Tumor Protein p63/Nuclear Factor κB Feedback Loop in Regulation of Cell Death

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Background: TP53 members and NF-κB respond to stresses. Results: TAp63α induced the expression of RelA and downstream genes involved in cell cycle arrest or apoptosis. TAp63α and RELA formed protein complexes resulting in their mutual stabilization, and TAp63α induced the RelA transcription. Conclusion: We defined a novel a feedback loop between NF-κB and TP63. Significance: TP53 members and NF-κB contribute to the regulation of cell death.

Tumor protein (TP)-p53 family members often play proapoptotic roles, whereas nuclear factor κB (NF-κB) functions as a proapoptotic and antiapoptotic regulator depending on the cellular environment. We previously showed that the NF-κB activation leads to the reduction of the TP63 isoform, ΔNp63α, thereby rendering the cells susceptible to cell death upon DNA damage. However, the functional relationship between TP63 isotypes and NF-κB is poorly understood. Here, we report that the TAp63 regulates NF-κB transcription and protein stability subsequently leading to the cell death phenotype. We found that TAp63α induced the expression of the p65 subunit of NF-κB (REL) and target genes involved in cell cycle arrest or apoptosis, thereby triggering cell death pathways in MCF10A cells. RELA was shown to concomitantly modulate specific cellular survival pathways, making it dispensable for the TAp63α-dependent regulation of cell death. We showed that TAp63α and RELA formed protein complexes resulted in their mutual stabilization and inhibition of the RELA ubiquitination. Finally, we showed that TAp63α directly induced RelA transcription by binding to and activating of its promoter and, in turn, leading to activation of the NF-κB-dependent cell death genes. Overall, our data defined the regulatory feedback loop between TAp63α and NF-κB involved in the activation of cell death process of cancer cells.

TP53 members and NF-κB respond to a variety of intrinsic stresses, such as DNA damage, hypoxia, starvation, and cytokine activation (1–4). TP53 family members often play proapoptotic roles, whereas NF-κB functions as a proapoptotic and antiapoptotic regulator (1, 3, 5–7). However, any of these transcription factors are frequently deregulated in cancer cells (8). TP53 proteins promote cell death through senescence, cell cycle arrest, apoptosis, and autophagy and promote metabolic changes. NF-κB, however, initiates the cellular responses utilizing specific metabolic pathways resulting in the synthesis of substrates for cell division (9–28). NF-κB consists of two subunits, p50 (NF-κB1) and p65 (REL, NF-κB3), activated by cleavage and phosphorylation leading to a nuclear accumulation (29).

TAp63 encodes several protein isotypes with the long transactivation domain (TD)2 (TA-) and the short TD (AN-) (1, 2). ΔNp63 isotypes often function as a dominant negative inhibitor toward TAp63 isotypes and TP53 exerting the opposing transcriptional functions (1, 2). However, a few reports showed that ΔNp63 isotypes could play the proactive role in regulation of gene transcription, RNA splicing, and signaling leading to modulation of cell survival/cell death, tumorigenesis, and drug resistance (30–33). Recent findings further showed that the treatment of cancer cells with cisplatin generated phosphorylated (p)-ΔNp63α that appears to act similar to TAp63 isotypes or p53 by activating genes implicated in apoptosis and autophagy (34–36).

We and others previously showed that the NF-κB activation is a potential mechanism by which levels of ΔNp63α are reduced, thereby rendering the cells susceptible to cell death in the face of cellular stress (37–39). Although the NF-κB was found to regulate TAp63 promoter activity (40), the functional relationship between TAp63 isotypes and NF-κB is poorly understood. We showed that TAp63 and NF-κB form protein complexes and TAp63 regulates the NF-κB transcription and protein stability leading to a cell death phenotype.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—Dimethyl sulfoxide (D8418) and MG-132 (C2211) were from Sigma-Aldrich. Dulbecco's modi-
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well in 96-well plates were cultured in 5% FBS for 24 h. 10 μl of MTT labeling reagent (5 μg/ml) was added to the culture medium without FBS, and the mix was then incubated in the dark for 4 h at 37 °C. Cells were lysed by the addition of 100 μl of an SDS-based detergent reagent, incubated for 2 h at 37 °C, and measurements (A_{570 nm} to A_{650 nm}) were obtained on a Spectra Max 250 96-well plate reader (Molecular Devices). Each assay was carried out in triplicate and repeated at least three times. Diagrams indicated the extent of cell viability expressed as a percentage of control.

Ubiquitination Assay—MCF10A cells were co-transfected with the pCMV-HA-Ub and pCMV4-RelA expression cassettes along with an empty vector, pCMV-TAp63α-FLAG plasmid or TAp63α siRNA. 48 h after transfection, cells were treated with 25 μM MG-132 and lysed in a buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing protease and phosphatase inhibitors (Sigma). Ubiquitinated proteins were recovered with the red anti-HA affinity gel (E6779; Sigma) and analyzed with the anti-RelA antibody.

NF-κB Luciferase Assay—MCF10A cells and H1299 cells were transfected with the pCMV-TAp63α-FLAG or control vector using FuGENE HD in the presence or absence of the pRelA-MetLuc2 reporter vector or pMetLuc2 control vector, and after 24–48 h, the RelA promoter-driven luciferase activity was determined by a Ready-To-Glow pNF-κB MetLuc luciferase reporter system (Clontech). Each transfection was performed in duplicate at least three times.

Measurement of RELA Half-life—100 μg/ml of cycloheximide (Sigma) was added to MCF10A cells 24 h after transfection with the indicated combination of plasmids. Protein levels were monitored at the indicated time points. To confirm equal loading, the membrane was reprobed with the β-actin antibody.

Promoter Luciferase Activity Assays—MCF10A cells were transiently transfected with the Bbc3-luc and pCDKN1A-luc reporter plasmids along with 500 ng of the pCMV-TAp63α expression plasmid (where indicated) in the presence or absence of the 1.0 μg pCMV4-RelA expression plasmid and the empty pGL3-Basic vector. For the NF-κB luciferase assay, MCF10A cells were transiently transfected with the RelA luciferase reporter plasmids along with the 500 ng of pCMV-TAp63α expression plasmid in the presence or absence of the 1.0 μg of pCMV4-RelA expression plasmid and the RelA empty vector. Transfection efficiency was determined by the Renilla luciferase gene-containing PRL-CLM plasmid (Promega). 48 h after transfection, cells were lysed in a lysis buffer (5× PLBR; Promega) with gentle shaking at room temperature for 20 min, and then total lysates were spun down at 13,000 rpm for 2 min to pellet the cell debris. The supernatants were transferred to a fresh tube, and the Dual luciferase activity in 2 μl (2 μg) of the cell lysates was measured according to the manufacturer’s protocol (Promega). Firefly luciferase activity was monitored by a luminometer programmed as described elsewhere (38, 39). After stopping the firefly luciferase activity, the Renilla luciferase activity was then measured. Each experiment was repeated at three times with duplicate transfections.

Statistical Analysis—Data represent mean ± S.D. from independent experiments, and statistical analysis was performed using a Student’s t test at a significance level of p < 0.05 to < 0.001.

RESULTS

TAp63α Regulates NF-κB/RelA Expression and Cell Death—TP63 plays a critical role in various cellular processes, including cell proliferation, differentiation, apoptosis, and senescence. We and others recently showed that NF-κB regulates ΔNp63α and TAp63α functions in cellular response to external stimuli (38, 39, 40). Because both TAp63α and NF-κB have been implicated in regulation of cell proliferation/differentiation, cell death/survival, we focused our studies on whether TAp63α plays a role in regulating NF-κB expression.

We initially examined how the TAp63α forced expression affects the RELA endogenous expression in MCF10A cells and H1299 cells (Fig. 1). We found that the RELA protein levels markedly increased by the ectopic TAp63α (0–1.5 μg) in a dose-dependent manner (Fig. 1, A and C). We further showed that TAp63α induced levels of CDKN1A and BBC3 proteins (Fig. 1, A and C), and subsequently increased cell death, as observed by the CASP3 and PARP cleavage (Fig. 1, A and C), and MTT assay (Fig. 1, B and D). Because MCF10A expresses detectable levels of the endogenous TAp63α, we next transfected MCF10A cells with the scrambled siRNA or TAp63α siRNA and found that the RELA protein levels markedly decreased upon the TAp63α silencing (Fig. 1E), whereas no changes were found under control (scrambled) conditions. Similarly, the TAp63α knockdown exclusively decreased the CDKN1A and BBC3 protein levels (Fig. 1E) and dramatically inhibited the cell death, as observed by a reduced CASP3 and PARP cleavage and MTT assay (Fig. 1F). These findings suggested that TAp63α could regulate RELA expression.

We next examined whether overexpression or down-regulation of RELA regulated CDKN1A, BBC3 protein levels, as well as cell survival. We showed that with the increased NF-κB expression (0–1.5 μg) up-regulated the TAp63α, CDKN1A, and BBC3 protein levels, increased the CASP3 and PARP cleaved product levels (Fig. 2A), and markedly reduced the cell viability (Fig. 2B). At the same time, siRNA-mediated RELA knockdown decreased the TAp63α, CDKN1A, and BBC3 protein levels (Fig. 2C). To eliminate the off-target effect of RelA shRNA, the shRNA-resistant pEGFP-p65R construct (42) was added to the cells (Fig. 2D). Expression of p65R in the RelA knocked-down MCF10A cells exhibited a “rescue” effect on TAp63α, CDKN1A, and BBC3 protein levels (Fig. 2D). However, shRNA-mediated knockdown of RELA dramatically reduced the cell death, as observed by the decreased CASP3 and PARP cleavage (Fig. 2C), or by cell viability assay (Fig. 2D), whereas co-transfection with the pEGFP-p65R plasmid increased cell death (Fig. 2E). These data strongly suggest the specific effect of RelA down-regulation on TAp63α expression and cell death.

To examine further the role for TAp63α/RelA axis in the cell death regulation, we transfected MCF10A cells with 20 nM TAp63α siRNA followed by increasing concentration of the ectopic RelA expression cassette (0–1.5 μg). In addition, we transfected MCF10A cells with 1 μg of RelA shRNA and then with the TAp63α expression cassette (0–1.5 μg). We found
that the RELA up-regulation following the TAp63α down-regulation increased the CDKN1A, BBC3 protein levels, PARP cleavage, and induced cell death (Fig. 3, A and B). However, the TAp63α up-regulation following the RELA down-regulation failed to affect the CDKN1A, BBC3 protein levels, PARP cleavage, and cell death induction (Fig. 3, C and D). This observation suggests that TAp63α regulates cell viability of MCF10A cells through a RELA overexpression.

**TAp63α Binds to RELA and Depresses Its Ubiquitin-mediated Degradation**—To explore the molecular mechanism underlying the TAp63α/NF-κB functional relationship, we transfected MCF10A cells with the TAp63α-FLAG expression cassette. We precipitated total lysates with an anti-FLAG antibody and probed TAp63α protein complexes with an antibody to RELA and vice versa. We showed that TAp63α and RELA, indeed, formed protein complexes in MCF10A cells (Fig. 4A).

We previously reported that IKKβ promotes a proteasomal degradation of ΔNp63α through a NF-κB-dependent pathway. Here, we further examined whether the physical interaction of TAp63α and RELA leads to a degradation of any of them. We transfected MCF10A cells with the scrambled siRNA and TAp63α siRNA and subsequently treated cells with the proteasome inhibitor, MG-132 or control medium. We found that the TAp63α down-regulation resulted in a decrease of the endogenous RELA, whereas the MG-132 treatment significantly restored the RELA levels (Fig. 4B), suggesting a proteasome-de-
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**FIGURE 2. Both RelA and TAp63α mediated cell death pathways.** A and B, MCF10A cells transfected with the pCMV4-RelA expression cassette (0–1.5 μg) for 24 h. A, immunoblotting with the indicated antibodies. B, MTT assay. C and D, MCF10A cells transfected with the RelA shRNA (0–1.5 μg) for 48 h followed by immunoblotting with the indicated antibodies. C, MCF10A cells were transfected with the 1 μg RelA or p65Res constructs for 24 h followed by transfection with RelA shRNA (0–1.5 μg) for 24 h and immunoblotted with the indicated antibodies. D, MTT assay. *, p ≤ 0.05 (n = 3) compared with control (untransfected cells or scrambled shRNA). Error bars, S.D.

**FIGURE 3. TAp63α induced cell death pathways through RelA.** MCF10A cells were transfected with the 20 nM TAp63α siRNA for 24 h followed by the increasing concentration of the ectopic RelA expression cassette (0–1.5 μg) for 24 h. A, immunoblotting with the indicated antibodies. B, MTT assay. MCF10A cells were transfected with the 1 μg of RelA shRNA followed by the TAp63α expression cassette (0–1.5 μg). C, immunoblotting with indicated antibodies. D, MTT assay. For the MTT assay, values from control cells transfected with the empty vector or control (scrambled) siRNA/shRNA were taken as 100%. *, p ≤ 0.05 (n = 3) compared with control (untransfected cells). Error bars, S.D.
dependent mechanism for the RELA reduction induced by TAp63α knockout.

To assess directly the role for TAp63α in regulation of RELA protein stability, we analyzed the effect of the TAp63α forced expression on the RELA half-life in MCF10A cells. Cells were co-transfected with the RELA and TAp63α expression cassettes or with scrambled and TAp63α siRNAs for 24 h. Cells were further exposed to cycloheximide for an additional 24 h. Immunoblot analysis of RELA at serial time intervals showed that the overexpression of the exogenous TAp63α increased the RELA half-life, whereas the down-regulation of the endogenous TAp63α dramatically modulated the RELA half-life (Fig. 4, C–E), supporting the notion that TAp63α positively regulates the RELA half-life in MCF10A cells.

Because TAp63α interacts with RELA and decreases its protein degradation, we further examined the role for ubiquitin-proteasome pathway in this process (43). We transfected MCF10A cells with the RelA and HA-Ub expression cassettes along with the increasing concentrations of the TAp63α or its siRNA for 36 h, and then cells were treated with MG-132 for an additional 10 h. Total lysates were precipitated with anti-HA-matrix and blotted with the anti-RELA antibody. Immunoblot analysis for β-actin showed the loading levels.

**FIGURE 4.** TAp63α-RELA protein interaction modulated the RELA protein level in MCF10A cells. A, complex formation between the TAp63α with RELA proteins. Top left panel, MCF10A cells were transfected with the TAp63α-FLAG or an empty FLAG vector. Immunoprecipitation (IP) was performed using anti-FLAG matrix and blotted with the anti-RELA antibody. Top right panel, MCF10A cells were transfected with the RelA-FLAG cassette or an empty FLAG-vector. Total lysates were precipitated with either control matrix or anti-RelA matrix and blotted with an anti-TP63 antibody. Immunoblotting for TAp63α and β-actin levels was used as input and loading controls, respectively. B, MCF10A cells were transfected with the scrambled siRNA or TAp63α siRNA for 48 h. Cells were treated with the 10 μm proteasome inhibitor, MG-132, for the indicated time periods. Total lysates were immunoblotted with the anti-RELA or anti-p63 antibodies. C–E, MCF10A cells were transfected with the 1.5 μg of RelA expression cassette along with the 1.5 μg of empty vector (C), 1.5 μg of TAp63α expression plasmid (D), or 20 nM TAp63α siRNA (E) for 48 h, as indicated. Cells were then treated with 100 μg/ml cycloheximide (CHX) at the indicated time points, total lysates were analyzed by immunoblotting with indicated antibodies. F, MCF10A cells were co-transfected with the RelA and HA-Ub expression plasmids, without or with the TAp63α expression vector or TAp63α siRNA for 36h and then treated with MG-132 for an additional 10 h. Total lysates were precipitated with anti-HA-matrix and blotted with the anti-RELA antibody. Immunoblot analysis for β-actin showed the loading levels.
**TAp63α Can Activate RelA Transcription**—To exert its transcriptional activity, NF-κB is activated by phosphorylation and then translocates into the nucleus (29, 44). We showed that TAp63α overexpression led to an increasing Ser-536 phosphorylation of RELA and subsequent translocation of activated RELA from cytoplasm to nucleus (Fig. 5A). We next performed the ChIP analysis of the endogenous RelA promoter (supplemental Fig. S1) in MCF10A cells transfected with an empty vector and TAp63α expression cassette or scrambled siRNA. Using the anti-TP63 antibody, we found that TAp63α displayed a strong binding enrichment to the specific region (−1900 to −1551 bp, supplemental Fig. S1) of the RelA promoter, whereas no binding with the nonspecific region (−600 to −351 bp, supplemental Fig. S1), designated as ChIP. We next performed the ChIP analysis of the endogenous RelA promoter (supplemental Fig. S1) in MCF10A cells transfected with an empty vector and TAp63α expression cassette or scrambled siRNA. Using the anti-TP63 antibody, we found that TAp63α displayed a strong binding enrichment to the specific region (−1900 to −1551 bp, supplemental Fig. S1) of the RelA promoter, whereas no binding with the nonspecific region (−600 to −351 bp, supplemental Fig. S1) of the RelA promoter (Fig. 5C). However, the siRNA-mediated...
TAp63α knockdown caused a significant decrease in its binding to the RelA promoter (Fig. 5C). Altogether, these data supported the notion that TAp63α directly regulates RelA transcription.

To examine further the effect of TAp63α on the RelA transcription, we transfected MCF10A cells and H1299 cells with the RelA luciferase reporter plasmid along with the TAp63α or RelA expression constructs. Using the luciferase reporter assay, we found that TAp63α dramatically increased the RelA promoter-driven luciferase activity in both MCF10A and H1299 cells (Fig. 5D). To confirm the activation of RelA after TAp63α up-regulation, we transfected MCF10A cells and H1299 cells with 1 μg of the TAp63α expression cassette along with the pRelA-MetLuc2 reporter construct or pMetLuc2 control construct (Fig. 6, A and B). Resulting cells were subjected to luciferase reporter assay (45). We thus found that the TAp63α over-expression significantly increased the RelA promoter-enhancer activity in both H1299 and MCF10A cells (Fig. 6, A and B).

Next, we showed that both TAp63α and RELA concomitantly induced the expression of cell cycle arrest and pro-apoptotic genes, such as pCdkn1a and Bbc3 (46, 47). MCF10A cells were transfected with the basic TA-luc, pCdkn1a-luc, or Bbc3-luc constructs along with the Renilla luciferase plasmid and the TAp63α or RelA or basic vector for 24 h. Immunoblotting for each sample was performed for p63, RelA, and β-actin. Firefly luciferase activity was normalized for the Renilla luciferase activity. Data presented as relative-fold change units (RFU) from the basic TA-luc activity are designated as 1. *p ≤ 0.001 (n = 5) compared with basic TA-luc activity, or pCdkn1a-luc (C) or Bbc3-luc (D) activities alone.

FIGURE 6. TAp63α increased levels of RelA-dependent genes. MCF10A cells were transfected with the TAp63α expression plasmid (0–1.5 μg). A and B, Ready-To-Glow™ Secreted Luciferase assay for the NF-κB/RelA response element-driven Metridia luciferase activity. 12 h after transfection of H1299 (A) and MCF10A (B) cells with the indicated constructs, the medium was replaced, and then after 16 h, samples of the medium were analyzed for secreted Metridia luciferase activity. The fold induction was calculated following the substrate addition. C and D, luciferase reporter assay for the Cdkn1a (C) and Bbc3 (D) promoters. MCF10A cells were transfected with the basic TA-luc, pCdkn1a-luc, or Bbc3-luc constructs along with the Renilla luciferase plasmid and the TAp63α or RelA or basic vector for 24 h. Immunoblotting for each sample was performed for p63, RelA, and β-actin. Firefly luciferase activity was normalized for the Renilla luciferase activity. Data presented as relative-fold change units (RFU) from the basic TA-luc activity are designated as 1. *p ≤ 0.001 (n = 5) compared with basic TA-luc activity, or pCdkn1a-luc (C) or Bbc3-luc (D) activities alone.
Regulation of RelA by TAp63α

We then found that RELA is indispensable for the TAp63α-dependent regulation of cell death by forming TAp63α–REL A protein complexes resulting in mutual stabilization, as well as in decrease of RELA ubiquitination. On the contrary, the ΔNp63α–REL A functional relationship leads to a down-regulation of both ΔNp63α or REL A in squamous cell carcinoma cells (38, 39), whereas the forced ΔNp63α expression in MCF10A cells failed to affect the REL A and BBC3 protein levels. PARP cleavage Reduced CDKN1A level (supplemental Fig. S2). Finally, we determined that TAp63α directly induced the RelA transcription by binding to and activating of its promoter and, in turn leading to activation of the NF-κB-dependent target genes and contributing to regulation of cell survival. Our data support the notion that TAp63α and NF-κB exert a functional cooperation leading to regulation of the NF-κB-dependent downstream target genes playing a decisive role in cell cycle arrest/cell death (52–54). They also form a regulatory feedback loop that leads to a tune up-regulation of TAp63 or ΔNp63 expression by NF-κB and control of NF-κB expression by TAp63 or ΔNp63 under specific stress conditions and, in turn activate or repress death pathways in cancer cells upon ligand binding to transmembrane death receptors on the target cell (extrinsic caspase cascade pathway), or upon DNA damage/ stress (intrins ic mitochondrial pathway associated with the release of cytochrome c, apoptosis-inducing factor, and Smac/Diablo proteins) (for review, see Refs. 55–57). Many components of these pathways are downstream transcriptional targets of TAp63 and NF-κB (e.g. Apaf1, Bax, Bbc3, Bcl2, Bin1, Casp1, Casp8, Casp11, Dapk1, Fas/CD95, FasL, Fadd, Gadd45B, Parp1, p53aip1, Pmaip1, Tnfr1, Tnfrsf6, Trail, and Xiap), as described elsewhere (58–65), suggesting the interplay between both apoptotic pathways.

The TP53 family and NF-κB proteins function as key regulators of cell death and survival, respectively (3, 7, 16, 27, 53, 55, 57). Under specific conditions, however, TP53 members can promote cell survival, whereas NF-κB can induce the cell death (7, 8, 22, 25, 26, 28). Given the transactivation ability for both NF-κB/REL A and TP53 members, it is very likely that the TP53 member-NF-κB protein complexes are essential for the transcriptional regulation of cell death/survival genes (7, 8, 22, 25, 28, 53, 54). Because TP53, TP63, or TP73 binds to the same or similar cis-elements (32), a recent cooperation between NF-κB and TP73 (20) should be extended to TP53 (22, 25, 28) and TP63 (37, 39). By transcriptional regulation and protein interactions, TP53 members and NF-κB contribute to the regulation of cell death, metabolism, and mitochondrial integrity, thereby emerging as important regulators of metabolic homeostasis (7, 8, 15–28, 50, 53, 59, 66, 67).

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