Supplemental Methods

Patient data and sample ascertainment

Patients included in this study were diagnosed with colorectal cancer at the University of Chicago between 1992 and 2012. This included 155 patients who were selected from a previously described cohort (1992-2002) of 448(1) and 305 patients from a cohort (2008-2012) of 489 based on availability of genetic material or formalin-fixed paraffin embedded uninvolved normal resections in addition to comprehensive tumor and survival data obtained from the University of Chicago Cancer Registry. Clinical information included age, sex, race, American Joint Committee on Cancer (AJCC) stage, grade, location of primary tumor, survival and vital status at last follow-up. This study was approved by the Institutional Review Board at the University of Chicago.

Pathologic assessment and DNA extraction

Tumor characteristics were assessed by a trained pathologist at diagnosis. For DNA extraction from FFPE tissues, 1mm cores from uninvolved resected tissues were extracted. Paraffin was removed using octane and methanol and DNA was extracted from tissue according to the manufactures instructions (Qiagen). Quality of DNA was assessed by UV spectroscopy and gel electrophoresis.

Genotyping

TaqMan allelic-discrimination assay designed around a single-nucleotide polymorphism (SNP) was used to genotype rs2241880 (part number: 4351379, Applied BioSystems, Foster City, CA). Allelic discrimination was performed using a Roche LightCycler 480. PCR was performed in a final volume of 5μl containing 2.5μl TaqMan Universal PCR Master Mix, 0.25μl TaqMan SNP Genotyping Probe, and 10ng genomic DNA suspended in 2.25μl nuclease free water. PCR conditions included an initial enzyme activation

(95°C 10 min) followed by 40 cycles of allelic discrimination (denature at 95°C for 15 sec, anneal/extend at 60°C for 1 min). Ramp rates were set to 1.6°C/s. Fluorescence was measured after each cycle and endpoint genotyping was performed using Roche LightCycler 480 Instrument Software Version 1.5.

Statistical analysis

For genetic studies data all analyses were performed using Stata SE (Version 12). Categorical variables were tested using $\chi 2$ test and Fisher's exact test. Continuous variables were analyzed by one-way ANOVA. Multi-factorial logistic regression was used to test the effect of independent categorical variables on a dichotomous dependent variable (stage IV vs. stage I-III). The Kaplan-Meier method was used to estimate survival functions and Cox proportional hazards model was used to adjust for covariates. Prognostic factors were tested by univariate analysis using log-rank test for equality of survival functions and stratified log-rank tests. Cox regression was used for multivariate models employing Breslow's method for ties. For all other experiments data are presented as mean ± SEM. Results were analyzed using PRISM software by either Student's t-test when comparing two groups or one/two-way ANOVA when appropriate. A P value less than 0.05 was considered significant for all statistical tests. Hardy-Weinberg equilibrium was tested using the calculator at (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20%20HW%-20calculator.xls) and analyzed using the appropriate χ^2 test.

Antibodies

ATG16L1 and p62/SQSTM1 antibodies were from MBL International. Actin antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). COXIV and VDAC antibodies were

from Thermo Scientific (Hanover Park, IL). RIG-I, MDA5, MAVS, ATG5 and FLAG antibody were from Cell Signaling (Danvers, MA). Tubulin, LC3, and TUFM antibodies were from Sigma (St. Louis, MO). TUFM antibody was from Sigma. MxA antibody was purchase from Germany. ISGF3γ antibody was from (BD bioscience).

Cell lines and cell culture reagents

HCT116 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). ATCC authenticated this cell line by isoenzymology and short tandem repeat (STR) profiling. HCT116 cells were not passaged more than 3 months following resuscitation. HCT116 cells were maintained (37°C, 5%CO_2) in McCoy's 5A medium (Life Technologies, Rockville, MD) containing 10% fetal bovine serum. HEK293 cells were cultured (37°C, 5%CO_2) in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100mg/ml), and streptomycin (100U/ml) (Life Technologies, Rockville, MD). Human WISH cells were obtained from ATCC (CCL-25) and were cultured in minimum essential media supplemented with NEAA HEPES (Life Technologies) and 10% fetal bovine serum in a 37°C humidified incubator containing 5% CO_2. WISH cells were established as a HeLa cell line by ATCC using isoenzymology and STR profiling. To generate ATG16L1 deficient or 300A expressing HCT116 cells the ATG16L1 locus was targeted through homologous recombination using an adeno-associated virus (2) to deliver targeting vectors as previously described(3, 4).

Generation of lentiviral constructs and viral particles

Full-length ATG16L1 was amplified and cloned into a lentiviral vector (pSMPUW) containing an IRES and puromycin resistance using PCR. MAVS and scrambled shRNA constructs were purchased from Sigma (product #236029 and #236030). Lentiviral

particles were generated according to protocols published by the Tronolab RNAi Consortium using VSVG and p89.1 envelop and packaging plasmids. Lentivirus was harvested in 2 rounds and supernatants were concentrated using Lenti-X (Clontech). Concentrated lentivirus was used to transduce HCT116 cells prior to antibiotic selection in culture media containing puromycin (1ug/ml). Protein expression or knock-down was confirmed by immunoblotting.

Immunoblotting

Cells were lysed in radioimmunoprecipitation (5) buffer (1% Triton, 0.25% DOC, 0.05% SDS, 50mM Tris-HCl [pH 8.0], and 150mM NaCl) or 1% Triton Lysis butter (50mM Hepes, 150mM NaCl, 1.5mMMgCl_2, 1mM EGTA, 20mM N-Ethylmaleimide, 10mM 1,10 phenanthroline monohydrate, and 1X protease inhibitor cocktail (Roche, Basel, Switzerland)). Protein concentration was determined by BCA assay (Thermo Pierce). Lysates were boiled in Laemmli buffer for 10 minutes and then resolved by SDS-PAGE (NuPAGE Novex 4-12% Bis-Tris Polyacrylamide gel (Invitrogen)) and then transferred to Polyvinylidene Fluoride (PVDF) FL membranes (Millipore). Membranes were incubated with blocking buffer (LI-COR BioSciences, Lincoln, NE) diluted in PBS containing 0.1% Tween 20 (PBST) prior to overnight incubation in primary antibody at 4°C. Membranes were washed in PBST and incubated with fluorophore-conjugated secondary antibody, and finally imaged using the Odyssey LI-COR Infrared Imaging System (LI-COR).

Immunohistochemisty and immunofluorescence

FFPE sections were deparaffinized in xylene and progressively hydrated through an alcohols. Endogenous peroxidase was blocked by incubating hydrated sections in 0.3% hydrogen peroxide for 15 min at room temperature. Slides were then demasked by

briefly boiling in 10mM sodium citrate pH 6 and held at temperature for 20 min in a vegetable steamer. After cooling, slides were blocked for 45 min in 5% bovine serum albumin in PBS. Anti-MxA antibody (purchased from Germany) was diluted 1:100 in 1% BSA in PBS and incubated with sections overnight at 4°C. Slides were washed 3 times for 10 min in PBS + 0.1% Tween20 before incubation with secondary HRP polymer-conjugated anti-mouse IgG (Dako) for 45 min. Slides were wased 3 times for 10 min in PBS + 0.1% Tween20 prior to development in buffer containing 3,3'-diaminobenzidine for 10 min. Finally slides were washed twice in PBS + 0.1% Tween20 and stained for 20 sec in diluted hematoxylin prior to dehydration in alcohols and mounting.

For immunofluorescence, FFPE sections were processed as described above with the omission of peroxidase block. For ISGF3g staining cells were grown on collagen coated coverslips (BD bioscience) and treated with human recombinant IFN-β for 6 hours. Then cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed twice in PBS, and permeabilized in ice cold methanol for 30 min at -20°C. Coverslips were washed 3 times in PBS and blocked using 5% BSA diluted in PBS. Anti-ISGF3γ antibody (BD bioscience) was diluted 1:200 in 1%BSA in PBS and coverslips were incubated overnight at 4°C. Coverslips were washed 3 times in PBS + 0.1% Tween20 and incubated with anti-mouse Alexa488-conjugated secondary antibody (Invitrogen) diluted 1:200 in PBS + 0.1% Tween20 for 45 min at room temperature. Slides were washed 3 times in PBS + 0.1% Tween20 and mounted using Prolong Gold anti-fade aqueous media (Invitrogen).

Real-time PCR

RNA was extracted using Trizol according to the manufacturer's instructions (Invitrogen) and cDNA synthesis performed using the Transcriptor First Strand cDNA synthesis kit

and random primers (6). Real-time PCR was performed in 10μl reaction volume containing 10ng cDNA, 5μl 2x SYBR green Master Mix, and 1μM each forward and reverse primer. Samples were pipetted in triplicate. PCR cycling was performed according to manufacturer's instructions using a 56°C annealing temperature. Primer sequences are as follows: interferon beta forward 5'-CACGACAGCTCTTTCCATGA-3', reverse 5'-AGCCAGTGCTCGATGAATCT-3'(7); beta actin forward 5'-GTACCACTGGCATCGTGATGGACT-3', reverse 5'-CCGCTCATTGCCAATGGTGAT-3(8)'; Mx1 forward 5′-TACCAGGACTACGAGATTG-3′, reverse 5′-TGCCAGGAAGGTCTATTAG-3′; C1orf29 forward 5′-AATCAGACAGAACAGTTAATCCTC-3′, reverse 5′-TCAACCATATCTTCAATGCTACC-3′(9). Relative gene expression was calculated using the ΔΔCt method normalized to beta actin.

Mitochondrial mass and flow cytometry

Mitochondrial mass was evaluated in live cells after staining with MitoTracker Green FM according to the manufacturer's protocol (Invitrogen). Immediately following staining, cells were washed twice in sterile PBS and dissociated using Accutase (Innovative Cell Technologies). Suspended cells were washed once in PBS containing 10% fetal calf seurm, resuspended in 500μl PBS + 10% FCS and analyzed by flow cytometry (BD FACSCalibur). Single cells were gated and mean fluorescence intensity was used to quantify MitoTracker Green FM fluorescence.

Supplemental Figure Legends

Figure S1. Adjusted Kaplan-Meier estimates of survival in CRC according to ATG16L1 genotype. Footnote: Data was centered around mean age for each genotype and adjusted for race and location of the primary tumor by cox regression. *Stratified log-rank test and KM estimates were generated using STATA.

Figure S2. MxA expression and scoring in human colon adenocarcinoma. A, immunohistochemistry against MxA (upper panels) and corresponding H/E (lower panels). Representative section depicting MxA+ve score. B, MxA expression in human adenocarcinoma and adjacent non-dysplastic epithelium. Immunohistochemistry against MxA (lower) and corresponding H/E (upper panel).C, immunohistochemistry against MxA (right) and corresponding H/E in normal colon epithelium.

Figure S3. Autophagy function in ATG16L1 T300A cells. A, immunoblots of whole-cell lysates prepared from ATG16L1+/+ and Δ/Δ (left panel) or ATG16L1 300T/300T and 300A/300A cells (right panel. Cells were treated with 50mM ammonium chloride for 4 hours prior to lysis to arrest lysosomal digestion (n=2). B, immunoblots of whole-cell lysates prepared from ATG16L1 cell panel (left) and ATG16L1Δ/Δ cells stably transduced with full-length ATG16L1 (right) (n=2). C, mean fluorescence of ATG16L1 cell panel stained with MitoView and analyzer by flow cytometry (n=3).

Figure S4. ATG16L1 regulates RIG-I/MDA5 expression in human CRC cells. A, immunoblots from cytosolic and mitochondrial fractions prepared from ATG16L1Δ/Δ cells stably transduced with empty vector (EV) or full-length ATG16L1 (n=2).

Figure S5. IFN-β promotes RIG-I expression in human CRC cells. A, immunofluorescence against ISGF3γ (green) and hoescht (blue) in ATG16L1+/+ cells treated with human recombinant IFN-β for 6 hours as indicated (n=2). B, immunoblots from whole-cell lysates prepared from ATG16L1+/+ cells treated with human recombinant IFN-β for 6 hours as indicated (n=2).

Figure S6. ATG16L1 regulates poly(I:C) induced IFN-I in human CRC cells. A, real-time PCR following stimulation with 1000ng/ml poly(I:C) for 4 hours in ATG16L1Δ/Δ cells stably transduced with empty vector (EV) or full-length ATG16L1 (n=2).

Supplementary Tables.

Table S1. ATG16L1 T300A frequency with respect to ethnicity

Table S2. Assoiciation of ATG16L1 genotype with clinicopathologic characteristics in colorectal cancers

Table S3. Association of ATG16L1 genotype with tumor grade and stage in colon cancer

Table S4. Cox regression analysis of prognostic factors with respect to overall survival

Supplemental References

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