SCO2 Induces p53-Mediated Apoptosis by Thr^{845} Phosphorylation of ASK-1 and Dissociation of ASK-1-TRX Complex

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FIGURE LEGENDS:

Figure S1. Western blot analysis in KB cells shows that p53 regulates the expression of SCO2 at high doses of UV stress. UV-50 treatment (lane 5) shows a significant increase in SCO2 protein level in comparison to the untreated cells (lane 2). UV-50 induced increase in SCO2 level was p53-dependent since p53 gene silencing using p53 siRNA abolished the increase in SCO2 protein (n=5) (SCO2 cDNA (lane1) is used as a control).

Figure S2. The role of SCO2 protein in tamoxifen-induced cellular ROS was observed. Tam-100 treatment led to significant increase in cellular ROS in both the p53-Wt KB and MCF-7 cells. Interestingly the tamoxifen induced increase in ROS was SCO2-dependnet as Tam-100-treated KB and MCF-7 cells showed significant increase in the cellular ROS content **(lane 2)**. SCO2 gene silencing resulted in a 4-fold decrease in the cellular ROS content **(lane 3)**. Similarly p53 gene silencing in the Tam-100-treated KB cells resulted in 5-fold decrease in the cellular ROS **(lane 4)**. These results suggest that tamoxifen might be generating ROS in cancer cells via a p53 and SCO2-dependent pathway (n=7) (S.D, ANOVA).

Figure S3. The role of SCO2 in inducing apoptosis was observed using TUNEL assay in the KB, MCF-7, A-431, H1299, HCT p53 (+/+) and HCT p53 (-/-) cells. Results show that ectopic expression of SCO2 cDNA results in a significant increase in TUNEL positive cells thus cellular apoptosis in these cells. ROS-quenching via NAC treatment abolishes SCO2-induced apoptosis. Tamoxifen is used as positive control. (* $p< 0.023$) (n=6) (S.D, ANOVA).

Figure S4. The role of SCO2-induced ROS in SCO2-induced tumor regression is observed using A-431, MCF-7, HCT p53 (+/+) and HCT p53 (-/-) tumor xenografts. Results show that ectopic expression of SCO2 in these tumor xenografts results in significant reduction in tumor size in 4 week time (black line). Interestingly upon ROS-quenching in these tumors the SCO2-induced tumor regression was abolished (red line). The untreated tumors served as controls (green line) (n=6) (S.D, ANOVA).

Figure S5. **SCO2 induces activation of the MAP2K3, MAP2K6, MAP2K4, MAP2K7, p38 and JNK kinases. (A)** Western-blot analysis was used to determine the total kinase and its phosphorylated form. Results show that the exogenous addition of SCO2 protein results in significant increase in the expressions of total kinase and its phosphorylated form (lane 2). ROS-quenching by NAC treatment results in significant decrease in the expression of both total and phosphorylated forms of these kinases (lane 3). Tamoxifen treatment was used as a positive control (n=3). **(B)** In HCT p53 (+/+) tumor xenografts, exogenous addition of SCO2 cDNA resulted in a significant increase in the total and phosphorylated forms of these kinases (lane 2) and this effect was reversed upon ROS quenching (lane 3; n=4).

MATERIAL AND METHODS

UV exposure and drug treatment: Experimental mice and exponentially growing cells were exposed to UV at a dose of 25 (low) or 50 (high) J/cm^2 with a Stratalinker 2400 UV crosslinker (Stratagene, La Jolla, CA). To equalize the irradiation conditions, medium was removed from the culture dishes during UV irradiation. Immediately after irradiation, the cells were incubated under standard culture conditions for 24 hr. The optimal concentration of tamoxifen was established by standard curve (5–100nM) and set as the maximal non-toxic effect at 10 and 100nM. Indicated doses were applied topically on shaved dorsal skin of mice in the inter-scapular region of 2 cm^2. Tissue from the exposed area of mice was extracted; homogenates prepared and assayed for protein expression. The cells were treated for 24 h with selected doses of tamoxifen. Cells were harvested, centrifuged and assayed for mRNA and protein expression. In each protocol, experiments were repeated at least three times and the representative data were displayed in the results.

Animal and Diet: All animal experiments were performed according to the guidelines approved by the IACUC of the Ohio State University (1). Swiss albino mice (male, 6-8 weeks old, 10 - 12 g body weight) were obtained from ULAR, Ohio State University (OSU, Columbus, USA) animal breeding colony. Animals were quarantined for 1 week on a 12/12 hour light/dark cycle and were fed solid pellet diet and water ad libitum.

Animals were divided in two groups as control and experimental with 10 mice in each group. Separate batches (for eight separate experiments) of BALB/c nu/nu, athymic mice (*n* = 10)/experiment, aged 5 to 6 weeks old, were maintained in micro-isolator cages (2/cage) within a pathogen-free isolation facility with 12 light/dark cycle at 22 to 24°C and 50% humidity. The basal diet (BD) was based on the AIN-93G formulation, modified to have a high fat content (20% corn oil) at the expense of corn starch. The FS diet was BD supplemented with 10% freshly ground FS corrected for the contribution of FS to fat, fiber, and protein components so that the energy values of the diets were maintained.

Reverse transcriptase PCR: Cells were lysed in appropriate amount of trizol (1 ml trizol per well of a 6 well plate for cultured cells). Cells were repeatedly and vigorously pipetted. Cells were then kept at room temperature for 5-10 minutes, and then 200 ul of chloroform per 1 ml of trizol was added and mixed thoroughly. The cells were again left at room temperature for 10 minutes. Cells were then centrifuged at 12,000 rpm at 4°C for 15 minutes and the upper aqueous colorless layer was transferred to a fresh eppendorf tube. To this eppendorf tube 75 µl LiCl (lithium chloride) followed by 1ml chilled EtOH (ethanol) were added and kept at -20°C for 2-3 hours. The eppendorf tube was centrifuge at maximum speed (>=12000 rpm) for 15 minutes at 4°C. The supernatant was discarded and 250 micro lt of 70% ethanol was added and the tube was kept at room temperature for 2 minutes. The tube was again centrifuged at 7500 rpm for 5 minutes at 4°C, finally the supernatant was discarded and the pellet was re-suspended in RNA grade water till it was completely dissolved. Single strand c-DNA was synthesized for incubation with sense and anti-sense primers using revert aid TM h minus first strand cDNA synthesis kit from fermentas. The resulting cDNA was diluted 1:10 before proceeding with the PCR reaction. PCR was conducted in master-cycler gradient (Brinkmann Instruments). Each PCR reaction used 50 µl cDNA, 2.5 U Taq polymerase (Eppendorf Scientific), 0.2 mMdNTPs and 0.5 µM primer. PCR products were resolved on 2% agarose gel containing 0.01% (v/v) ethidium bromide and visualized by UV illuminator. The size of the PCR amplicon was determined by comparison with 100-bp DNA ladder (Promega).

RT-PCR PRIMERS: β-actin forward primer: ATGAAGTGTGACGTTGACATCCG; β-ctin: reverse primer: GCTTGCTGATCCACATCTGCTG; SCO2 forward primer: CGGAATTCAGAACAGTTTTCCCAAGGATCTCC; SCO2 reverse primer: CGGAATTCAACCTTAGCGAGTTTCAGTCAGTCC.

Real-time PCR: Real-time PCR was performed using the 7500 fast real-time system (Applied Biosystems) using TaqMan probe (Applied Biosystems) (98). GAPDH served as an endogenous control to normalize expression. Each sample was analyzed in quadruplicate. Relative expression and standard error were calculated by the supplied fast 7500 real-time system software.

Western blotting: Western blot analysis is performed as described previously by Gogna et al (1-6, 8). Whole-cell lysates were prepared using RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mMNaCl, 1% NP-40, 1 mM EDTA, 0.1% SDS, and 1 mM DTT). Proteins were resolved by 10% or 12% SDS-PAGE and transferred onto PVDF membranes (Invitrogen). Membranes were stripped according to the molecular weight of the proteins, which were subjected to analysis. Incubations with primary antibodies were followed by incubations with the appropriate secondary antibodies (Amersham) and detection by ECL (Amersham).

Protein ELISA: The wells of a PVC microtiter plate were coated with polyclonal antibodies at a concentration of 1-10 µg/ml in carbonate/bicarbonate buffer (pH 7.4). The plate was covered with an adhesive plastic and incubated overnight at 4°C. Next the coating solution was removed and the plate was washed twice by filling the wells with 200 µl PBS. The solutions or washes were removed by flicking the plate over a sink. The remaining drops were removed by patting the plate on a paper towel. The remaining protein-binding sites in the coated wells were blocked by adding 200 µl blocking buffer, 5% non-fat dry milk/PBS, per well. Again the plate was covered with an adhesive plastic and incubated overnight at 4°C. 100 µl of appropriately diluted cell suspension was added to each well and incubated for 90 min at 37°C. 100 µl (0.5 µg/ 100 µl) of diluted detection antibody (monoclonal) was added to each well. The plate was covered with an adhesive plastic and incubated for 2 h at room temperature. The plates were washed four times with PBS. 100 µl of secondary

antibody conjugated with horse raddish peroxidase was added to the plate. The plate was covered with an adhesive plastic and incubated for 1-2 h at room temperature. The plate was washed four times with PBS.

Chromatin immunoprecipitation: Formaldehyde was added at a final concentration of 1% directly to cell culture media. Fixation proceeded at 22°C for 10 min and was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were collected by centrifugation and rinsed in cold phosphate-buffered saline. The cell pellets were re-suspended in swelling buffer (10 mM potassium acetate, 15 mM magnesium acetate, 0.1 M Tris [pH 7.6], 0.5 mMphenylmethylsulfonyl fluoride, and100 ng of leupeptin and aprotinin/ml), incubated on ice for 20 min, and then Dounce homogenized. The nuclei were collected by microcentrifugation and then re-suspended in sonication buffer(1% sodium dodecyl sulfate, 10 mM EDTA, 50 mMTris-HCl [pH 8.1],0.5 mMphenylmethylsulfonyl fluoride, and 100 ng of leupeptin and aprotinin/ml) and incubated on ice for 10 min. Prior to sonication,0.1 g of glass beads (212- to 300-μm diameter; Sigma) was added to each sample. The samples were sonicated on ice with an Ultrasonics sonicator at setting 10 for six 20-s pulses to an average length of approximately 1,000 bp and then micro-centrifuged. The chromatin solution was pre-cleared with the addition of Staphylococcus aureus protein A-positive cells for 15 min at 4°C. Prior to use, the Staph A cells were blocked with 1 μg of sheared herring sperm DNA/μl and 1 μg of bovine serum albumin/μl for at least 4 h at4°C. Pre-cleared chromatin from 10^7 cells was incubated with 1 μg of affinity-purified rabbit polyclonal antibody or no antibody and rotated at 4°C for approximately 12 to 16 h. p53 Antibodies were used for immunoprecipitation, washing, and elution of immune complexes was carried out as previously described (3). Prior to the first wash, 20% of the supernatant from the reaction with no primary antibody for each time point was saved as total input chromatin and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Cross-links were reversed by the addition of NaCl to a final concentration of 200 mM, and RNA was removed by the addition of10 μg of RNase A per sample followed by incubation at 65°C for4 to 5 h. The samples were then precipitated at −20°C overnight by the addition of 2.5 volumes of ethanol and then pelleted by micro-centrifugation. The samples were re-suspended in 100 μl of Tris-EDTA (pH 7.5), 25 μl of

5× proteinase K buffer (1.25% sodium dodecyl sulfate, 50 mMTris [pH 7.5], and 25 mM EDTA), and 1.5 µl of proteinase K (Boehringer Mannheim) and incubated at 45°Cfor 2 h. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) followed by extraction with chloroform-isoamyl alcohol and then precipitated with 1/10 volume of 3 M NaOAc (pH5.3), 5 µg of glycogen, and 2.5 volumes of ethanol. The pellets were collected by micro-centrifugation, re-suspended in 30 µl ofH_2O, and analyzed by PCR using primers as previously described by Matoba et al (7).

ASK-1 kinase activity assay: After treatments, cells were scraped and extracted in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 12 mM β-glycerophosphate, 150 mMNaCl, 5 mM EGTA, 1 mMNa_3VO_4, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 20 µg/ml aprotinin, 3 mMdithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The extracts were vortexed and centrifuged at 17,000 × *g* for 12 min at 4 °C. The clarified supernatants were frozen on dry ice and stored at −80 °C. The further steps were carried out at 4 °C. To assay Ask1 activity, an equal volume of Ask1 cell lysate normalized for Ask1 protein was incubated with 10 µl of anti-Ask1 antibody for 1 h and harvested with 15 µl of protein A-Sepharose 50% v/v in lysis buffer. After 30 min, the samples were centrifuged for 15 s and washed twice with 300 µl of 20 mMTris-HCl, pH 7.5, containing 250 mM NaCl, 5 mMEGTA, 1% Triton X-100, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. This was followed by two washes with 20 mM Tris-HCl, pH 7.5, containing 5 mM EGTA, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitate was directly used for the kinase assays. The assay was carried out in 20 µl of kinase buffer (100 µM ATP, 3 µCi of [γ-^{32}P]ATP, 20 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 6.5 µg of MBP). ASK-1 activity was assayed for 12 min at 30 °C and was stopped by the addition of 10 µl of SDS-PAGE loading buffer. Kinase activity was evaluated by measuring incorporation of the radioactivity into MBP after resolution by SDS-PAGE and quantification using a PhosphorImager (Molecular Dynamics).

Luciferase Assay: Cells were plated in 35 mm petri dishes the day before transfection so that they reached 60–80% confluence upon transfection. Reporter plasmids (1.0–1.5 mg/well) were transfected with

effectene transfection reagent (Qiagen) as per the manufacturer's instructions. After desired incubation period the cells were washed in cold PBS three times and lysed with 200ml of the lysis buffer by a freeze-thaw cycle, and lysates were collected by centrifugation at 14,000 rpm for 2 minutes in a bench top centrifuge. Twenty micro liter of supernatant was used for the assay of luciferase activity using a kit (Promega) as per the manufacturer's instruction.

Annexin-V staining: Beckton Dickinson flow cytometer was used to detect the apoptotic cell surface shift of phosphatidylserine by the binding of fluorescein isothiocyanate (FITC) conjugated annexin V to the outer membrane of intact cells. Floating cells were collected by centrifugation and these, as well as the attached cells were washed in PBS (Ca^{2+} or Mg^{2+} free)/0.1% EDTA. The attached cells were gently scraped off the dish and centrifuged. After centrifugation, the cells were treated with 500µl binding buffer (abcam) then 5 µl of annexin V-FITC and 5 µl propidium iodide was added. Cells were treated at room temperature for 5 mins. These cells were then filtered through 70m mesh, to eliminate cell aggregates and analyzed by flow cytometry.

TUNEL Assay: TdT-mediated dUTP Nick End Labeling (TUNEL) assay was performed using apop-tag fluorescein *in situ* apoptosis detection kit (invitrogen). Briefly, the cell lines were grown on glass-bottomed dishes and were first washed in equilibration buffer, treated with TdT enzyme in a humidified chamber at 37°C for 1h; cells were then washed and treated (RT, 30mins) in the dark with fluorescein-conjugated anti-digoxigenin.

ROS Measurement: ROS levels were determined by incubating the cells in phosphate buffered saline (PBS) containing 10 µM 2',7' di-chloro-dihydro-fluorescein-diacetate (H-DCFDA, Molecular Probes) for 30 mins at 37° C. DCFDA was metabolized by non-specific esterases to the non-fluorescence product, 2',7'-di-chloro-dihydro-fluoresceine, which was oxidized to the fluorescent product, DCF, by ROS. The cells were washed twice in PBS, trypsinized, re-suspended in PBS, and measured for their ROS using Beckton Dickinson flow cytometer.

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Figure S1

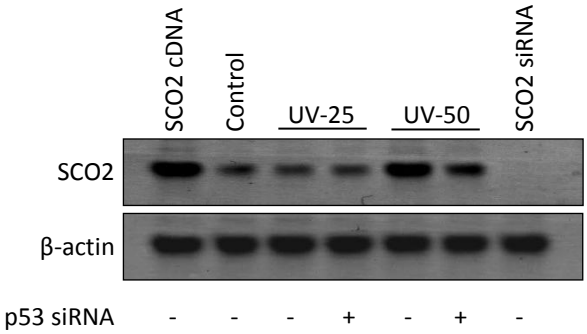
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Figure S2

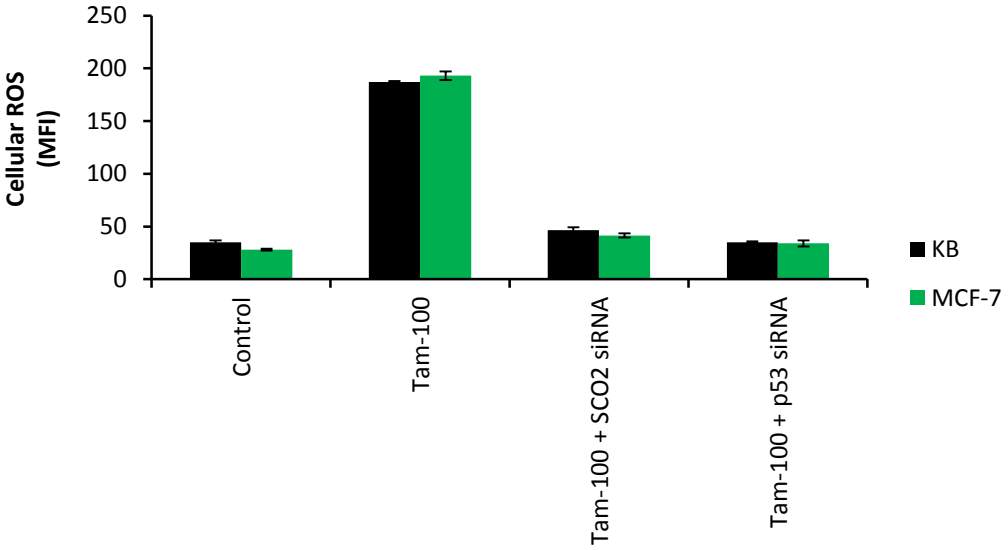
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Figure S3

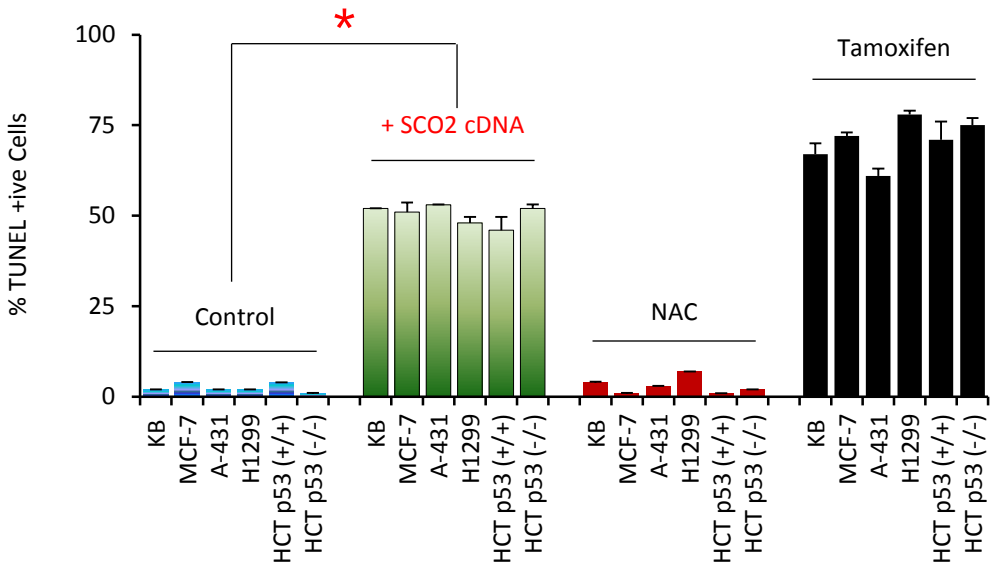
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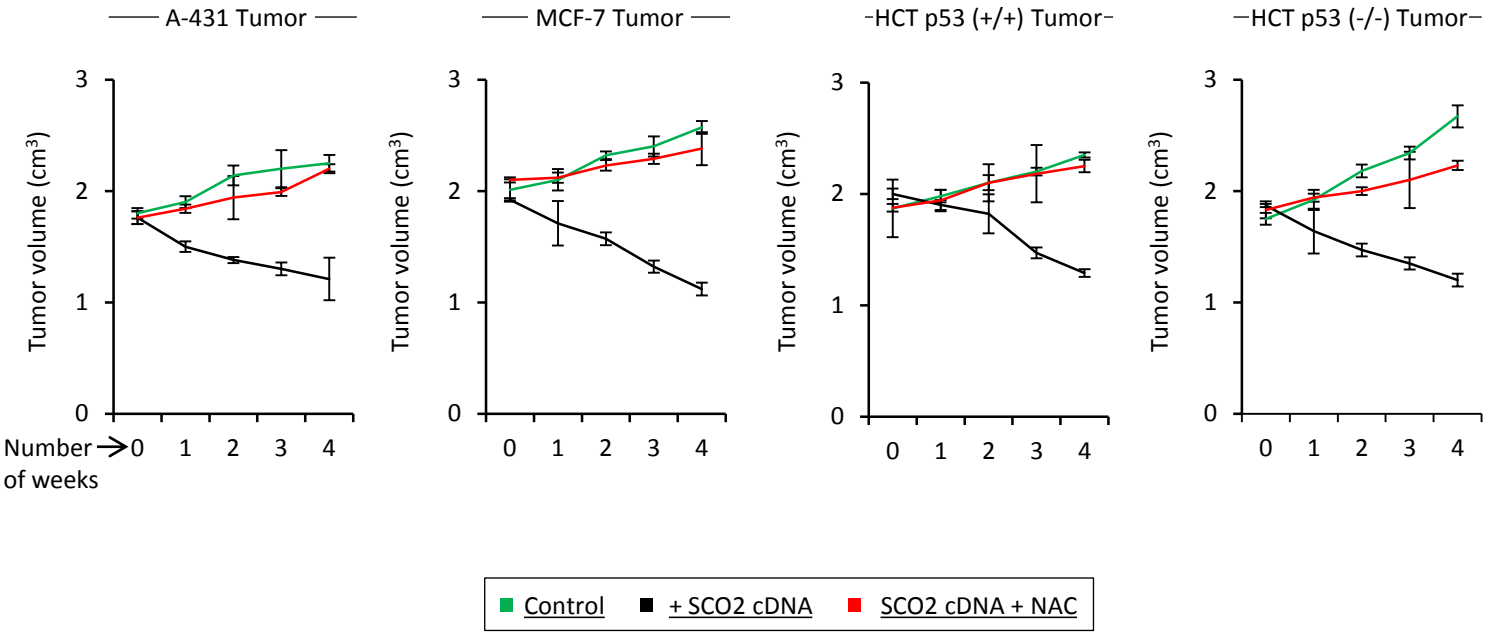
Figure S4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MCF-7 Tumor
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3
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Number of weeks
Control
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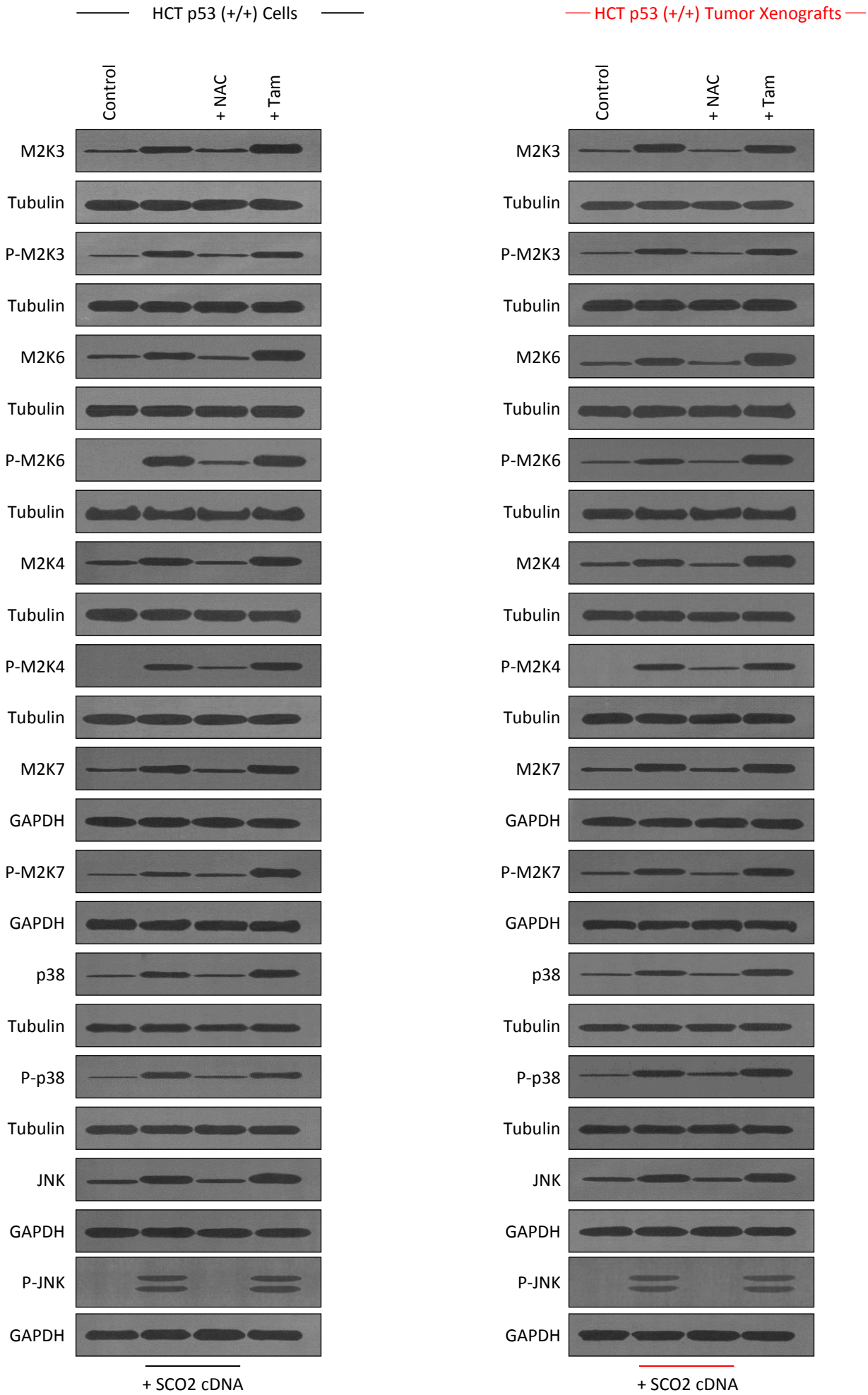
SCO2 cDNA + NAC

Figure S5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+ SCO2 cDNA
+ SCO2 cDNA