Supporting Information

Khutornenko et al. 10.1073/pnas.0910885107

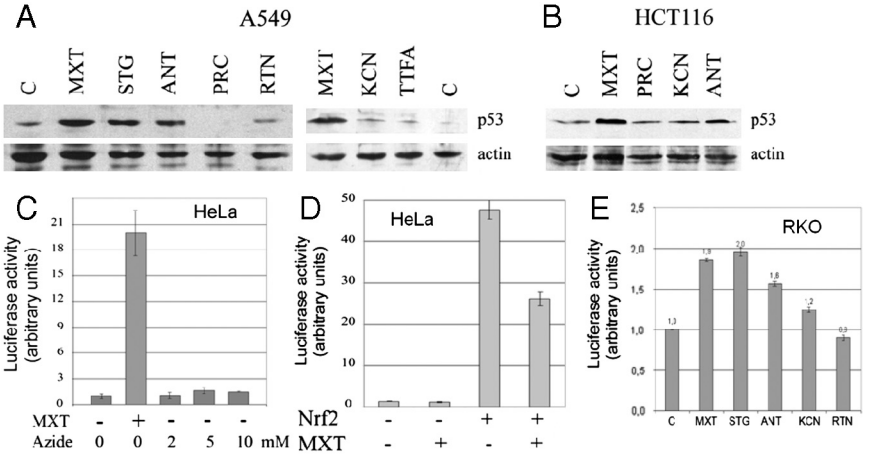

Fig. S1. Mitochondrial ETC complex III inhibitors up-regulate p53 protein levels and activity. (*A* and *B*) Western analysis of p53 protein levels in A549 cells (*A*) and HCT116 cells (*B*) exposed to the indicated ETC inhibitors: 2 μM myxothiazol (MXT), 1 μM stigmatellin (STG), 2 μM antimycin A (ANT), 2 μM piericidin (PRC), 2 μM rotenone (RTN), 5 mM KCN (KCN), 100 μM TTFA (TTFA), and nontreated (*C*) for 18 h. (*C*) Luciferase reporter assay of p53-dependent transcription in HeLa cells exposed to 2 μM myxothiazol (MXT) or indicated concentrations of sodium azide (Azide) for 22 h. The experiments were performed in triplicates. Data are represented as mean +/− SEM. (*D*) Myxothiazol does not have a general positive effect on transcription. Luciferase reporter assay of ARE-dependent transcription in response to 2 μM myxothiazol (MXT) for 20 h. Transcription of ARE-dependent reporter gene was induced by ectopic expression of transcription factor Nrf2 but was not elevated after treatment with myxothiazol (MXT). Luciferase activity was measured in HeLa cells co-transfected with reporter plasmids and Nrf2-encoding plasmid (1) or empty vector as indicated. The experiments were performed in triplicates. Data are represented as mean +/− SEM. (*E*) Influence of ETC inhibitors on p53 protein activity in RKO cells. Luciferase reporter assay of p53-dependent transcription in RKO cells exposed to 2 μM myxothiazol (MXT), 1 μM stigmatellin (STG), 2 μM antimycin A (ANT), 5 mM KCN (KCN), 2 μM rotenone (RTN), or without drugs (*C*) for 20 h. The experiments were performed in triplicates. Data are represented as mean +/− SEM.

1. Karapetian RN et al. (2005) Nuclear oncoprotein prothymosin alpha is a partner of Keap1: Implications for expression of oxidative stress-protecting genes. *Mol Cell Biol* 25(3):1089–1099.

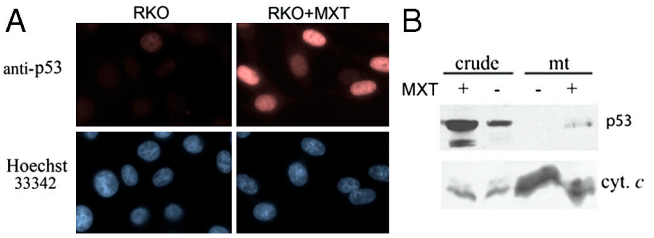

Fig. S2. Influence of myxothiazol on p53 protein level and intracellular localization. (*A*) RKO cells grown on coverslips were treated with 1 μM myxothiazol for 18 h, 1 μg/ml Hoechst 33342 (Sigma) for 30 min, then fixed and processed for microscopy as described previously (2). Anti-p53 monoclonal antibodies (DO-1, Santa Cruz Biotechnology) at a dilution of 1:200 were used as primary antibodies. Rhodamin-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology) were used as secondary antibodies at a dilution of 1:400. The stained cells were observed and photographed with Carl Zeiss AxioVision 200 fluorescent microscope equipped with Hamamatsu Orca camera. (*B*) Western analysis of p53 protein levels in mitochondrial fraction (mt) and crude cell lysates (crude) of RKO cells exposed to 2 μM myxothiazol for 18 h. Mitochondrial protein cytochrome c (cyt. c) was also measured as a loading control. Mitochondria were isolated as described (3); antibodies to cytochrome C (6H2.B4) were from BD Biosciences Inc.).

2. Evstafieva AG et al. (2003) Apoptosis-related fragmentation, translocation, and properties of human prothymosin alpha. *Exp Cell Res* 284(2):211–223.
3. Mihara M & Moll UM (2003) Detection of mitochondrial localization of p53. *Methods Mol Biol* 234:203–209.

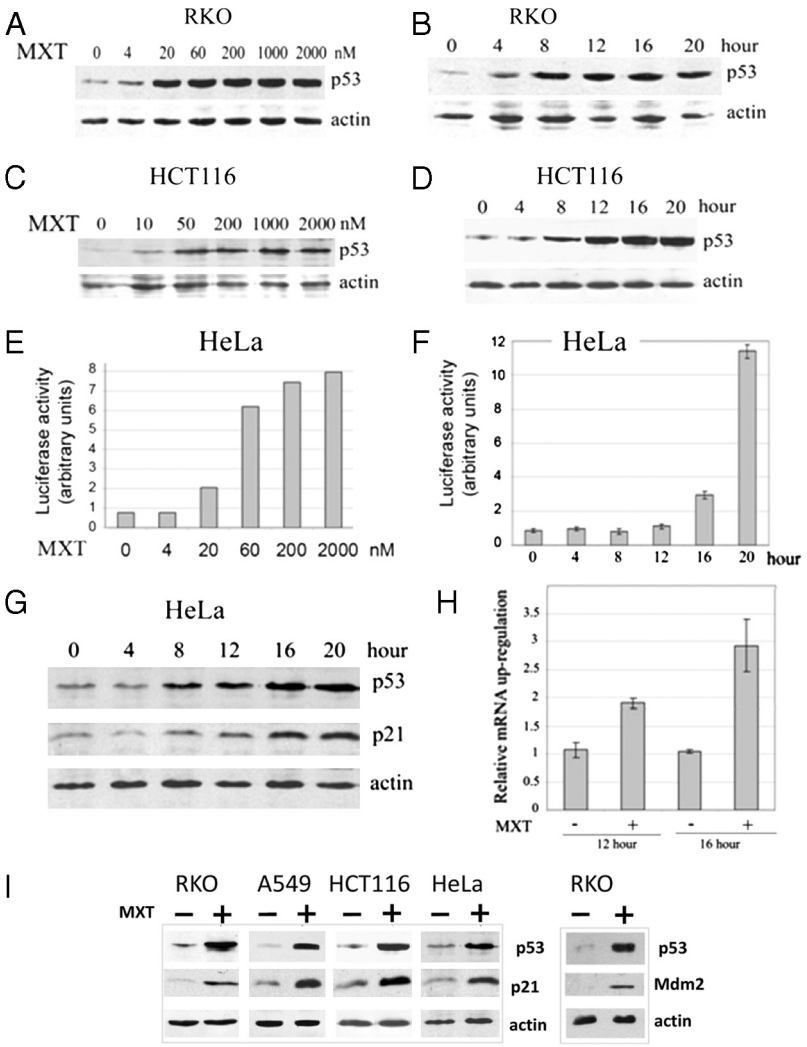

Fig. S3. Time- and dose-dependence of p53 accumulation and activation by myxothiazol. Western analysis of p53 protein levels in RKO (*A*) and HCT116 (*C*) cells exposed to indicated concentrations of myxothiazol for 18 h. Western analysis of p53 protein levels in RKO (*B*), HCT116 (*D*), and HeLa (*G*) cells exposed to 2 μM myxothiazol for indicated intervals of time. Luciferase reporter assay of p53-dependent transcription in HeLa cells exposed to indicated concentrations of myxothiazol for 20 h (*E*) or exposed to 2 μM myxothiazol for indicated intervals of time (*F*). Real-time PCR detection of CDKN1A (p21) mRNA (*H*) was performed using the SYBR-Green protocol with primers: 5'-GGGATGAGTTGGGA and 5'-AATCTGTCATGCTGG. Total RNA was isolated from RKO cells treated with 2 μM myxothiazol for 12 or 16 h or untreated. The relative mRNA up-regulation for CDKN1A was determined by comparing with the mRNA level of the control (untreated) cells, which was arbitrarily set as 1. The mRNA level of CDKN1A was normalized with the level of GAPDH mRNA. Each experiment was repeated three times. Accumulation of p53 and p53-dependent p21 and Mdm2 proteins was detected 16 h after treatment with 2 μM myxothiazol. Western analysis (*I*) was performed with 20 μg of protein extracts from RKO, A549, HCT116 and HeLa cells and developed with anti-p53 DO-1 and anti-p21 F-5 monoclonal antibodies (Santa Cruz Biotech).

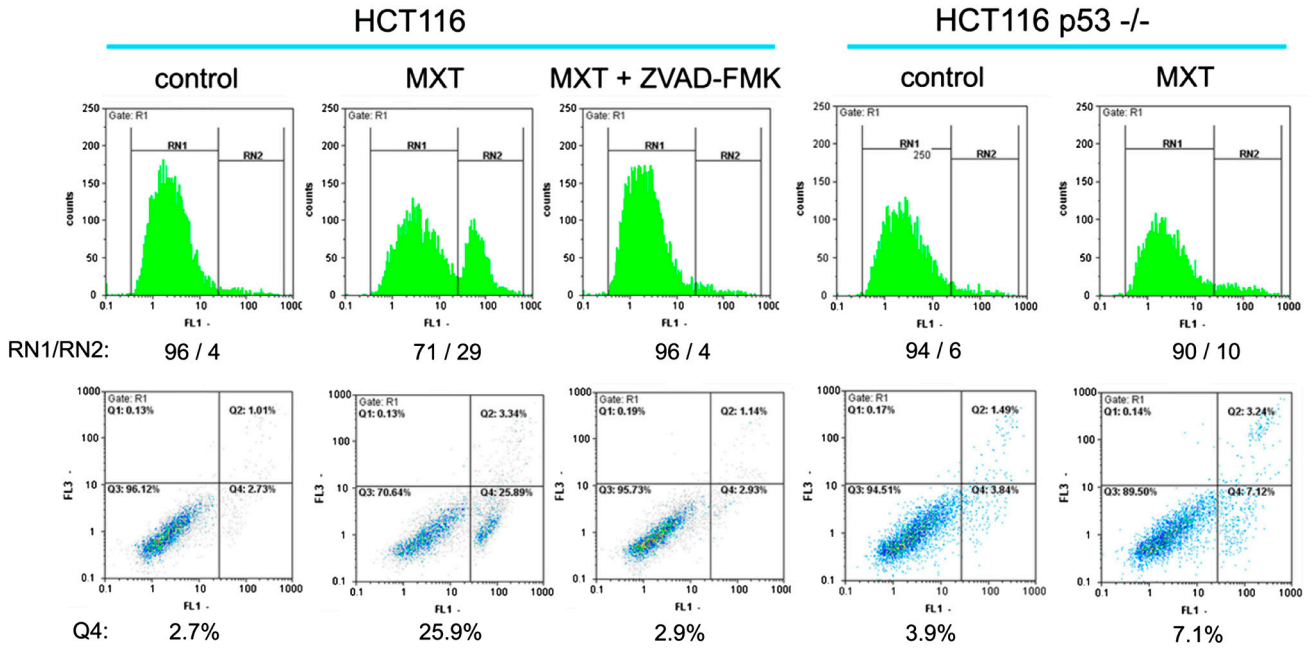

Fig. S4. Mitochondrial ETC complex III inhibitor myxothiazol stimulates p53-dependent apoptosis. Flow cytometry analysis of apoptosis in HCT116 wt and p53 −/− cells not treated (control) or treated with 2 μM myxothiazol (MXT), or 2 μM myxothiazol + 50 μM ZVAD-FMK for 20 h. The attached cells were harvested, washed with PBS, and stained with Annexin V AlexaFluor 488 conjugate (Invitrogen) (FL1) and propidium iodide (100μg/ml) (FL3) for 15 min in the dark. The cells were analyzed with Flow cytometer (Partec PASIII). Proportion of Annexin Vnegative (RN1) to Annexin V-positive (RN2) cells and percentage of Annexin V-positive, propidium iodide negative (Q4) cells is indicated.

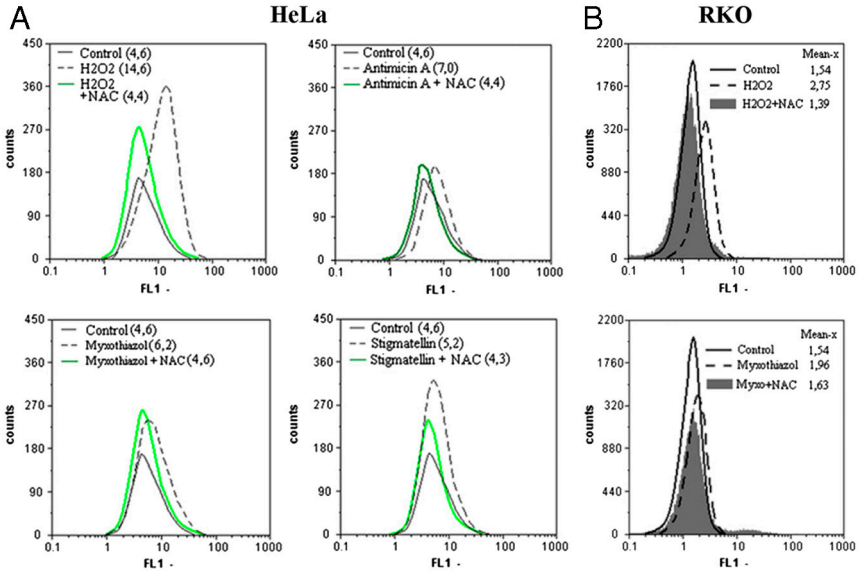

Fig. S5. Induction of intracellular ROS by ETC complex III inhibitors. (*A*) Flow cytometry analysis of ROS in HeLa cells not treated (control) or treated with 200 μM hydrogen peroxide for 2 h or 2 μM antimycin A, 2 μM myxothiazol, 1 μM stigmatellin for 18 h in the absence or in the presence of NAC. 10 mM NAC was added 1 h before treatment with ETC inhibitors or hydrogen peroxide. The cells were stained with 10 μM dihydrorhodamine 123 (Invitrogen Molecular Probes) for 30 min. Fluorescence intensity (FL1) of peak positions is indicated. (*B*) Flow cytometry analysis of ROS in RKO cells not treated (control) or treated with 300 μM hydrogen peroxide (H2O2) or 1 μM myxothiazol (Myxo) for 20 h. Where indicated 10 mM NAC was added 1 h before treatment with ETC inhibitor or hydrogen peroxide. The cells were stained with 10 μM CM-H2DCFDA (Invitrogen Molecular Probes) for 30 min. Fluorescence intensity (FL1) of peak positions is indicated.

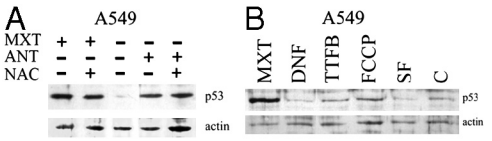

Fig. S6. ROS scavenger NAC does not prevent up-regulation of p53 in response to ETC complex III inhibitors; protonophorous uncoupling reagents do not induce p53 up-regulation comparable to the effect of myxothiazol. (*A*) Western analysis of p53 protein levels in A549 cells exposed for 18 h to 2 μM myxothiazol (MXT), 2 μM antimycin A (ANT) or not treated. Where indicated 10 mM NAC was added 1 h before treatment with ETC inhibitors. (*B*) Western analysis of p53 protein levels in A549 cells exposed for 18 h to 2 μM myxothiazol (MXT) or uncoupling reagents 0.4 mM dinitrophenol (DNF), 5 μM TTFB (TTFB), 1 μM FCCP (FCCP), 1 μM SF (SF) or not treated (C).

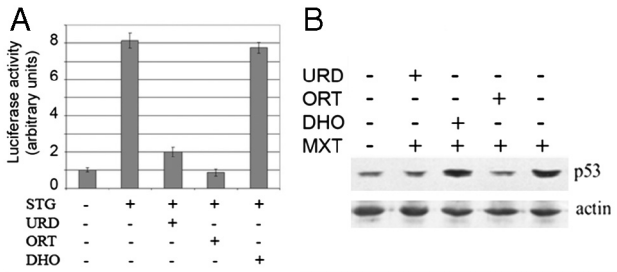

Fig. S7. The effects of exogenous uridine, orotate, and dihydroorotate on the p53 activation by stigmatellin and myxothiazol. (*A*) Luciferase reporter assay of p53-dependent transcription in HeLa cells exposed to 1 μM stigmatellin (STG) in the presence of 50 μg/ml uridine (URD), 1 mM orotate (ORT), or 1 mM dihydroorotate (DHO) for 22 h. The experiments were performed in triplicates. Data are represented as mean +/− SEM. (*B*) Western analysis of p53 in HeLa cells exposed to 1 μM myxothiazol (MXT) in the presence of 50 μg/ml uridine (URD), 1 mM orotate (ORT), or 1 mM dihydroorotate (DHO) for 22 h.

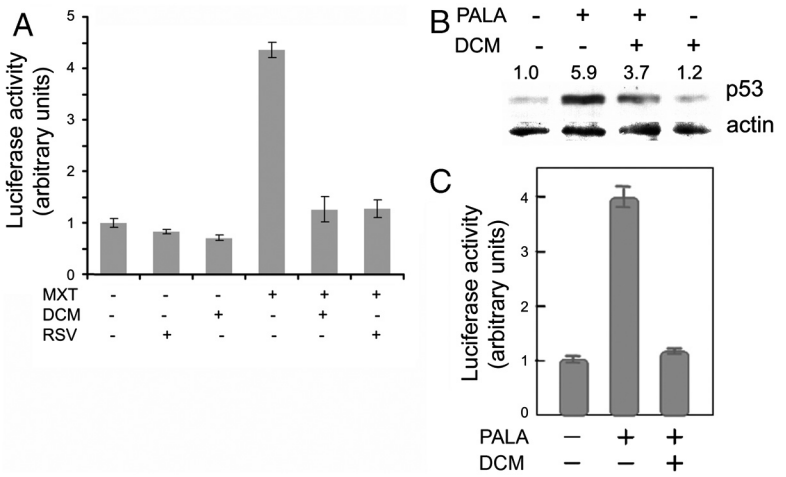

Fig. S8. The p53 induction in response to myxothiazol and PALA is mitigated by the NQO1 inhibitor dicoumarol and NQO2 inhibitor resveratrol. (*A*) Luciferase assay for p53-dependent transcription in HeLa cells exposed to 2 μM myxothiazol (MXT) for 18 h or/and 300 μM dicoumarol (DCM) for 4 h or 50 μM resveratrol (RSV) for 18 h. (*B*) Western analysis of p53 in RKO cells treated with 250 μM PALA for 12 h and 300 μM dicoumarol (DCM) for 4 h. (*C*) Luciferase assay for p53− dependent transcription in HeLa cells exposed to 250 μM PALA for 20 h or/and 300 μM dicoumarol (DCM) for 4 h.

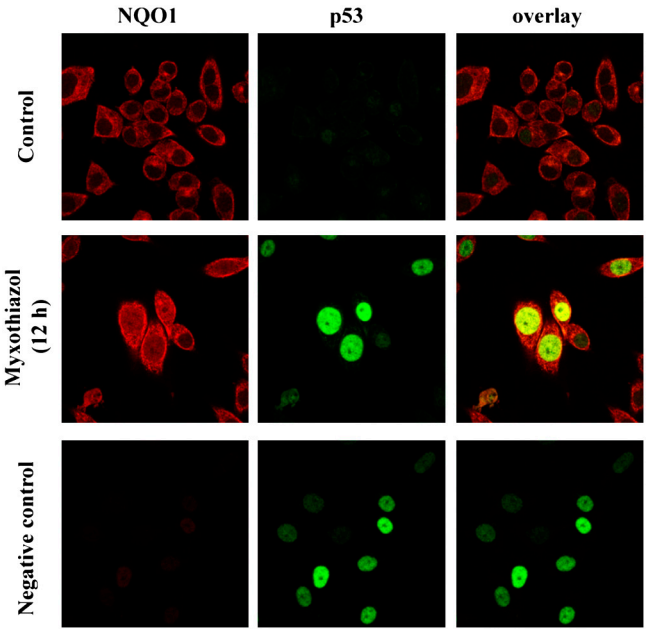

Fig. S9. Co-localization of p53 and NQO1 after bc1 complex inhibition in RKO cells. Cells were incubated with 2 μM myxothiazol (Myxothiazol and Negative control) or without myxothiazol (control) for 12 h, and stained with antibodies to NQO1 (red) and p53 (green). Then, pictures with red and green fluorescence were overlaid. Yellow color depicts co-localization of two proteins inside the cells. Negative control was performed to confirm the absence of antibodies crossreactivity (see *Experimental Procedures* for details).

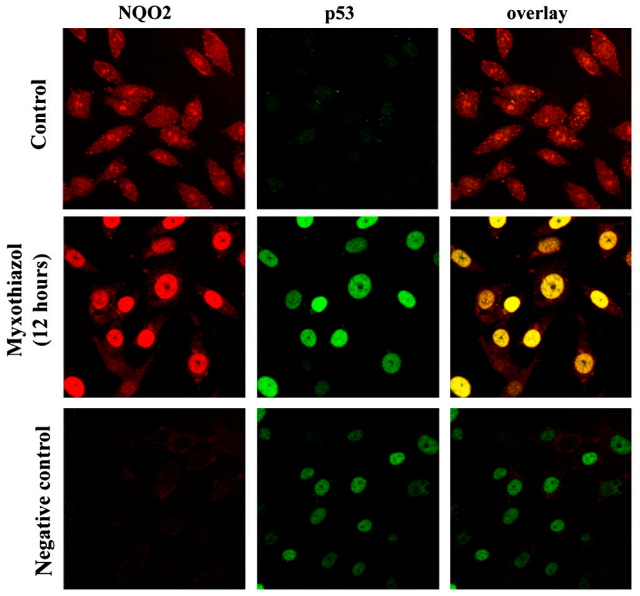

Fig. S10. Co-localization of p53 and NQO2 after bc1 complex inhibition in RKO cells. Cells were incubated with 2 μM myxothiazol (Myxothiazol and Negative control) or without myxothiazol (control) for 12 h, and stained with antibodies to NQO2 (red) and p53 (green). Then, pictures with red and green fluorescence were overlaid. Yellow color depicts co-localization of two proteins inside the cells. Negative control was performed to confirm the absence of antibodies crossreactivity (see *Experimental Procedures* for details).