MATERIALS AND METHODS

Antibody and reagent

Actin (sc-8432), SOD2 (sc-137254), Heme Oxygenase 1 (HO-1, sc-390991) and NAD(P)H quinone oxidoreductase 1 (NQO1, sc-32793) were from Santa Cruz Biotechnology (Dallas, TX). ZnT10 (SLC30A10) was from Lifespan Biosciences (Seattle, WA). Ki-67 (12202T), cleaved caspase 3 (CC3, 9664L), and secondary antibodies against rabbit (7074S) or mouse IgG (7076S) were from Cell Signaling Technology (Danvers, MA). Tempol (4-Hydroxyl, 176141) was from Sigma-Aldrich (St. Louis, MO).

Mice

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico Health Sciences Center (#23-201434-HSC). Adenomatous polyposis coli *(APC)* is the major driver mutation occurred in roughly 80% of CRC patients (Aghabozorgi et al, 2019). Mice with a disruption of *Apc* specifically in the colon have a robust increase in colon adenomatous polyps (Hinoi et al, 2007). We generated mouse models for our study by crossing *Gpx4* floxed mice ($Gpx4^{F/F}$, Strain #: 027964, Jax) with a tamoxifen-inducible colon tumor model, the colon-specific caudal type homeobox 2 (*Cdx2*) ^{CreERT2} $Apc^{F/+}$ mice (el Marjou et al., 2004). This crossbreeding resulted in two key strains: $Cdx2^{CreERT2}$ $Apc^{F/+}$ $Gpx4^{F/F}$ mice and $Cdx2^{CreERT2}$ $Gpx4^{F/F}$ mice.

Induction of colitis and colorectal cancer

Both male and female mice, specifically $Cdx2^{CreERT2}$ $Gpx4^{F/F}$ and $Gpx4^{F/F}$ mice aged 6-8 weeks, were subjected to treatment with 2% dextran sodium sulfate (DSS, J63606-22, Thermo Scientific, MA) dissolved in sterile, distilled water *ad libitum* for a duration of 7 days, followed by a subsequent 3-day period of normal drinking water. Body weights were monitored daily. To assess the influence of diet on the response to colitis, mice were exposed to various diets,

including a standard chow diet, and diets supplemented with different concentrations of iron (3.5ppm, D03072501i; 40ppm, D03072502i; and 1000ppm, D18082901i), manganese (1ppm, D18901i; 60ppm, D10001i; and 300ppm, TD220455) from Research Diets Inc (New Brunswick, NJ). Additionally, vitamin E-deficient (TD.88163) and vitamin E control (TD.99455) diets were obtained from Envigo Teklad (Madison, WI). Colorectal tumor was induced as we described before (Liu et al, 2023). Briefly, *Cdx2* ^{CreERT2} *Apc*$^{F/+}$ and *Cdx2* ^{CreERT2} *Apc*$^{F/+}$ *Gpx4*$^{F/F}$ mice received 100 mg kg−1 tamoxifen for three consecutive days, followed by a 7-day treatment with 1.5% DSS. Subsequently, they were returned to regular drinking water for 28 days. After sacrifice, colons were longitudinally opened, and tumor number and size were assessed under a dissection microscope.

Metabolomics Analysis

Tissue metabolite extraction and metabolite measurement by liquid chromatography-mass spectrometry (LC-MS) were conducted as previously outlined (TeSlaa et al., 2021). In brief, frozen tumor tissue samples were ground using a Retsch CryoMill system (RETSCH GmbH, Haan, Germany) connected to liquid nitrogen. The extraction buffer (acetonitrile: methanol: water, 4:4:2, v:v:v) pre-cooled at -20 °C was added to the ground tissue at a concentration of 25 mg/mL, followed immediately by vortexing for 10 seconds. The mixture was then incubated at 4 °C for 10 min before centrifugation at 16,000 g at 4 °C for 10 min. The supernatant was transferred to an LC-MS vial and analyzed on the LC-MS. A pooled quality control (QC) sample was prepared by mixing 20 µL from each experimental sample.

Metabolite extracts were analyzed using a quadrupole-orbitrap mass spectrometer coupled with hydrophilic interaction chromatography (HILIC). Chromatographic separation was achieved on an XBridge BEH Amide XP Column (2.5 µm, 2.1 mm × 150 mm) with a guard column (2.5 µm, 2.1 mm × 5 mm) (Waters, Milford, MA). For the gradient, mobile phase A was water: acetonitrile

95:5, and mobile phase B was water: acetonitrile 20:80, both phases containing 10 mM ammonium acetate and 10 mM ammonium hydroxide. The linear elution gradient was: 0 ~ 3 min, 100% B; 3.2 ~ 6.2 min, 90% B; 6.5 ~ 10.5 min, 80% B; 10.7 ~ 13.5 min, 70% B; 13.7 ~ 16 min, 45% B; and 16.5 ~ 22 min, 100% B, with a flow rate of 0.3 mL/min. The autosampler was set at 4 °C. The column temperature was 30 °C. The injection volume was 5 µL. Needle wash was applied between samples using acetonitrile: methanol: water at 4:4:2 (v:v:v).

For mass spectrometry, a Q Exactive HF (Thermo Fisher Scientific, San Jose, CA) mass spectrometer was used. For MS1 acquisition, the MS scanned from 70 to 1000 m/z with switching polarity at a resolution of 120,000 for all experimental samples. The relevant parameters were sheath gas, 40; auxiliary gas, 10; sweep gas, 2; spray voltage, 3.5 kV; capillary temperature, 300 °C; S-lens, 45. The resolution was set at 120,000 (at m/z 200). Maximum injection time (max IT) was set at 500 ms, and automatic gain control (AGC) was set at 3 × 106. Full scan-MS/MS (top 10) data were acquired by data-dependent acquisition (DDA) using pooled QC samples with separated modes. The full scan condition for positive or negative ion mode was set as follows: resolution, 120,000; scan ranges, 60-260, 250-460, 450-660, 650-1000; max IT, 300 ms; AGC, 3 × 106. The DDA parameters were resolution, 30,000; AGC, 1 × 105; max IT, 100 ms; loop count, 10; isolation window, 1.0 m/z; HCD collision energy was set at 10, 20, 40%; minimum AGC, 8 × 103. MS1 raw data files were converted into mzXML using msconvert and imported to El-Maven (Elucidata, Cambridge, MA) for targeted metabolomics. Metabolites were identified based on accurate mass and retention time with an in-house library. Additionally, MS1 and MS/MS raw data files were used for untargeted metabolomics. LC-MS peak extraction, identification, and alignment were performed using MS-DIAL (5.1.221218) with default settings. The libraries used for identification were ESI (+)-MS/MS from standards+bio+in silico (16,995 unique compounds); ESI (-)-MS/MS from standards+bio+in silico (15,245 unique compounds); from the website: http://prime.psc.riken.jp/compms/msdial/main.html#MSP.

Metabolites identified by MS-DIAL were exported and subjected to further processing: 1) Mass error was calculated in an Excel sheet, and ions with a mass error larger than 10 ppm were excluded; 2) redundant identified metabolites from MS-DIAL analysis were excluded when the metabolites were detected and in our in-house library.

Real-time qPCR analysis

Real-time qPCR analysis was performed using RNA extracted with the IBI reagent Kit (IB47602, IBI Scientific, Dubuque, IA). Following reverse transcription, gene expression was assessed by a SYBR green qPCR protocol using the Applied Biosystems QuantStudio 3 qPCR System (0.1mL). Normalization of all gene expression data was carried out using 18s as a reference. The primer sequences are provided in **Table S1**.

Western blot analysis

Animal tissues were lysed and homogenized in radioimmunoprecipitation assay (RIPA) buffer, followed by a 15-minute ice incubation. After centrifugation at 4 °C for 15 minutes, the supernatant was collected, and protein concentration was determined using the Bradford assay. Equal amounts (30–50 μg) of protein were loaded onto SDS-PAGE gels and run for 1 hour at 120V. Proteins were then transferred to nitrocellulose membranes for 1 h at 100 V. Following blocking with 5% milk, membranes were incubated with primary antibodies overnight at 4 °C. After incubation with secondary antibodies, membranes were developed with ECL reagent (Thermo Scientific) and imaged using the Chemidoc Image machine (BioRad).

Histology and immunofluorescence staining

Colon tissues, following removal of intestinal content, were fixed with 10% formalin at room temperature for 48 hours and embedded in paraffin blocks. Paraffin slides (5 μm) underwent hematoxylin and eosin (H&E) staining and were scored by a gastrointestinal pathologist (Dr.

David R Martin) in a blinded manner for histology. In the colitis model, inflammation was scored based on the following scheme: Grade 1, scattered neutrophils in lamina propria; Grade 2, scattered neutrophils plus lymphocytes or plasma cells in lamina propria; Grade 3, dense mixed inflammatory infiltrates involving mucosa plus submucosa. Crypt damage score was based on the following scheme: Grade 1, severely crypt attenuation; Grade 2, basal crypt atrophy; Grade 3, near complete crypt atrophy/drop off; Grade 4, full thickness mucosal drop out. The percentage of involved colons was also scored, and the final score was obtained by multiplying the percentage with the summary score of inflammation and crypt injury. For the tumorigenesis model, tissues were classified as tubular adenoma with low-grade (LG, score 1) versus high-grade (HG, score 2) dysplasia, and the involved lesion percentage was estimated. The final pathological score was achieved by multiplying the lesion percentage with the grade score. For immunofluorescence analysis, after antigen unmasking, tissues were incubated overnight with the primary antibody at 4 °C. After washing with 1x PBS containing 0.1% Tween-20 three times for 5 minutes each, slides were incubated with the secondary antibody, and images were captured with a fluorescence microscope (EVOS, Thermo Fisher Scientific).

Inductively coupled plasma mass spectrometer (ICP-MS).

Paraffin slides (5 μm thick) of colon tumors from *Cdx2* ^{CreERT2} *Apc*$^{F/+}$ mice fed diets containing 1 ppm or 60 ppm Mn were prepared for metal imaging using Laser Ablation ICP-MS as described before (Hare et al, 2017). Metal levels in colon tumor lysates were quantified using Agilent 7900 ICP-MS.

Statistical analysis

Experiments were replicated three times. Data were expressed as mean ± SD. Statistical analyses were conducted using Student's t-test, one-way, or two-way analysis of variance (ANOVA) using Graphpad Prism. A p-value less than 0.05 was deemed statistically significant.

SUPPLEMENTAL FIGURES

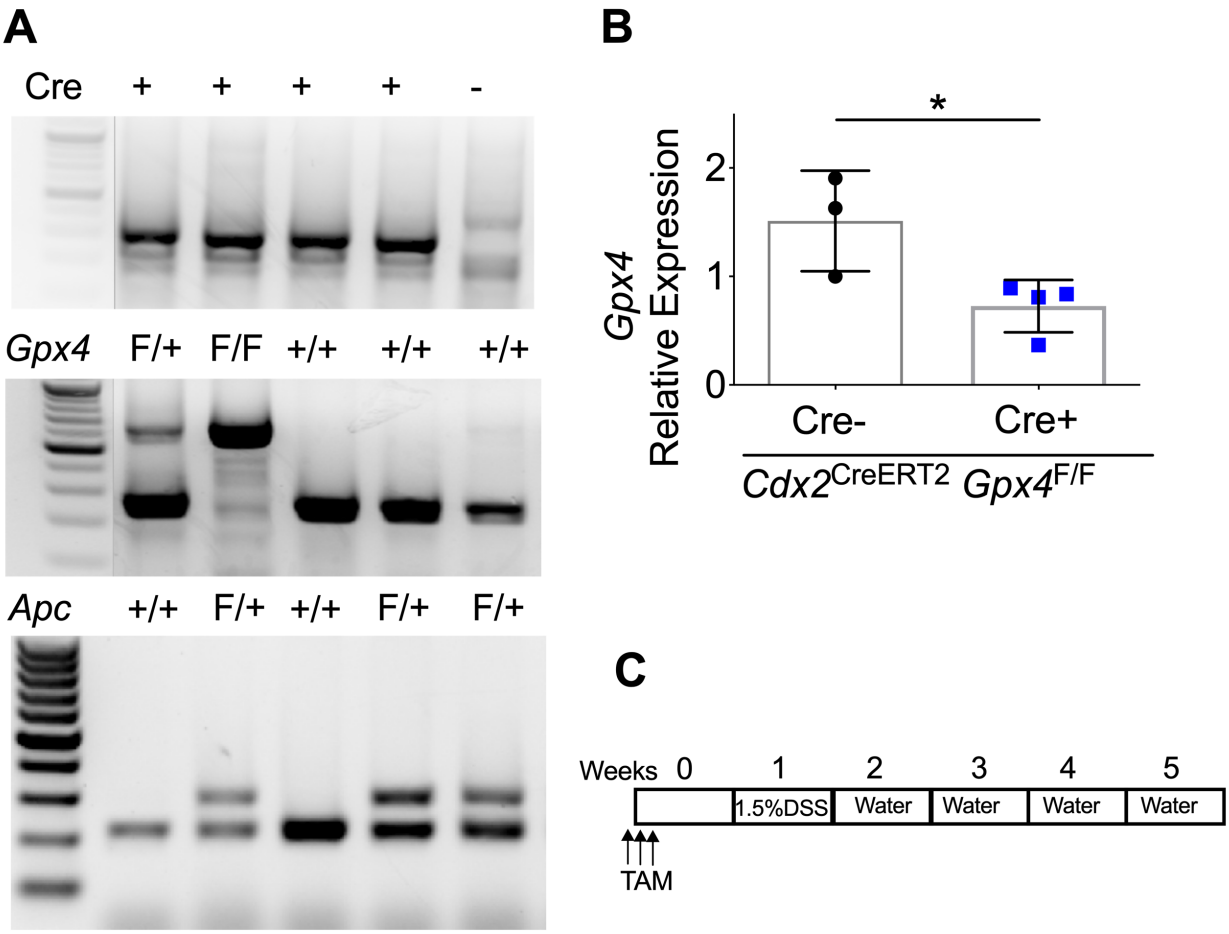

Figure S1 Mouse Model Validation and Treatment Scheme for the Colorectal Cancer Model.

(**A**) Representative genotyping results for the Cre/*Gpx4/Apc* genes.

(**B**) qPCR analysis of *Gpx4* mRNA expression in colon tissues from *Gpx4*$^{F/F}$ (Cre-) and *Cdx2* ^{CreERT2} *Gpx4*$^{F/F}$ (Cre+) mice. *p < 0.05, Unpaired Student t test.

(**C**) Outlines the time schedule for the colorectal cancer model, involving the daily administration of Tamoxifen (100mg/kg) for 3 consecutive days, followed by a one-week treatment with 1.5% dextran sulfate sodium (DSS). After a four-week recovery period, the mice are euthanized, and colorectal tumors are examined and collected for analysis.

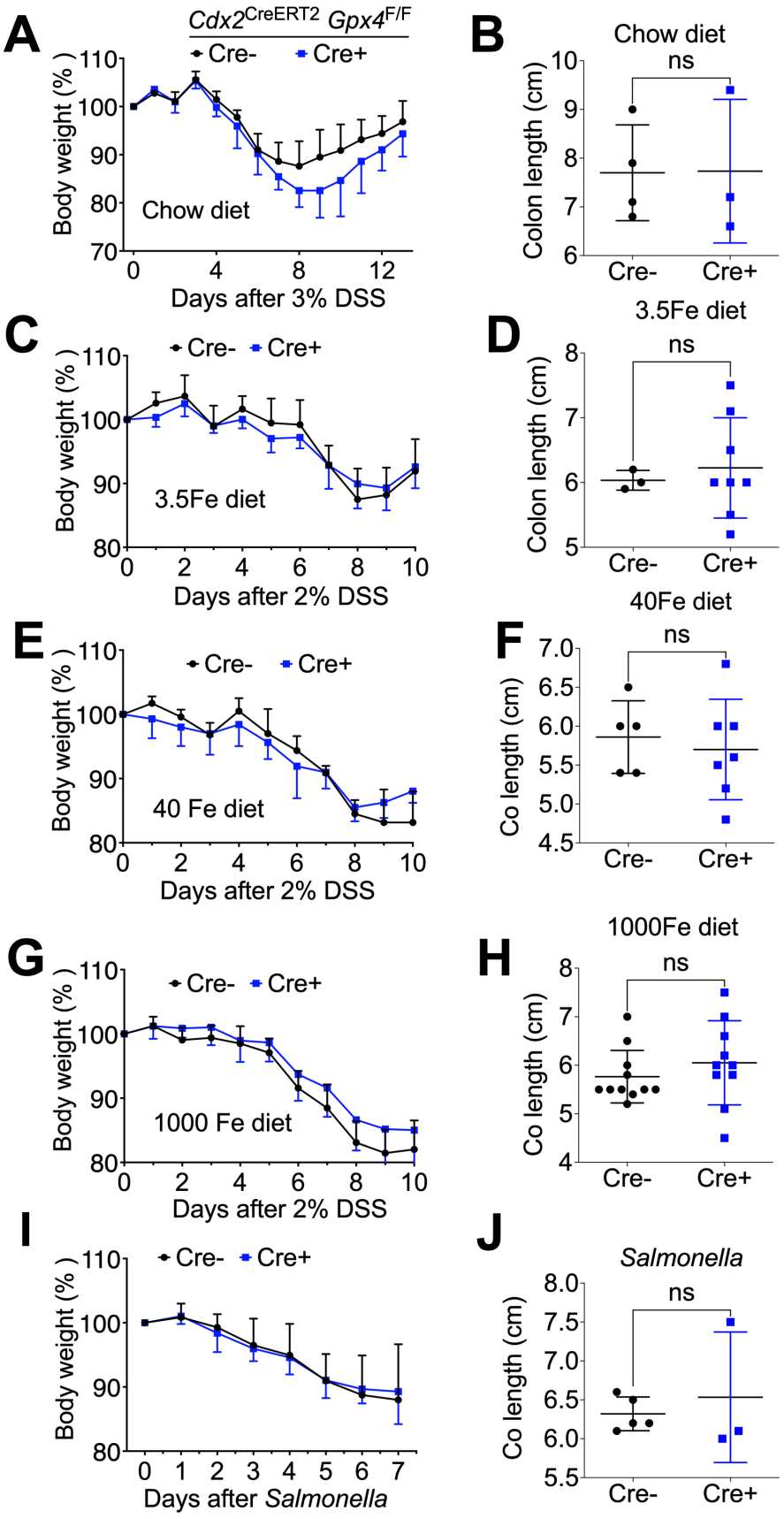
A
Cdx2CreERT2 Gpx4F/F
Cre-
Cre+
Body weight (%)
Chow diet
Days after 3% DSS
B
Chow diet
ns
Colon length (cm)
Cre-
Cre+
C
Body weight (%)
3.5Fe diet
Days after 2% DSS
D
3.5Fe diet
ns
Colon length (cm)
E
Body weight (%)
40 Fe diet
Days after 2% DSS
F
40Fe diet
ns
Co length (cm)
G
Body weight (%)
1000 Fe diet
Days after 2% DSS
H
1000Fe diet
ns
Co length (cm)
I
Body weight (%)
Days after Salmonella
J
Salmonella
ns
Co length (cm)

Figure S2 GPX4 Depletion Specifically in Colon Epithelial Cells Doesn't Change Mouse Sensitivity to Colitis under Different Treatments.

(**A**) Body weight loss curve and (**B**) colon length when mice were fed with a chow diet and treated with 3% DSS for 7 days.

(**C**) Body weight loss curve and (**D**) colon length when mice were fed with a 3.5ppm low iron diet and treated with 2% DSS for 7 days.

(**E**) Body weight loss curve and (**F**) colon length when mice were fed with a 40ppm normal iron diet and treated with 2% DSS for 7 days.

(**G**) Body weight loss curve and (**H**) colon length when mice were fed with a 1000ppm normal iron diet and treated with 2% DSS for 7 days.

(**I**) Body weight loss curve and (**J**) colon length when mice were fed with a chow diet and treated with *Salmonella*.

NS: Not significant. Unpaired Student t-test.

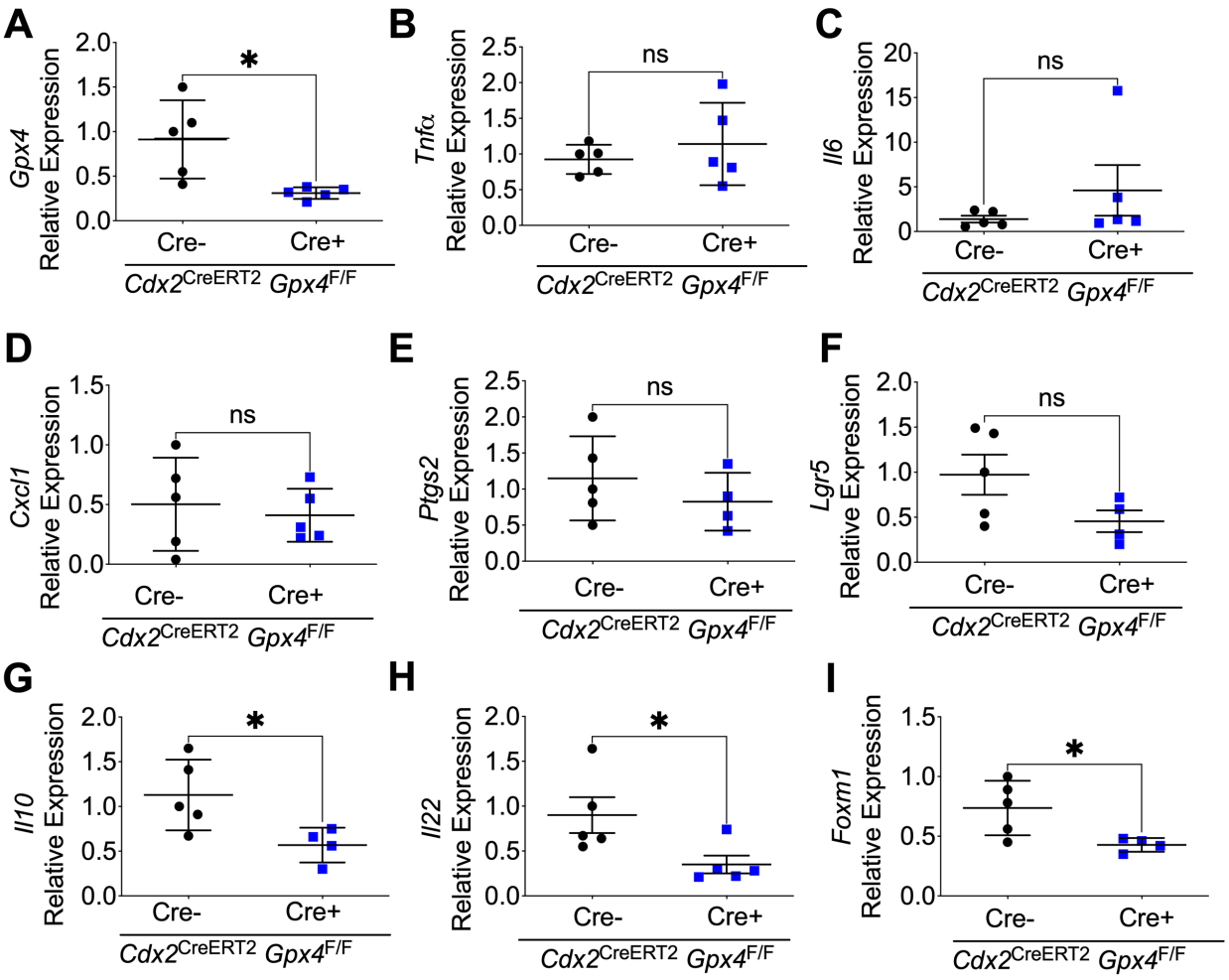

Figure S3 GPX4 Depletion Specifically in Colon Epithelial Cells Doesn't Change Proinflammatory Cytokines but Reduces Anti-inflammatory Cytokines in Mouse Colon Tissues from Mice Fed with a Vitamin E-Deficient Diet.

qPCR analysis of the mRNA expression: (**A**) *Gpx4*, proinflammatory cytokines such as (**B**) *Tnf*, (**C**) *Il6*, (**D**) *Cxcl1*, and (**E**) *Ptgs2*, (**F**) stem cell marker *Lgr5*, as well as anti-inflammatory cytokines like (**G**) *Il10*, (**H**) *Il22*, and (**I**) transcription factor *Foxm1*. *p < 0.05, NS: Not significant. Unpaired Student t-test.

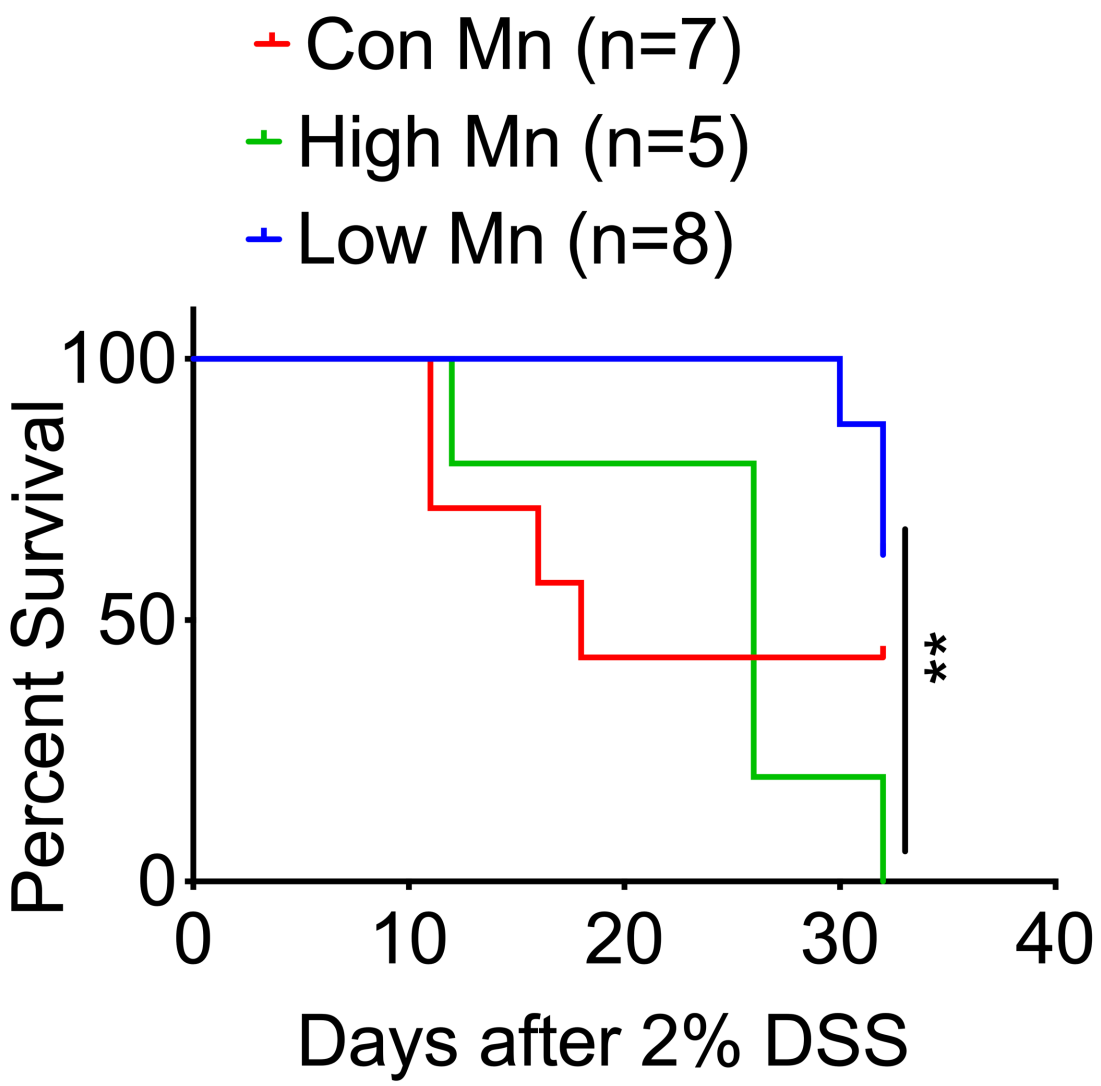

Figure S4. The High Mn Diet is Associated with Reduced Mouse Survival under DSS Treatment.

The survival curve depicts the outcomes of *Cdx2*^{CreERT2} *Apc*$^{F/+}$ mice subjected to 2% DSS treatment for 7 days under varying Mn diets, including a low (1ppm) Mn diet, a normal (60ppm) Mn diet, and a high (300ppm) Mn diet.

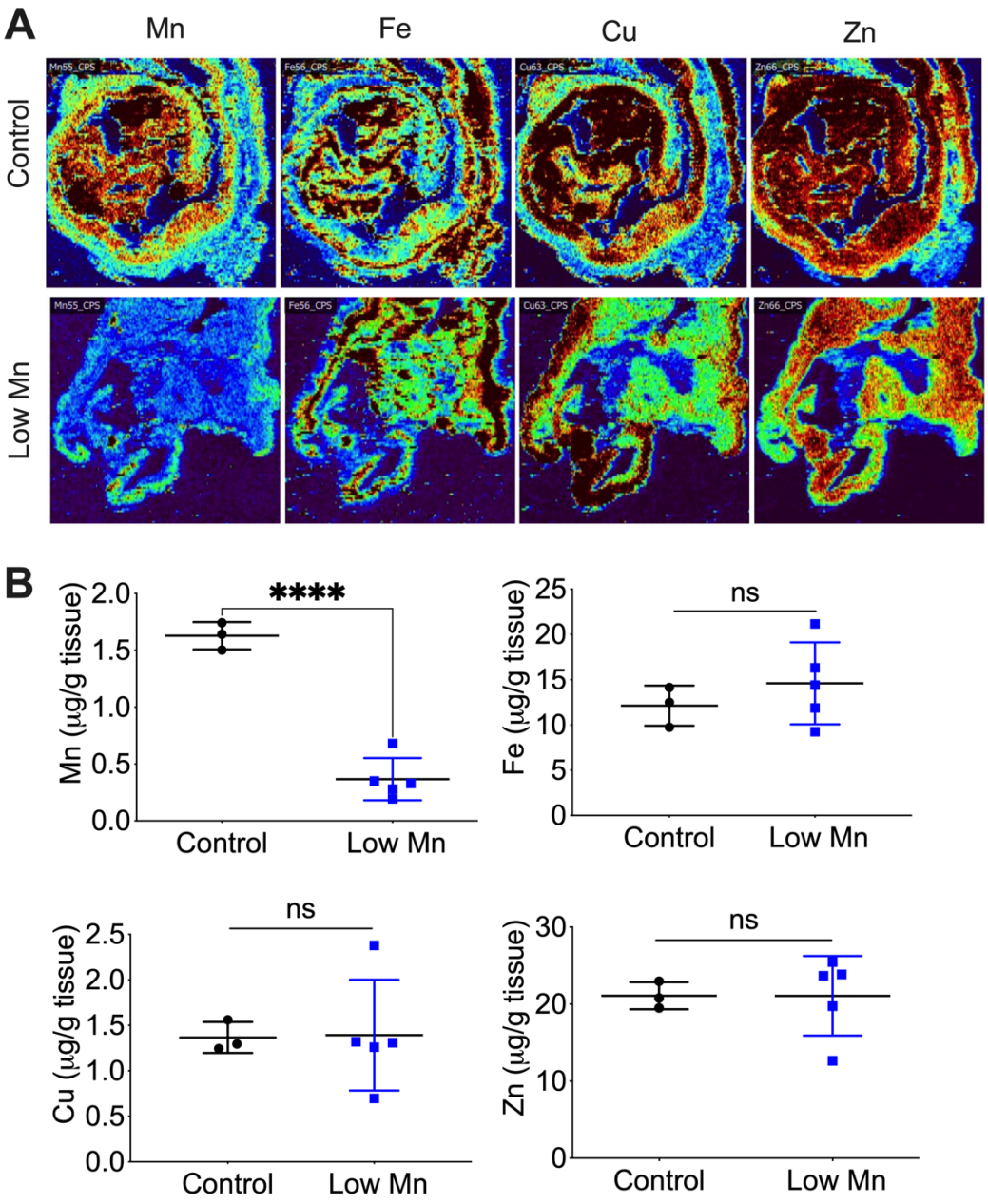

Figure S5 A Low Mn Diet Specifically Reduces Mn Concentration in Colon Normal and Tumor Tissues.

(**A**) Representative laser ablation ICP-MS imaging in colons and (**B**) ICP-MS quantification of metals in colon tumors from a colon tumor-prone *Cdx2*$^{ERT2\text{-}Cre}$ *Apc*$^{F/+}$ mouse model fed with a control Mn diet (60ppm, n=3) or a low Mn diet (1ppm, n=5) for a month. ****p < 0.0001, ns: not significant, Student's t-test.

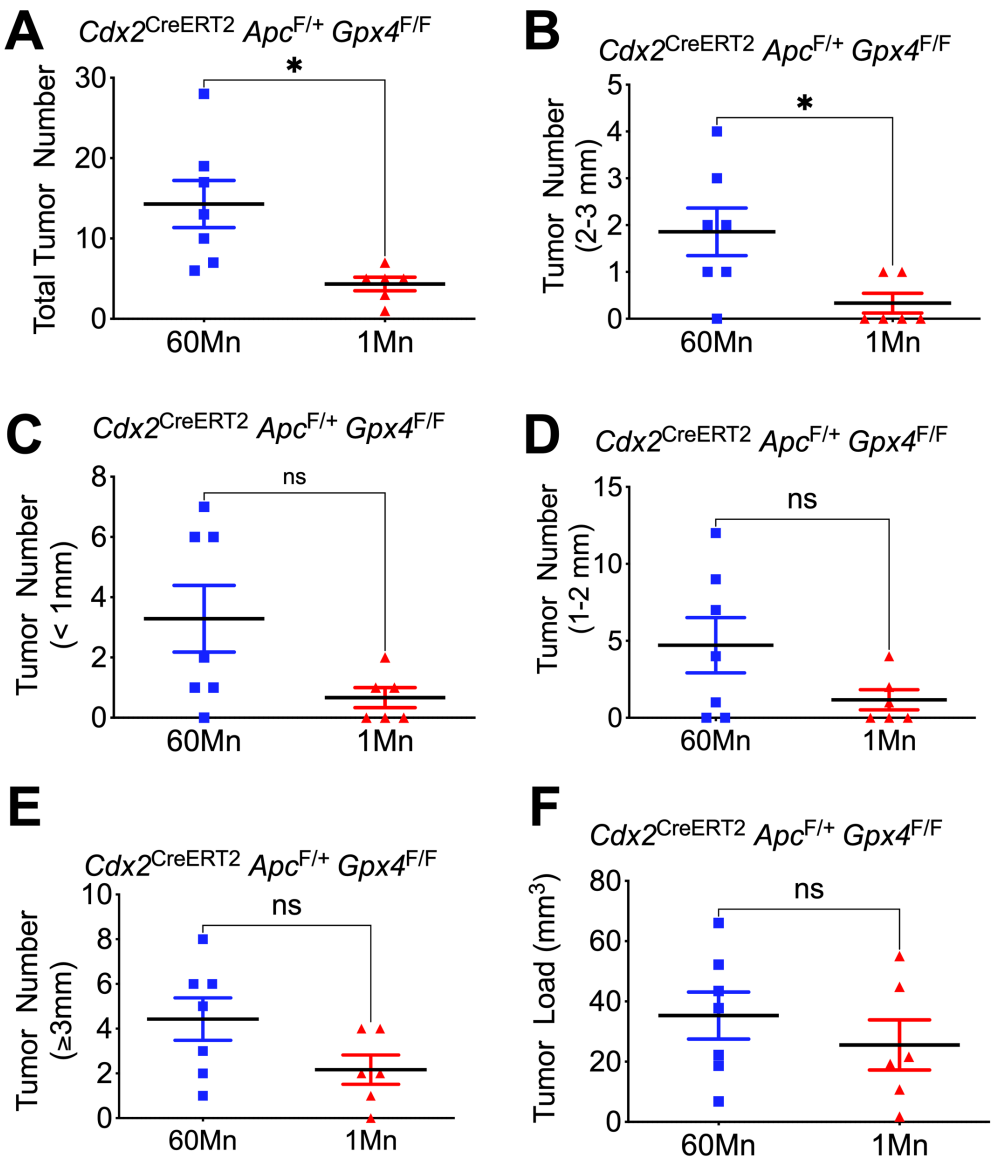

Figure S6 A Low Mn Diet Reduces Tumor Formation in Mice with Colon Epithelial Cell-Specific *Gpx4* Depletion.

(**A**) Total tumor number, (**B**) the number of tumors with a diameter between 2 to 3 mm, (**C**) the number of tumors with a diameter less than 1 mm, (**D**) the number of tumors with a diameter ranging from 1 to 2 mm, (**E**) the number of tumors with a diameter greater than 3 mm, and (**F**) tumor load in *Cdx2* ^{CreERT2} *Apc*$^{F/+}$ *Gpx4*$^{F/F}$ mice compared to *Cdx2* ^{CreERT2} *Apc*$^{F/+}$ mice fed with a control Mn diet (60ppm, n=7) or a low Mn diet (1ppm, n=6) for a month. *$p < 0.05$, NS: Not significant. Unpaired Student t-test.

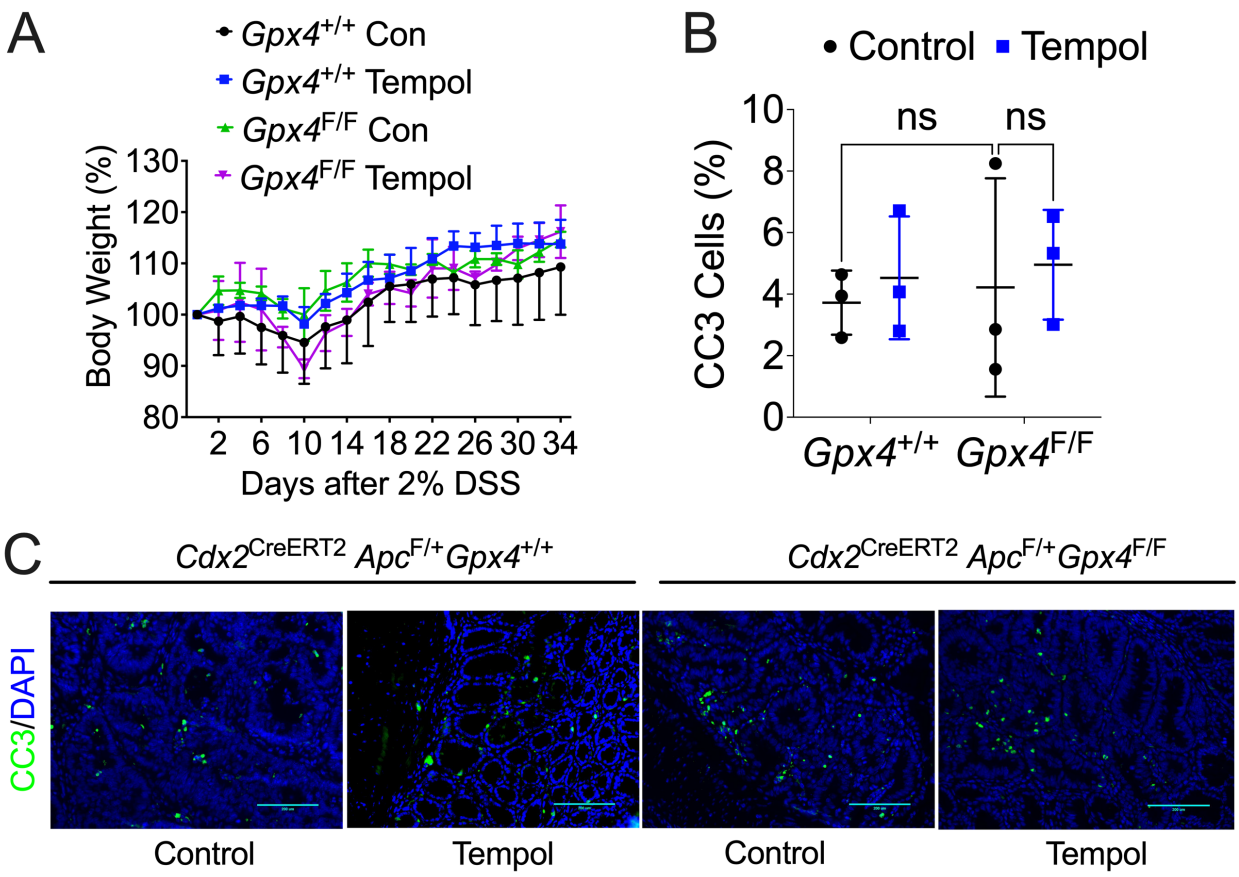

Figure S7. The Impact of a Superoxide Dismutase Mimetic, Tempol Treatment, on Body Weight and Apoptotic Marker in the Colon Tissue of Mice with Colon Epithelial Cell-Specific *Gpx4* Depletion.

(**A**) Depiction of body weight curves illustrating changes over time after treatment with either a vehicle or tempol. (**B**) Quantification and representative images of immunohistochemistry staining for the apoptotic marker cleaved caspase-3 (CC3) in colon tissue samples from *Cdx2* ^{CreERT2} *Apc*$^{F/+}$ *Gpx4*$^{F/F}$ mice and *Cdx2* ^{CreERT2} *Apc*$^{F/+}$ mice treated with either the vehicle or tempol. NS: Not significant. Two-way ANOVA followed by Tukey's multiple comparisons test.