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Supplemental Information

ΔNp63 Inhibits Oxidative Stress-Induced Cell Death, Including Ferroptosis, and Cooperates with the BCL-2 Family to Promote Clonogenic Survival

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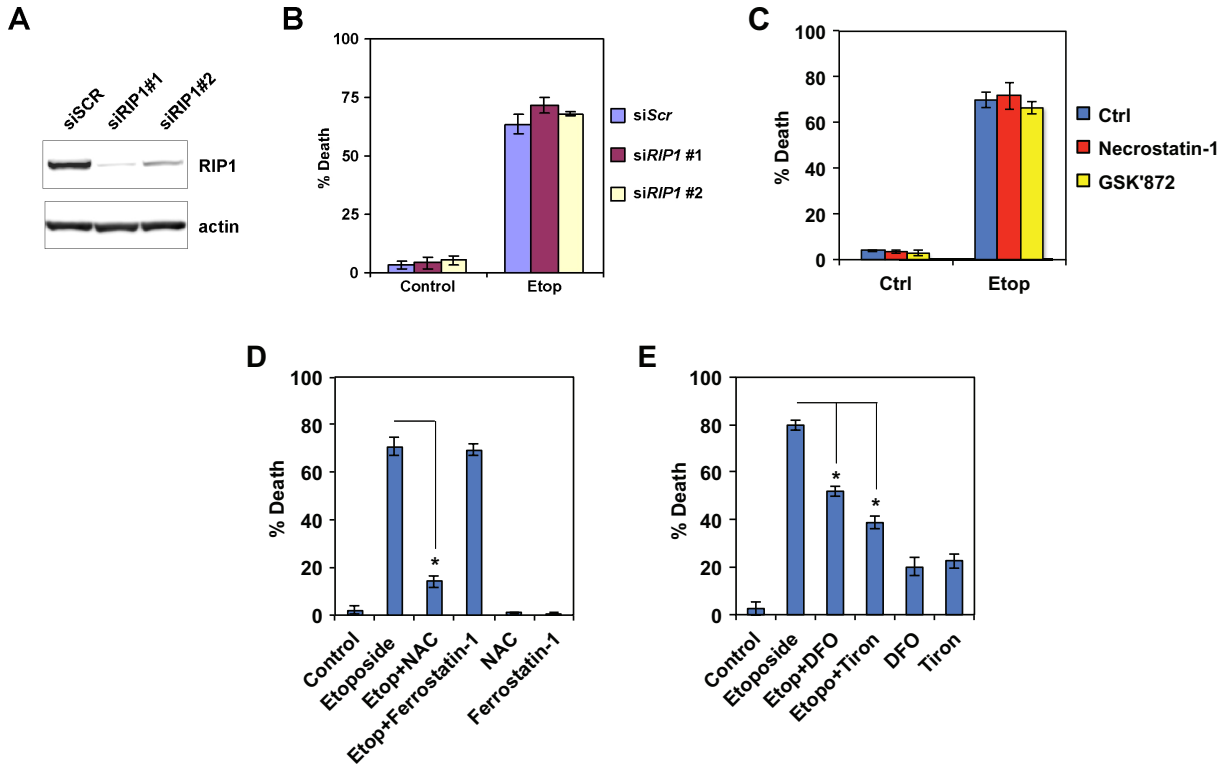
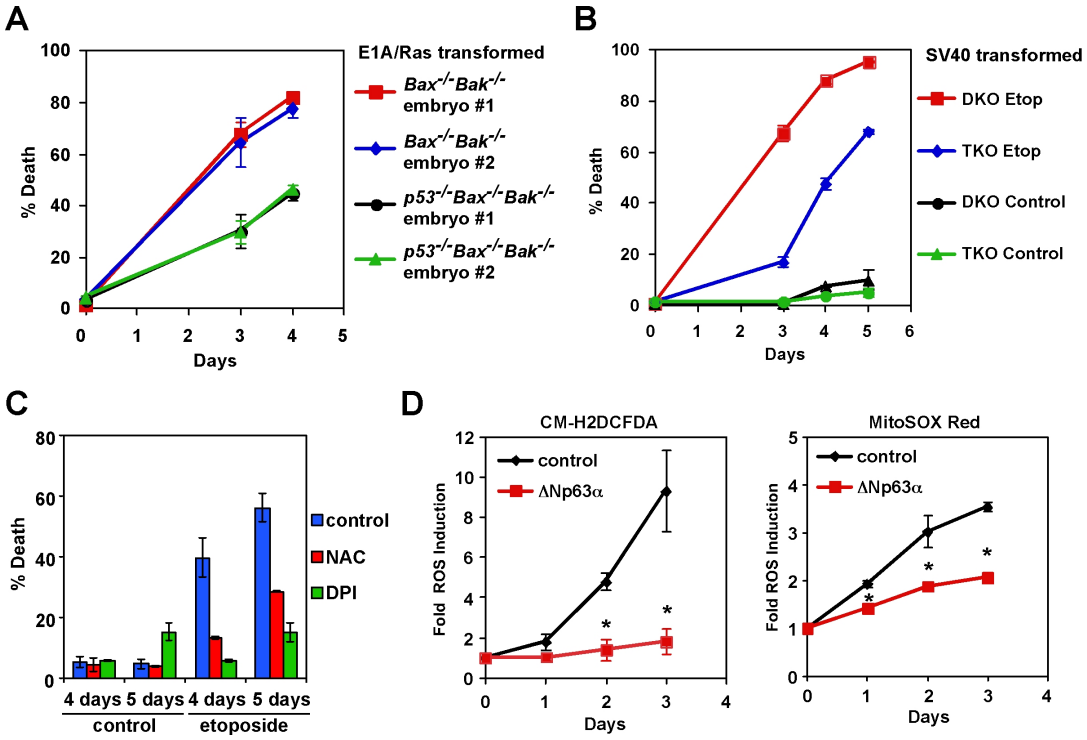
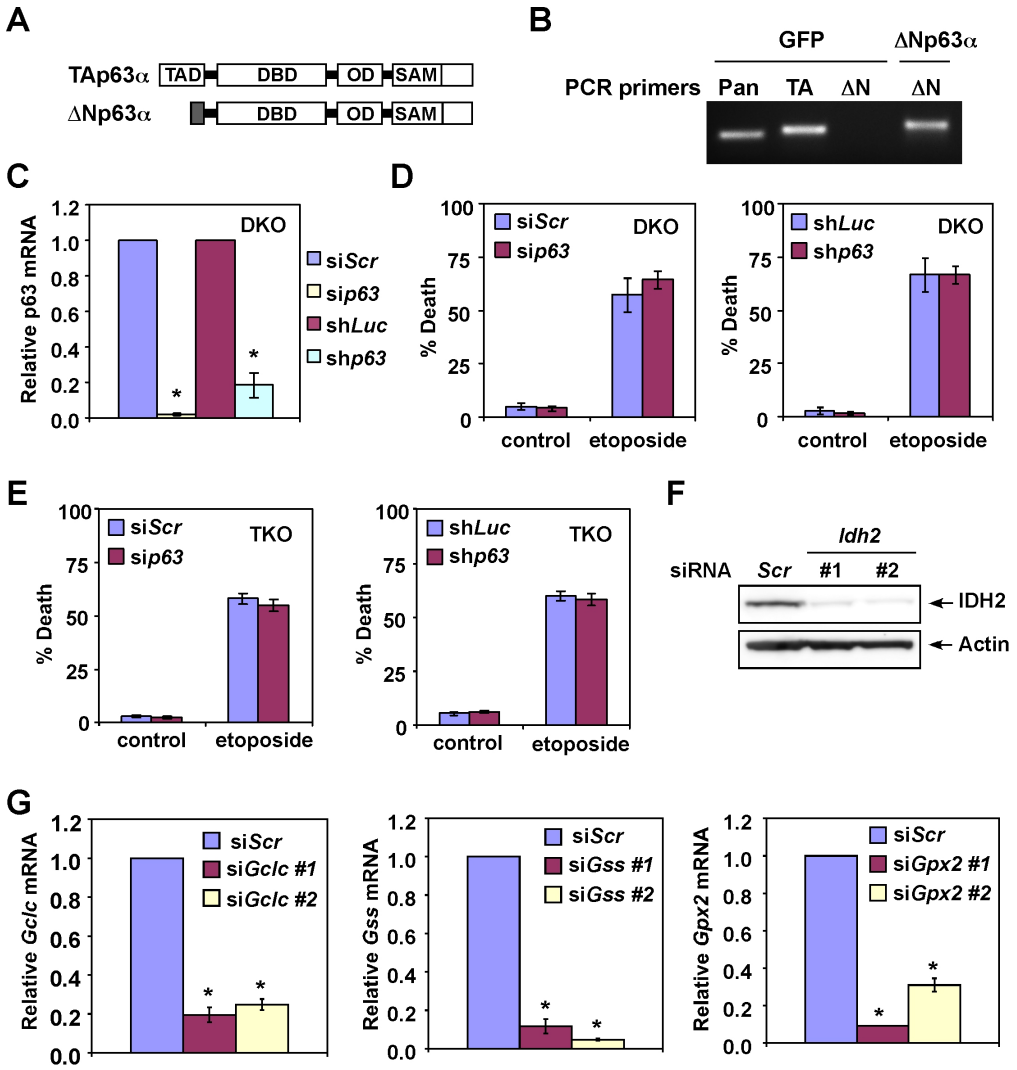
Supplemental Information



Supplemental Figure S1. Characterization of DNA damage-induced programmed necrotic death in *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs. (A) SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs were transfected with scrambled siRNA (siScr) or siRNA against *RIP1*. Cell lysates were analyzed by immunoblots using the indicated antibodies. (B) Cells described in (A) were treated with etoposide (10 μg/ml) for 3 days. (C) SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs were treated with the indicated agents for 3 days. (D) SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO cells were treated with etoposide, etoposide plus N-acetyl-L-cysteine (NAC, 25 mM), or etoposide plus ferrostatin-1 (10 μM) for 3 days. (E) SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO cells were treated with etoposide, etoposide plus deferoxamine (DFO, 80 μM), or etoposide plus Tiron (10 mM) for 3 days. Cell death in (B-E) was quantified by flow cytometric analysis following propidium iodide staining (mean ± s.d., n = 3 independent experiments). * $P < 0.05$.

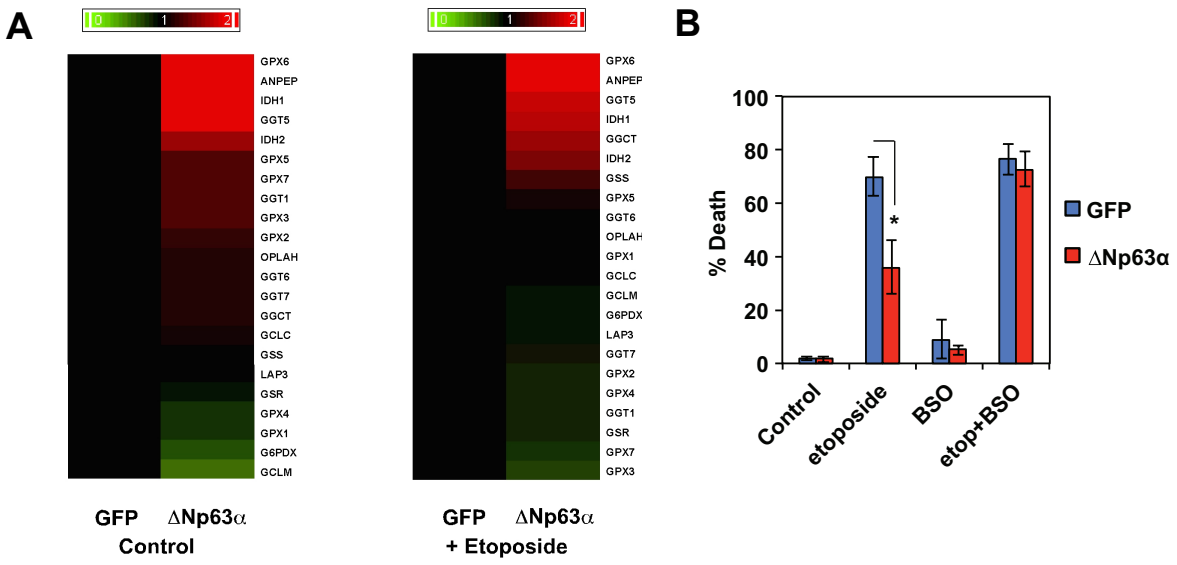


Supplemental Figure S2. **Characterization of DNA damage-induced programmed necrotic death in *p53*$^{-/-}$*Bax*$^{-/-}$*Bak*$^{-/-}$ TKO MEFs.** (A) Primary MEFs isolated from two *Bax*$^{-/-}$*Bak*$^{-/-}$ embryos and two *p53*$^{-/-}$*Bax*$^{-/-}$*Bak*$^{-/-}$ embryos were transformed by E1A and Ras. E1A/Ras-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ and *p53*$^{-/-}$*Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs were treated with etoposide (10 μg/ml). Cell death was quantified by annexin-V staining at the indicated times (mean ± s.d., n = 3). (B) SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ or *p53*$^{-/-}$*Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs were untreated or treated with etoposide. Cell death was quantified by propidium iodide staining at the indicated times (mean ± s.d., n = 3). (C) SV40-transformed *p53*$^{-/-}$*Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs were treated with etoposide or etoposide plus N-acetyl-L-cysteine (NAC, 20 mM) or etoposide plus diphenyleneiodonium (DPI, 100 nM) for the indicated times. Cell death was quantified by propidium iodide staining at the indicated times (mean ± s.d., n = 3). (D) ΔNp63α prevents DNA damage-induced ROS accumulation in *p53*$^{-/-}$*Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. SV40-transformed *p53*$^{-/-}$*Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs transduced with control or ΔNp63α-expressing retrovirus were mock treated or treated with etoposide. Oxidation of the ROS-sensitive dye CM-H_2DCFDA or MitoSOX Red was quantified by flow cytometric analysis. Data shown are fold increase of ROS after etoposide treatment (mean ± s.d., n = 3). * $P < 0.05$.

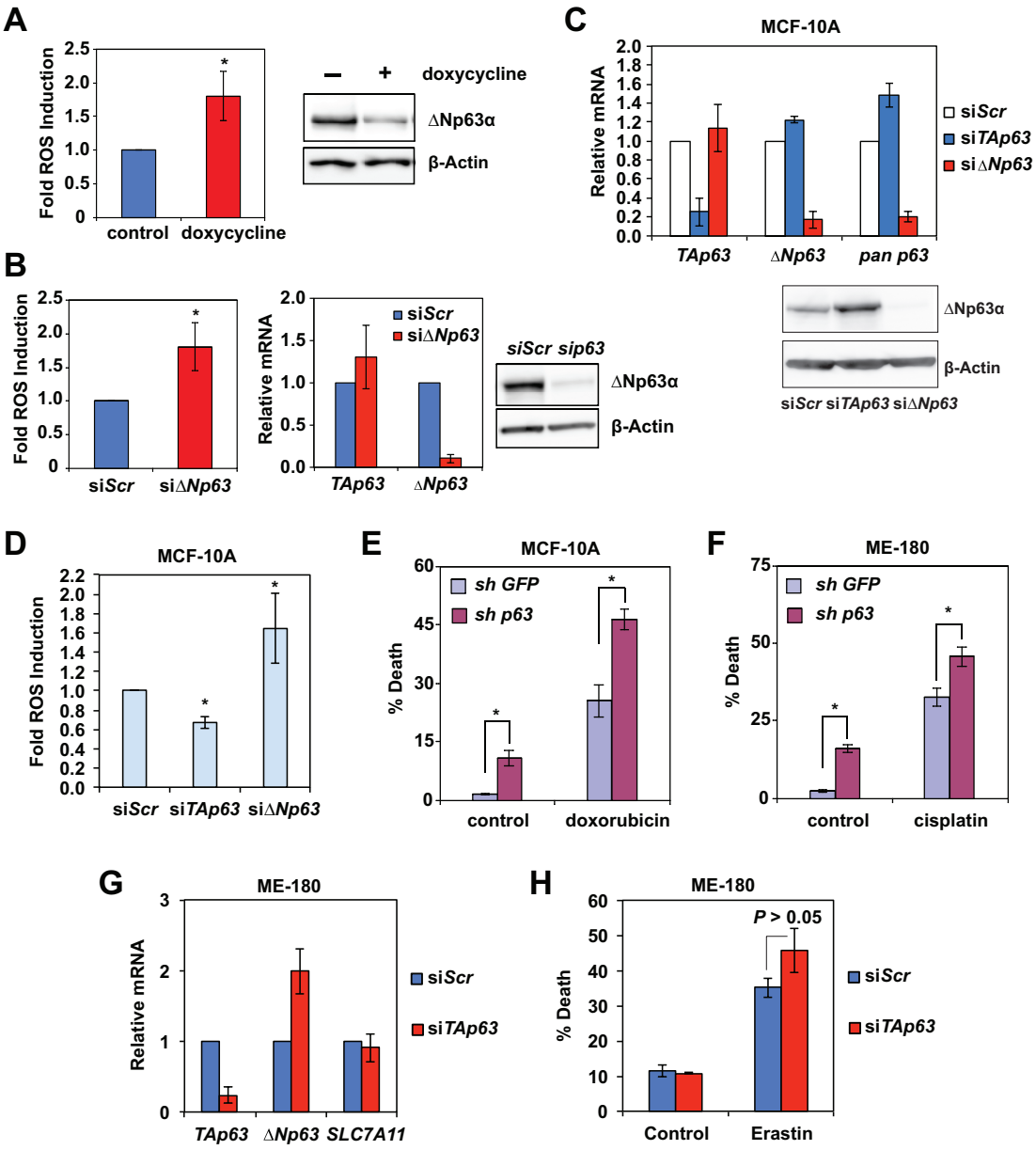


Supplemental Figure S3. **Characterization of p63 isoform expression and validation of knockdown in MEFs**. (A) A schematic of modular structure of the p63 isoforms. (B) cDNA prepared from *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs transduced with GFP or ΔNp63α-expressing retrovirus was subjected to PCR using primers specific for all isoforms of p63 (Pan), TA isoforms of p63 (TA), or ΔN isoforms of p63 (ΔN). (C) SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs transduced with retrovirus expressing shRNA against luciferase or p63, or transfected with scrambled siRNA (siScr) or siRNA against p63 were subjected to quantitative RT-PCR analysis for p63 expression. Data presented as mean ± SD from three independent experiments. (D) SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs, transfected with scrambled siRNA (siScr) or siRNA against p63 for 3 days, were mock treated or treated with etoposide (10 μg/ml) for 3 days. DKO

MEFs transduced with retrovirus expressing shRNA against luciferase or p63 were mock treated or treated with etoposide for 3 days. Cell death was quantified by propidium iodide staining (mean ± s.d., n = 3). (E) SV40-transformed *p53*$^{-/-}$*Bax*$^{-/-}$*Bak*$^{-/-}$ TKO MEFs, transfected with scrambled siRNA (siScr) or siRNA against p63 for 3 days, were mock treated or treated with etoposide for 5 days. TKO MEFs transduced with retrovirus expressing shRNA against luciferase or p63 were mock treated or treated with etoposide for 5 days. Cell death was quantified by propidium iodide staining (mean ± s.d., n = 3). (F) *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs transfected with scrambled siRNA (siScr) or siRNA against *Idh2* were analyzed by immunoblots using the indicated antibodies. (G) *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs transfected with scrambled siRNA (siScr) or siRNA against *Gclc*, *Gss*, or *Gpx2* were analyzed by qRT-PCR using the indicated gene-specific primers. Data presented as mean ± SD from three independent experiments. * $P < 0.05$.

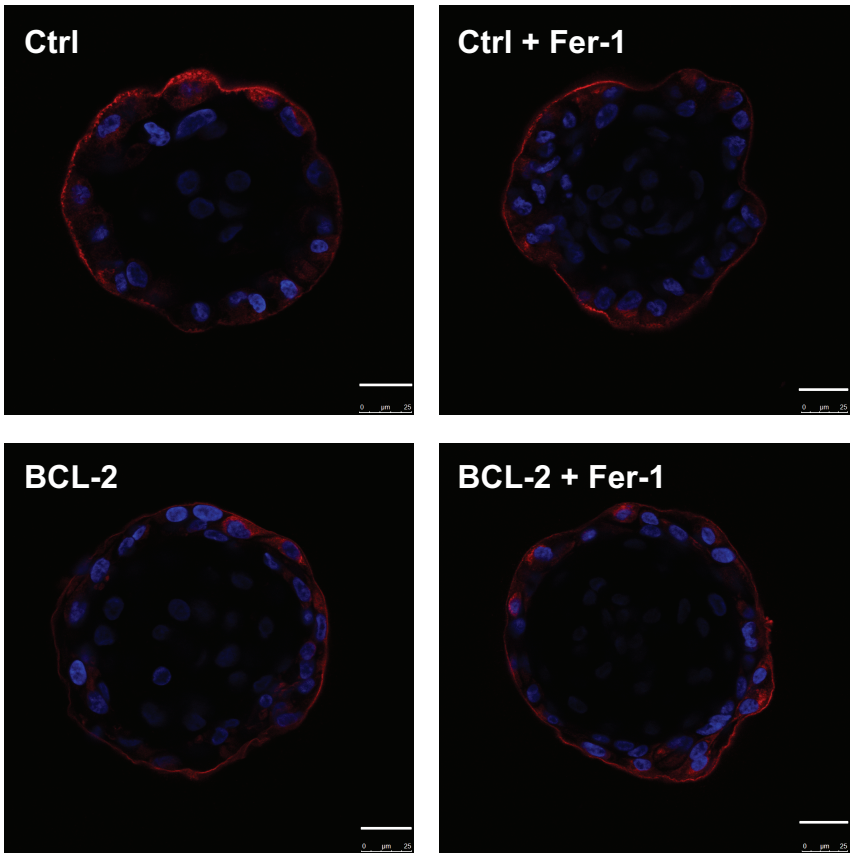


Supplemental Figure S4. **ΔNp63α upregulates glutathione metabolic pathway genes and the protective effect of ΔNp63α against oxidative stress-induced cell death is mitigated by the GCLC inhibitor buthionine sulfoximine (BSO).** (A) A heat map representation of glutathione metabolism genes differentially regulated by ΔNp63α. *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO cells transduced with retrovirus expressing GFP or ΔNp63α were mock treated or treated with etoposide (10 μg/ml) for 6 hours. The gene expression profiles were assessed using the Affymetrix GeneChip Mouse Gene 1.0 ST array and analyzed for genes involved in glutathione metabolism. (B) SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ DKO MEFs stably expressing GFP or ΔNp63α were treated with the indicated agents for 3 days. Cell death was quantified by propidium iodide staining (mean ± s.d., n = 3 independent experiments). * $P < 0.05$.

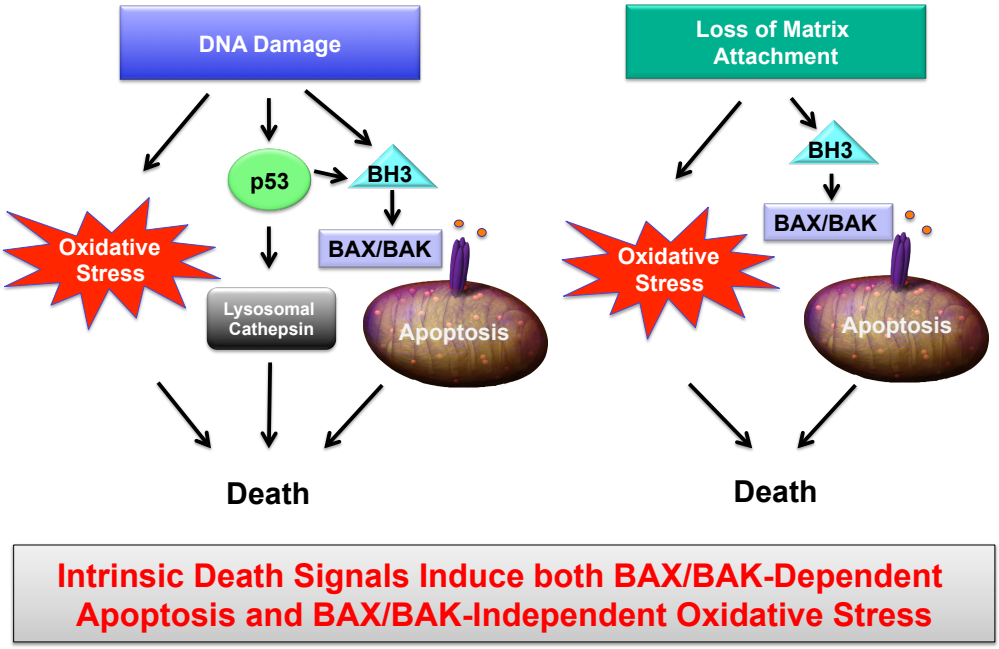


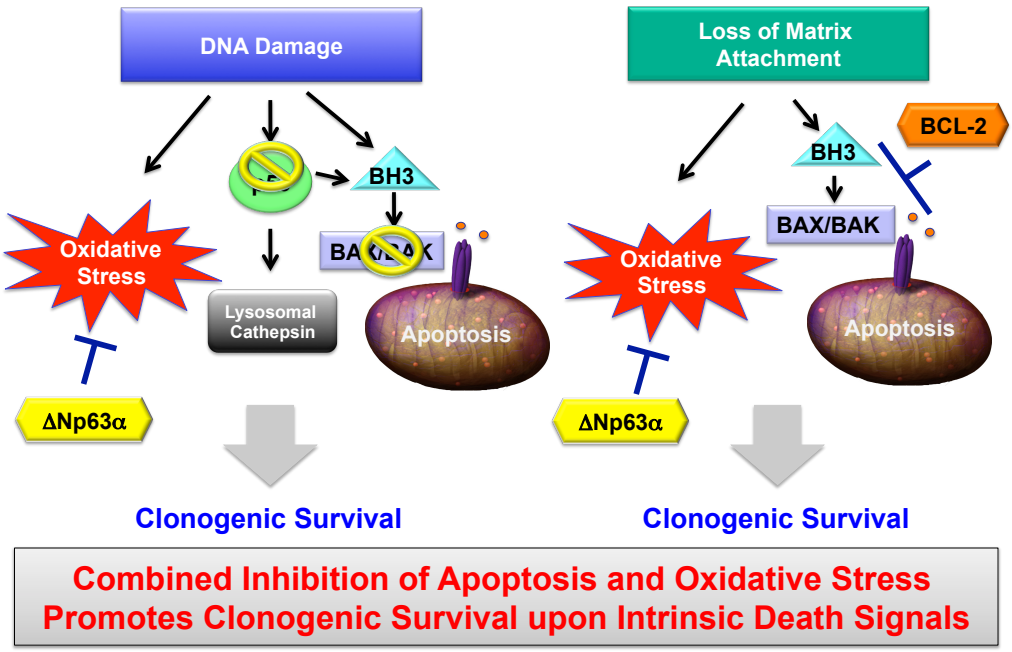
Supplemental Figure S5. Knockdown of ΔNp63α but not TAp63 induces ROS accumulation and sensitizes cells to chemotherapeutic agent-induced cell death. (A) ME-180 cells were transduced with lentivirus expressing doxycycline-inducible miR30-based shRNA against all p63 isoforms. Cells were untreated or treated with doxycycline (2 μg/ml) for three days and analyzed for ROS production and immunoblots using the indicated antibodies. Oxidation of the ROS-sensitive dye H_2DCFDA was quantified by flow cytometric analysis. Data shown are fold increase of ROS induced by knockdown of p63 (mean ± s.d., n = 3). (B) ME-180 cells transfected with scrambled

siRNA (siScr) or siRNA against *ΔNp63* for 3 days were subjected to analysis for ROS production or immunoblot analysis using the indicated antibodies. Oxidation of the ROS-sensitive dye H_2DCFDA was quantified by flow cytometric analysis. Data shown are fold increase of ROS induced by knockdown of *ΔNp63* (mean ± s.d., n = 3). (C) MCF-10A cells, transfected with scrambled siRNA or siRNA against *TAp63* or *ΔNp63*, were subjected to qRT-PCR analysis for the indicated p63 isoform as well as immunoblot analysis using the indicated antibodies. qRT-PCR data were normalized against GAPDH (mean ± s.d., n = 3). (D) MCF-10A cells, transfected with scrambled siRNA or siRNA against *TAp63* or *ΔNp63*, were subjected to CM-H_2DCFDA staining followed by flow cytometric analysis. Data shown are fold increase of ROS induced by knockdown of *TAp63* or *ΔNp63* (mean ± s.d., n = 3). (E) MCF-10A cells transduced with lentivirus expressing shRNA against GFP or p63 for 48 hours were untreated or treated with doxorubicin (1 μg/ml) for 27 hours. Cell death was quantified by propidium iodide (PI) staining (mean ± s.d., n = 3). (F) ME-180 cells transduced with lentivirus expressing shRNA against GFP or p63 for 48 hours were mock treated or treated with cisplatin (10 μM) for 32 hours. Cell death was quantified by propidium iodide (PI) staining (mean ± s.d., n = 3). (G) ME-180 cells, transfected with scrambled siRNA or siRNA against *TAp63*, were subjected to qRT-PCR analysis of *TAp63*, *ΔNp63*, and *SLC7A11* mRNA. Data were normalized against GAPDH (mean ± s.d., n = 3). (H) ME-180 cells, transfected with scrambled siRNA or siRNA against *TAp63*, were mock treated or treated with erastin (20 μM) for 72 h. Cell death was quantified by propidium iodide staining (mean ± s.d., n = 3). *, $P < 0.05$.



Supplemental Figure S6. **Ferrostatin-1 has minimal effect on the luminal clearance of mammary acini in three-dimensional culture.** MCF-10A cells transduced with retrovirus expressing GFP or BCL-2 were cultured in reconstituted basement membrane (Matrigel) ± ferrosatin-1 for 28 days. Acini were fixed and stained for Laminin 5 (red) and nuclear stain DAPI (blue). Representative confocal microscopy images from two independent experiments are shown. Scale bar, 25 μm.





Supplemental Figure S7. Model depicts the apoptotic and non-apoptotic cell death pathways activated by DNA damage and loss of matrix attachment, respectively.

Gene	Cat#	Sequences
mouse *Idh2* #1	Ambion s114463	5'-AGACUGACUUCGACAGGAAtt
mouse *Idh2* #2	Ambion 95489	5'-GGAAUAAGAUCUGGUAUGAtt
mouse *Gclc* #1	Ambion s66718	5'-CGGUAUGACUCAAUAGAUAtt
mouse *Gclc* #2	Ambion s201400	5'-GGGUGAUCCUCUCAUACAAtt
mouse *Gss* #1	Ambion s67110	5'-GCCCAGUCAGUAUAAUUCAtt
mouse *Gss* #2	Ambion s67112	5'-CCAUCAAAAAGGACGACUAtt
mouse *Gpx2* #1	Dharmacon siGenome-02	5'-UCAAUGAGCUGCAAUGUCG
mouse *Gpx2* #2	Dharmacon siGenome-03	5'-CAACUACCCGGGACUACAA
mouse *Nrf2*	Ambion s70523	5'-CAUUUUUACUCAUCGAUCUtt
mouse *p63*	Dharmacon siGenome-04	5'-CCACCGAACUGAAGAAGCU
human *ΔNp63*	Dharmacon siGenome-08	5'-CGACAGUCUUGUACAAUUU
human *TAp63*	Dharmacon siGenome-06	5'-CAAACAAGAUUGAGAUUAG
mouse *Rip1* #1	Ambion s72975	5'-GGUGGUACCCUUUACUACAtt
mouse *Rip1* #2	Ambion s72977	5'-GGAUUUGGAACUACAGGUAtt

Table S1. Summary of siRNA oligos.

Gene	Sequences of Primers for qRT-PCR
mouse p63 pan-isoform	5’-CCTTTCCGTCAGAATACACACGGA-3’
mouse p63 pan-isoform	5’-GTTTCTGAAGTAGGTGCTGGTGCT-3’
mouse *TAp63* isoform	5’-CCTATATGCTCAGTACAGCCCATCG-3’
mouse *TAp63* isoform	5’-CTATTCTGTGCGTGGTCTGTGTTGT-3’
mouse *ΔNp63* isoform	5’-TCTTAGAAGATTCGCAGCGCAAGG-3’
mouse *ΔNp63* isoform	5’-CTATTCTGTGCGTGGTCTGTGTTGT-3’
mouse *Gclc*	5’-TACACCTGGATGATGCCAACGA-3’
mouse *Gclc*	5’-GTCAACCTTGGACAGCGGAATG-3'
mouse *Gss*	5’-CCTGATGCTAGAGAGATCTCGTGC-3'
mouse *Gss*	5’-CTCTCTCCTCACTGTCCTTCAGC-3'
mouse *Idh2*	5’-CCTGATGACATCTGTGCTGGTCT-3'
mouse *Idh2*	5’-GAGCTCTGTCCAGGTTGCTCTT-3'
mouse *Nrf2* (*Nfe2l2*)	5’-ATCCAGACAGACACCAGTGGATC-3’
mouse *Nrf2* (*Nfe2l2*)	5’-CAAACTTGCTCCATGTCCTGCTC-3’
mouse *Slc7a11*	5'-GTGGTGTGTTCGCTGTCTCCA-3'
mouse *Slc7a11*	5'-CGGAGAAGAGCATCACCATCGTC-3'
human p63 pan-isoform	5'-AGAACGGTGATGGTACGAAGCG-3'
human p63 pan-isoform	5'-GTACTGCATGAGTTCCAGGGACTC-3'
human *TAp63* isoform	5'-AAGATGGTGCGACAAACAAG-3'
human *TAp63* isoform	5'-AGAGAGCATCGAAGGTGGAG-3'
human *ΔNp63* isoform	5'-GGAAAACAATGCCCAGACTC-3'
human *ΔNp63* isoform	5'-GTGGAATACGTCCAGGTGGC-3'
human *GSS*	5'-GACCAGCGTGCCATAGAGAATGA-3'
human *GSS*	5'-CATGTGACCTCTCCAGCAGTAGAC-3'
human *IDH2*	5'-GATGGGAAGACGATTGAGGCTGA-3'
human *IDH2*	5'-TCAGGAAGTGCTCGTTCAGCTT-3'
human *GSR*	5'-CCAACGTCAAAGGCATCTATGCAG-3'
human *GSR*	5'-ATCTTCCGTGAGTCCCACTGTC-3'
human SLC7A11	5'-GTTGCGTCTCGAGAGGGTCA-3'
human SLC7A11	5'-GTCGAGGTCTCCAGAGAAGAGC-3'

Table S2. Summary of Primers for qRT-PCR.