Supplemental Information

RNF20 Links Histone H2B Ubiquitylation with Inflammation and Inflammation-Associated Cancer

Ohad Tarcic, Ioannis S. Pateras, Tomer Cooks, Efrat Shema, Julia Kanterman, Hadas Ashkenazi, Hana Boocholez, Ayala Hubert, Ron Rotkopf, Michal Baniyash, Eli Pikarsky, Vassilis G. Gorgoulis, and Moshe Oren
Figure S2, related to Figure 2

A

![Graph A1](image)

![Graph A2](image)

B

![Graph B1](image)

![Graph B2](image)

C

|            | No TNF-α | TNF-α 30 min |
|------------|----------|--------------|
| siLacZ     | siRNF20  | siLacZ       | siRNF20     |
| β-tubulin  |          |              |             |
| IκB/β-tubulin: | 1.00 | 0.9          | 0.2         | 0.2         |
| IκB        |          |              |             |

D

| siLacZ | siRNF20 |
|--------|---------|
| ![Image D1](image) | ![Image D2](image) |
| No TNF-α | TNF-α 30 minutes |
| ![Image D3](image) | ![Image D4](image) |
| ![Image D5](image) | ![Image D6](image) |
| ![Image D7](image) | ![Image D8](image) |
| ![Image D9](image) | ![Image D10](image) |
| ![Image D11](image) | ![Image D12](image) |
| ![Image D13](image) | ![Image D14](image) |
| ![Image D15](image) | ![Image D16](image) |
| ![Image D17](image) | ![Image D18](image) |
| ![Image D19](image) | ![Image D20](image) |
| ![Image D21](image) | ![Image D22](image) |
| ![Image D23](image) | ![Image D24](image) |

![Graph D1](image)

![Graph D2](image)
Figure S3, related to Figure 3

A. RNF20 mRNA expression over time in WT and RNF20+/- cells.

B. Western blot analysis showing protein expression levels of RNF20, GAPDH, H2Bub1, and H2B.

C. Cytokine mRNA expression in WT and RNF20+/- cells over time.

D. Cytokine mRNA expression in WT and RNF20+/- cells under TNF treatment.

E. RNF20 mRNA expression in WT and RNF20+/- cells over time.

F. Cytokine mRNA expression in WT and RNF20+/- cells under TNF treatment.

- siLacZ
- siRNF20
- WT
- RNF20+/-
- Mip2
- IL22
- RNF20
- IL23
- CCL2
- IL6
- KC
Figure S4, related to Figure 4

A) Colon length (cm)- day 180
B) Spleen Weight (g)- day 180
C) Water DSS
WT Water
n=8
+/- Water
n=10
WT DSS
n=17
+/- DSS
n=19

D) Weight index

E) mRNA levels

F) RNF20 mRNA

G) RNF20 mRNA

H) H2Bub1 protein

I) Secreted IFN-γ

J) Spearman correlation = -0.42

K) Spearman correlation = 0.67

L) Spearman correlation = -0.746
Figure S5, related to Figure 4

A

B

C

D

E

F

G

H

I

J
Figure S6, related to Figure 6

A

WT  

RNF20 +/-

B

Carcereous crypt  

Normal crypts

H2B  

H2Bub1
Figure S7, related to Figure 7

A

IL8 mRNA

RNF40 mRNA levels

B

IL6 mRNA

RNF40 mRNA levels

C

H2Bub1/H2B

Non-specific colitis

Normal

Cancerous

H2Bub1/H2B

Epithelial compartment

Non-epithelial compartment

D

H2Bub1/H2B

Epithelial compartment

Non-epithelial compartment

○ Normal

○ Cancerous

△ Normal

△ Cancerous
Supplemental figure legends

Figure S1 - related to figure 1. RNF20 affects the response of MCF10A cells to TNF-α

A. MCF10A cells were transfected for 48 hours with siRNA targeted against either RNF20 or LacZ as control, and then treated with 10ng/ml TNF-α for different durations. Global H2Bub1 and H2B levels were monitored by Western blot analysis. Similar results were obtained in nine independent experiments.

B. MCF10A cells were treated with TNF-α for the indicated durations, harvested and subjected to chromatin immunoprecipitation (ChIP) with H2B and H2Bub1 antibodies. Precipitated DNA was subjected to qRT-PCR analysis with primers corresponding to the promoter κB site (κB) and the first or second exons of the IL8, CXCL1 and IL6 genes, as well as a low H2Bub1 low-transcribed region (con). H2Bub1 ChIP values were normalized to H2B values. Error bars = SD. Similar results were obtained in three independent experiments.

C. Knockdown efficiency of RNF20 was validated by qRT-PCR. Values were normalized to GAPDH mRNA in the same sample. Error bars = SD. Similar results were obtained in nine independent experiments.

D. MCF10A cells were transfected and treated with TNF-α as in (A) for the indicated durations. RNA was extracted, and the indicated cytokine mRNAs were quantified by qRT-PCR and normalized to GAPDH mRNA in the same sample. *= p-value<0.05, **=p-value<0.01. Error bars = SD. Similar results were obtained in five independent experiments.

E. MCF10A cells were transfected and treated with TNF-α as in (A) for 1 hour. RNA was extracted, and the indicated cytokine mRNAs were quantified by qRT-PCR and normalized to GAPDH mRNA in the same sample. *= p-value<0.05. Error bars = SD. Similar results were obtained in five independent experiments.

F. MCF10A cells were transfected and treated with TNF-α as in (A) for the indicated durations. RNA was extracted, and p53 and BCL2 mRNA was quantified by qRT-PCR and normalized to GAPDH mRNA in the same sample. Error bars = SD. Similar results were obtained in three independent experiments.

G. MCF10A cells were transfected for 48 hours with siRNA targeted against either RNF40 or LacZ as control, and treated with TNF-α as in (A) for the indicated durations. RNA was extracted, and cytokine mRNA levels were determined by qRT-PCR as in (D). Error bars = SD. Similar results were obtained in three independent experiments.

H. MCF10A cells were transfected for 48 hours with siRNA targeted against either LacZ as control or RAD6A, and treated with TNF-α as in (A) for the indicated durations. RNA was extracted, and cytokine mRNA levels were determined by qRT-PCR as in (D). Error bars = SD. Similar results were obtained in three independent experiments.

I. MCF10A cells were transfected for 48 hours with 3 different single siRNA oligonucleotide targeted against RNF20 or with LacZ siRNA (siCon) as control, and then treated with 10ng/ml TNF-α for
30 minutes. RNA was extracted, and RNF20 knockdown efficiency as well as expression of the indicated cytokine mRNAs were quantified by qRT-PCR and normalized to GAPDH mRNA in the same sample.

J. MCF10A cells were treated with TNF-α for the indicated durations, harvested and subjected to chromatin immunoprecipitation (ChIP) with H3 and H3K9me3 antibodies. Precipitated DNA was subjected to qRT-PCR analysis with primers spanning the κB site of the IL8, CXCL1 and IL6 genes. H3K9me3 ChIP values were normalized to H3 values. Error bars = SD. Similar results were obtained in three independent experiments.

Figure S2- related to figure 2. RNF20 knockdown increases NF-κB-dependent cytokine gene expression but does not affect p65 and p50 expression, IκB degradation or p65 localization
A. MCF10A cells were transfected for 48 hours with siRNA targeted against either RNF20, p65, RNF20 plus p65 (dKD) or LacZ as control, and then treated with 10ng/ml TNF-α for different durations. IL8, Cxcl1 and p65 mRNA was quantified by qRT-PCR as in Fig. S1D. Error bars = SD. Similar results were obtained in four independent experiments.
B. MCF10A cells were transfected for 48 hours with siRNA targeted against either RNF20, p50, RNF20 plus p50 (dKD) or LacZ as control, and then treated and analyzed for IL8, Cxcl1 and p50 mRNA expression as in (A). Error bars = SD. Similar results were obtained in four independent experiments.
C. MCF10A cells were transfected for 48 hours with siRNA targeted against RNF20 or LacZ as control, and then treated with 10 ng/ml TNF-α for 30 minutes. Cell extracts were subjected to Western blot analysis with the indicated antibodies. Similar results were obtained in three independent experiments.
D. MCF10A cells were transfected with the indicated siRNA oligonucleotides, treated with 10ng/ml TNF-α for the indicated time periods, fixed and stained with p65 (green) or RNF20 (red) specific antibodies or with DAPI. Similar results were obtained in three independent experiments.

Figure S3- related to figure 3. Analysis of RNF20 and cytokine expression in YAMC cells, small intestine organoids, peritoneal inflammatory leukocytes and bone marrow-derived macrophages
A. YAMC cells were transfected with siRNA targeted against RNF20 or LacZ as control. 48 hours later cells were treated with 10 ng/ml TNF-α for 1 hour or left untreated (0). RNF20 mRNA was quantified by qRT-PCR. Values were normalized to β-actin mRNA in the same sample. Error bars = SD. Similar results were obtained in five independent experiments.
B. Lungs of either RNF20+/− or WT mice were homogenized and proteins were extracted and subjected to Western blot analysis for RNF20, GAPDH, H2Bub1 and total H2B. The expected size of the truncated RNF20 protein is around 97kD.
C. Small intestine organoids were treated with 10 ng/ml TNF-α for different durations. RNF20 mRNA was quantified by qRT-PCR, employing primers derived from exon 18 of the RNF20 gene. Values were
normalized to β-actin mRNA in the same sample. Error bars = SE. The graph represents average of four independent experiments.

D. Small intestine organoids from WT or RNF20+/− mice were treated with 10 ng/ml TNF-α for different durations. Cytokine mRNA levels were determined by qRT-PCR and normalized to β-actin mRNA in the same sample. *=p-value<0.05. Error bars = SE. The graph represents average of four independent experiments.

E. Peritoneal inflammatory leukocytes from WT and RNF20+/− mice were derived and treated as in Fig. 3E. RNF20 mRNA levels were determined by qRT-PCR, employing primers derived from exon 18 of the RNF20 gene, and normalized to β-actin mRNA in the same sample. Error bars = SD. Similar results were obtained in three independent experiments.

F. Bone marrow-derived macrophages from WT and RNF20+/− mice were prepared as described (Foster, Hargreaves et al. 2007) and treated with 10 ng/ml TNF-α for 30 minutes. mRNA levels of the indicated genes were determined by qRT-PCR, and normalized to β-actin mRNA in the same sample. *=p-value<0.05. **=p-value<0.01. Error bars = SE. The graph represents average of four independent experiments.

Figure S4- related to figure 4. RNF20+/− mice develop more severe colonic inflammation following DSS treatment

A. 8 week old male mice weighing 22-28g were treated with 2% DSS as described in Fig 4A. After 65 days mice were sacrificed and colon length was compared.

B. Colons of either WT or RNF20+/− mice, treated as in Fig. 4A, were collected at day 180 and colon length was measured. *= p-value<0.05. Error bars = SE. n= number of mice in indicated group.

C. Spleens of WT and RNF20+/− mice, treated as in Fig. 4A, were collected at day 180 and weighed. *= p-value<0.05. Error bars = SE. n= number of mice in indicated group.

D. 8 week old male WT or RNF20+/− mice weighing 22-28 g were treated with 2% DSS as outlined in Fig 4A. Mice were weighed every 3-4 days, and weight index was calculated for each mouse relative to its weight at the start of the experiment (day 0). The number of mice in each group is indicated above the graph. **=p-value<0.01.

E. Colons of either RNF20+/− or WT mice were homogenized and RNA was extracted as described in supplemental experimental procedures. p53, Cingulin and c-myc mRNA levels were determined by qRT-PCR and normalized to β-actin mRNA in the same sample. Error bars = SE. *= p-value<0.05, **= p-value<0.01

F. Lungs of either RNF20+/− or WT mice were homogenized and RNA was extracted as described in supplemental experimental procedures. RNF20 mRNA levels were determined by qRT-PCR, employing primers derived from exon 18 of the RNF20 gene, and normalized to β-actin mRNA in the same sample. *=p-value<0.05. n= number of mice in indicated group. Error bars = SE.
G. Colons of either RNF20+/− or WT mice were homogenized and RNA was extracted as described in supplemental experimental procedures. RNF20 mRNA levels were determined by qRT-PCR, employing primers derived from exon 18 of the RNF20 gene, and normalized to β-actin mRNA in the same sample. Error bars = SE.

H. RNA extracted from the lungs of WT and RNF20+/− mice was subjected to qRT-PCR analysis of RNF20 mRNA, employing primers derived from exon 18 of the RNF20 gene. In parallel, proteins were extracted from another section of the same lung and subjected to Western blot analysis for H2Bub1; H2Bub1 band intensities were normalized to H2B in the same sample. For each mouse, normalized H2Bub1 level was plotted against the corresponding RNF20 mRNA level. Spearman correlation coefficient =0.77, **=p-value<0.01.

I. Lung RNF20 mRNA was quantified at day 7 as in (F). In parallel, the colons of the same mice were incubated in PBS for 24 hours at 37°C, and the supernatant was collected for IFN-γ secretion analysis by ELISA. Levels of secreted IFN-γ were then plotted against RNF20 mRNA. Spearman correlation coefficient =-0.70, **=p-value<0.01.

J. MIP2 mRNA in the colons of individual mice was quantified at day 26, and values were plotted against those of lung RNF20 mRNA in the same mice. Spearman correlation coefficient =-0.42, *=p-value <0.05.

K. Lung RNF20 mRNA was quantified at day 180 as in (F). Colon lengths were measured in parallel, and plotted against RNF20 mRNA. Spearman correlation coefficient=0.67, *=p-value<0.05.

L. Lung RNF20 mRNA was quantified at day 180 as in (F). Individual mouse spleen weights were plotted against lung RNF20 mRNA in the same mouse. Spearman correlation coefficient=-0.746, **=p-value<0.01.

Figure S5- related to figure 4. RNF20+/− mice have increased immune infiltrates and myeloid-derived suppressor cells following DSS treatment

A. Colons of WT and RNF20+/− mice, either untreated or treated with 2% DSS, were collected at day 65 and subjected to histopathological analysis. Arrows denote patches of infiltrating immune cells. Scale bar= 500µm.

B. Quantitative analysis of immune patches. *=p-value<0.05. n= number of mice per group. Error bars = SE.

C. Colons of DSS-treated WT and RNF20+/− mice collected at days 26 and 65 were subjected to IHC staining for F4/80. Insets show higher magnification of dashed area. Lower magnification scale bar = 100µm, higher magnification scale bar =50 µm.

D. Quantitative analysis of F4/80 staining. HPF= high power field. *= p-value<0.05, **= p-value<0.01. Error bars = SE.

E. Colons of DSS-treated WT and RNF20+/− mice collected as in (C) were subjected to IHC staining for CD3. Arrowheads depict CD3+ lymphocytes within the epithelium. Scale bar = 50µm.

F. Quantitative analysis of CD3 staining. HPF= high power field. *= p-value<0.05. Error bars = SE.
G. 8 week old mice weighing 22-28 g were treated with 2% DSS as outlined in Fig. 4A. At day 26 spleens were removed and analyzed by FACS for CD11b/GR1 positive cells, corresponding to myeloid-derived suppressor cells (MDSCs). Each dot represents a single mouse. A representative FACS analysis is shown in the right panels. * = p-value<0.05. Error bars = SE.

H. Mice were treated as in (G). Spleens were removed at day 26 and secretion of ROS and NO from MDSCs was quantified. Each dot represents a single mouse. *= p-value<0.05. Error bars = SE.

I. WT and RNF20+/− mice were treated as in (G). Spleen MDSCs were isolated as described in Supplemental experimental procedures. Arg1 (Arginase 1) mRNA was quantified by qRT-PCR and normalized to β-actin mRNA in the same sample. *** = p-value<0.001. Error bars = SE.

J. Spleens of mice treated as in (G) were subjected to FACS-based analysis of CD247 (T cell receptor zeta chain) and CD3 epsilon within CD3+ cells. Each dot represents a single mouse. ** = p-value<0.01. Error bars = SE.

Figure S6- related to figure 6. RNF20+/− mice develop more tumors than WT mice upon DSS-AOM treatment

A. 8 week old male mice weighing 22-28 g were treated with 1µg/g AOM plus 2% DSS as described in Fig 6A. Representative images from a colonoscopy performed at day 180, with polyps visible.

B. Colons of RNF20+/− mice treated with DSS were collected at day 180 and subjected to histopathological analysis to monitor carcinoma development. Shown is a representative case of mucinous adenocarcinoma. Note that cancerous glands invade the muscularis propria (T2 tumor stage). The right panel shows a higher magnification of the rectangle in the left panel. Scale bars = 600 µm and 100 µm (higher magnification). Asterisks denote mucin pools.

C. Colons of mice treated with DSS+AOM were collected at day 180 and subjected to IHC staining of H2B or H2Bub1. Arrows depict invasive cancerous glands. Scale bars = 100µm.

Figure S7- related to figure 7. Reduced RNF40 mRNA and H2Bub1 in human colorectal cancer

A. The correlation between RNF40 mRNA and IL8 mRNA across cases of human CRC was determined using the Spearman correlation test. Data is from TCGA (www.cbioportal.org/public-portal/). *=p-value<0.05.

B. The correlation between RNF40 mRNA and IL6 mRNA across cases of human CRC was determined using the Spearman correlation test. Data is from TCGA (www.cbioportal.org/public-portal/). ** = p-value<0.01.

C. Colon sections from 25 human non-specific colitis patients, 19 ulcerative colitis patients and 24 human colitis-associated cancer (CAC) patients were stained for H2Bub1 or total H2B. Representative pictures from two CAC patients are shown on the left, and quantification of relative H2Bub1 staining intensity (H2Bub1/H2B) is shown in the right panel. Inset shows higher magnification of the dashed area.
Lower magnification scale bar = 100µm, higher magnification scale bar = 25µm. **= p-value<0.01. Error bars = SE.

D. Colons of CRC patients were stained for H2Bub1 and H2B and the relative H2Bub1 staining intensity (H2Bub1/H2B) was quantified. For each patient, relative H2Bub1 intensity in the epithelial compartment and in the adjacent stroma was compared between matched cancerous and non-cancerous (normal) areas.

Table S1- related to figure 7. *RNF20* and *RNF40* mRNA are inversely correlated with inflammatory cytokine expression in human CRC samples

Correlation between *RNF20* and *RNF40* mRNA levels and cytokine mRNA levels was calculated using a Spearman correlation test and data from TCGA (www.cbioportal.org/public-portal/).

|             | r Value | p Value |
|-------------|---------|---------|
| IL8 vs RNF20 | -0.382857 | 0.006067 |
| IL6 vs RNF20 | -0.357887 | 0.010718 |
| CXCL1 vs RNF20 | -0.356062 | 0.011154 |
| IL8 vs RNF40 | -0.310636 | 0.02812 |
| IL6 vs RNF40 | -0.378535 | 0.006716 |
| CXCL1 vs RNF40 | -0.246387 | 0.084554 |
| IL10 vs RNF40 | 0.310156 | 0.028377 |
Supplemental experimental procedures

RNA purification and real time PCR
For quantitative reverse transcriptase real time PCR (qRT-PCR), RNA was purified from cells using the NucleoSpin kit (Macherey Nagel, Germany). Subsequently, cDNA was prepared using MMLV reverse transcriptase (Promega, Madison, USA) and random hexamer primers (Applied Biosystems, USA). Real time PCR was done using SYBER Green PCR supermix (Invitrogen Carlsbad, California, USA), employing a StepOnePlus instrument (Applied Biosystems). For hnRNA analysis, primers spanning both exonic and intronic regions were used, thus allowing the analysis of nascent, non-spliced RNA, which corresponds to primary transcript.

SDS-PAGE and Western blotting
For protein extraction, cells were lysed in NP40 lysis buffer (150mM NaCl, 50mM Tris pH8, 1% NP40) freshly supplemented with protease inhibitor mix (Sigma-Aldrich, USA). Lysates were centrifuged at 14,000 RPM for 10 minutes at 4°C, and the soluble fraction was taken for further analysis. Protein concentration evaluation was done with the BCA kit (Thermo Fisher Scientific, Chicago, USA) according to the manufacturer’s protocol. Next, protein sample buffer (3% SDS, 10% glycerol, 5% β-mercaptoethanol, 62mM Tris pH6.8) was added and aliquots containing equal amounts of total protein were heated at 95°C for 5 minutes and vortexed vigorously three times, followed by centrifugation at 14,000 RPM. Supernatants were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% gel, followed by transfer to a nitrocellulose membrane. Membranes were blocked in 5% milk for 1 hour, incubated with antibodies overnight at 4°C, washed three times in PBST (0.05% Tween-20 in PBS) and incubated for 1 hour with horseradish-peroxidase (HRP)-conjugated IgG, followed by 3 washes with PBS-T. Finally, membranes were incubated for 1 minute with ECL mix (GE healthcare, Little Chalfont, UK).

For chromatin bound/unbound fractions cells were harvested in buffer A (100mM NaCl, 300mM sucrose, 3mM MgCl2, 10mM PIPES pH6.8, 1mM EGTA, 0.2% triton x-100), followed by a 3 minute 1500 rpm spin. Supernatants were collected as soluble unbound fraction, while pellets were suspended in buffer B (50mM NaCl, 300mM sucrose, 3mM MgCl2, 10mM PIPES pH6.8, 1mM EGTA) supplemented with DNase I (final concentration = 0.5 U/µl) and incubated for 30 minutes at 37°C.

Chromatin immunoprecipitation
For H2Bub1, H2B, H3K9me3, H3 and Pol II phospho-ser 5 ChIP, cells were fixed by adding 0.1 volume of an 11% formaldehyde solution directly to the medium for 10 minutes at room temperature, followed by addition of 0.06 volumes of 2.5M glycine. Next, cells were washed twice with PBS, harvested and pelleted at 3,000 RPM. To extract chromatin, cells were resuspended in 1ml of cell lysis buffer (50mM PIPES, 85mM KCl, 0.5% NP-40); nuclei were pelleted by centrifugation at 4,000 RPM at 4°C, and resuspended in 100µl of nuclei lysis buffer (50mM Tris-Cl, 10mM EDTA, 1% SDS). Next, samples were diluted 1:4 in dilution buffer (0.01% SDS, 1.1%...
Triton, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl) and subjected to 15 cycles of sonication to generate 200-500 bp fragments. Each cycle included 20 seconds of sonication and 60 seconds of interval. Sonicated chromatin was blocked with 50% slurry of protein A Sepharose beads with single strand salmon sperm DNA (Trevigen, Gaithersburg, USA) at 4°C for 2 hours. At this stage 50 µl of each sample were kept as input, and the rest was incubated with 50% slurry of protein A Sepharose beads and single strand salmon sperm DNA and the appropriate antibody at 4°C overnight. Immunoprecipitates were washed with dilution and washing buffer (100 mM Tris-Cl, pH 8, 500 mM LiCl, 1% NP-40, 1% Deoxycholic acid and fresh protease inhibitors). IP samples and inputs were eluted with 50 mM NaHCO3, 1% SDS. DNAse-free RNase (50 ng/ml) was then added, followed by incubation for 30 minutes at 37°C. Next, proteinase-K (200 ng/ml) was added and incubated at 45°C for 3 hours, followed by incubation overnight at 65°C in order to reverse cross-linking. DNA was purified using a QIAGEN PCR purification kit (QIAGEN industries, Venlo, Netherlands) according to the manufacturer’s protocol, and then subjected to Real-time qPCR analysis.

For p50 and p65 ChIP, cells were fixed and harvested as above, and lysed in 400 µl ice cold lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM PMSF) for 10 minutes at 4°C. Samples were then added with 800 µl dilution buffer which contained 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1. Following preclearing with protein A Sepharose beads, 50 µl of each sample was kept as input, and the rest was incubated at 4°C overnight with 3 µg of the appropriate antibody in a final concentration of 0.1% BRIJ-35. Immune complexes were collected by incubation with 50 µl protein A-Sepharose (50% slurry) and 2 µg salmon sperm DNA for 1 hour at 4°C. Beads were washed sequentially for 5 minutes each at 4°C in Wash Buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), Wash Buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and Wash Buffer 3 (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and twice with Tris borate-EDTA (TBE) buffer, and eluted with 100 µl Elution Buffer (1% SDS, 0.1 M NaHCO3). Crosslinks were reversed by incubation in 0.2 M NaCl at 65 °C overnight. Supernatants were incubated for 1 hour at 45°C with Proteinase K (20 µg each), 40 mM Tris-HCl, pH 6.5, and 10 mM EDTA, and DNA was purified as above.

For Re-ChIP experiments 25 µl of ReChIP buffer (Dilution Buffer with 10 mM DTT) was added to washed beads and incubated at 37°C for 30 minutes. The sample was then diluted 40 times in dilution buffer and subjected to immunoprecipitation, washes and elution as above.

For ChIP of mouse colons, epithelial cells were isolated from the colon as described (Guma, Stepniak et al. 2011) and ChIP was performed as above.

**Immunofluorescence staining**

For immunofluorescence staining cells were plated on 12 mm coverslips and fixed with ice-cold methanol for 1 minute at room temperature. Next, coverslips were washed three times with PBS. Subsequently, coverslips were blocked with 10% FBS in PBS for 30 minutes, incubated for 1 hour with primary antibody in washing buffer (PBS with 2% FBS), and washed 3 times with washing buffer followed by 30 minutes incubation with secondary
antibody or 5mg/ml DAPI (Sigma-Aldrich). Following further washing with washing buffer, coverslips were mounted on slides for microscopy.

**Cytokine analysis:**
Peritoneal leukocytes were plated or colon specimens were harvested and washed in sterile 20mM PBS, pH7.4, and then cut longitudinally and horizontally into 1cm fragments. Colon fragments were spread into 6-well dishes and incubated for 24 hours with 1ml PBS. Levels of secreted Interferon-γ (IFN-γ) and TNF-α in the supernatants were measured by an enzyme-linked immunosorbent assay (ELISA) (Biolegend, San Diego, USA) according to the manufacturer’s instructions.

**Antibodies:**
The following primary antibodies were used for Western blot analysis and ChIP: anti-H2B (07-371, Millipore), anti-RNF20 (ab32639, Abcam), anti p50 (ab7971, Abcam), anti p65 (sc372, Santa Cruz), anti IkB (ab7217, Abcam), anti β-tubulin (T7816, Sigma-Aldrich), anti H3K9me3 (ab8898, Abcam), anti H3 (ab1791, Abcam), anti Pol II phospho ser5 (ab5131, Abcam). Anti H2Bub1 was described before (Minsky, Shema et al. 2008).

**Generation of RNF20+/- mice**
An ES cell line containing an insert between exon 17 and exon 18, relatively close to the 3’ end of the RNF20 gene, was obtained from the Gene Trap Consortium. The predicted protein product lacks the catalytic RING domain of RNF20, but retains most of the rest of the protein. Hence, a set of primers targeting RNF20 exon 18 indicate the levels of full length (FL) RNF20, and a set of primers targeting exon 8 indicate both FL and truncated RNF20 together. These ES cells were employed in order to generate chimeric mice with germline transmission. RNF20+/- heterozygous mice were then generated by additional crosses and confirmed by tail DNA PCR genotyping.

**MDSC analysis:**
Isolated mouse splenocytes and peripheral blood (PBLs) were subjected to cell surface staining for 30 min at 4°C, using the following antibodies (Biolegend): FITC-labeled anti-Gr1, and anti-CD11c; PE-labeled anti-F4/80, anti-CD3ε and anti-Gr1; and biotinylated anti-CD11b detected with streptavidin-Cy5. For intracellular staining of CD247, cells were first fixed for 20 min with 1% paraformaldehyde at 4°C and permeabilized for 10 min with 0.1% saponin (Sigma Aldrich). Cells were washed and then incubated for 30 min at 4°C with FITC-labeled anti-CD247 (clone H146). For intracellular NO- and ROS detection, diaminofluorescein-2 diacetate (DAF-2DA) reagent (NOS 200-1; Cell Technology) and aminophenyl fluorescein (APF) (4011; Cell Technology) were used respectively according to the manufacturer’s instructions and determined by flow cytometry analysis. Monoclonal antibodies specific for CD16 and CD32 (93; Biolegend) were used for blockade of Fc receptors before staining. Samples were analyzed by FACSCalibur using Cell Quest software (BD). For *in vitro* co-incubation experiments and RNA analysis, MDSCs were isolated from mouse spleens by MACS cell
separation using anti-Gr1 antibodies with a magnetic column separation system (Miltenyi Biotec) as previously described (Ezernitchi, Vaknin et al. 2006). For functional analysis, MDSCs were co-incubated with naïve splenic T cells for 20h as previously described (Ezernitchi, Vaknin et al. 2006).

Immunohistochemistry

For IHC analysis the following antibodies were employed: anti-p65/RelA (C-20, sc-372, Santa Cruz; 1:100), anti-p65/RelA (Ab-1, RB-1638-P, Thermo Scientific/Pierce; 1:100), anti-p50/p105 (E381, ab32360, Abcam), anti-phospho-histone H2AX (Ser139, 05-636, Millipore; 1:1000), anti-p53 (NCL-p53-CM, Novocastra, 1:400), anti-p21 (F-5, sc-6246, Santa Cruz, 1:200), anti-histone H2B (ab52484, Abcam; 1:1500), anti-ubiquityl-histone H2B (56, 05-1312, Millipore; 1:1500), anti-CD3 (ab5690, Abcam; 1:400), anti-F4/80 (c1:A3, MCA497G, Serotec; 1:100), anti-CD206 (ab64693, Abcam; 1:1500), anti-p53 (NCL-p53-CM, Novocastra, 1:400), anti-p21 (F-5, sc-6246, Santa Cruz, 1:200), anti-histone H2B (ab52484, Abcam; 1:1500), anti-CD3 (ab5690, Abcam; 1:400), anti-F4/80 (c1:A3, MCA497G, Serotec; 1:100), anti-CD206 (ab64693, Abcam; 1:1500), anti-CD11b (ab75476, Abcam; 1:500) and anti-Gr1 (RB6-8C5, ab25377, Abcam; 1:50). IHC was performed on paraffin-embedded tissues. Unmasking of the antigen retrieval was performed by heat-mediated antigen retrieval method in 10mM citric acid (pH6.0). For anti-p65/RelA, anti-p50/p105, anti-phospho-histone H2AX, anti-histone H2B, anti-ubiquityl-histone H2B, analysis the UltraVision LP Detection System was employed (#TL-060-HD, Thermo Scientific, Bioanalytica, Greece) according to the manufacturer’s instructions. For anti-p53, anti-p21, anti-CD3 (Abcam), anti-CD45R/B220, anti-F4/80, anti-CD206, anti-CD11b and anti-Gr1 the indirect streptavidin-biotin-hyperoxidase method was employed. Sections were incubated with 3% hydrogen peroxide to quench endogenous peroxidase. Prior to the addition of the primary antibody all sections were incubated with a streptavidin and a biotin solution for 15 minutes each time, in order to block endogenous biotin employing the Streptavidin/Biotin kit (#SP2002) from Vector. Incubation with the secondary antibody was performed using a biotin-conjugated antibody at a 1:200 dilution for 30 minutes. The next step comprised 30 minutes of incubation in Streptavidin HRP Conjugate (18-152, Millipore; 1:200). For color development 3,3’-diaminobenzidine tetrahydrochloride (DAB, Sigma) was employed and hematoxylin was used as counterstain. Evaluation of p65, γΗ2ΑΧ, p53 and p21 was performed as previously described (Gorgoulis, Vassiliou et al. 2005; Cooks, Pateras et al. 2013). Previously characterized cases served as positive controls for p65, p53 and p21 staining (Cooks, Pateras et al. 2013). HeLa cells served as positive control for p50 immunostaining. For γH2AX staining non-irradiated and irradiated mice tissues served as negative and positive controls respectively. Assessment of H2Bub1 and H2B immunostaining was performed based on Wang, Yang et al. 2013. In particular, an immunoreactivity score (IRS) was calculated by multiplying the staining index (SI) by the labeling index (LI), IRS = SI x LI. SI was defined based on a four-tiered scale: no staining: 0; weak staining: 1; moderate staining: 2; strong staining:3. LI was defined according to a five-based tiered scale: 0-9%=0; 10-25%= 1;26-50%=2; 51-75%=3; >75%=4. For each case we measured (H2Bub1 IRS)/(H2B IRS) ratio. For non-epithelial compartment analysis, 1,000 cells were counted and analyzed as above. In mice, the evaluation of CD3, F4/80, CD206, CD11b, and Gr1(Lys6g) was performed by counting the number of the corresponding positive immune cells in 10 high power fields (magnification 400x). Kupffer cells in mouse liver served as
positive control for F4/80 and CD206 staining. Three independent observers carried out slide examination, with minimal inter-observer variability.

**Statistical analysis of expression data from public databases**

*RNF20* and *RNF40* mRNA levels in a previously described gene expression array (Sen and Bhaumik 2013) were compared between healthy patients and UC patients using one-way ANOVA. Data from TCGA, comparing *RNF20* and *RNF40* mRNA levels within the same individuals, was tested using paired t-tests. All statistical tests were done using Statsoft’s Statistica, v. 12.

**Primers**

For mRNA amplification the following primers were used:

- **p53**: Fw 5’CCCAAGCAATGGATGATTGTA,  
  Rv 5’GGCATTTGGAGCTTCTAGCT;  
- **p65**: Fw 5’CTCCGCGGGCAAGCAT  
  Rev 5’TCCTGTGTAACATTGTATTGCTGAT  
- **CXCL1**: Fw 5’AGTCATAGCCACACTCAAGAATGG  
  Rv 5’GATGCAAGATTGGAGCAAGC  
- **IL-8**: Fw 5’GGCACGCTTTCTGATTTCAGTTG  
  Rv 5’CTTGGCAAACGCTGCACCTTCA  
- **RNF20**: Fw 5’GAACACGCGACTCAACCCGACA  
  Rv 5’GGAATTACCCCGGTTCCTAGGACTT  
- **GAPDH**: Fw 5’AGCCTCAAGATCATCAGCAATG  
  Rv 5’CACGATACCAAAGGTGGTCATGGAT  
- **IL-6**: Fw 5’AGCCCTGAGAAGGGAGACATGTA  
  Rv 5’TCTGCCAGCTGGCTCTCTGCT  
- **Bcl2**: Fw 5’GGCTGGGATGCTTTGCTGGTTGGTG  
  Rv 5’CAGCCAGGGAATCACAACAAGA  
- **p50**: Fw 5’GGATCTACTAGAAGTCACATCTGCTTGTGTTG,  
  Rv 5’AGGGCGTCTGGTGACAGATCATT  
- **Rad6**: Fw 5’TCAAGAGGGTTGGCAGGAGGATCC  
  Rv 5’CGAAAAATGACCCCGGTCCA  
- **RNF40**: Fw 5’AGGCCGAGGTTGAGCT  
  Rv 5’GCCTCTTGCTCTGCTCTCCTT  
- **IL-1β**: Fw 5’GCCTGAAGCCCTTGCTGTAGT  
  Rv 5’GGGCGTCTGGTGAGACATGTA  
- **IL12b**: Fw 5’GCTGGTGCGCTGACGACCAT  
  Rv 5’TCAAGAGGGTTGGCAGGAGGATCC  
- **IL23a**: Fw 5’TCAAGAGGGTTGGCAGGAGGATCC  
  Rv 5’TCAAGAGGGTTGGCAGGAGGATCC
Rv 5’: CCGATCCTAGCAGCTTCTCATAA

For mouse mRNA amplification the following primers were used:
RNF20 (exon 8): Fw 5’CCCGGAAGTTTGAGGAAATG
Rv 5’CGGTTCTGAGCCAAACTCTTTG
RNF20 (exon 18): Fw 5’CACCAAGAAGCCAGACAATGTG
Rv 5’CCGTGCCTTGTAGTCTCTTTATCTC
GAPDH: Fw 5’TTCTTGTGCAATGGCCAGCC,
Rv 5’CACCGACCTTCACCATTGT
KC (cxcl1): Fw 5’GCTGGGAGGACACCTTTAGCA
Mip2: Fw 5’GCCAAGGTTGACTTCA
Rv 5’TGTCTGGGCGCAGTG
TNF-α: Fw 5’AGGGATGAGAAGTTCCCAAATG
Rv 5’TGTGAGGGTCTGGGCCATA
IL6: Fw 5’TCCGGAGGCTTAATTACACATGT
Rv 5’TGCCATTGCACAACCTCTTTCT
Beta-actin: Fw 5’ CACTATTGGCAACGAGCGGT
Rv 5’ GCCACAGGATTCACCATAC
Cingulin: Fw 5’: GCACCCTTCGGGACGAAC
Rv 5’: TGGACCCAAAGGAGTCAGT
p53: Fw 5’: AAGGAAAAAACCACCTTGATGGAGGT
Rv 5’: CGGAACATCTCAGGAGCGTT
Myc: Fw 5’: GCCCATCTTAGGTGACTGAG
Rv 5’: CCACAGACACCATCAATTCT
IL23a: Fw 5’ GCGGGACATATGAATCTACTAAGAGA
Rv 5’ CAACCATCTTCGACACTGGGATACG
CCL2: Fw 5’ GCAGCAGGTGTCCCAAAGAA
Rv 5’ GGTCAGCAGACCTCTCTCTCTTG
Arginase1: Fw 5’ GCACAGAAAGCAGAAGTCCTT
Rv 5’ CCTCGAGGGCTGCTCCTTTGA

For hnRNA amplification primers targeting intron-exon junctions were used:
Intronic IL-8: Fw 5’ TTGGAATTAGAAGGAAAGTAGCTGG
Rv 5’TGATTTATCAACAGGCACAGCTC
Intronic IL6: Fw 5’ CCACAAGTAAGTGAGGAAAATCC
Rv 5’CACACCCCTCCCTCACACA
Intronic CXCL1: Fw 5’ CGTCCCCACCCCTGGTTCT
Rv 5’ CCTCAATGAAGGCAGTTTTACCT

For amplification of cytokine sites in ChIP the following primers were used:
IL-8 κB site: Fw: 5’TGGGCCATCAGTTGCAAA
Rv 5’ACTTATGCACCCCTCATCTTTTCATT
Cxcl1 κB site: Fw 5’CGATCTGGAACCTCAGGAAT
Rv 5’AGATCCGCGAAACCTTTTATA
IL-6 κb site: Fw 5’AGCCTCAATGACGACCTAAGCT
Rv 5’CGTCCTTTAGCATGACCTTCTCT
IL-8 1st intron: Fw: 5’ TGACCTACAGCGTTTTTTCTATGTC
Rv 5’TGTTACCAAAGCATCAAGAATAGCTT
Cxcl1 1st intron: Fw 5’ TCGCTCAGTCAGTGAGTCTCTCTCT
Rv 5’GCCTGACGCCGAGTT
IL-6 2nd intron: Fw 5’TGCCAAAACCAGCCTTGTGA
Rv 5’ACTGAGTGCTCCAGAGTTGGA
p21 (con): Fw 5’AGCAGGCTGTGGCTCTGATT
Rv 5’CAAATAGCCACCAGCCTCTCTTCT
Low transcribed area (con): Fw 5’ TTTTCCCCCATCTGTTGGCT
Rv 5’TCTCCAGCCTGCAACACCT
GAPDH: Fw 5’ CCGGGAGAAGCTGAGTCATG
Rv 5’TTTGCGGTGGAAATGTCTCTT
Supplemental References:

Cooks, T., I. S. Pateras, et al. (2013). "Mutant p53 prolongs NF-kappaB activation and promotes chronic inflammation and inflammation-associated colorectal cancer." Cancer Cell 23(5): 634-646.

Ezernitchi, A. V., I. Vaknin, et al. (2006). "TCR zeta down-regulation under chronic inflammation is mediated by myeloid suppressor cells differentially distributed between various lymphatic organs." J Immunol 177(7): 4763-4772.

Gorgoulis, V. G., L. V. Vassiliou, et al. (2005). "Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions." Nature 434(7035): 907-913.

Guma, M., D. Stepniak, et al. (2011). "Constitutive intestinal NF-kappaB does not trigger destructive inflammation unless accompanied by MAPK activation." J Exp Med 208(9): 1889-1900.

Minsky, N., E. Shema, et al. (2008). "Monoubiquitinated H2B is associated with the transcribed region of highly expressed genes in human cells." Nat Cell Biol 10(4): 483-488.

Sen, R. and S. R. Bhaumik (2013). "Transcriptional stimulatory and repressive functions of histone H2B ubiquitin ligase." Transcription 4(5).

Cooks, T., I. S. Pateras, et al. (2013). "Mutant p53 prolongs NF-kappaB activation and promotes chronic inflammation and inflammation-associated colorectal cancer." Cancer Cell 23(5): 634-646.

Ezernitchi, A. V., I. Vaknin, et al. (2006). "TCR zeta down-regulation under chronic inflammation is mediated by myeloid suppressor cells differentially distributed between various lymphatic organs." J Immunol 177(7): 4763-4772.

Foster, S. L., D. C. Hargreaves, et al. (2007). "Gene-specific control of inflammation by TLR-induced chromatin modifications." Nature 447(7147): 972-978.

Gorgoulis, V. G., L. V. Vassiliou, et al. (2005). "Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions." Nature 434(7035): 907-913.

Guma, M., D. Stepniak, et al. (2011). "Constitutive intestinal NF-kappaB does not trigger destructive inflammation unless accompanied by MAPK activation." J Exp Med 208(9): 1889-1900.

Minsky, N., E. Shema, et al. (2008). "Monoubiquitinated H2B is associated with the transcribed region of highly expressed genes in human cells." Nat Cell Biol 10(4): 483-488.

Sen, R. and S. R. Bhaumik (2013). "Transcriptional stimulatory and repressive functions of histone H2B ubiquitin ligase." Transcription 4(5).