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Δ Np63 inhibits oxidative stress-induced cell death, including ferroptosis, and cooperates with the BCL-2 family to promote clonogenic survival

Gary X. Wang

Washington University in St. Louis

Ho-Chou Tu

Memorial Sloan Kettering Cancer Center

Yiyu Dong

Memorial Sloan Kettering Cancer Center

Anders Jacobsen Skanderup

National University of Singapore

Yufeng Wang

Memorial Sloan Kettering Cancer Center

See next page for additional authors

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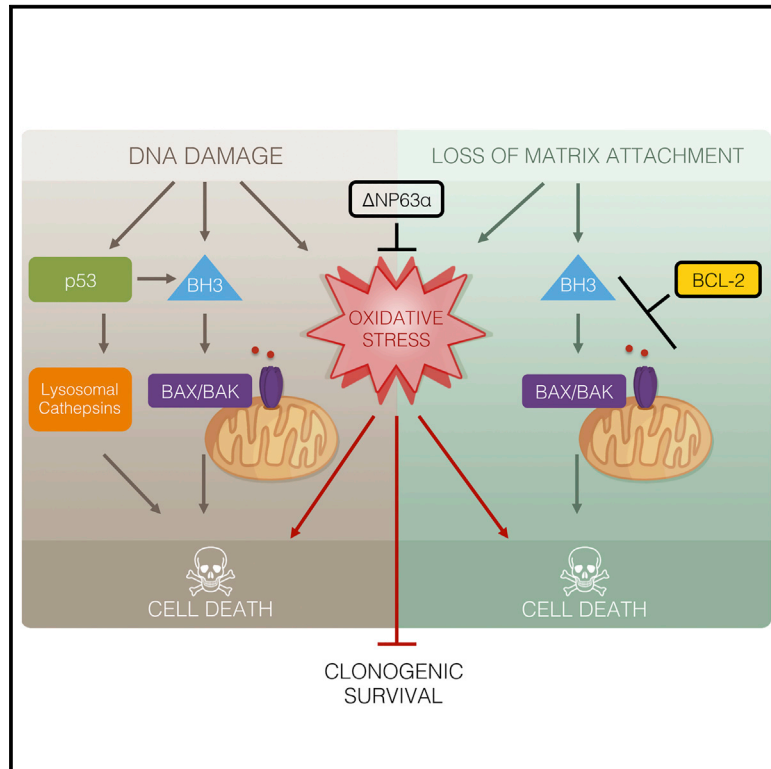
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Authors

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Graphical Abstract



Authors

Gary X. Wang, Ho-Chou Tu, Yiyu Dong, ..., Han Liu, James J. Hsieh, Emily H. Cheng

Correspondence

cheng1@mskcc.org

In Brief

Apoptosis-defective cells remain vulnerable to oxidative stress, which limits long-term survival. Wang et al. identify Δ Np63 α as a central regulator of redox homeostasis through transcriptional control of a tightly coupled glutathione metabolic circuit. Δ Np63 α alleviates oxidative stress and cooperates with the BCL-2 family to promote both long-term cellular well-being and cancer metastasis.

Highlights

- Δ Np63 α is a key cellular guardian against oxidative stress, including ferroptosis
- Δ Np63 α inhibits oxidative stress through regulation of glutathione metabolism
- Δ Np63 α cooperates with the BCL-2 family to promote long-term clonogenic survival
- TP63 amplification upregulates glutathione metabolism to promote tumorigenesis

Data and Software Availability

GSE106214



Δ Np63 Inhibits Oxidative Stress-Induced Cell Death, Including Ferroptosis, and Cooperates with the BCL-2 Family to Promote Clonogenic Survival

Gary X. Wang,^{1,2} Ho-Chou Tu,¹ Yiyu Dong,¹ Anders Jacobsen Skanderup,³ Yufeng Wang,¹ Shugaku Takeda,¹ Yogesh Tengarai Ganesan,¹ Song Han,¹ Han Liu,¹ James J. Hsieh,⁴ and Emily H. Cheng^{1,5,6,7,*}

¹Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

²Division of Biology & Biomedical Sciences, Washington University, St. Louis, MO 63110, USA

³Genome Institute of Singapore, National University of Singapore, 60 Biopolis St., 138672 Singapore, Singapore

⁴Molecular Oncology, Department of Medicine, Siteman Cancer Center, Washington University, St. Louis, MO 63110, USA

⁵Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

⁶Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, Cornell University, New York, NY 10065, USA

⁷Lead Contact

*Correspondence: chenge1@mskcc.org

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SUMMARY

The BCL-2 family proteins are central regulators of apoptosis. However, cells deficient for BAX and BAK or overexpressing BCL-2 still succumb to oxidative stress upon DNA damage or matrix detachment. Here, we show that Δ Np63 α overexpression protects cells from oxidative stress induced by oxidants, DNA damage, anoikis, or ferroptosis-inducing agents. Conversely, Δ Np63 α deficiency increases oxidative stress. Mechanistically, Δ Np63 α orchestrates redox homeostasis through transcriptional control of glutathione biogenesis, utilization, and regeneration. Analysis of a lung squamous cell carcinoma dataset from The Cancer Genome Atlas (TCGA) reveals that *TP63* amplification/overexpression upregulates the glutathione metabolism pathway in primary human tumors. Strikingly, overexpression of Δ Np63 α promotes clonogenic survival of *p53*^{-/-}*Bax*^{-/-}*Bak*^{-/-} cells against DNA damage. Furthermore, co-expression of BCL-2 and Δ Np63 α confers clonogenic survival against matrix detachment, disrupts the luminal clearance of mammary acini, and promotes cancer metastasis. Our findings highlight the need for a simultaneous blockade of apoptosis and oxidative stress to promote long-term cellular well-being.

INTRODUCTION

Proper execution of cell death ensures normal biological processes, and its deregulation causes human diseases ranging from cancer to neurodegenerative disorders (Thompson, 1995). The evolutionarily conserved signaling cascade, consisting of the BCL-2 family, the adaptor protein Apaf-1, and the caspase family, outlines the quintessential apoptotic network (Wang,

2001). In response to apoptotic signals, the “activator” BH3-only molecules, including BID, BIM, PUMA, and NOXA, trigger homo-oligomerization of BAX and BAK to permeabilize mitochondria, leading to efflux of cytochrome c for caspase activation (Chen et al., 2015; Cheng et al., 2001; Inoue-Yamauchi et al., 2017; Kim et al., 2006, 2009; Ren et al., 2010; Wang, 2001; Wei et al., 2001). Although apoptosis has long been considered as the major cell death mechanism required for the successful development and maintenance of tissue homeostasis in metazoans, double deficiency of *Bax* and *Bak* only disrupts the development and homeostasis in restricted sets of tissues (Lindsten et al., 2000), suggesting the existence of BAX/BAK-independent cell death mechanism(s) in maintaining a tissue homeostatic state.

In exploring this cell death conundrum, we discovered that apoptosis-deficient *Bax*^{-/-}*Bak*^{-/-} double-knockout (DKO) mouse embryonic fibroblasts (MEFs) underwent a regulated form of necrotic cell death in response to DNA damage induced by topoisomerase inhibitors (Tu et al., 2009). Surprisingly, this type of necrotic cell death requires active transcription/translation (Tu et al., 2009). Because this DNA damage-induced programmed necrotic death (PND) does not require RIP1 and is not blocked by inhibitors of RIP1 and RIP3, it is distinct from tumor necrosis factor (TNF)-induced “necroptosis” (Pasparakis and Vandenabeele, 2015; Sun and Wang, 2014; Yuan and Kroemer, 2010; Figure S1). Furthermore, we have reported that this death is independent of caspases, the mitochondrial permeability transition pore (PTP), autophagy, and poly(ADP-ribose) polymerase (PARP) (Tu et al., 2009). Notably, DNA alkylation induces PARP-dependent necrotic death, whereas double-strand DNA breaks induce PARP-independent necrotic death in DKO cells (Tu et al., 2009; Zong et al., 2004). Mechanistically, we have delineated a p53-cathepsin axis that cooperates with ROS (reactive oxygen species) to activate PND in DKO cells undergoing double-strand DNA breaks (Tu et al., 2009).

Similar to DNA damage-induced cell death, it was reported that inhibition of apoptosis by BCL-2 is insufficient to provide long-term clonogenic survival against anoikis (Schafer et al., 2009), a form of cell death that is induced by detachment from



the extracellular matrix in anchorage-dependent cells. Interestingly, the antioxidant Trolox cooperates with BCL-2 to enhance clonogenic survival and prevent the luminal clearance of acini in 3D culture of mammary epithelial cells (Schafer et al., 2009). Hence, although apoptosis is the fastest mechanism for eliminating cells upon death stimuli, inhibition of apoptosis is insufficient to confer long-term clonogenic survival, which is required to prevent the pathological loss of cells during disease processes. ROS appears to play a critical role in abrogating long-term clonogenic survival. The importance of ROS in regulating cell death is further exemplified by the recent characterization of ferroptosis, an iron-dependent, oxidative form of PND that is triggered by the depletion of intracellular glutathione or inhibition of GPX4, leading to accumulation of lipid hydroperoxides (Conrad et al., 2016). Of note, ferroptosis is not involved in DNA damage-induced death of *Bax*^{-/-}*Bak*^{-/-} cells because ferrostatin-1, an inhibitor of ferroptosis, failed to protect DKO cells from etoposide (Figure S1). In contrast, iron chelators protected *Bax*^{-/-}*Bak*^{-/-} cells from etoposide-induced death (Figure S1). In this study, we sought to identify a master regulator of ROS and determine whether the identified guardian of oxidative stress can cooperate with the gatekeepers of mitochondrial apoptosis (i.e., BCL-2 family proteins) to promote clonogenic survival against intrinsic cell death signals.

Here we demonstrate that long-term clonogenic survival of apoptosis-defective cells in response to DNA damage or loss of matrix attachment could be achieved through overexpression of the putative oncogene Δ Np63 α , the major isoform of p63. Δ Np63 α increases cellular resistance against oxidative stress and ferroptosis by regulating the synthesis, utilization, and regeneration of glutathione, a central component of the cellular antioxidant defense system. The regulation of the glutathione metabolic pathway by Δ Np63 α was further demonstrated in human squamous cell lung cancer, in which *TP63* is frequently amplified. Amplification of *TP63* appears to serve as a mechanism by which squamous cell lung cancer evades oxidative stress and promotes tumorigenesis. In summary, simultaneous inhibition of mitochondrion-dependent apoptosis and ROS-induced cell death can confer long-term cellular survival, a strategy likely hijacked by cancer.

RESULTS

Δ Np63 α Confers Clonogenic Survival of *p53*^{-/-}*Bax*^{-/-}*Bak*^{-/-} Triple-Knockout Cells against DNA Damage through Inhibition of Oxidative Stress

Our prior studies of *Bax*^{-/-}*Bak*^{-/-} cells showed that genotoxic stress-induced, ROS-dependent necrotic death requires active transcription (Tu et al., 2009), suggesting that genotoxic stress-induced ROS can be regulated by specific transcription factor(s). To search for such candidates, we examined several transcription factors known to regulate cell death or the DNA damage response, which led to the discovery of Δ Np63 α as a key transcriptional regulator of ROS. Overexpression of Δ Np63 α prevented etoposide-induced ROS accumulation in transformed *Bax*^{-/-}*Bak*^{-/-} MEFs (Figure 1A). Three ROS-sensitive dyes were employed to interrogate intracellular ROS levels: 2',7'-dichlorofluorescein diacetate (H₂DCFDA), a broad-spectrum ROS

indicator; dihydroethidium (DHE), a specific indicator for superoxide; and MitoSOX Red, a specific indicator for mitochondrial matrix superoxide. Overexpression of Δ Np63 α also reduced etoposide-induced lipid peroxidation (Figure 1B). Consistent with our previous characterization of p53 and ROS as two independent effectors in executing necrotic death (Tu et al., 2009), overexpression of a dominant-negative mutant of p53 (p53 DN) did not prevent ROS induced by genotoxic stress (Figure 1A). Furthermore, overexpression of either Δ Np63 α or p53 DN provided only a short-term survival advantage against etoposide (Figure 1C), whereas co-expression of both Δ Np63 α and p53 DN enhanced clonogenic survival of DKO cells (Figure 1D). Of note, we have previously shown that transformation of MEFs by the SV40 genome does not inactivate p53 (Tu et al., 2009).

To evaluate whether Δ Np63 α regulates ROS independently of p53, *p53*^{-/-}*Bax*^{-/-}*Bak*^{-/-} triple-knockout (TKO) MEFs were generated. Although transformed *p53*^{-/-}*Bax*^{-/-}*Bak*^{-/-} TKO MEFs were more resistant to DNA damage than *Bax*^{-/-}*Bak*^{-/-} DKO MEFs (Figure S2), TKO cells were eventually killed by DNA damage because of oxidative stress, which could be alleviated by the antioxidants N-acetyl-L-cysteine (NAC) and diphenyleneiodonium (DPI) (Figure S2). Importantly, overexpression of Δ Np63 α protected TKO cells from etoposide-induced ROS accumulation and cell death (Figures 1E and 1F; Figure S2). Furthermore, inhibition of oxidative stress by Δ Np63 α conferred clonogenic survival of TKO cells against DNA damage (Figure 1G). Because BCL-X_L amplification/overexpression and p53 mutations can co-occur in human cancers, we next examined whether Δ Np63 α can inhibit etoposide-induced death in BCL-X_L-overexpressing p53-deficient MEFs, which resemble *p53*^{-/-}*Bax*^{-/-}*Bak*^{-/-} TKO MEFs. Indeed, overexpression of both BCL-X_L and Δ Np63 α , but not either alone, completely blocked etoposide-induced death of *p53*^{-/-} MEFs (Figure 1H). Notably, overexpression of BCL-X_L provided more protection than Δ Np63 α because BIM-mediated activation of BAX/BAK occurs in p53-deficient cells.

It has been reported that another p63 isoform, TAp63, is induced by DNA damage in MEFs (Flores et al., 2002) and that deficiency of TAp63, but not p53, protects oocytes from ionizing irradiation (Suh et al., 2006). Of note, MEFs only express TAp63 but not Δ Np63, as determined by qRT-PCR (Figure S3). However, TAp63 protein was below the immunoblot detection limit. Knockdown of TAp63 in DKO or TKO MEFs had no effect on etoposide-induced cell death (Figure S3), indicating that endogenous TAp63 is not involved in DNA damage-induced necrotic death and that Δ Np63 α does not inhibit oxidative stress through antagonizing endogenous TAp63 in MEFs. Consistent with the reported pro-death activity of TAp63 α (Suh et al., 2006), we were unable to stably express TAp63 α in DKO cells. However, when expression of TAp63 α was induced in DKO cells using a lentiviral tetracycline-inducible system, it neither enhanced nor blocked DNA damage-induced PND in DKO cells (Figure 1I). Moreover, expression of a human ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome-derived R244H Δ Np63 α mutant (Dötsch et al., 2010; van Bokhoven et al., 2001) that loses DNA binding activity failed to protect DKO cells from DNA damage-induced cell death (Figure 1J). These results indicate that binding to DNA, but not

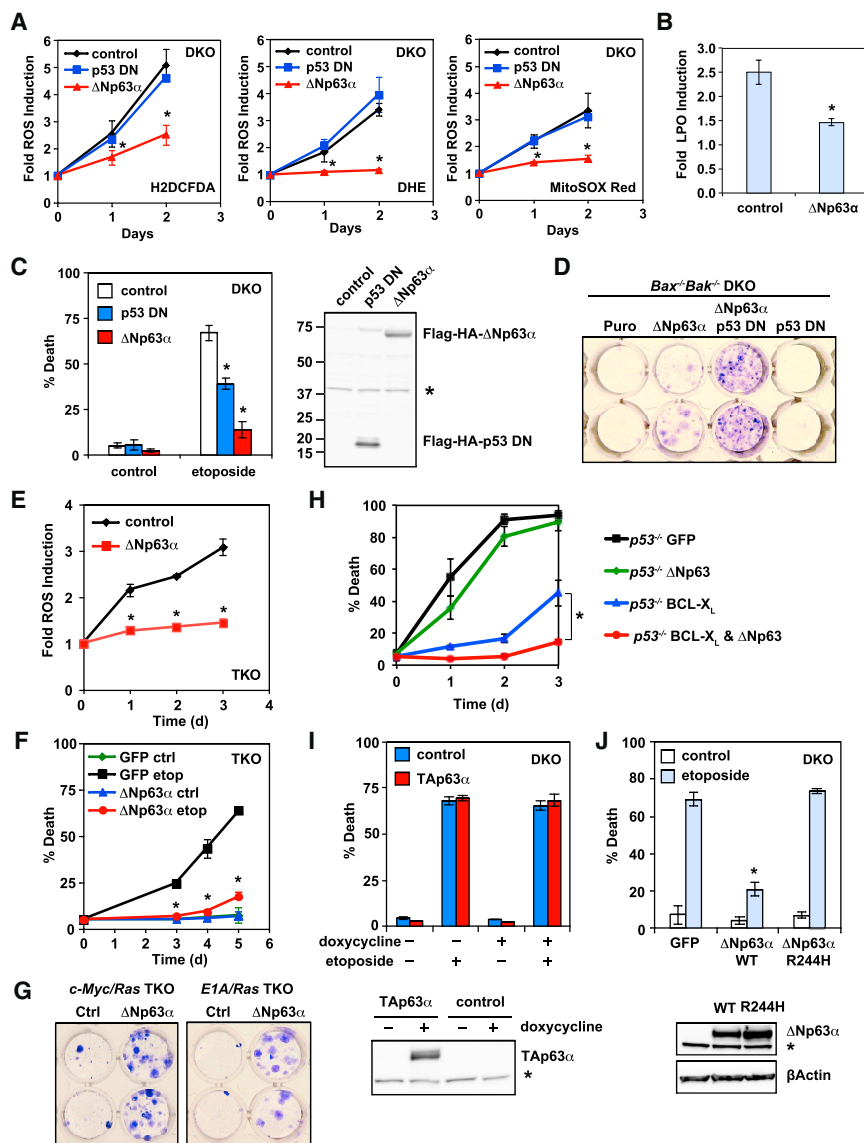


Figure 1. Δ Np63 α Alleviates DNA Damage-Induced Oxidative Stress and Enhances Clonogenic Survival of Transformed $p53^{-/-}$ $Bax^{-/-}$ $Bak^{-/-}$ TKO MEFs

(A) SV40-transformed $Bax^{-/-}$ $Bak^{-/-}$ MEFs transduced with the indicated retrovirus were untreated or treated with etoposide (10 μ g/mL). Oxidation of the ROS-sensitive dyes H₂DCFDA, DHE, or MitoSOX Red was quantified by flow cytometry. Data show the fold increase of ROS after etoposide treatment (mean \pm SD, n = 3).

(B) Cells as described in (A) were subjected to staining using the lipid peroxidation probe C11-BODIPY^{665/676}. Oxidation of C11-BODIPY was quantified by flow cytometry. Data show the fold increase of lipid peroxidation (LPO) after etoposide treatment (mean \pm SD, n = 3).

(C) Cell death was quantified by propidium iodide staining 3 days after etoposide treatment (mean \pm SD, n = 3). The expression of N-terminal FLAG-HA-tagged Δ Np63 α and dominant-negative p53 was assessed by an anti-FLAG immunoblot. The asterisk denotes a cross-reactive band serving as a loading control.

(D) SV40-transformed $Bax^{-/-}$ $Bak^{-/-}$ MEFs transduced with the indicated retrovirus were treated with etoposide for 14 hr. Colonies were stained with crystal violet after 12 days. The data shown are representative of three independent experiments.

(E) SV40-transformed $p53^{-/-}$ $Bax^{-/-}$ $Bak^{-/-}$ MEFs transduced with the indicated retrovirus were left untreated or treated with etoposide. Oxidation of the ROS-sensitive dye DHE was quantified by flow cytometry. Data show the fold increase of ROS after etoposide treatment (mean \pm SD, n = 3).

(F) Cell death was quantified by propidium iodide staining at the indicated times after etoposide treatment (mean \pm SD, n = 3).

(G) c-Myc/Ras- or E1A/Ras-transformed $p53^{-/-}$ $Bax^{-/-}$ $Bak^{-/-}$ MEFs transduced with the indicated retrovirus were treated with etoposide for 9 hr. Colonies were stained with crystal violet after 10 days.

(H) SV40-transformed $p53^{-/-}$ MEFs transduced with the indicated retrovirus were left untreated or treated with etoposide. Cell death was quantified by propidium iodide staining at the indicated times (mean \pm SD, n = 3).

(I) SV40-transformed $Bax^{-/-}$ $Bak^{-/-}$ MEFs transduced with the indicated retrovirus were left untreated or treated with doxycycline for 2 days, followed by etoposide treatment for 3 days. Cell death was quantified by propidium iodide staining (mean \pm SD, n = 3). The expression of TAp63 α was assessed by an anti-p63 immunoblot. The asterisk denotes a cross-reactive band serving as a loading control.

(J) SV40-transformed $Bax^{-/-}$ $Bak^{-/-}$ MEFs transduced with the indicated retrovirus were left untreated or treated with etoposide. Cell death was quantified by propidium iodide staining 3 days after etoposide treatment (mean \pm SD, n = 3). The expression of WT or mutant Δ Np63 α was assessed by an anti-p63 immunoblot. The asterisk denotes a cross-reactive band serving as a loading control.

*p < 0.05 (Student's t test).

p53 family members, is critical for Δ Np63 α to prevent DNA damage-induced necrotic death.

Δ Np63 α Orchestrates Redox Homeostasis through Transcriptional Control of Glutathione Metabolism Genes

We envisioned that, if Δ Np63 α prevents DNA damage-induced oxidative stress by increasing antioxidant capacity, then Δ Np63 α could protect cells against exogenous oxidants.

Indeed, overexpression of Δ Np63 α protected wild-type (WT) MEFs from thiol oxidant diamide and the lipid oxidant tert-butyl hydroperoxide (TBH) (Figure 2A). Given that glutathione is the major antioxidant produced by the cell and plays a central role in regulating the intracellular redox state (Meister, 1995; Trachootham et al., 2009), one testable thesis is that Δ Np63 α mitigates oxidative stress through transcriptional regulation of glutathione metabolism genes. Glutathione is synthesized in two sequential steps that are catalyzed by glutamate-cysteine

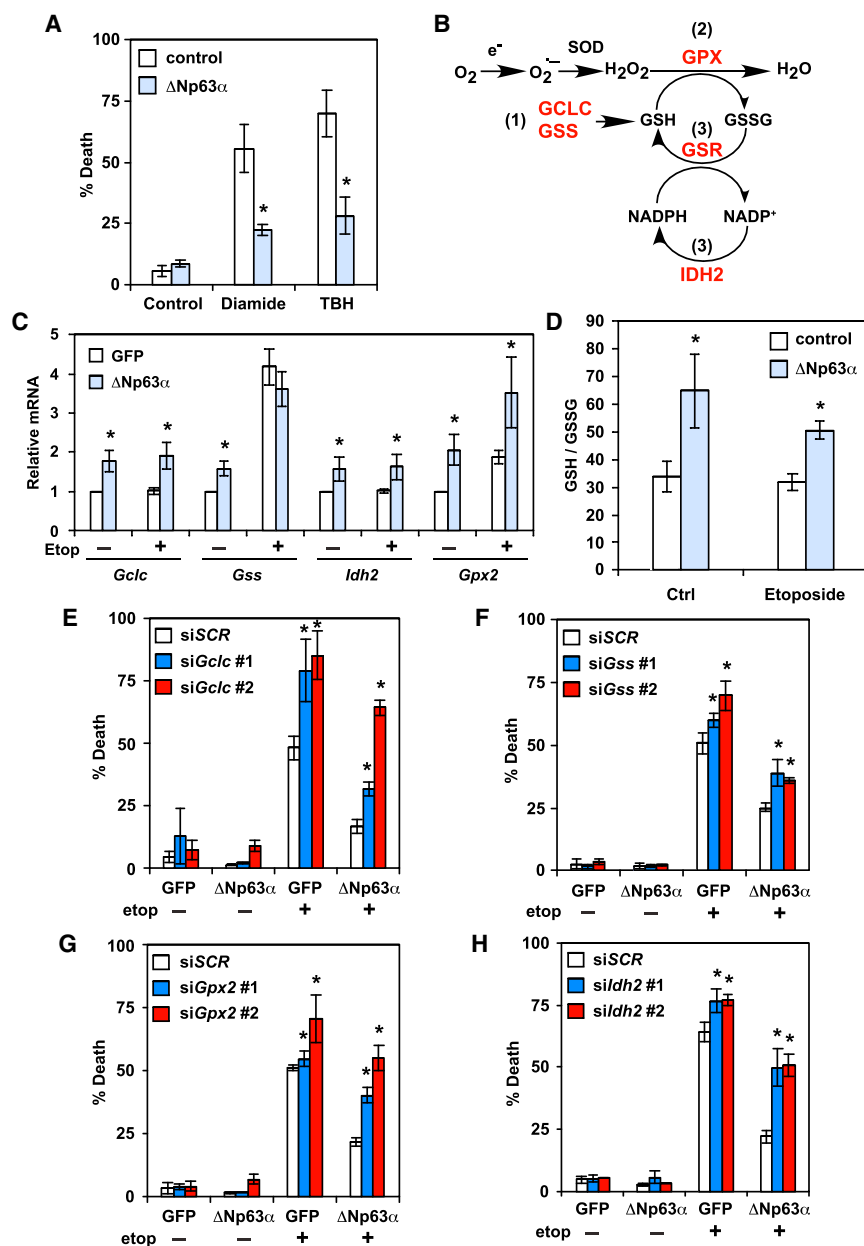


Figure 2. Δ Np63 α Controls the Homeostasis of Intracellular Redox through Transcriptional Control of Glutathione Metabolism Genes

(A) SV40-transformed WT MEFs transduced with the indicated retrovirus were left untreated or treated with diamide (100 μ M) for 4 hr or tert-butyl hydroperoxide (TBH, 50 μ M) for 7 hr. Cell death was quantified by propidium iodide staining (mean \pm SD, n = 3).

(B) A schematic of the effect of Δ Np63 α on glutathione metabolism. Overexpression of Δ Np63 α in cells generates a more reduced intracellular redox state and increases resistance to oxidative damage through coordinated regulation of glutathione metabolism genes (highlighted in red). Specifically, Δ Np63 α upregulates enzymes responsible for glutathione biosynthesis, ROS detoxification by glutathione, and regeneration of GSH from GSSG. (C) SV40-transformed *Bax*^{-/-}*Bak*^{-/-} MEFs transduced with the indicated retrovirus were left untreated or treated with etoposide for 6 hr. The mRNA levels of the indicated genes were analyzed by qRT-PCR (mean \pm SD, n = 3).

(D) SV40-transformed *Bax*^{-/-}*Bak*^{-/-} MEFs transduced with the indicated retrovirus were left untreated or treated with etoposide for 24 hr. Intracellular reduced (GSH) and oxidized (GSSG) glutathione concentrations were determined using an enzyme-cycling assay and normalized against protein concentration. Normalized [GSH] and [GSSG] were used to calculate the GSH/GSSG ratio (mean \pm SD, n = 3).

(E–H) SV40-transformed *Bax*^{-/-}*Bak*^{-/-} MEFs stably expressing GFP or Δ Np63 α were transfected with scrambled siRNA (siScr) or siRNA against *Gclc* (E), *Gss* (F), *Gpx2* (G), or *Idh2* (H). Cells were subsequently left untreated or treated with etoposide for 3 days. Cell death was quantified by propidium iodide staining (mean \pm SD, n = 3).

*p < 0.05 (Student's t test).

ligase (GCL) and glutathione synthetase (GSS), respectively (Meister, 1995; Figure 2B). It exists in both reduced (glutathione [GSH]) and oxidized (glutathione disulfide [GSSG]) states (Meister, 1995). GSH participates in the detoxification of hydrogen peroxide and lipid hydroperoxides through various glutathione peroxidases (GPXs) (Figure 2B). During this process, GSH is oxidized to GSSG, which can then be recycled back to GSH through the action of glutathione reductase (GSR) at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Figure 2B). NADPH can be generated by several enzymes, including isocitrate dehydrogenase 2 (IDH2). Significantly, gene expression profiling revealed that genes involved in the glutathione redox pathway were upregulated

in Δ Np63 α -overexpressing cells both before and after DNA damage (Figure S4). qRT-PCR confirmed that GCLC (the catalytic subunit of GCL), GSS, IDH2, and GPX2 were upregulated by Δ Np63 α (Figure 2C). Hence, it appears that Δ Np63 α orchestrates a transcription program to coordinate the biosynthesis of glutathione, the utilization of GSH as an antioxidant, and the regeneration of GSH from GSSG (Figure 2B), the outcome of which would be an increased GSH-to-GSSG ratio, indicating a more reduced redox state. Indeed, overexpression of Δ Np63 α significantly increased the GSH/GSSG ratio in cells both before and after etoposide treatment (Figure 2D). The functional significance of these Δ Np63 α -induced glutathione metabolism genes in regulating ROS-induced cell death was interrogated by small interfering RNA (siRNA)-mediated knockdown (Figure S3). Knockdown of GCLC, GSS, GPX2, or IDH2 compromised the ability of Δ Np63 α to protect *Bax*^{-/-}*Bak*^{-/-} DKO cells against DNA damage to varying extents (Figures

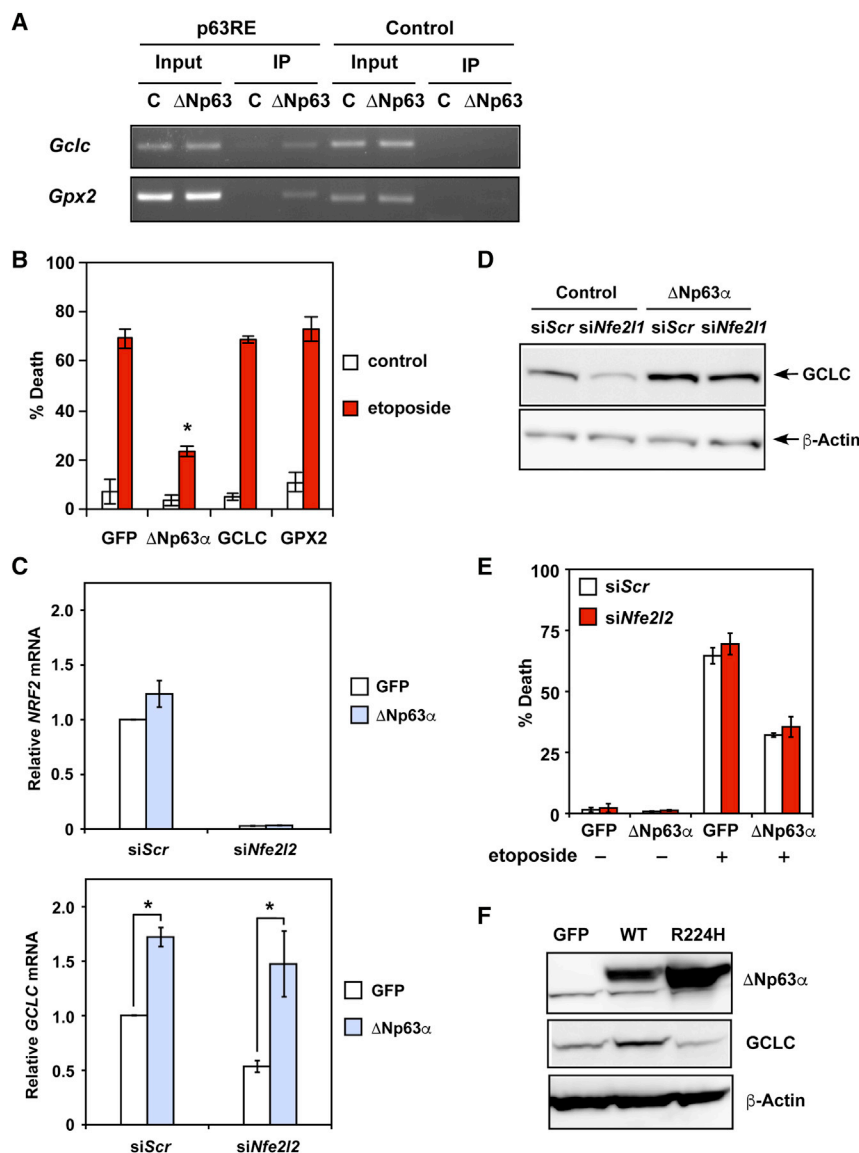


Figure 3. ΔNp63α Regulates Glutathione Metabolism and Oxidative Stress-Induced Cell Death Independent of NRF2

(A) SV40-transformed *Bax*^{-/-} *Bak*^{-/-} MEFs transduced with a control or ΔNp63α-expressing retrovirus were subjected to chromatin immunoprecipitation using the anti-p63 antibody, followed by PCR amplification of intron 1 of *Gclc* and *Gpx2*, respectively. A 5' sequence located ~3–4 kb upstream of the transcription start site of *Gclc* or *Gpx2* served as a negative control.

(B) SV40-transformed *Bax*^{-/-} *Bak*^{-/-} DKO MEFs transduced with the indicated retrovirus were left untreated or treated with etoposide. Cell death was quantified by propidium iodide staining (mean ± SD, n = 3).

(C) SV40-transformed *Bax*^{-/-} *Bak*^{-/-} MEFs transduced with retrovirus expressing GFP or ΔNp63α were transfected with siScr or siRNA against *Nrf2* (*Nfe2l2*). The mRNA levels of *Nrf2* (*Nfe2l2*) and *Gclc* were analyzed by qRT-PCR (mean ± SD, n = 3).

(D) Protein lysates from cells described in (C) were analyzed by immunoblots using the indicated antibodies.

(E) SV40-transformed *Bax*^{-/-} *Bak*^{-/-} MEFs stably expressing GFP or ΔNp63α were transfected with scrambled siRNA or siRNA against *Nrf2* (*Nfe2l2*). Cells were subsequently left untreated or treated with etoposide for 3 days. Cell death was quantified by propidium iodide staining (mean ± SD, n = 3).

(F) SV40-transformed *Bax*^{-/-} *Bak*^{-/-} MEFs transduced with the indicated retrovirus were analyzed by immunoblots using the indicated antibodies.

*p < 0.05 (Student's t test).

2E–2H). The protective effect of ΔNp63α was also mitigated by the GCLC inhibitor buthionine sulfoximine (BSO) (Figure S4). Of note, knockdown of these genes also enhanced etoposide-induced cell death in control DKO cells, but to a lesser degree (Figures 2E–2H), supporting the involvement of oxidative stress in this form of cell death. Collectively, our data show that ΔNp63α orchestrates a tightly coupled glutathione metabolic circuit to alleviate oxidative stress (Figure 2B), protecting apoptosis-defective cells from ROS-mediated necrotic cell death.

Because DNA binding activity is required for ΔNp63α to alleviate oxidative stress, we searched for the p63 response element in glutathione metabolism genes (Hoh et al., 2002; Perez et al., 2007). Potential p63 binding sites were identified in intron 1 of GCLC and GPX2. Direct targeting of ΔNp63α to these sites was demonstrated by chromatin immunoprecipitation (ChIP)

results highlight the importance of ΔNp63α in orchestrating a tightly coupled glutathione metabolic circuit in maintaining redox homeostasis. Because NRF2 (NFE2L2) is the most studied transcription factor that controls the expression of antioxidant genes, including GCLC (Motohashi and Yamamoto, 2004), we investigated whether ΔNp63α regulates GCLC through NRF2. Knockdown of *Nrf2* (*Nfe2l2*) had a minimal effect on ΔNp63α-mediated upregulation of GCLC even though it partially reduced GCLC in control cells (Figures 3C and 3D). Consequently, the protective effect of ΔNp63α against DNA damage-induced necrotic death was not affected by NRF2 loss (Figure 3E). Furthermore, the DNA-binding incompetent R224H mutant of ΔNp63α failed to upregulate GCLC (Figure 3F). In summary, these data not only illustrate an NRF2-independent regulation of GCLC by ΔNp63α but also indicate that ΔNp63α is a central regulator of glutathione biogenesis.

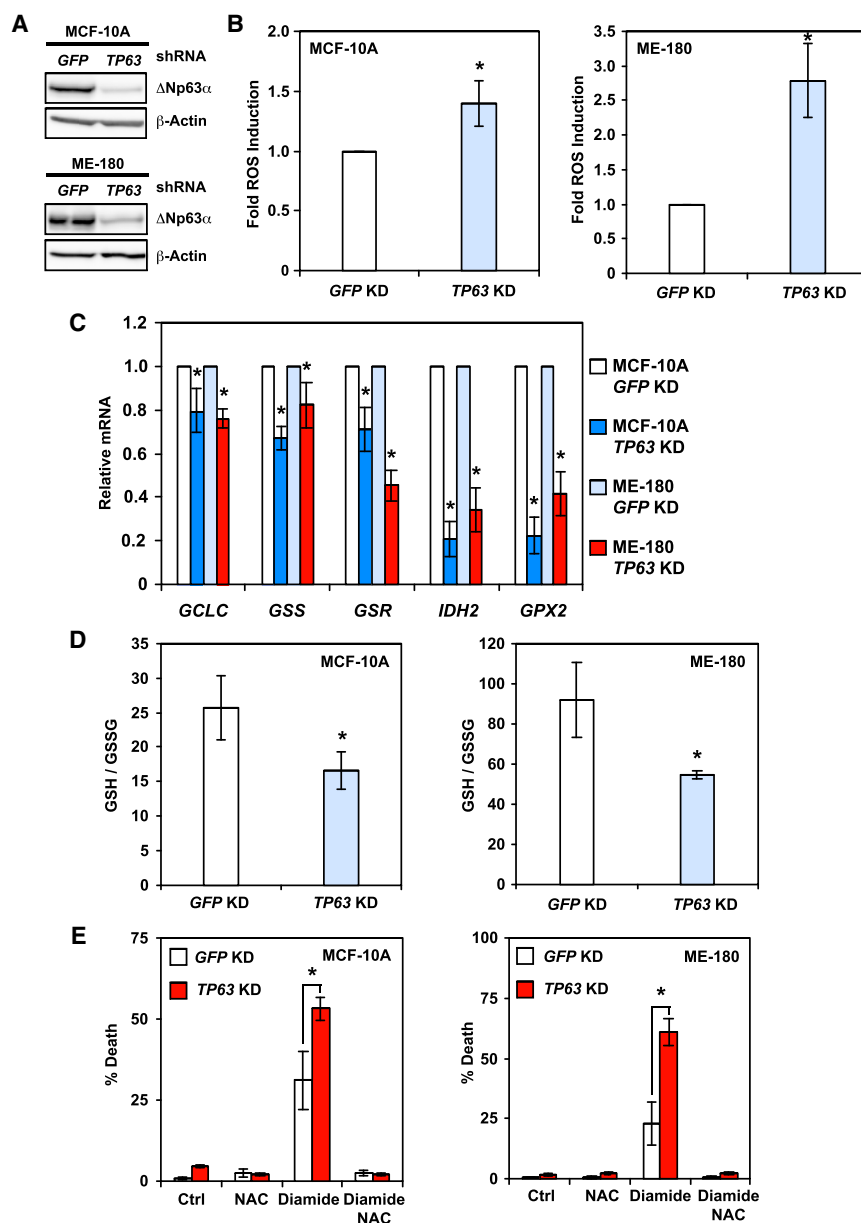


Figure 4. Deficiency of $\Delta Np63\alpha$ Generates a More Oxidized Intracellular Redox State and Sensitizes Cells to Oxidative Stress

(A) MCF-10A or ME-180 cells transduced with lentivirus expressing shRNA against GFP or *TP63* were analyzed by immunoblots using the indicated antibodies.

(B) MCF-10A or ME-180 cells transduced with lentivirus expressing shRNA against GFP or *TP63* were stained with CM- H_2 DCFDA or H_2 DCFDA. Oxidation of the respective dyes was quantified by flow cytometry. Data show the fold increase of ROS induced by knockdown of p63 (mean \pm SD, $n = 3$).

(C) MCF-10A or ME-180 cells were transduced with lentivirus expressing shRNA against GFP or *TP63*. The mRNA levels of the indicated genes were analyzed by qRT-PCR (mean \pm SD, $n = 3$).

(D) MCF-10A or ME-180 cells transduced with lentivirus expressing shRNA against GFP or *TP63* were analyzed for intracellular reduced (GSH) and oxidized (GSSG) glutathione concentrations using an enzyme-cycling assay. Normalized [GSH] and [GSSG] were used to calculate the GSH/GSSG ratio (mean \pm SD, $n = 3$).

(E) MCF-10A or ME-180 cells transduced with lentivirus expressing shRNA against GFP or *TP63* were left untreated or treated with the oxidant diamide in combination with the antioxidant NAC for 6 hr (MCF-10A) or 4 hr (ME-180). Cell death was quantified by propidium iodide staining (mean \pm SD, $n = 3$).

* $p < 0.05$ (Student's *t* test).

Deficiency of $\Delta Np63\alpha$ Generates a More Oxidized Intracellular Redox State and Sensitizes Cells to Oxidative Stress

To investigate the role of endogenous $\Delta Np63\alpha$ in regulating redox homeostasis, knockdown of p63 using a validated small hairpin RNA (shRNA) that targets all p63 isoforms (Godar et al., 2008) was performed in cell lines where $\Delta Np63\alpha$ but not TAp63 protein was detected by immunoblots (Figure 4A). Consistent with our gain-of-function studies, knockdown of endogenous $\Delta Np63\alpha$ led to increased intracellular ROS in the human mammary epithelial cell line MCF-10A and the cervical carcinoma cell line ME-180 (Figure 4B). Knockdown of $\Delta Np63\alpha$ using miR30-based shRNA or siRNA specific for $\Delta Np63$ also increased ROS (Figure S5). Because MCF-10A cells

have been reported to express low levels of TAp63 that are below the immunoblot detection limit (Figure 4A), knockdown of either TAp63 or $\Delta Np63\alpha$ was performed in MCF-10A cells using isoform-specific siRNA. Knockdown of $\Delta Np63\alpha$ increased ROS, whereas knockdown of TAp63 increased $\Delta Np63$ expression and slightly reduced ROS (Figure S5). Together, these results support that $\Delta Np63\alpha$ suppresses ROS accumulation. Importantly, qRT-PCR showed that $\Delta Np63\alpha$ knockdown downregulated GCLC, GSS, IDH2, and GPX2 (Figure 4C)—the same set of genes that was upregulated by $\Delta Np63\alpha$ (Figure 2C). Of note, GSR was suppressed by loss of endogenous $\Delta Np63\alpha$ in human cells but not induced by $\Delta Np63\alpha$ overexpression in MEFs (Figure 4C and data not shown), which might reflect cell-type-specific regulation. Consistent with downregulation of glutathione metabolism genes by $\Delta Np63\alpha$ knockdown, the GSH/GSSG ratio was reduced in cells deficient for $\Delta Np63\alpha$ (Figure 4D), indicating a more oxidized redox state. Consequently, $\Delta Np63\alpha$ knockdown enhanced diamide-induced cell death, which was blocked by NAC (Figure 4E). Furthermore, $\Delta Np63\alpha$ knockdown slightly increased baseline cell death over time and sensitized cells to chemotherapeutic agent-induced cell death (Figure S5). Altogether, our loss-of-function studies revealed

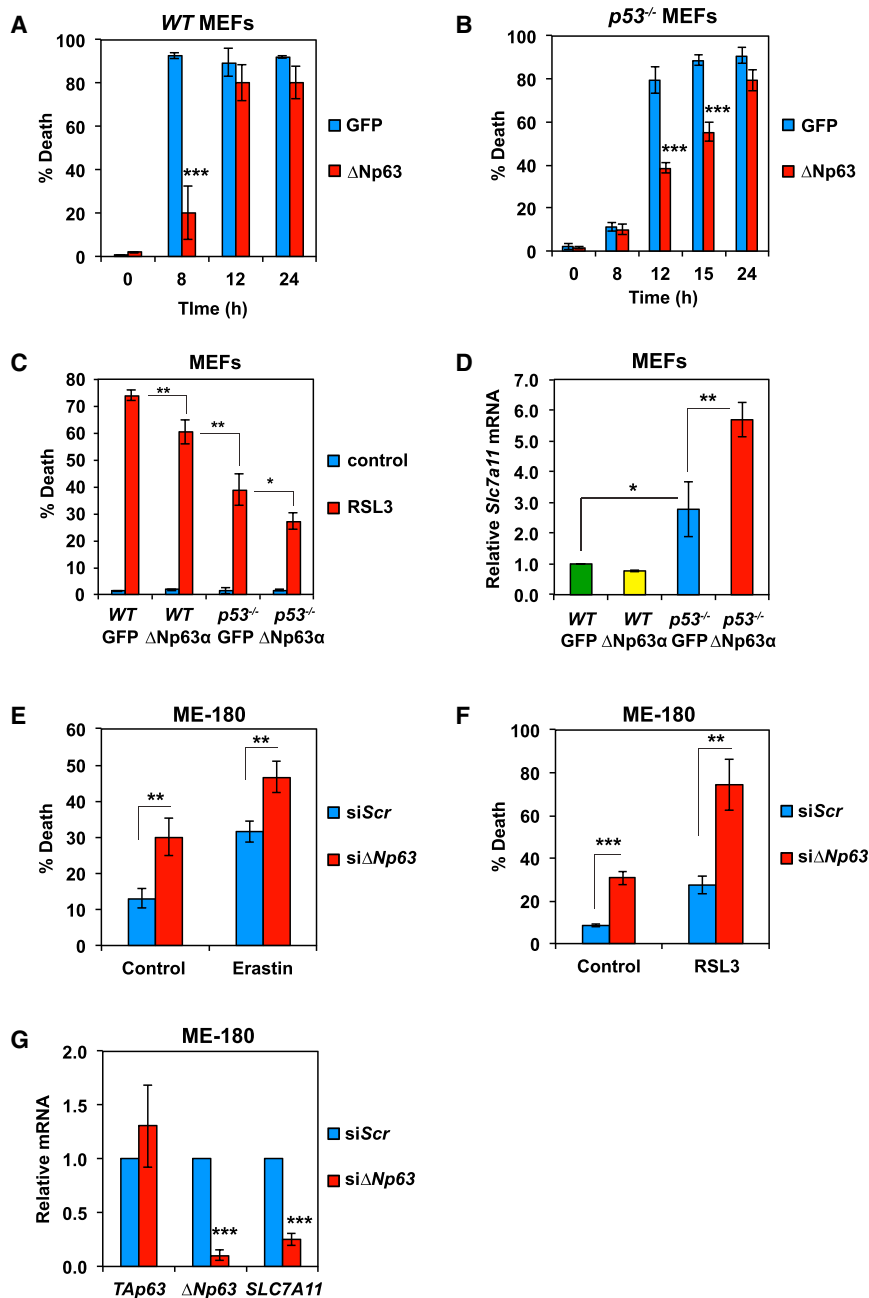


Figure 5. Δ Np63 α Can Inhibit Ferroptosis Independent of p53

(A) SV40-transformed WT MEFs transduced with GFP or Δ Np63-expressing retrovirus were left untreated or treated with erastin (4 μ M) for the indicated times. Cell death was quantified by propidium iodide staining (mean \pm SD, n = 3). (B) SV40-transformed p53^{-/-} MEFs transduced with GFP or Δ Np63-expressing retrovirus were left untreated or treated with erastin (4 μ M) for the indicated times. Cell death was quantified by propidium iodide staining (mean \pm SD, n = 3). (C) SV40-transformed WT or p53^{-/-} MEFs transduced with GFP or Δ Np63-expressing retrovirus were left untreated or treated with RSL3 (1 μ M) for 8 hr. Cell death was quantified by propidium iodide staining (mean \pm SD, n = 3). (D) SV40-transformed WT or p53^{-/-} MEFs transduced with retrovirus expressing GFP or Δ Np63 were subjected to qRT-PCR analysis of *Slc7a11* mRNA. Data were normalized against β -actin (mean \pm SD, n = 3). (E) ME-180 cells, transfected with scrambled siRNA or siRNA against Δ Np63, were left untreated or treated with erastin (20 μ M) for 72 hr. Cell death was quantified by propidium iodide staining (mean \pm SD, n = 3). (F) ME-180 cells, transfected with scrambled siRNA or siRNA against Δ Np63, were left untreated or treated with RSL3 (10 μ M) for 48 hr. Cell death was quantified by propidium iodide staining (mean \pm SD, n = 3). (G) ME-180 cells, transfected with scrambled siRNA or siRNA against Δ Np63, were subjected to qRT-PCR analysis of the indicated genes (mean \pm SD, n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t test).

an important role of endogenous Δ Np63 α in maintaining redox homeostasis.

Δ Np63 α Can Inhibit Ferroptosis Independent of p53

Given that Δ Np63 α orchestrates glutathione homeostasis and glutathione depletion induces ferroptosis, we envisioned that Δ Np63 α might inhibit ferroptosis. Because p53 is reported to positively regulate ferroptosis through transcriptional repression of *Slc7a11* (Jiang et al., 2015), a central component of the cystine-glutamate antiporter (system X_c⁻), and Δ Np63 α can potentially function as a dominant-negative regulator of p53,

we compared the effect of Δ Np63 α on ferroptosis in both WT and p53^{-/-} MEFs. Consistent with the published results (Jiang et al., 2015), p53 deficiency protected MEFs from ferroptosis induced by erastin but only at an early time point (Figures 5A and 5B). Overexpression of Δ Np63 α protected both WT and p53^{-/-} MEFs from erastin-induced death, suggesting that the anti-ferroptotic activity of Δ Np63 α is independent of p53. Similar results were observed in RSL3-induced ferroptosis (Figure 5C). Although the effect of Δ Np63 α on glutathione homeostasis is probably sufficient to inhibit ferroptosis, Δ Np63 α may also regulate the expression of *Slc7a11* directly or indirectly through p53. Interestingly, Δ Np63 α upregulated *Slc7a11* expression in p53^{-/-} but not in WT MEFs, where p53 suppressed *Slc7a11* expression (Figure 5D), suggesting that Δ Np63 α is unable to abrogate p53-mediated transcriptional repression of *Slc7a11*. Together, these findings suggest that the protective effect of Δ Np63 α against ferroptosis is probably due to its transcriptional control of glutathione metabolism genes in WT MEFs, whereas its regulation of *Slc7a11* further

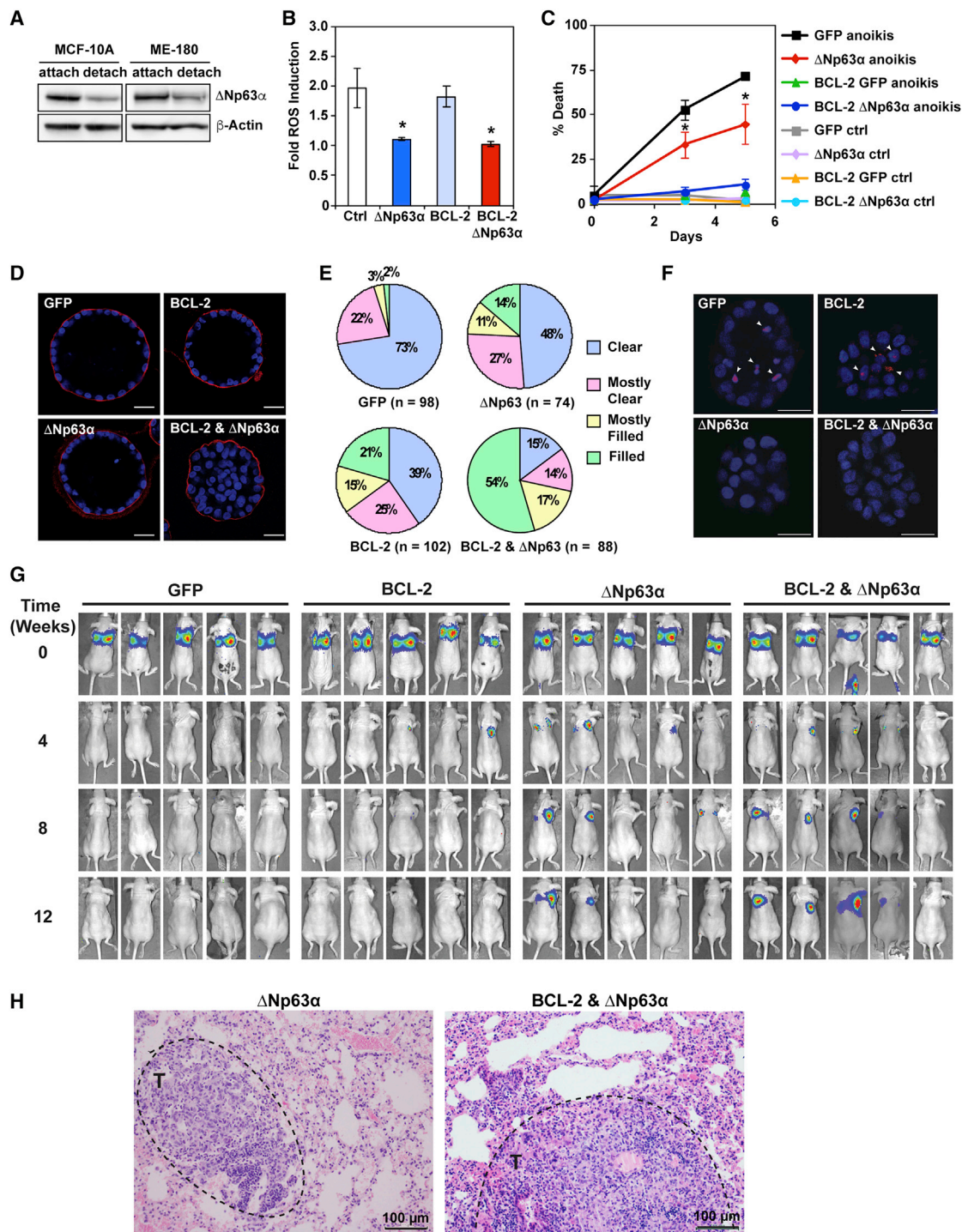


Figure 6. ΔNp63α Mitigates Anoikis-Induced Oxidative Stress and Cooperates with Anti-Apoptotic BCL-2 to Disrupt the Luminal Clearance of Mammary Acini and Promote Cancer Metastasis

(A) MCF-10A or ME-180 cells were grown on poly-HEMA-coated plates in the presence of 1% methylcellulose for 12 hr to induce anoikis. Paired samples grown under either regular or detachment conditions were assessed by anti-p63 and anti-actin immunoblots.

(B) MCF-10A cells transduced with the indicated retrovirus were grown on poly-HEMA-coated plates in the presence of 1% methylcellulose to induce anoikis. Paired samples grown under either regular or detachment conditions were stained with DHE at 1.5 days. Oxidation of DHE was quantified by flow cytometry. Data show the fold increase of ROS induced by anoikis (mean ± SD, n = 3).

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contributes to its anti-ferroptotic activity in p53-deficient MEFs. We subsequently performed loss-of-function studies of $\Delta Np63\alpha$ in ME-180 cells that are HPV-positive. $\Delta Np63\alpha$ knockdown enhanced ferroptosis and downregulated *SLC7A11* expression in ME-180 cells (Figures 5E–5G), which is consistent with our gain-of-function studies in MEFs. In contrast, *TAp63* knockdown in ME-180 cells neither reduced *SLC7A11* expression nor enhanced ferroptosis (Figure S5). Overall, our data suggest that $\Delta Np63\alpha$ can inhibit ferroptosis independent of p53.

$\Delta Np63\alpha$ Alleviates Anoikis-Induced Oxidative Stress and Cooperates with BCL-2 to Disrupt the Luminal Clearance of Mammary Acini and Promote Metastasis

The observation that antioxidants and anti-apoptotic BCL-2 work in concert to promote long-term clonogenic survival against matrix detachment (Schafer et al., 2009) prompted us to investigate whether $\Delta Np63\alpha$ can mitigate oxidative stress associated with anoikis and whether $\Delta Np63\alpha$ can cooperate with BCL-2 to prevent the luminal clearance of acini in 3D culture of MCF-10A cells. Interestingly, loss of matrix attachment led to downregulation of $\Delta Np63\alpha$ in both MCF-10A and ME-180 cells, where $\Delta Np63\alpha$ knockdown induced oxidative stress (Figures 6A and 4B). Notably, overexpression of $\Delta Np63\alpha$, but not BCL-2, prevented matrix detachment-induced ROS accumulation in MCF-10A cells even though $\Delta Np63\alpha$ provided less protection against cell death than BCL-2 (Figures 6B and 6C). To investigate long-term survival upon loss of matrix attachment, MCF-10A cells were seeded onto a reconstituted basement membrane under conditions that enable the formation of 3D acini, where the inner, matrix-deprived cells are eliminated to facilitate lumen formation (Schafer et al., 2009). Overexpression of either BCL-2 or $\Delta Np63\alpha$ only weakly inhibited luminal clearance, whereas co-expression of BCL-2 and $\Delta Np63\alpha$ markedly blocked luminal clearance (Figures 6D and 6E). Luminal clearance of MCF-10A acini was evident within 2 weeks after seeding, whereas co-expression of BCL-2 and $\Delta Np63\alpha$ potently blocked luminal clearance for up to 1 month (Figures 6D and 6E). Importantly, overexpression of $\Delta Np63\alpha$, but not BCL-2, suppressed ROS accumulation in the matrix-deprived luminal cells at early time points before these cells were eliminated (Figure 6F). Both *TAp63* and $\Delta Np63\alpha$ have been shown to regulate the expression of adhesion molecules, and adenovirus-mediated knockdown of p63 was reported to induce anoikis (Carroll et al., 2006). However, we did not observe obvious adhesion problems when p63 was silenced, which is in accordance with a previous report

(Senoo et al., 2007). Notably, only $\Delta Np63\alpha$, but not *TAp63*, is capable of regulating oxidative stress (Figure 1; Figure S5). Ferrostatin-1 had minimal effect on the luminal clearance of mammary acini in 3D culture (Figure S6).

Because resistance to anoikis has been proposed to promote cancer metastasis, and oxidative stress has been shown to limit cancer metastasis (Buchheit et al., 2014; Piskounova et al., 2015), we next examined whether overexpression of $\Delta Np63\alpha$ and/or BCL-2 promotes lung metastasis of a triple-negative breast cancer cell line, MDA-MB-231, that is transduced with luciferase for bioluminescence imaging. Tail vein injection of $\Delta Np63\alpha$ -overexpressing, luciferase-transduced MDA-MB-231 cells into immunodeficient mice resulted in lung metastases in 2 of 5 mice, whereas GFP-expressing control cells failed to metastasize to the lungs (Figures 6G and 6H). These findings are consistent with a report showing that NAC increases the metastatic melanoma burden because of increased ROS and reduced GSH/GSSG ratios in metastatic tumors (Piskounova et al., 2015). Although overexpression of BCL-2 alone did not increase lung metastasis, co-expression of BCL-2 and $\Delta Np63\alpha$ further increased lung metastases, and 4 of 5 mice, following injection, developed lung metastases (Figures 6G and 6H). In summary, combined inhibition of apoptosis and oxidative stress through co-expression of BCL-2 and $\Delta Np63\alpha$ confers clonogenic survival against anoikis and promotes lung metastasis.

Amplification of TP63 in Human Squamous Cell Lung Cancer Upregulates Glutathione Metabolism Genes to Promote Anchorage-Independent Growth

In contrast to the tumor suppressor function of p53, $\Delta Np63\alpha$ has been implicated as an oncogene and is frequently amplified and/or overexpressed in human squamous cell carcinoma of the lung and of the head and neck (Dötsch et al., 2010; Keyes et al., 2011; Tonon et al., 2005). Because cancers often have an increased demand for antioxidant capacity compared with normal tissues, likely because of inherent metabolic derangements that increase ROS production (Trachootham et al., 2009), $\Delta Np63\alpha$ might promote tumorigenesis by reducing oxidative stress. To explore this possibility, we analyzed a lung squamous cell carcinoma dataset from The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Research Network, 2012) to determine whether amplification/overexpression of p63 upregulates the glutathione metabolism pathway in primary human tumors. Consistent with previously published reports (Dötsch et al., 2010; Tonon et al., 2005), *TP63* amplification is a frequent

(C) MCF-10A cells transduced with the indicated retrovirus were grown on poly-HEMA-coated plates in the presence of 1% methylcellulose to induce anoikis. Cell death was quantified by annexin-V staining at the indicated times (mean \pm SD, $n = 3$).

(D and E) MCF-10A cells transduced with the indicated retrovirus were cultured in a reconstituted basement membrane (Matrigel) for 31 days. Acini were fixed and stained for Laminin 5 (red) and the nuclear stain DAPI (blue). The luminal cellularity of acini was scored using confocal microscopy: clear, 0%–25% of the luminal space filled with cell nuclei; mostly clear, 25%–50%; mostly filled, 50%–75%; filled, 75%–100%. Representative confocal microscopy images from three independent experiments are shown in (D). Scale bars, 25 μ m. The data shown in (E) are the average of three independent experiments.

(F) MCF-10A cells transduced with the indicated retrovirus were cultured in Matrigel for 6–8 days. Acini were stained with DHE (red) and Hoechst 33342 (blue). Representative confocal microscopy images are shown. Arrowheads indicate DHE-high cells. Scale bars, 25 μ m.

(G) Luciferase-transduced MDA-MB-231 cells overexpressing GFP, BCL-2, $\Delta Np63\alpha$, or both $\Delta Np63\alpha$ and BCL-2 were injected into the tail vein of immunodeficient mice, and lung colonization was analyzed by *in vivo* bioluminescence imaging ($n = 5$ for each group).

(H) Representative histopathological images of lungs with metastatic tumors (black dashed circle) from mice injected with luciferase-transduced MDA-MB-231 cells overexpressing $\Delta Np63\alpha$ or both $\Delta Np63\alpha$ and BCL-2. Scale bars, 100 μ m.

* $p < 0.05$ (Student's *t* test).

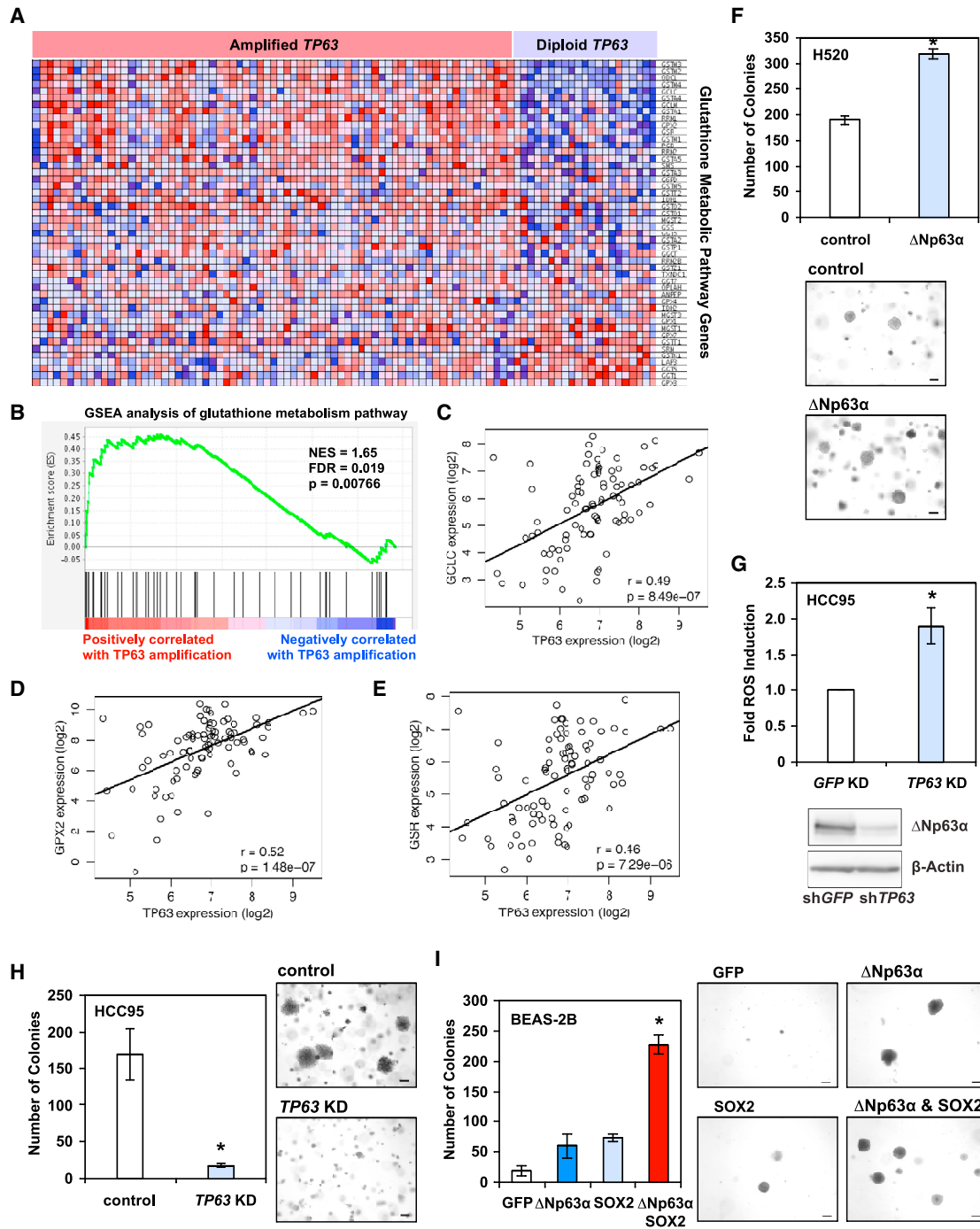


Figure 7. Amplification of *TP63* in Human Squamous Cell Lung Cancer Upregulates Glutathione Metabolism Genes and Promotes Anchorage-Independent Growth

(A) Heatmap of expression levels of Kyoto Encyclopedia of Genes and Genomes (KEGG) glutathione metabolism pathway genes in the TCGA lung squamous cell carcinoma samples harboring amplified or diploid *TP63* as indicated. The expression levels are plotted in a red-blue color scale, with red indicating high expression and blue indicating low expression.

(B) GSEA enrichment plot demonstrating enrichment of the KEGG glutathione metabolism pathway in TCGA lung squamous cell carcinoma samples with amplified *TP63* compared with diploid samples. Nominal $p = 0.00766$. False discovery rate (FDR) $q = 0.019$.

(C–E) Pairwise comparison of mRNA expression of *TP63* versus *GCLC* (C), *GPX2* (D), or *GSR* (E) in TCGA lung squamous cell carcinoma samples harboring amplified *TP63* with Pearson correlation and p values shown.

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event in human lung squamous cell carcinoma (~30%), and the majority of *TP63*-amplified tumors overexpress Δ Np63. We further interrogated the database using gene set enrichment analysis (GSEA) and found that tumor samples harboring *TP63* amplification and overexpressing Δ Np63 had significant enrichment of glutathione metabolic pathway genes compared with diploid tumor samples (Figures 7A and 7B). Furthermore, within the *TP63*-amplified subset, Δ Np63 expression levels positively correlated with the expression of several genes that were discovered through our mechanistic studies, including GCLC (pairwise correlation coefficient [r] = 0.49, p = 8.49e-07), GPX2 (r = 0.52, p = 1.48e-07), and GSR (r = 0.46, p = 7.29e-06) (Figures 7C–7E).

Given that oxidative stress is induced in response to matrix detachment, the ability of cells to form colonies in soft agar is not only determined by their proliferative potential but also by their resistance to oxidative stress. We next examined whether Δ Np63 α promotes anchorage-independent growth by inhibiting oxidative stress. Indeed, overexpression of Δ Np63 α in a squamous cell lung cancer cell line, H520, with low Δ Np63 α expression increased soft agar colony formation without enhancing cell proliferation (Figure 7F and data not shown). In contrast, Δ Np63 α knockdown in a squamous cell lung cancer cell line, HCC95, that highly expresses Δ Np63 α led to increased ROS accumulation and reduced soft agar colony formation (Figures 7G and 7H). Because genomic amplification on chromosome 3q26.33 harbors an oncogene, *SOX2*, in addition to *TP63* (Bass et al., 2009), we envisioned that *SOX2* and Δ Np63 α might cooperate to promote oncogenesis. Consistent with a previous report (Bass et al., 2009), overexpression of *SOX2* alone only slightly increased colony formation in soft agar (Figure 7I). Significantly, *SOX2* and Δ Np63 α synergized to transform normal human bronchial epithelial cells, BEAS-2B (Figure 7I). Collectively, these data support the notion that amplification of *TP63* may be a strategy by which squamous cell lung cancer evades oxidative stress and, thereby, promotes tumorigenesis.

DISCUSSION

Here we report that Δ Np63 α prevents oxidative stress and cooperates with apoptotic inhibition to promote long-term cellular survival against DNA damage and extracellular matrix detachment, highlighting the quintessence of suppressing oxidative stress in promoting long-term survival (Figure S7). Caspase-dependent apoptosis has long been considered the predominant pathway for cell death. Apoptosis can be initiated through either intrinsic death signals, such as DNA damage, loss of matrix attachment, cytokine/growth factor deprivation, and

endoplasmic reticulum (ER) stress, or extrinsic death signals that are mediated by the ligand engagement of death receptors, including FAS and TNF-R1. Studies of extrinsic cell death discovered a necrotic program, necroptosis, when apoptosis is inhibited (Pasparakis and Vandenabeele, 2015; Sun and Wang, 2014; Yuan and Kroemer, 2010). The discovery of programmed forms of necrotic death represents a significant conceptual advance for cell death research. However, it remains unsettled whether intrinsic death signals also activate necrosis and whether intrinsic necrosis, if it exists, is also programmed. We and others have previously shown that cells incompetent of undergoing apoptosis still succumb to oxidative stress upon DNA damage or loss of matrix attachment and display morphological features of necrosis (Schafer et al., 2009; Tu et al., 2009). The current study showed that oxidative stress-induced necrotic death in these settings could be blocked by overexpression of Δ Np63 α through coordinated regulation of glutathione biosynthesis, utilization, and regeneration. In fact, Δ Np63 α guards against oxidative stress triggered by a wide array of death stimuli in several different cell lines, including intrinsic apoptotic signals, thiol or lipid oxidants, and ferroptosis-inducing agents. These data support the hypothesis that Δ Np63 α is a key cellular guardian against oxidative stress analogous to antiapoptotic BCL-2 family proteins that act at the convergence of diverse apoptosis-inducing signaling pathways. Overall, our data support the theory that intrinsic death signals can trigger necrosis when apoptosis is inhibited and intrinsic necrosis is also programmed.

Inhibition of oxidative stress may be especially critical to the well-being of long-lived cells such as stem cells (Holmström and Finkel, 2014; Kobayashi and Suda, 2012). Because stem cells must remain capable of both self-renewal and repletion of lost progeny throughout an organism's lifespan, they are expected to be more tolerant to the long-term exposure to ROS and oxidative stress. In fact, Δ Np63 α is important in maintaining the stem cell populations of stratified epithelia (Dötsch et al., 2010; Keyes et al., 2011; Senoo et al., 2007). In addition to sustaining the proliferative capacity of epithelial stem cells as proposed previously (Keyes et al., 2011; Senoo et al., 2007), our data imply that Δ Np63 α may also promote stem cell survival through its antioxidant function. Intriguingly, *p63*^{+/-} mice have a shortened lifespan and display features of accelerated aging, and *p63*-deficient cells exhibit heightened cellular senescence (Keyes et al., 2005), all of which could be caused by oxidative damage (Balaban et al., 2005) and may be linked to oxidative stress resulting from the loss of Δ Np63 α .

Our findings highlight the need for a simultaneous blockade of apoptosis and oxidative stress to promote long-term cellular

(F) H520 cells transduced with control retrovirus or retrovirus expressing Δ Np63 α were plated in soft agar for 18 days. Colonies with diameters larger than 200 μ m were counted (mean \pm SD, n = 3). Representative images are shown (scale bars, 200 μ m).

(G) HCC95 cells transduced with lentivirus expressing shRNA against GFP or *TP63* were stained with CM-H₂DCFDA or subjected to immunoblot analysis using the indicated antibodies. Oxidation of CM-H₂DCFDA was quantified by flow cytometry. Data show the fold increase of ROS induced by knockdown of *TP63* (mean \pm SD, n = 3).

(H) HCC95 cells transduced with lentivirus expressing control or shRNA against *TP63* were plated in soft agar for 12 days. Colonies with diameters larger than 200 μ m were counted (mean \pm SD, n = 3). Representative images are shown (scale bars, 200 μ m).

(I) BEAS-2B cells transduced with the indicated retrovirus were plated in soft agar for 18 days. Colonies with diameters larger than 200 μ m were counted (mean \pm SD, n = 3). Representative images are shown (scale bars, 200 μ m).

* p < 0.05 (Student's t test).

well-being, a strategy likely hijacked by cancer. To evade apoptotic checkpoints, cancer cells often overexpress anti-apoptotic BCL-2 family proteins through chromosome translocation involving *BCL-2* (Korsmeyer, 1992) or amplification of *BCL-X_L* and *MCL-1* (Beroukhi et al., 2010). Likewise, amplification of *TP63* can be a strategy for cancer cells to evade oxidative stress-induced cell death. The importance of antioxidant defense in tumorigenesis is also supported by the discovery of frequent loss-of-function mutations of *KEAP1* and gain-of-function mutations of *NRF2* in human cancers (Hayes and McMahon, 2009). *NRF2* controls cellular adaptation to oxidative stress by inducing antioxidant and detoxification genes, whereas *KEAP1* sequesters *NRF2* in the cytoplasm and promotes the degradation of *NRF2*. Here we showed that Δ Np63 α could regulate *GCLC*, the rate-limiting GSH biosynthetic enzyme, through an *NRF2*-independent manner, supporting a central role of Δ Np63 α in glutathione biogenesis. In addition, we showed that Δ Np63 α could inhibit ferroptosis independent of p53. Given the recent discovery of ferroptosis regulation as one of the tumor suppressor mechanisms of p53 (Jiang et al., 2015), suppression of ferroptosis by Δ Np63 α may contribute to its oncogenic properties. Our study highlights the importance of evading two cell death mechanisms in tumorigenesis, which may provide a selection pressure for genetic/epigenetic alterations with oncogenic advantages.

Although a detailed blueprint of the core apoptotic pathway has been constructed through biochemical and genetic studies over the past two decades, we are still unable to fully rescue cells from most death stimuli. The identification of oxidative stress as a critical mechanism compromising long-term survival of apoptosis-defective cells may open new avenues for therapeutic interventions aimed at preventing excessive cell loss during disease processes. Conversely, targeting the antioxidant pathway may hold promise for the future development of anti-cancer therapeutics by eliminating cancer cells that evade apoptotic checkpoints.

EXPERIMENTAL PROCEDURES

Plasmid Construction, Retrovirus Production, and siRNA

Murine Δ Np63 α was dually tagged with FLAG and hemagglutinin (HA) at the N terminus and cloned into murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-Puro or tagged with HA at the N terminus and cloned into MSCV-IRES-GFP. The target sequence of shRNA against mouse p63 is 5'-AGCACACGATCGAAACGTA-3'. The target sequence of scramble shRNA is 5'-GCGCGCTTTGTAGGATTTCG-3'. Lentivirus-mediated knockdown constructs for human p63 and GFP were described previously (Godar et al., 2008; Sancak et al., 2008). Lentiviral tetracycline-inducible miR30-based shRNA against human p63 was obtained from Open Biosystems. The target sequence is 5'-TCCGAGCTATGTCAGTACTATT-3'. The siRNA oligos were purchased from Thermo Fisher Scientific (Silencer Select oligos) or Dharmicon and summarized in Table S1.

Cell Culture and Viability Assays

Generation and transformation of MEFs were performed as described previously (Chen et al., 2015; Cheng et al., 2001). The HCC95 cell line was provided by Dr. John Minna at University of Texas Southwestern Medical Center. To induce anoikis, cells were grown on plates coated with poly(2-hydroxyethyl methacrylate) (HEMA; Sigma) in the presence of 1% methylcellulose (Sigma). Cell death was quantified by annexin-V (BioVision Technologies) or propidium iodide (Sigma) staining, followed by flow cytometric analysis using either a

FACSCalibur (BD Biosciences) or an LSRFortessa (BD Biosciences). Data were analyzed using CellQuest Pro (BD Biosciences) or FACSDiva (BD Biosciences).

Measurement of ROS

Production of ROS was monitored by flow cytometry using redox-sensitive dyes (Invitrogen), including H₂DCFDA, 5-(and-6)-chloromethyl-H₂DCFDA (CM-H₂DCFDA), DHE, C11-BODIPY^{665/676}, or MitoSOX Red. Oxidation of the ROS-sensitive dyes was quantified by flow cytometry. Median fluorescence in the appropriate detection channels was assessed by FlowJo (Tree Star). Fold induction of ROS was obtained by dividing the median fluorescence of experimental samples by that of control samples.

Reverse Transcription and qPCR

Total RNA was extracted from cells using TRIZOL (Invitrogen), and reverse transcription was performed with oligo-dT plus random decamer primers (Ambion) using Superscript II (Invitrogen). Quantitative PCR was performed with SYBR Green Master Mix (Applied Biosystems) using gene-specific primers, summarized in Table S2. For TaqMan probes, quantitative PCR was performed with TaqMan PCR Master Mix (Applied Biosystems). Quantitative PCR was performed on an ABI Prism 7300 sequence detection system (Applied Biosystems) or a ViiA 7 real-time PCR system (Applied Biosystems). Data were analyzed by normalization against GAPDH, β -actin, or 18S rRNA as described previously (Tu et al., 2009). The TaqMan probes for mouse GPX2 (TaqMan assay ID Mm00850074_g1) and human GCLC (TaqMan assay ID Hs00155249_m1) were obtained from Invitrogen.

3D Culture of MCF-10A Cells and Indirect Immunofluorescence

Microscopy

3D culture of MCF-10A cells was performed as described previously (Debnath et al., 2003). Briefly, 10⁴ cells were seeded onto one chamber of a 4-well chamber slide pre-coated with Matrigel (BD Biosciences). After 31 days, cells were fixed using 4% paraformaldehyde (Fisher Scientific), permeabilized using 0.5% Triton X-100, and sequentially incubated with anti-Laminin-5 (D4B5, EMD Millipore), Alexa Fluor 568-conjugated goat anti-mouse immunoglobulin G (IgG) (Invitrogen), anti-GFP (ab13970, Abcam), Alexa Fluor 488-conjugated goat anti-chicken IgG (Invitrogen), and the nuclear stain DAPI (Sigma). Images were acquired using the Leica TCS SP5-II confocal upright and the PerkinElmer UltraVIEW ERS confocal microscopes at the Molecular Cytology Core Facility at Memorial Sloan Kettering Cancer Center (MSKCC) and analyzed by MetaMorph software (Molecular Devices). To measure ROS levels, 3D culture of MCF-10A cells after 6–8 days was incubated with Hank's balanced salt solution (HBSS, Invitrogen) containing 5 μ M DHE (Invitrogen) and 3 μ g/mL Hoechst 33342 (Invitrogen) at 37°C for 30 min in a humidified 5% CO₂ incubator, washed once with HBSS, and then imaged immediately.

Reagents, Antibodies, and Immunoblot Analysis

The following chemicals were used: etoposide (Sigma), diamide (Sigma), TBH (Sigma), DPI (Sigma), NAC (Sigma), ferrostatin-1 (Sigma), necrostatin-1 (Sigma), GSK'872 (EMD Millipore), deferoxamine (Sigma), and Tiron (Sigma). The following antibodies were used: anti-FLAG (M2, Sigma), anti-p53 (FL393, Santa Cruz Biotechnology), anti-p63 (4A4, Santa Cruz Biotechnology), anti-GCLC (HPA036359, Sigma), anti-IDH2 (ab55271, Abcam), anti-RIP1 (610458, BD Biosciences), and anti- β -actin (A1978, Sigma). Cell lysates were resolved by NuPAGE gels (Invitrogen) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore). Antibody detection was accomplished using the enhanced chemiluminescence method (Western Lightning, PerkinElmer) and the LAS-3000 imaging system (Fujifilm).

Glutathione Assays

Measurement of intracellular GSH and GSSG was performed using the 5,5'-dithio-bis(2-nitrobenzoic acid)-glutathione reductase recycling method as described previously (Rahman et al., 2006). Briefly, the rate of 5-thio-2-nitrobenzoic acid (TNB) formation was determined by measuring the rate of change of absorbance at 412 nm. Linear regressions based on values obtained from standard curves were used to calculate concentrations of total and oxidized glutathione, which were then used to calculate the concentration

of reduced glutathione. Glutathione concentrations were normalized based on protein concentration determined using the BCA Kit (Pierce).

Gene Expression Profiling and Heatmap Plot

Total RNA was extracted from cells using TRIZOL (Invitrogen) and subjected to cleanup using the RNeasy Mini Kit (QIAGEN). RNA samples were submitted to the Laboratory for Clinical Genomics at the Washington University in St. Louis for microarray analysis using the GeneChip Mouse Gene 1.0 ST array (Affymetrix). The heatmap plot was generated using the Partek Genomics Suite (Partek).

Gene Set Enrichment and mRNA Expression Correlation Analysis

GSEA (Subramanian et al., 2005) was used to statistically evaluate the extent to which genes in the glutathione metabolism pathway (Kyoto Encyclopedia of Genes and Genomes, KEGG_GLUTATHIONE_METABOLISM, MSigDB v3.0) were dysregulated in TCGA lung squamous cell carcinoma tumor samples with *TP63* amplification. We first identified tumor samples with diploid ($n = 25$) and amplified *TP63* (inferred gain of ≥ 2 copies, $n = 91$). All genes expressed in tumor samples ($n = 107$) were sorted by mRNA expression change in *TP63*-amplified versus diploid samples (sorting from down- to up-regulation, Wilcoxon rank-sum test), and GSEA was used to evaluate the null hypothesis that genes in the glutathione metabolism gene set were not differentially expressed in amplified versus diploid samples (using 1,000 permutations). Pairwise correlations between *TP63* mRNA expression and mRNA expression levels of genes in the glutathione metabolism pathway in samples with *TP63* amplification were evaluated using the Pearson correlation coefficient, and regression lines were estimated using the ordinary least-squares method.

Xenograft Studies

Animal experiments were performed in accordance with the MSKCC Institutional Animal Care and Use Committee. Tail vein injection was performed as described previously (Minn et al., 2005). Briefly, 2×10^5 cells were injected into the lateral tail vein of athymic nude mice. Successful injections were confirmed by bioluminescence imaging. For the imaging, 75 mg/kg of D-Luciferin (Xenogen) in PBS was injected retro-orbitally into anesthetized mice. Bioluminescence images were obtained with the IVIS imaging system (Xenogen) and analyzed using Living Image software (Xenogen).

Statistical Analysis

Cell death, ROS induction, qRT-PCR, and glutathione assays were analyzed for statistical significance using unpaired Student's *t* test with $\alpha = 0.05$. Statistical analysis for GSEA is described above.

DATA AND SOFTWARE AVAILABILITY

The accession number for the microarray data reported in this paper is GEO: GSE106214.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.11.030>.

AUTHOR CONTRIBUTIONS

G.X.W. designed and conducted experiments and analyzed data. E.H.C. designed the research, analyzed data, and supervised the project. H.-C.T., Y.D., Y.W., S.T., Y.T.G., S.H., and H.L. conducted some experiments. A.J.S. analyzed data. J.J.H. supervised some experiments.

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