Supplemental Materials and Methods

**RNAi**

Control shRNA and HMGB1 shRNA (Sigma) were transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Control siRNA and p53 siRNA were transfected into cells using X-tremeGENEsiRNA reagent (Roche Applied Science) according to the manufacturer’s instructions. At the end of the RNAi treatment (48 h), the levels of p53 or HMGB1 were analyzed by western blot.

**Western blotting**

Proteins in the whole cell, nuclear and cytosolic lysates were resolved on 4-12% Criterion XT Bis-Tris gels (Bio-Rad, USA) and transferred to a nitrocellulose membrane. Following blocking, the membrane was incubated overnight at 4°C with individual primary antibodies. After incubation with peroxidase-conjugated secondary antibodies for 1 h at 25°C, the signals were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. The relative band intensity was quantified using the Gel-pro Analyzer® software (Media Cybernetics, Bethesda, MD, USA).

**Immunoprecipitation analysis**

Cell lysates were cleared by centrifugation (12000 g, 10 min). Concentrations of proteins in the supernatant were determined by the bicinchoninic acid assay. Prior to immunoprecipitation, samples containing equal amount of proteins were pre-cleared with
Protein A or protein G agarose/sepharose (4°C, 3 h) and subsequently incubated with various control IgG or specific antibodies in the presence of protein A or G agarose/sepharose beads for 2 h or overnight at 4°C with gentle shaking. Following incubation, agarose/sepharose beads were washed extensively with PBS, and proteins were eluted by boiling in 2× sodium dodecyl sulfate (SDS) sample buffer before SDS-PAGE electrophoresis.

**Subcellular fractionation**

Subcellular fractionation of cells was carried out with a mitochondrial and nuclear isolation kit obtained from Pierce (Rockford, IL, USA) according to the manufacturer’s instructions.

**Immunofluorescence analysis of LC3**

Cells were cultured on glass cover-slips and fixed in 3% formaldehyde for 15 min at room temperature prior to detergent extraction with 0.1% Triton X-100 for 5 min at 25°C. Cover slips were saturated with 2% bovine serum albumin in phosphate buffered saline for 1 h at room temperature and processed for immunofluorescence with primary antibodies followed by Alexa Fluor 488 or Cy3 (Invitrogen). Nuclear morphology was resolved with application of the fluorescent dye Hoechst 33342 (Invitrogen). Images were taken with an Olympus Fluoview 1000 confocal microscope (Olympus Corp, Tokyo, Japan), and fluorescence signals were measured using Image-Pro Plus platform (Media Cybernetics, Bethesda, MD, USA). The average LC3 punctae per cell was determined from 15 random fields.
**Ultrastructural analysis**

Transmission electron microscopy (TEM) assessment of autophagy was performed as previously described (1). In brief, cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), followed by postfixation for 6 h in 1% O₃O₄. After dehydration with graded alcohols, the samples were embedded in epoxy resin (Epon). Then, thin sections (70 nm) were cut with a microtome (Leica Ultracut R), mounted on copper grids and post-stained with 2% uranyl acetate and 1% lead citrate, dried, and analyzed using a transmission electron microscope at 25°C (JEOL 100CX, Peabody, MA, USA). Thick sections were cut (300 nm) and stained with 1% toluidine blue. Images were acquired digitally from a randomly selected pool of 10 to 15 fields under each condition.

**Apoptosis assays**

Apoptosis in cells was assessed using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) by flow cytometric analysis. Caspase-9 activity was assayed using the Caspase-9 Colorimetric Assay Kit (Calbiochem, USA) according to the manufacturer’s instructions. Bax and Cytochrome C in the cytosolic or mitochondrial fraction were detected by Western blotting.

**Tissue microarray analysis**

Prepared as previously described (2). Samples were stained with p53/HMGB1 and p62/HMGB1. 119 samples from 29 patients with normal colon and adenocarcinoma of the colon were scored by two independent scientists who were blinded to the histologic
diagnosis and grade (manual scoring) and the Cellomics software analysis (automated analysis). The scores for the quality of sample and quantity of each stain were normalized and averaged from these three sources. Total survival and survival from first recurrence were obtained with approval from the University of Pittsburgh Institutional Review Board on deidentified specimens with a suitable firewall: IRB Protocol # PRO11100423. Linear regression with comparison of F-statistics corrected for multiple comparison was used to determine if HMGB1 or p53 expression was associated with total survival and survival from first recurrence.

**Statistical analysis**

Data are expressed as means ±s.d of two or three independent experiments performed in triplicate. One-way ANOVA was used for comparison among the different groups. When the ANOVA was significant, post hoc testing of differences between groups was performed using an LSD test. A paired T-test was used for analysis of the tissue microarray samples to compare colorectal cancer samples with normal colon. A $p$-value < 0.05 was considered significant.

**Reference**