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

Highlights

- RNF20 and H2Bub1 modulate the NF-κB response
- RNF20-deficient mice are prone to colonic inflammation and colorectal cancer
- Reduced H2Bub1 may create a pro-tumoral microenvironment
- Reduced H2Bub1 is associated with colitis and colorectal cancer in humans

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In Brief

Ubiquitination of histone H2B (H2Bub1), primarily by the E3 ligase RNF20, is reduced in many advanced cancers. Tarcic et al. report that downregulation of RNF20 and H2Bub1 promotes chronic colonic inflammation and inflammation-associated colorectal cancer in mice and humans, partly by augmenting NF-κB activity and attenuating the antitumoral T cell response.
RNF20 Links Histone H2B Ubiquitylation with Inflammation and Inflammation-Associated Cancer

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SUMMARY

Factors linking inflammation and cancer are of great interest. We now report that the chromatin-targeting E3 ubiquitin ligase RNF20/RNF40, driving histone H2B monoubiquitylation (H2Bub1), modulates inflammation and inflammation-associated cancer in mice and humans. Downregulation of RNF20 and H2Bub1 favors recruitment of p65-containing nuclear factor κB (NF-κB) dimers over repressive p50 homodimers and decreases the heterochromatin mark H3K9me3 on a subset of NF-κB target genes to augment their transcription. Concordantly, RNF20−/− mice are predisposed to acute and chronic colonic inflammation and inflammation-associated colorectal cancer, with excessive myeloid-derived suppressor cells (MDSCs) that may quench antitumoral T cell activity. Notably, colons of human ulcerative colitis patients, as well as colorectal tumors, reveal downregulation of RNF20/RNF40 and H2Bub1 in both epithelium and stroma, supporting the clinical relevance of our tissue culture and mouse model findings.

INTRODUCTION

Inflammation is a crucial protective response conserved in all multicellular organisms (Medzhitov, 2010). It entails activation of many cell types through several signaling pathways including nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3), resulting in release of cytokines and chemokines. Inflammation is tightly regulated and is effectively turned off when no longer needed (Lawrence et al., 2001; O'Dea and Hoffmann, 2010). Malfunction of the shutdown machinery might result in chronic inflammation, promoting pathologies such as inflammatory bowel disease (IBD) including ulcerative colitis (UC) and Crohn’s disease (Majno and Joris, 2004; Kumar and Robbins, 2007). Although proper inflammatory response may suppress cancer (Mantovani et al., 2008), chronic inflammation can promote tumor initiation and progression (Brigati et al., 2002; Pikarsky et al., 2004; Griwennikov et al., 2010; Schetter et al., 2010; Ben-Neriah and Karin, 2011; Hanahan and Weinberg, 2011). Notably, IBD patients carry an increased cancer risk (Eaden et al., 2001).

NF-κB transcription factors consist of different dimeric combinations of p50, p52, p65 (RelA), c-Rel, and RelB, binding DNA as either hetero- or homodimers (DiDonato et al., 2012). Since only p65, c-Rel, and RelB contain transcriptional activation domains (TAD), dimers comprising these subunits can activate transcription while the others repress transcription unless they recruit specific co-activators (Heinz et al., 2013; Kogure et al., 2013). Additionally, NF-κB regulates diverse processes such as apoptosis, cell proliferation, differentiation, and tumorigenesis (DiDonato et al., 2012; Perkins, 2012).

Eukaryotic DNA is packaged in nucleosomes consisting of four core histones (H2A, H2B, H3, and H4), which undergo many post-translational modifications (PTM) that control chromatin dynamics and transcription (Campos and Reinberg, 2009). One such PTM is monoubiquitylation of mammalian histone H2B on lysine 120 (H2Bub1), executed primarily by the RNF20 (hBRE1)/RNF40 E3 ligase complex (Hwang et al., 2009). H2Bub1 resides mainly downstream to transcription start sites (TSS), in preferential association with highly transcribed genes (Minsky et al., 2008). It participates in RNA polymerase II (RNA Pol II)-dependent transcription, cooperatively with the facilitates chromatin transcription (FACT) and polymerase-associated factor (PAF) complex (Pavri et al., 2006). H2Bub1 can also negatively regulate transcription and may contribute
to heterochromatin silencing (Zhang et al., 2008). In HeLa cells, H2Bub1 modulates transcription of specific gene sets (Shema et al., 2008): one subgroup, enriched in proto-oncogenes, is upregulated upon partial RNF20 depletion, while the other, including the p53 tumor suppressor, is downregulated. Furthermore, H2Bub1 impedes recruitment of TFIIIS, a factor capable of relieving paused RNA Pol II (Shema et al., 2011). Conversely, H2Bub1 upregulates other transcripts by favoring recruitment of the SWI/SNF chromatin remodeling complex (Shema-Yacoby et al., 2013).

Notably, RNF20 displays tumor suppressor features (Shema et al., 2008). Moreover, RNF20 and H2Bub1 are reduced in many cancers (Prenzel et al., 2011; Chernikova et al., 2012; Hahn et al., 2012; Urasaki et al., 2012; Thompson et al., 2013; Wang et al., 2013; Bedi et al., 2014). H2Bub1 levels are low in both embryonic and adult stem cells but increase during differentiation and may be a prerequisite for proper execution of the differentiation program (Fuchs et al., 2012; Kariuki et al., 2012). Additionally, RNF20 and H2Bub1 are implicated in the DNA damage response, replication stress, and chromosomal stability (Kari et al., 2011; Moyal et al., 2011; Nakamura et al., 2011; Shiloh et al., 2011; Chernikova and Brown, 2012; Thompson et al., 2013).

H2Bub1 is coupled to ongoing transcription (Pavri et al., 2006; Zhang et al., 2008). Its modulation affects the transcriptional response to epidermal growth factor (EGF) (Shema et al., 2008), estrogen (Prenzel et al., 2011; Bedi et al., 2014), interferon gamma (Buro et al., 2010), and androgens (Jääskeläinen et al., 2012). We now report that RNF20 depletion, leading to decreased H2Bub1, augments the ability of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) to upregulate a panel of inflammation-associated genes. Importantly, mice with reduced RNF20 and H2Bub1 are predisposed to chronic colonic inflammation. Notably, colons of UC patients tend to underexpress RNF20 and RNF40 mRNA, in conjunction with H2Bub1 downregulation in both epithelial and stromal compartments. Moreover, RNF20-low mice succumb preferentially to inflammation-associated colorectal cancer (CRC) and their tumors exhibit reduced H2Bub1, as observed also in human CRC. Hence, attenuation of RNF20 and H2Bub1 may facilitate the emergence of chronic inflammation and subsequent cancer.

RESULTS

RNF20 Restricts NF-κB Target Gene Transcription in Response to TNF-α
To explore links between H2Bub1 and inflammation, we monitored H2Bub1 levels in cells exposed to TNF-α, employing non-transformed human mammary epithelial MCF10A cells. TNF-α downregulated transiently H2Bub1, both globally (Figures 1A and S1A) and at promoters and bodies of several cytokine genes (Figure S1B). This was not due to a decrease in RNF20 mRNA (Figure S1C) or protein (Figure 1A). As expected, RNF20 knockdown caused substantial H2Bub1 loss (Figure S1A, siRNF20).

TNF-α transactivated many genes including IL8 and IL6, as revealed by analysis of heterogeneous nuclear RNA (hnRNA) corresponding to primary transcripts (Figure 1B). Notably, RNF20 knockdown (Figure S1C) accelerated and prolonged this effect (Figure 1B), upregulating mature cytokine mRNA (Figures S1D and S1E). In contrast, RNF20 knockdown downregulated the non-inflammatory genes p53 and Bcl2 (Figure S1F). Hence RNF20, presumably through H2Bub1, restricts the activation of a set of inflammation-associated genes. Notably, knockdown of additional H2Bub1 machinery genes (RNF40 and hRAD6A) elicited similar effects (Figures S1G and S1H). Augmented cytokine gene expression was seen with three different RNF20 small interfering RNA (siRNA) oligos (Figure S1I), ruling out off-target effects.

In non-stimulated state, promoter regions of several proinflammatory cytokine genes are decorated with H3K9me3 (Figures 1C and S1J); this heterochromatin mark decreased transiently upon TNF-α exposure, concomitantly with the transcription wave (Figure S1J). Remarkably, H3K9me3 was selectively depleted from such promoters upon RNF20 knockdown, along with increased binding of paused (Ser5-phosphorylated) RNA Pol II (Figure 1D); this may underpin the accelerated transcriptional response to inflammatory stimuli (Figure 1B).

RNF20 Favors p50 Binding and Disfavors p65 Binding to xB Sites
Transcriptional induction of cytokine genes by TNF-α is largely mediated through NF-κB activation (Hayden and Ghosh, 2008; Ben-Neriah and Karin, 2011). Indeed, p65 depletion practically ablated the response (Figure S2A). The canonical NF-κB response to TNF-α employs primarily p65/p50 dimers (Hayden and Ghosh, 2012). Interestingly, rather than compromising IL8 and CXCL1 expression, p50 knockdown upregulated those genes (Figure S2B). Hence, in this system, repressive p50 homodimers may outweigh activating p65-containing dimers, while partial p50 depletion alleviates repression and promotes transcription by favoring p65-containing dimers. We therefore employed chromatin immunoprecipitation (ChIP)-qPCR to assess the association of p65 and p50 with relevant xB sites. In non-treated cells, p65 occupancy was very low, just above a control gene (p21/CDKN1A) lacking xB sites (Figure 2A). TNF-α increased p65 occupancy, which was further augmented by RNF20 depletion in IL8 and CXCL1. RNF20 knockdown did not affect TNF-α-induced kB degradation (Figure S2C) or p65 nuclear translocation (Figure S2D). However, it reduced basal and TNF-α-induced p50 binding, modestly in IL6 and more strongly in IL8 (Figure 2B), and affected differentially the global recruitment of NF-κB subunits to chromatin upon TNF-α treatment, favoring p65 over p50 (Figure 2C).

We next performed sequential ChIP (“re-ChIP”), reacting the chromatin first with anti-p65 and then anti-p50 antibodies. As expected, TNF-α increased the abundance of putative p65:p50 heterodimers (Figure 2D). Notably, this was further moderately augmented by RNF20 knockdown. Thus, RNF20 depletion alters the ratio between chromatin-associated NF-κB subunits in favor of p65, facilitating transcriptional activation.

RNF20 Downregulation Augments the TNF-α Response in Mouse Colonocytes, Intestinal Organoids, and Innate Immune Cells
To extend our observations to non-cancerous mouse epithelial cells, young adult mouse colonocytes (YAMC) (Whitehead
et al., 1993) cells were employed. As in MCF10A cells, TNF-α elicited a rapid transient decrease in H2Bub1 also in YAMC cells (Figure 3A). As expected, RNF20 KD (Figure S3A) diminished H2Bub1 (Figure 3A). Notably, it augmented the induction of IL6 and KC (a mouse homolog of IL8) (Figure 3B).

To investigate whether RNF20 affects inflammation in vivo, we attempted to generate RNF20 knockout mice utilizing embryonic stem cells carrying a gene trap insertion within the RNF20 gene (see Supplemental Experimental Procedures); the insert is located between exons 17 and 18, and the predicted 97 kDa
protein should lack the C-terminal RING domain. Such truncated protein was undetectable (Figure S3B), suggesting its intrinsic instability. Our attempts to obtain viable homozygous knockout progeny failed, owing to preimplantation embryonic lethality.

Heterozygous RNF20+/− mice were viable and appeared normal. We derived small intestinal 3D organoid cultures (Sato et al., 2011) from wild-type (WT) and RNF20+/− mice. As expected, RNF20+/− organoids had less RNF20 mRNA and H2Bub1 than WT organoids (Figures 3C and S3C). Importantly, TNF-α elicited a rapid decrease in H2Bub1 (Figure 3C), and NF-κB target gene induction was augmented in RNF20+/− relative to WT organoids (Figures 3D and S3D). Likewise, peritoneal inflammatory leukocytes (mostly monocytes) and bone marrow-derived macrophages from RNF20+/− mice, underexpressing RNF20 (Figures S3E and S3F), displayed an augmented TNF-α response at both RNA (Figures 3E and S3F) and secreted cytokine (Figure 3F) levels.

Thus, in non-cancerous epithelial cells, intestinal organoids, and innate immune cells, reduced RNF20/H2Bub1 augments the proinflammatory transcriptional response.
A. Time following TNF-α

<table>
<thead>
<tr>
<th></th>
<th>siLacZ</th>
<th>siRNF20</th>
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<tr>
<td>TNF-α (minutes):</td>
<td>-</td>
<td>30</td>
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H2Bub1: 1.00 0.185 0.523 0.043

H2B: 1.00 0.307 0.285 0.207

H2Bub1/H2B:

B. IL6 mRNA

Time (hours) 0 1

siLacZ 0.5 1.0

siRNF20 0.7 1.5

C. KC mRNA

Time (hours) 0 1

siLacZ 1.0 3.0

siRNF20 1.5 4.0

D. TNF-α mRNA

Time (hours) 0 1 2 3 4

WT 1.0 2.0 3.0 4.0

RNF20 +/- 1.0 2.0 3.0 4.0

E. TNF-α mRNA

Time (minutes) 0 30

WT 1.0 4.0

RNF20 +/- 1.0 4.0

F. TNF-α secretion (pg/ml)

Time (hours) 0 0.5 1 3

WT 100 200 300 400

RNF20 +/- 100 200 300 400

IFN-γ secretion (pg/ml)

Time (hours) 0 0.5 1 3

WT 50 100 150 200

RNF20 +/- 50 100 150 200

(legend on next page)
RNF20+/− colons than in WT colons (Figure 4B). Although inflammation subsided partially from day 65 onward, it remained higher in the heterozygotes. Moreover, by day 26 RNF20+/− mice displayed augmented splenomegaly (Figure 4C), and by day 65 they had shorter colons compared to WT mice (Figures 4D and S4A); both features persisted till day 180 (Figures S4B and S4C). Concordantly, DSS-treated RNF20+/− mice lost more weight than WT mice (Figure S4D). Thus, RNF20+/− mice are prone to augmented acute and chronic inflammation.

RNF20+/− mice express less RNF20 in all tissues. To assess the contribution of the bone marrow (BM)-derived compartment, we performed BM transplantation studies where BM of each genotype was transplanted into irradiated mice of each genotype. Reassuringly, WT mice reconstituted with WT BM were less inflamed than RNF20+/− mice reconstituted with RNF20+/− BM (Figure 4E). Interestingly, both WT mice reconstituted with RNF20+/− BM and RNF20+/− mice reconstituted with WT BM displayed intermediate inflammation, suggesting that both the intestinal epithelial compartment and the myeloid compartment contribute to the increased inflammation in RNF20+/− mice, consistent with the augmented cytokine responses in both cell types in vitro (Figures 3 and S3).

Remarkably, RNF20+/− colons had compromised barrier function, resulting in increased fluorescence in the blood of mice force-fed dextran FITC (Figure 4F). This intestinal leakiness, perhaps due to reduced expression of tight junction genes (e.g., cingulin, Figure S4E), may offer an additional explanation for the epithelial contribution to the augmented inflammation. Interestingly, c-Myc expression was elevated in RNF20+/− colons (Figure S4E).

Although average RNF20 mRNA levels in RNF20+/− colons and lungs (Figures S4F and S4G) was approximately half that of WT mice, it varied greatly among individual mice (Figure 4G). H2Bub1 correlated well with RNF20 mRNA (Figure S4H). Remarkably, regardless of genotype, RNF20 mRNA levels (measured in the non-inflamed lungs) were strongly inversely correlated with leukocyte inflammatory score, resulting in increased fluorescence in the blood of mice prone to RNF20+/− mice, mirroring the increased cytokine responses in both cell types in vitro (Figures 3 and S3).

Increased MDSC Activation in RNF20+/− Mice

Under inflammatory conditions, immune cells infiltrate the colonic epithelium. Notably, immune patches composed mainly of T cells, B cells, and macrophages were more abundant in DSS-treated RNF20+/− than in WT mice (Figures S5A and S5B). RNF20+/− mice displayed more infiltrating macrophages (F4/80 positive, Figures S5C and S5D), including M2 macrophages (CD206+, Figure 4H), as well as more T cells (CD3+, Figures S5E and S5F), at both day 26 and 65. Importantly, RNF20-deficient mice had more myeloid-derived suppressor cells (MDSCs), identified by IHC staining for CD11b+ and Gr1 (Figure 4I) and CD11b positivity by FACS (Figure S5G). Those MDSCs were more active, producing excessive ROS and nitric oxide (Figure S5H) and overexpressing Arginase 1 (Figure S5I). Furthermore, T cell receptor ζ chain was downregulated in RNF20+/− splenic T cells (Figure S5J), indicative of impaired T cell function. CD3 epsilon was comparable in WT and RNF20+/− mice (Figure S5J), further arguing that the reduced T cell functionality in the latter is mediated by MDSCs. Importantly, RNF20+/− MDSCs revealed a significantly greater capacity to downregulate ζ chain levels in co-incubated naive T cells (Figure 4J). Hence, reduced RNF20/H2Bub1 might promote immunosuppression via increased MDSC activation.

Elevated NF-κB Activity and DNA Damage in RNF20+/− Mice

Consistent with the enhanced NF-κB activation in cultured cells, inflamed colons of RNF20+/− mice revealed stronger nuclear p65 staining than WT mice, while p50 exhibited an opposite trend (Figure 5A). Nuclear γ-H2AX foci, indicative of DNA damage, were more abundant in RNF20+/− colons (Figure 5B), probably reflecting the combined effect of increased DNA-damaging ROS (Schetter et al., 2010) and compromised DNA double strand break repair (Chernikova et al., 2010; Moyal et al., 2011; Nakamura et al., 2011). Notably, despite the marked increase in apparent DNA damage, p53 was only mildly elevated (Figure 5B); p21, the canonical p53 target gene, was hardly induced at all. This suggests a compromised p53 response, probably due to p53 mRNA downregulation in RNF20+/− mice (Figure S4E). Thus, while RNF20+/− colonicocytes accumulate more DNA damage, their ability to mount a full DNA damage response is compromised, potentially exacerbating genomic instability.

Figure 3. Reduced RNF20 Augments the TNF-α Response in Mouse Cells, Intestinal Organoids, and Peritoneal Inflammatory Leukocytes

(A) YAMC (young adult mouse colonocytes) cells were transfected with siRNA targeted against RNF20 or LacZ as control and either treated or not treated with 10 ng/ml TNF-α for 30 min. H2B and H2Bub1 levels were assessed by Western blot analysis. Similar results were obtained in five independent experiments.

(B) YAMC cells were transfected as in (A). Forty-eight hours later, cells were treated with 10 ng/ml TNF-α for 1 hr. IL6 and KC mRNA levels were determined by qRT-PCR, and normalized to β-actin mRNA in the same sample. *p < 0.05. Error bars, SD. Similar results were obtained in five independent experiments.

(C) Small intestine organoids from WT or RNF20+/− mice were either treated or not treated with 10 ng/ml TNF-α for 30 min. H2B and H2Bub1 levels were assessed by western blot analysis. The H2B and H2Bub1 lanes are from two separate gels loaded with identical aliquots of the same extract. Similar results were obtained in 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 < 0.05, **p < 0.01. Error bars, SE. The graph represents average of four independent experiments.

(E) Mice were injected intraperitoneally with 4% thioglycolate. Four days later, peritoneal inflammatory leukocytes were collected and either treated or not treated with 10 ng/ml TNF-α for 30 min. Cytokine mRNA levels were determined by qRT-PCR and normalized to β-actin mRNA in the same sample. *p < 0.05. Error bars, SD. Similar results were obtained in three independent experiments.

(F) Mice were injected intraperitoneally with 4% thioglycolate. Four days later, peritoneal inflammatory leukocytes were removed and treated with 10 ng/ml TNF-α for different durations. Secreted cytokines were quantified by ELISA. Similar results were obtained in three independent experiments.

See also Figure S3.
**RNF20++/++ Mice Are Predisposed to Inflammation-Associated Colorectal Tumors**

Chronic colonic inflammation promotes colorectal cancer (Terzić et al., 2010; Ullman and Itzkowitz, 2011). In the mouse, this is greatly enhanced by combining DSS with the chemical carcinogen azoxymethane (AOM) (Ward et al., 1973; Tanaka et al., 2003). We therefore injected mice with AOM (1 μg/g body weight) 7 days prior to DSS exposure (Figure 6A). Colonoscopy revealed more polyps in RNF20++/++ mice than in WT mice (Figure 6B; representative images in Figure S6A). Moreover, while RNF20++/++ adenomas often progressed to carcinoma by day 180, none did in WT mice (Figure 6C, DSS-AOM). Likewise, DSS alone elicited carcinomas by day 180 in several RNF20++/++ but no WT animals (Figure 6C, DSS). Some RNF20++/++ mice developed mucinous carcinoma with neoplastic glands invading the submucosa and penetrating the muscularis propria (T2), indicative of an aggressive course (Figure S6B). Hence, reduced RNF20 promotes both initiation and progression of inflammation-associated CRC in mice. Notably, the impact of partial RNF20 depletion on tumor progression was more pronounced than on inflammation. Thus, beyond augmented inflammation, additional effects of RNF20 shortage might also promote cancer in this setting.

While total H2B staining was similar in tumors and non-tumorous crypts (Figure 6D, left panels), polyps and carcinomas in both genotypes often displayed reduced H2Bub1 in both epithelial and stromal compartments (Figures 6D, 6E, and S6C, arrows), implying that colorectal tumorigenesis is associated with further H2Bub1 decrease.

**Lower H2Bub1 May Promote Human Ulcerative Colitis and Colorectal Cancer**

To evaluate the human relevance of our findings, we analyzed a gene expression array including data from 27 healthy people, 35 UC patients with non-inflamed colons, and 38 UC patients with inflamed colons (Sen and Bhaurik, 2013). For patients sampled multiple times, mean values were used. Remarkably, UC colons had reduced RNF20 and RNF40 mRNA (Figure 7A). Moreover, RNF20 and RNF40 were inversely correlated with several inflammatory cytokine mRNAs, including IL6 and IL8 (Figures S7A and S7B; Table S1), consistent with the notion that H2Bub1 downregulation drives chronic inflammation and NF-κB activation also in humans. In agreement, H2Bub1 was markedly reduced in IBD patient colonic epithelium, and to a lesser degree, also in the stromal compartment, relative to non-specific mild chronic colitis (NSC) (Figures 7B and 7C). This raises the intriguing possibility that constitutively reduced RNF20/RNF40, leading to partial H2Bub1 depletion, may predispose humans to particular chronic inflammatory diseases.

Mouse colorectal tumors exhibited reduced H2Bub1 (Figure 6D). Consistent with earlier observations (Urasaki et al., 2012), a similar pattern was revealed in human CRC tissue microarrays (Figures 7D and 7E) and colitis-associated cancer (CAC) samples (Figure S7C). Specifically, H2Bub1 in the cancer cells declined along disease progression (Figures 7F and S7D). Interestingly, partial H2Bub1 downregulation was often seen also in the tumor stroma, although no further reduction occurred as cancer progressed (Figures 7D and 7F). Significantly, analysis of TCGA (http://www.cbioportal.org/public-portal/) colon adenocarcinoma gene expression data, comparing normal and tumor samples from the same patients, revealed lower RNF20 and RNF40 mRNA in the cancer tissue (Figure 7G). Thus, H2Bub1 is often reduced in human CRC, partly owing to compromised RNF20/40 expression, corroborating the human relevance of our mouse model.

**DISCUSSION**

In cultured cells, TNF-α elicits a rapid transient decrease in global H2Bub1, preceding the induction of NF-κB target genes. RNF20 depletion enhances NF-κB-dependent transcription of specific cytokine genes, suggesting that TNF-α-induced H2Bub1 downregulation facilitates the ensuing NF-κB response.

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**Figure 4. RNF20++/++ Mice Are More Prone to Acute and Chronic Colonic Inflammation**

(A) Timeline of the chronic DSS protocol. (B) Eight-week-old male WT or RNF20++/++ mice weighing 22–28 g were treated with 2% DSS as outlined in (A). To monitor inflammation, mice were anesthetized with 10% xylene and 10% ketamine, and colonoscopies were performed as indicated. Inflammation scores were calculated as described in Experimental Procedures. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars, SE. At least eight mice of each group were analyzed.

(C) Spleens of either WT or RNF20++/++ mice were collected at day 26 and weighed. *p < 0.05. n, number of mice per group. Error bars, SE.

(D) WT and RNF20++/++ mice were sacrificed at day 65 and colon lengths were measured. *p < 0.01. n, number of mice per group. Error bars, SE.

(E) Six-week-old male mice were sub-lethally irradiated to deplete bone marrow (BM) cells, and injected with BM cells derived from either WT or RNF20++/++ mice. Mice were then treated with DSS as outlined in (A). Inflammation was monitored as in (B). *p < 0.05, **p < 0.01. Error bars, SE. At least six mice of each group were analyzed.

(G) RNA was extracted from lungs of WT and RNF20++/++ mice as described in the Supplemental Experimental Procedures. RNF20 mRNA levels in lungs of individual mice were plotted against the corresponding colonoscopy scores. Spearman correlation coefficient was −0.775. ***p < 0.001.

(H) Colonos of DSS-treated RNF20++/++ and WT mice collected at day 26 were subjected to IHC staining for CD206. Lower panels show a higher magnification of the dashed area in upper panels. Arrowheads indicate positive nuclei. Lower magnification scale bars, 100 μm; higher magnification scale bars, 50 μm; bottom, quantification based on six WT mice and five RNF20++/++ mice; HFP, high power field. *p < 0.05, **p < 0.01. Error bars, SE.

(I) Colonos of DSS-treated WT and RNF20++/++ mice collected at day 26 were subjected to IHC staining for CD11b and Gr1. Insets: a higher magnification of the dashed area. Lower magnification scale bar, 100 μm; higher magnification scale bar, 50 μm; bottom, quantification based on six WT mice and five RNF20++/++ mice; HFP, high power field. *p < 0.01. Error bars, SE.

(J) Spleens of DSS-treated WT and RNF20++/++ mice were collected at day 26. MDSCs were isolated and incubated with naive T cells from spleens of untreated RNF20++/++ mice as described in the Supplemental Experimental Procedures, at the indicated MDSC:T cell ratio. Cells were then subjected to FACS-based analysis of CD247 (T cell receptor zeta chain) within CD3+ cells. **p < 0.01. Error bars, SE. See also Figures S4 and S5.
The subsequent recovery of H2Bub1 might serve to tune down the NF-kB response. Consequently, constitutively reduced H2Bub1 might hamper efficient shutoff, resulting in prolonged “leaky” NF-kB activation. Notably, although H2Bub1 is the most likely mediator of the effects of RNF20, a contribution of additional RNF20 substrates cannot be ruled out presently.

The abundance of H2Bub1 on chromatin can dictate selective binding of numerous transcription-associated factors (Shema-Yaacoby et al., 2013). By altering chromatin architecture (Fierz et al., 2011), H2Bub1 may favor binding of p50 homodimers over p50-p65 heterodimers or p65 homodimers. Notably, down-regulation of RNF20 and H2Bub1 reduces the heterochromatin mark H3K9me3 in the promoter region of affected cytokine genes (Figure 1C) and enables efficient Pol II recruitment (Figure 1D). This might involve the histone H3K9 methyltransferase EHMT1, which interacts constitutively with p50 homodimers (Ea et al., 2012). TNF-α-induced downregulation of H2Bub1 might also facilitate the binding of the transcription elongation factor TFIIIS (Shema et al., 2011). Lacking a functional TAD, p50 homodimers might repress NF-κB activity (Heinz et al., 2013; Kogure et al., 2013), keeping cytokine genes off in the absence of activation signals. When encountering an activation signal, H2Bub1 levels rapidly decrease; we propose that this attenuates the association of p50 homodimers with chromatin, favoring recruitment of transactivation-competent p65-containing dimers. Interestingly, human tumors often underexpress p50, probably fostering cancer progression (Kravtsova-Ivantsiv et al., 2015).

The modular role of H2Bub1 is not limited to TNF-α. Indeed, H2Bub1 and RNF20 also affect the transcriptional response to EGF and IFN-γ (Shema et al., 2008; Buro et al., 2010). H2Bub1 is more dynamic than other histone modifications (Fuchs et al., 2014); hence, rapid alterations in H2Bub1 may guide changes in other histone modifications (Zhu et al., 2005; Nakachi et al., 2013).

RNF20+/− mice display increased susceptibility to colonic inflammation and inflammation-associated cancer. Since RNF20−/− mice are heterozygous in all tissues, the elevated inflammation may be due to H2Bub1-dependent changes not only in the gut epithelium but also in other host components.

Figure 5. Increased NF-κB Activation and DNA Damage in RNF20+/− Mice
(A) Colon sections of DSS-treated WT and RNF20+/− mice collected at day 26 were stained for p65 and p50. Arrowheads denote positive nuclei. Scale bar, 50 μm. LI%, labeling index. *p < 0.05. Error bars, SE. Right panel: quantification based on five WT and six RNF20+/− mice.
(B) Colon sections of DSS-treated WT and RNF20+/− mice collected at day 26 were subjected to IHC staining for γH2AX, p53 and p21. Arrowheads denote positive nuclei. Scale bar, 50 μm. LI%, labeling index. *p < 0.05, **p < 0.01. Error bars, SE. Right panel: quantification based on three WT and four RNF20+/− mice.
Figure 6. RNF20+/- Mice Are Predisposed to Colonic Tumorigenesis

(A) Timeline of the DSS-AOM protocol.

(B) Eight-week-old male mice weighing 22–28 g were treated with 1 μg/g AOM plus 2% DSS as described in (A). Polyps were monitored by colonoscopy on the indicated days. *p < 0.05. Error bars, SE.

(C) Colons of WT and RNF20+/- mice, treated as indicated at the bottom, were collected at day 180 and subjected to histopathological analysis. Bars depict the percentage of mice with the indicated lesions within a given treatment group; in mice with more than one type of lesion, only the most advanced lesion was considered.

(D) Immunohistochemistry showing H2B and H2Bub1 expression in WT and RNF20+/- colons. N, normal tissue; T, tumor tissue.

(E) Western blot analysis comparing H2Bub1 and H2B levels in normal and polyp tissues from WT and RNF20+/- mice. Bar values represent the relative quantification of H2Bub1/H2B expression (legend continued on next page)
such as immune cells, a notion supported by the enhanced TNF-α response of RNF20−/− inflammatory leukocytes and macrophages (Figures 3E, 3F, and S3F), as well as by the bone marrow chimera experiments. Furthermore, we cannot exclude RNF20/H2Bub1 effects on gut microbiota.

Notably, RNF20 mRNA levels varied greatly between individual mice (Figure 4G), and inflammation inversely correlated with RNF20 mRNA irrespective of genotype. This raises the intriguing possibility that in humans, where inter-individual differences in H2Bub1 levels are likely broader than in inbred mice, such inherent differences might affect susceptibility to chronic inflammatory diseases. Indeed, human ulcerative colitis (UC) is significantly associated with reduced RNF20/40 expression (Figure 7A), and lower RNF20/40 correlates with augmented inflammatory cytokine expression in human CRC (Figures S7A and S7B; Table S1).

Remarkably, both with DSS alone and with DSS+AOM, aggressive tumors were observed exclusively in RNF20−/− but not WT mice (Figure 6C). Indeed, as in our mouse models, many human sporadic CRC tumors exhibit reduced H2Bub1 (Urasaki et al., 2012), which declines as tumors progress (Figures 7D–7F). Reduced RNF20 and RNF40 mRNA in human CRC (Figure 7G) provides at least one plausible mechanistic explanation for the frequent depletion of H2Bub1. Conceivably, compromised H2Bub1 probably contributes to colorectal carcinogenesis by more than just augmenting inflammation. H2Bub1 facilitates DNA repair (Moyal et al., 2011; Nakamura et al., 2011; Shiloh et al., 2011); indeed, RNF20−/− mice display elevated γ-H2AX (Figure 5B), suggesting exacerbated DNA damage. Furthermore, RNF20−/− mice exhibit abundant M2 macrophages, providing a tumor supportive environment. Importantly, they display enhanced recruitment and augmented activation of MDSCs, which suppress anti-tumoral T cell activity and thereby facilitate tumor progression (Katoh et al., 2013). Such non-cell-autonomous effects of reduced RNF20 might underpin the increased tumor progression in RNF20−/− mice as well as the association between lower H2Bub1 and advanced human cancer.

Stem cells (SCs) typically display low H2Bub1, which increases markedly during SC differentiation and is required for their differentiation (Fuchs et al., 2012; Karpiuk et al., 2012). Hence, reduced H2Bub1 might also increase the tendency of colonic epithelial cells to adopt stem cell-like features. Of note, NF-κB has a crucial role in crypt stem cell expansion (Myant et al., 2013; Schвиталла et al., 2013). Intriguingly, like RNF20, the stem cell E3 ligase RNF43 has also been implicated in colonic tumor suppression (Koo et al., 2012).

In sum, our study highlights non cell-autonomous functions of H2Bub1 and implies that low H2Bub1 favors inflammation and cancer. It remains to be determined whether, like in mice, also in humans inter-individual variations in constitutive H2Bub1 levels may be a risk factor for UC and CRC and perhaps additional inflammatory diseases and inflammation-associated cancers.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and siRNA Transfections**

MCF10A cells were cultured at 37°C in DMEM/F-12 medium (Biological Industries) supplemented with penicillin and streptomycin as well as 10% horse serum, and 10 ng/ml EGF. YAMC cells were cultured at 32°C in RPMI-1640 (Biological Industries) supplemented with penicillin and streptomycin and 10−8 M hydrocortisone, 10−5 M α-thioglycerol, 1 μg/ml insulin, 5 units/ml mouse INF-γ, and 5% fetal calf serum. Small intestine organoids were prepared as described (Sato et al., 2011).

siRNA-mediated knockdown was with Dharmafect 1 reagent according to the manufacturer’s protocol (Dharmacon RNAi technologies, Thermo Fisher Scientific), siRNA oligonucleotide SmartPools (siLacZ, siRNF20, si-p65, si-p50, siRNF40, and siRad6) were from Dharmacon. Final concentration of all siRNA oligonucleotides was 25 nM. TNF-α from R&D Systems was applied at a final concentration of 10 ng/ml. Cells were washed twice with cold PBS, harvested, centrifuged for 2 min at 4,000 RPM, and frozen at −80°C until lysis.

**Mouse Handling**

DSS treatment, colonoscopy, and analysis of mouse tissues were as described (Cooks et al., 2013). Azoxymethane (Wako Chemicals, 011-20171; 1 μg/g) was injected intraperitoneally (i.p.) 7 days prior to the first DSS treatment. For bone marrow (BM) transplantation, mice were irradiated with 950 rad and injected intravenously with 5 × 106 BM-derived cells. Mice were then treated for 6 weeks with Ciprofloxin and subjected to DSS treatment. For intestinal permeability experiments mice were force fed by gavage with Dextran FITC (77816, Sigma-Aldrich; 0.6 g/g). After 6 hr, mice were bled and blood fluorescence intensity was measured. Procedures involving animals conformed to institutional guidelines approved by the Animal Ethics Committee of the Weizmann Institute (IAUC 00800211-1).

Inflammation severity and polyps were scored by colonoscopy according to a previously published five-parameter scoring system (Becker et al., 2006); data was analyzed with the R statistical analysis software, employing ANOVA. H&E slides of each mouse were analyzed by two independent pathologists, and tumors were scored according to their grade: low grade dysplasia (LGD), high grade dysplasia (HGD), carcinoma in situ (Tis), tumor invading the submucosa (T1), and tumor invading the muscularis propria (T2). To obtain peritoneal inflammatory leukocytes, mice were injected i.p. with 4% thioglycolate. Four days later, leukocytes were collected from mouse peritoneum and plated in a tissue culture dish.

**Human Ethical Issues**

Primary colon biopsies for IHC studies were obtained from Hebrew University-Hadassah Medical Center under approval by the Institutional Review Board (HMO-0145-13), verifying that informed consent is not required.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.020.
Author Contributions

O.T. designed and performed the experiments, and wrote the manuscript. T.C. provided advice and helped in conducting experiments. E.S. generated the RNF20 knockout mice. I.P. and V.G. conducted histological staining and analysis. E.P. and A.H. conducted pathological analysis and provided suggestions for further analyses. M.B., J.K., H.A., and H.B. conducted the experiments presented in Figures 4J, S5G, S5H, and S5J. R.R. conducted bio-statistical analysis of human data. M.O. supervised the work and wrote the manuscript.

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Figure 7. Reduced RNF20 and RNF40 mRNA and H2Bub1 in Human Colonic Pathologies

(A) Published microarray data (Sen and Bhaumik, 2013) was analyzed, comparing average RNF20 and RNF40 mRNA levels in colons of individuals with no colonic pathologies (Normal) to those of ulcerative colitis patients (UC), regardless of inflammation status. *p < 0.05, **p < 0.01. Error bars, SE.
(B) Colon samples from 25 non-specific mild chronic colitis (non-specific colitis [NSC]) patients and 19 ulcerative colitis (UC) patients were stained for H2Bub1 or total H2B. Representative cases are shown. Insets: higher magnification of dashed area. Lower magnification scale bar, 100 μm.
(C) IHC from (B) was quantified for relative H2Bub1 staining intensity in epithelium and stroma. ***p < 0.001. Error bars, SE.
(D) A tissue microarray from 75 human CRC patients, classified using the tumor node metastasis (TNM) system, was stained for H2Bub1 and total H2B. Scale on upper right indicates relative H2Bub1 intensity (H2Bub1/H2B). TNM stages: I, carcinoma invading the submucosa; II, carcinoma invading the muscularis propria; III, carcinoma invading through the muscularis propria into the pericolorectal tissue.
(E) Representative H2B and H2Bub1 staining of normal and cancerous human colonic tissue. Insets: higher magnification of dashed area. Lower magnification scale bar, 100 μm; higher magnification scale bar, 25 μm.
(F) Quantification of the data in (D). *p < 0.05, **p < 0.01.
(G) RNF20 and RNF40 mRNA levels were compared between colorectal cancer (CRC) and corresponding normal colonic tissue, using the TCGA database (http://www.cbioportal.org/public-portal). *p < 0.01. Error bars, SE.

See also Figure S7 and Table S1.

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