21 because of increased binding of TAp63γ to the p21 promoter, in turn leading to an increase in G1 phase cells. Further, βTrCP1 is able to ubiquitylate TAp63γ in vitro and in cells, and this ubiquitylation is needed for the functional activation of TAp63γ.

The literature on various E3 ligases in signaling, in terms not only of degradation but also of activation, is increasing at an incredible rate. Although our report is the first to demonstrate a direct role for SCFβTrCP1 in stabilization and activation of its substrate, βTrCP1 is involved in activation pathways, such as its well established role in aiding in the activation of the NF-κB pathway by degrading IkBα (27). Additionally, the association between transcriptional activators and E3 ligases is also well trodden experimental ground. In fact, another E3 ligase that forms a complex with the SCF core is Skp2, which co-activates the oncogene c-myc (28, 29). Our data clearly demonstrate that TAp63γ is stabilized exogenously at saturating levels of both TAp63γ and βTrCP1 and destabilized endogenously by knockdown of βTrCP1 and βTrCP2 (Fig. 1, C–E). Our endogenous data in primary MEFs shows that the TAp63γ protein level is markedly reduced with the loss of βTrCP1 (Fig. 1A). Consistent with this result, increased levels of βTrCP1 raise the steady-state levels of TAp63γ (Figs. 1B and 4B), again while the mRNA levels remain largely even (Figs. 1A and 4A). Taken together, these data demonstrate that the stabilization and activation of TAp63γ by SCFβTrCP1 are present endogenously.

An intriguing observation is the clear effect of the ubiquitin modification itself on TAp63γ stability. Our data show that the p63 isoforms have a distinctly differing degree of ubiquitylation, demonstrating that ubiquitylation could be used as one mechanism to distinguish specific activity of each p63 isoform (Fig. 7A). Further, TAp63γ is ubiquitylated in the presence of βTrCP1, but not ΔNβTrCP1 (Fig. 7, B and C), suggesting that this binding includes the entire SCF complex. Our data clearly show that significantly more TAp63γ is recruited to the p21 promoter in the presence of βTrCP1 (Figs. 5B and 8C) after its stabilization, accounting for the significant activation of p21 at both the mRNA and protein level (Figs. 4, A and B, and 8B). However, strong evidence in Fig. 8 shows that in each case when the ubiquitylation activity is lost through expression of the ΔFβTrCP1 mutant, which can bind the substrates of βTrCP1 but not carry them to the SCF complex for ubiquitylation, TAp63γ stability (Fig. 8A), its up-regulation of p21 (Fig. 8B), and its binding to the p21 promoter (Fig. 8C) are lost as well. Adding further detail to our model are the facts that the stability of TAp63γ is initiated off chromatin, since our IP data are taken from a whole cell lysate that would not contain chromatin-associated proteins, and that this signaling event most likely occurs in the nucleus, since βTrCP1 and TAp63γ both are nuclear proteins and are therefore aptly placed for activation (7, 10). Although ubiquitin is traditionally thought of as a degradation signal, the list of proteins that use ubiquitin as an activating stimulus is growing. Some ubiquitin linkages have been linked to activation, and a recent study shows that forked polyubiquitin chains created by some E2s, including UbcH5c, may also be a signal used for a function other than proteasomal degradation (27, 30). Further, p53 is targeted for ubiquitylation by E4F1, and this modification stabilizes it for a p21-specific activation cascade very similar to what we observe with p63 (31). It is also unsurprising that p63 is targeted by an SCF complex, since p53 is also recognized by a cullin-containing complex during viral infection (22). The characterization of βTrCP1 ubiquitylation of TAp63γ would be an interesting future study, especially with the conservation of this stabilizing role of ubiquitin among two of the three p53 family members (1). For now, what is clear in light of our data and earlier studies is that the relationship among p63 DNA binding, subsequent transactivation activity, and stability is most likely the result of subtle modifications and interactions tailored to each isoform.

Specific isoform interaction would be provided by binding affinity and presence of βTrCP1 in the cell. βTrCP1 binds TAp63γ and ΔNp63γ isoforms in vitro and in cells at both the N- and C-terminal segments of the protein (Figs. 2 and 3), regions known to be important for moderating stability among the p53 family members. It is quite intriguing that βTrCP1 makes both N- and C-terminal contacts with varying interaction at those regions, because the difference in binding strength could easily provide a mechanism for the modulation of isoform-specific activity. Other than conferring a selective TA isoform-specific advantage in binding to and subsequent activation by βTrCP1, this dual binding site may also serve three distinct purposes. First, the placement of the N-terminal site may occlude the binding of other substrates, allowing for selective activation of specific downstream targets involved in growth arrest, but not apoptosis, as our later mRNA/protein activation and cell cycle profile suggests (Figs. 4 and 6). In this respect, the lack of binding in the case of endogenous TAp63β may be explained in that other interacting proteins may have a higher affinity for TAp63β at those sites (Fig. 2C). Second, it could also prevent export to the cytoplasm, in the case of MDM2 occlusion, or prevent immediate degradation by ligases, such as Itch. Last, it is also possible that βTrCP1 binding facilitates the placement of other post-translational modifications on p63. Further, in each of these cases, not only the βTrCP1 affinity for the individual p63 isoforms, but also βTrCP1 affinity for its other substrates would play a critical role in its regulation of p63 under our model, since SCFβTrCP1 protein levels have been demonstrated as a limiting factor in endogenous signaling (10, 27). Our data show that βTrCP1 also binds TAp63α and ΔNp63α; this binding could provide another level of regulation through isoform competition. Likewise, future studies will probably elucidate the effect of the specific balance of p63 isoforms, as well as the other p53 family members, on this pathway in vivo, since these studies focus largely on TAp63γ and our binding and ubiquitylation data suggest that differences in
binding and ubiquitylation would be important for the regulation of p63 signaling through this mechanism. Therefore, most likely a fine balance exists between the availability of both βTrCP1 and p63 impacting the ability of βTrCP1 to act as a regulator of p63.

Thus, in light of our data, we propose a model (Fig. 9) in which SCFβTrCP1 associates with and stabilizes TAp63γ in the nucleus through the covalent addition of ubiquitin. The increased load of ubiquitylated TAp63γ at the p21 promoter then leads to an increased level of transcript and G1 cell cycle arrest. It will be an interesting future question to explore what upstream signal might begin this cascade endogenously and how this modification would interact with others as more regulators of p63 are found. Clearly, discerning more about such subtle regulations of the p63 isoforms will lead to a greater understanding of the role of p63 in differentiation and oncogenesis.

Acknowledgments—We thank Yetao Jin, Mushai Dai, and Joylene Gaska for help in editing and final preparation of the manuscript. The Myc-ΔNp63α construct and O3C cells were kindly provided by the laboratory of Dr. Molly Kulesz-Martin. The human keratinocyte lines were kindly provided by the laboratory of Dr. Mihail Iordanov.

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