SUPPLEMENTAL INFORMATION:

Manganese superoxide dismutase is a p53-regulated gene that switches cancers between early and advanced stages

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Supplemental Experimental Procedures:

Reagents

Unless stated otherwise, all antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and all chemicals were purchased from Sigma (St. Louis, MO). Anti-β-actin antibody was purchased from Sigma (St. Louis, MO). MnSOD, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies and chromatin immunoprecipitation (ChIP) assay kits were purchased from Upstate Biotechnologies (Lake Placid, NY).

Anesthesia

For non-invasive imaging, mice were anesthetized by intraperitoneal injection of 40 µl of ketamine and xylazine (4:1). Following non-invasive imaging analysis, animals were kept warm and allowed to recover from anesthesia before being returned to their normal environment.

Substrate and delivery to skin

Since the mice used for non-invasive imaging stably expressed the MnSOD-luciferase reporter gene, D-luciferin was used as the substrate for reporter gene activity detection and quantification. Seventy µL of D-luciferin substrate (12.5 mM) prepared in DMSO was applied onto the back of each mouse to monitor luciferase expression in the skin.

Construction of plasmid

A BamHI fragment (B7) containing a 3.4 kb 5'-flanking region was used to generate the –555 to +24 basal *MnSOD* promoter by PCR. To create this construct, PCR primers with recognition sequences of KpnI (upstream) and BglII (downstream) restriction sites were added for subcloning at the upstream of the luciferase reporter gene. To generate intronic fragment constructs (I_2E) (1742–2083), BamHI digested 39b -phage DNA containing the human *MnSOD*

gene (8074 bp) was used as the template for PCR amplification. The PCR product was then ligated to the BglII site of the promoter containing PGL3 basic vector, which yielded the natural orientation of the gene. The primer sequences used were: forward primer, (–555) 5'-CGGGGTACCCGCTGGCTCTACCCTCAGCTCATA-3'; reverse primer, (+24) 5'-GGAAGATCTGCCGAAGCCACCACAGCCACGAGT-3'; forward primer, (+1742) 5'-GGAAGATCTCGGGGTTATGAAATTTGTTGAGTA-3'; reverse primer, (+2083).

PCR amplified I_2E fragments were separately subcloned into the pGL3 vector containing the human *MnSOD* basal promoter. PCR constructs were verified by DNA sequencing. The I_2E containing luciferase plasmid DNA was used for generating transgenic animals. pcDNA3.1/Sp1, a cDNA clone for Sp1, was subcloned into the pcDNA3.1 expression vector.

ChIP assay

ChIP assays were carried out as described previously (Dhar et al., 2006; Dhar et al., 2007). Briefly, histone was cross-linked to DNA by adding formaldehyde to skin cells to reach a concentration of 1% and then incubated for 30 min at 37°C. Cells were then suspended in sodium dodecyl sulfate (SDS) lysis buffer with protease inhibitor. Chromatin was sheared by sonication to sizes with DNA lengths between 200 and 1000 bp and immunoprecipitated with appropriate antibodies. Antibody–chromatin complexes were analyzed by either western blotting or PCR. For PCR, DNA-protein complexes were eluted with elution buffer (1% SDS and 0.1 M NaHCO3). Reversal of cross-linking was performed, the proteins were digested, and DNA was then recovered by phenol:chloroform extraction and ethanol precipitation. The DNA recovered from immunoprecipitated DNA-protein complexes was analyzed by PCR amplification.

Tissue Pathology

Mice were humanely euthanized and were necropsied and tissue samples were collected and fixed in 4% formalin in phosphate buffer followed by paraffin embedding. Tissue sections were stained with hematoxylin and eosin; the morphologic analysis was carried out by the Department of Veterinary Medicine at the University of Wisconsin.

Southern blotting

To identify transgenic mice carrying the human *MnSOD* gene construct, genomic DNA was isolated from mouse tail, as described previously (Laird et al., 1991). Tail DNA was digested with the restriction enzymes Kpn1 and BglII, separated on a 1% formaldehyde agarose gel, and transferred onto a nylon membrane. The membrane was pre-hybridized and then hybridized with the ^{32}P-labeled intronic fragment (1742–2083) of the *MnSOD* gene probe, as described previously (St. Clair and Holland, 1991). The membrane was then exposed to Kodak XAR film at –80°C.

Transient transfection and luciferase assay

JB6 cells were transfected with plasmids following a Lipofectamin® transfection protocol according to the manufacturer's instructions. Cells were co-transfected with 2 μM plasmid DNA constructs containing the enhancer or promoter fragments of the human *MnSOD* gene in pGL3 vector. β-galactosidase-cDNA was used as an internal control at a concentration of 0.2 μM. Sp1 expression vectors (pcDNA3.1/Sp1) were co-transfected with the *MnSOD* promoter containing pGL3 reporter vector in cells. In a separate experiment, the Sp1 expression vector and p53 siRNA or control siRNA were co-transfected with the *MnSOD* promoter and enhancer containing pGL3 vector. The cells were then analyzed using the luciferase reporter assay system with a TD-20/20 luminometer (Turner Design Promega, Madison, WI) following the manufacturer's instructions.

Nuclear extract preparation

Nuclei were isolated from one million skin cells. Briefly, cells were re-suspended in buffer A containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and protease inhibitor. The cell suspension was incubated on ice for 15 min; 12.5 µl of 10% Nonidet P-40 was added, and the mixture was vortexed for 15 s. The cytoplasmic and nuclear fractions were separated by centrifugation at 17,000g at 4°C for 30 s. The nuclear pellets were resuspended in buffer B containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.9), 1.5 mM $MgCl^2$, 420 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid, 35% glycerol, 0.5 mM dithiothreitol and protease inhibitors. Nuclear proteins in the supernatant fraction were collected by centrifugation at 14,000g at 4°C for 2 min.

Western analysis

Samples were subjected to SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were incubated with the primary antibody for 2 h at room temperature or overnight at 4°C. The membranes were then incubated with the secondary antibody after being washed twice with Tris-buffered saline Tween-20. Proteins were detected using the enhanced chemiluminescence detection system (ECL®, Amersham Biosciences Piscataway, NJ). The Quantity One® Image Analyzer software program (Bio-Rad Hercules, CA) was used for quantitative analysis.

RNA isolation, cDNA synthesis and reverse transcription–PCR

Total RNA was isolated by using TRIzol reagent (Invitrogen Carlsbad, CA) according to the manufacturer's protocol. Briefly, the cells were suspended in 1 ml of TRIzol reagent and incubated at room temperature for 15 min. Chloroform (0.2 ml) was added to the sample, vortexed and then incubated at room temperature for 10 min. The RNA containing aqueous phase was extracted after centrifugation at 12,000g for 15 min at 4°C. RNA was precipitated

with isopropyl alcohol; the pellet was washed with 70% ethanol and then air-dried. RNA samples having an A_{260}:A_{280} ratio of 18.0 ± 0.5 were used for reverse transcription (RT)–PCR. For RT, cDNA was generated using 0.4 µg of total RNA, oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase, according to the manufacturer's instructions (RT-for-PCR kit, Clontech Laboratories Mountain View, CA), in a total volume of 20 µl. Two µL of cDNA were amplified using the primer sets for mouse *MnSOD* and β-actin (primer sequence is available upon request). PCR products were separated on an agarose gel and visualized by ethidium bromide.

Supplementary Table S1. Data supporting Figure 3 and 4.

Pathology findings in skin and tumor tissues treated with DMBA and TPA for 25 weeks. 1 mm^3 tissues were fixed with 4% formaldehyde and then embedded with paraffin. Tissue sections were prepared and pathological analyses were performed in the pathology laboratory.

Supplementary Table S2. Data supporting Figure 3 and 4.

Pathology findings in skin and tumor tissues during late-stage carcinogenesis. After 25 weeks of treatment, animals were observed for up to 64 weeks before being euthanized. 1 mm^3 tissues were fixed with 4% formaldehyde and then embedded with paraffin. Tissue sections were prepared and pathological analyses were performed in the pathology laboratory.

Supplementary Table S3 (Data supporting Figure 5): p53 siRNA sequences

Supplementary Figure S1 (data supporting Figure 5): Knock-down of p53 by p53 siRNAs

JB6 cells were transfected with control siRNAs or p53 siRNAs for 72 h. Cells were then collected and cell lysates were prepared and subjected to Western blot analysis. Knock- down

of p53 proteins was detected by using an antibody specific to mouse p53 protein following p53 siRNA transfection. p53 target proteins were also detected from the same experiment using specific antibodies to p21 or MnSOD (top panel). p53 knock-down by p53 siRNAs and its effect on p53 target genes were densitometrically quantified and are presented as bar graph (bottom panel). Each data point represents mean ± SD of three samples; statistically significant from individual control , * $p < 0.05$ and ** $p < 0.01$.