Title

The Histone Methyltransferase SETD2 Modulates Oxidative Stress to Attenuate Colonic Inflammation and Tumorigenesis in Mice

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Short Title: SETD2 Attenuates Colitis and Tumorigenesis

Abbreviations: AMP, antimicrobial peptide; AOM, azoxymethane; 7-AAD, 7-aminoactinomycin D; CD, Crohn's disease; CRC, colorectal cancer; DSS, dextran sodium sulfate; GO, gene ontology; H2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; H3K36, histone H3 lysine 36; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; NAC, N-acetyl-L-cysteine; 8-OHdG, 8-oxo-2'-

1 deoxyguanosine; **PRDX**, peroxiredoxin; **ROS**, reactive oxygen species; **RT-qPCR**,
2 Real-Time quantitative PCR; **UC**, ulcerative colitis; **ZO-1**, tight junction protein 1.

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5

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16

17 **Author Contributions**

18 M.L. mainly performed the experiments, analyzed the data and wrote the paper. H.R.,
19 X.L., J.L., W.F. and J.X. helped with the experiments. L.L. and W.Q.G. conceived the
20 concept, designed the experiments and drafted the manuscript. All authors had edited
21 and approved the final manuscript.

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1 Abstract

2 **BACKGROUND & AIMS:** Inflammatory bowel disease (IBD) is a complex and
3 relapsing inflammatory disease, and patients with IBD exhibit a higher risk of
4 developing colorectal cancer (CRC). Epithelial barrier disruption is one of the major
5 causes of IBD in which epigenetic modulation is pivotal. However, the epigenetic
6 mechanisms underlying the epithelial barrier integrity regulation remain largely
7 unexplored. Here, we investigated how SETD2, an epigenetic modifier, maintains
8 intestinal epithelial homeostasis and attenuates colonic inflammation and tumorigenesis.

9 **METHODS:** GEO public database and IBD tissues were used to investigate the clinical
10 relevance of SETD2 in IBD. To define a role of SETD2 in the colitis, we generated
11 mice with epithelium-specific deletion of *Setd2* (*Setd2^{Vil-KO}* mice). Acute colitis was
12 induced by 2% dextran sodium sulfate (DSS), and colitis-associated CRC was induced
13 by injecting azoxymethane (AOM), followed by three cycles of 2% DSS treatments.
14 Colon tissues were collected from mice and analyzed by histology,
15 immunohistochemistry and immunoblots. Organoids were generated from *Setd2^{Vil-KO}*
16 and control mice, and were stained with 7-AAD to detect apoptosis. A fluorescent probe,
17 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), was used to detect the levels
18 of ROS in intestinal epithelial cells (IECs) isolated from the two types of mice. RNA-
19 seq and H3K36me3 ChIP-seq analyses were performed to identify the mis-regulated
20 genes modulated by SETD2. Results were validated in functional rescue experiments
21 by N-acetyl-l-cysteine (NAC) treatment and transgenes expression in IECs.

22 **RESULTS:** SETD2 expression became decreased in IBD patients and DSS-treated

1 colitis mice. *Setd2*^{Vil-KO} mice displayed abnormal loss of mucus-producing goblet cells
2 and antimicrobial peptide (AMP)-producing Paneth cells, and exhibited pre-mature
3 intestinal inflammation development. Consistent with the reduced SETD2 expression
4 in IBD patients, *Setd2*^{Vil-KO} mice showed increased susceptibility to DSS-induced colitis,
5 accompanied by more severe epithelial barrier disruption and markedly increased
6 intestinal permeability that subsequently facilitated inflammation-associated CRC.
7 Mechanistically, deletion of *Setd2* resulted in excess reactive oxygen species (ROS),
8 which led to cellular apoptosis and defects in barrier integrity. NAC treatment in
9 *Setd2*^{Vil-KO} mice rescued epithelial barrier injury and apoptosis. Importantly, *Setd2*
10 depletion led to excess ROS by directly down-regulating antioxidant genes that inhibit
11 ROS reaction. Moreover, overexpression of antioxidant PRDX6 in *Setd2*^{Vil-KO} IECs
12 largely alleviated the overproductions of ROS and improved the cellular survival.

13 **CONCLUSIONS:** Deficiency of *Setd2* specifically in the intestine aggravates
14 epithelial barrier disruption and inflammatory response in colitis via a mechanism
15 dependent on oxidative stress. Thus, our results highlight an epigenetic mechanism by
16 which *Setd2* modulates oxidative stress to regulate intestinal epithelial homeostasis.
17 SETD2 might therefore be a pivotal regulator that maintains the homeostasis of the
18 intestinal mucosal barrier.

19 **Keywords:** IBD; SETD2; Oxidative Stress; ROS; Epithelial Barrier.

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1 Introduction

2 Inflammatory bowel disease (IBD) is the most commonly diagnosed inflammatory
3 disorder that can be sub-classified into Crohn's disease (CD) and ulcerative colitis
4 (UC)^{1,2}. Chronic inflammation inevitably results in tissue injury and tumorigenesis, and
5 IBD patients have higher risk of colorectal cancer development^{3,4}. IBD results from a
6 complex interactions between the mucosal barrier, commensal bacteria, and the
7 immune system⁵. The intestinal mucosal barrier consists of intestinal epithelial cells
8 (IECs), which are adhered to each other via epithelial tight junctions, and plays a critical
9 role in protecting the gastrointestinal tract⁶. Defects in the barrier function allow the
10 translocation of luminal pathogens and bacteria into the intestinal lamina propria and
11 trigger chronic inflammation and tissue injury, which occasionally causes dysplasia⁷.
12 IBD have been associated with abnormal intestinal epithelial barrier function^{8,9}.
13 However, the underlying molecular mechanisms of IBD remain largely ambiguous.

14
15 Intestinal inflammation is usually accompanied by excessive production of reactive
16 oxygen. High levels of reactive oxygen species (ROS) create oxidative stress within the
17 cells and lead to a loss of homeostasis¹⁰. Redox imbalance has been proposed as one
18 potential cause factor for IBD¹¹. Oxidative stress plays an important role in the
19 development and progression of IBD, and is associated with CRC pathogenesis¹²⁻¹⁴. In
20 the gastrointestinal tract, oxidative stress leads to damages of the intestinal mucosal
21 layer and epithelial cell apoptosis, which results in bacterial invasion in the gut and in
22 turn stimulates the immune response¹⁵⁻¹⁷. Clinical studies show that the increase of ROS

1 and biomarkers of oxidative injury contributes to tissue damage in IBD^{17, 18}. Our
2 previous work has also shown that excess ROS leads to defects in barrier integrity and
3 the subsequent inflammation¹⁹. In order to resist the oxidative damage, the intestinal
4 epithelium contains an extensive system of antioxidants^{20, 21}. Colitis is usually
5 associated with a decrease in the levels of antioxidants in colonic tissues¹⁸, whereas
6 overexpression of antioxidant enzymes results in attenuation of colitis in mice¹⁶. Thus,
7 a balance between oxidant and antioxidant mechanisms is necessary to maintain
8 intestinal epithelial homeostasis.

9

10 Epigenetic regulation disorder is important in the onset and pathogenesis of IBD²²⁻²⁴,
11 which may influence the maintenance of homeostasis in the intestinal epithelium.
12 Studies have shown that DNA methylation is correlated with disease susceptibility and
13 progression in IBD^{25, 26}. It is also known that inactive histone modification H3K27
14 trimethylation regulated by EZH2 promotes the inflammatory response and apoptosis
15 in colitis²⁷. Moreover, our previous study has also shown that BRG1, a key epigenetic
16 regulator, is required for the homeostatic maintenance of intestines to prevent
17 inflammation and tumorigenesis¹⁹. In addition, SETD2, a trimethyltransferase of
18 histone H3 lysine 36 (H3K36), is frequently mutated (17%) in UC samples with a high
19 risk of developing colorectal carcinoma²⁸, suggesting that SETD2 plays a potential role
20 in colitis and colitis-associated CRC. SETD2 has also been identified as a common
21 mutation across cancer types, including glioma²⁹, renal cell carcinoma³⁰, leukemia^{31, 32}
22 and lung cancer^{33, 34}. In addition, we have recently also reported that SETD2 is pivotal

1 for bone marrow mesenchymal stem cells differentiation, genomic imprinting and
2 embryonic development, initiation and metastasis of pancreatic cancer, and normal
3 lymphocyte development³⁵⁻³⁹. However, functions of SETD2 in IBD and inflammation-
4 associated CRC remain largely undefined.

5

6 To investigate a possible role of SETD2 in IBD, in the present work we generated
7 intestinal epithelium-specific *Setd2*-knockout mice, and found that deletion of *Setd2* in
8 intestinal tissues promotes DSS-induced epithelial damage and subsequent
9 tumorigenesis. Mechanistically, our results highlight SETD2 as a critical epigenetic
10 determinant in the prevention of colitis and tumorigenesis through modulation of
11 oxidative stress.

12

13 **Materials and Methods**

14 The full Materials and Methods section is included in the supplementary information.

15 ***Animal Experiments***

16 All animals were bred and maintained at an animal facility under specific pathogen-
17 free conditions. Sex-matched littermates were used in all studies. All studies with mice
18 were carried out by following the Guide for the Care and Use of Laboratory Animals,
19 which was approved by Bioethics Committee at the School of Biomedical Engineering,
20 Shanghai Jiao Tong University. *Setd2*^{Vil-KO} mice were generated by crossing *Setd2*-
21 *floxed* mice with *Villin-Cre* mice. Acute colitis was induced by adding 2% dextran
22 sodium sulphate (DSS; MP Biomedicals) for 5 days, which was then followed by 5 days

1 of regular drinking water. Body weights were monitored daily, and mice were sacrificed
2 5 days and 10 days after DSS treatment, except when indicated otherwise. Colitis-
3 associated CRC was induced by injecting AOM (10 mg/kg, Sigma), followed by three
4 cycles of 2% DSS treatments.

6 *Isolation of Intestinal Epithelial Cells and Culture*

7 For RNA and protein extraction analyses, IECs were isolated as follows: mice were
8 sacrificed and colonic specimens were dissected, opened longitudinally and cleared
9 from feces by washing extensively in cold PBS. Colons were cut in small pieces of 1
10 mm and incubated in 30 mM EDTA solution in PBS at 37°C for 10 min. 10 min later,
11 EDTA solution was replaced with ice-cold PBS and shaken vigorously for 30 s. This
12 process was repeated once more. Supernatants were collected and combined from both
13 incubations, then centrifuged at 1200 rpm for 5 min at 4°C. RNA and protein were
14 isolated from the pellet for further analysis. For culture purpose, the colons were cut
15 into pieces and washed by DMEM for three times, then incubated with digestion buffer
16 (250ug/ml collagenase type I and 500ug/ml Dispase II) for 1.5 h in 37°C. After the
17 incubation, the cell suspension was passed through 100 um cell strainers (Corning).
18 After washes, the cells were plated in dishes coated with rat tail tendon collagen type I
19 overnight and were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin.
20 The next day, the cells were treated with NAC (5 mM) for subsequent experiments.

22 *Statistical Analysis*

1 Data were analyzed using the student's t-test and results are presented as the mean $\pm$
 2 s.e.m (SEM) unless otherwise indicated. Pearson correlation coefficients were used to
 3 evaluate the relationships between SETD2 and gene expressions. Statistical analysis
 4 was performed using the GraphPad Prism software. A p value that was less than 0.05
 5 was considered statistically significant for all data sets.

6

7 ***Data Availability***

8 All data are available from the authors upon reasonable request. RNA-Seq and ChIP-
 9 Seq raw data have been deposited in the Gene Expression Omnibus (GEO) under
 10 accession number GEO: GSE 151968.

11

12 **Results**

13 **SETD2 Expression Becomes Decreased in IBD Patients and DSS Treated Mice**

14 To explore a possible role of SETD2 in IBD, we analyzed SETD2 mRNA expression
 15 in public datasets of UC samples. The results indicate that the level of SETD2 mRNA
 16 was reduced in these samples compared with that in healthy controls (Figure 1A). To
 17 expand these observations, we performed real-time quantitative PCR (RT-qPCR) assays
 18 to determine SETD2 expression in colonic biopsies from IBD patients and healthy
 19 specimens. Consistent with the results obtained from dataset GSE9452, RT-qPCR
 20 analysis indicated that the SETD2 mRNA levels were significantly decreased in the
 21 IBD biopsies compared with their normal specimens (Figure 1B). To further evaluate
 22 the relevance of SETD2 in IBD, we measured SETD2 expression level in a colitis

1 mouse model. DSS-induced colitis is a common mouse model that imitates the clinical
2 pathology of IBD^{40, 41}. We challenged wild-type mice with 3% DSS for 7 days and
3 analyzed 2 days later (Figure 1C). The DSS-treated wild-type mice exhibited
4 widespread damage and mild inflammation in the colon (Figure 1D). The excess
5 inflammatory response observed in the DSS-treated wild-type mice was associated with
6 a loss of mucus-producing goblet cells and antimicrobial peptide (AMP)-producing
7 Paneth cells (Figure 1E). The two types of cells play an important role in intestinal
8 antibacterial defense by releasing antimicrobial factors^{42, 43}. Consistently, the DSS-
9 treated colons produced significantly more proinflammatory cytokines and chemokines
10 than the colons from the control littermates (Figure 1F). These results indicated that we
11 successfully constructed a colitis mouse model. Subsequently, we evaluated SETD2
12 protein and RNA expressions in the intestine of colitis mice. Similar to what was seen
13 in IBD patients, SETD2 was down-regulated in the intestine of DSS-induced colitis
14 mice (Figure 1G-1H). Together, these findings suggest a causal link between SETD2
15 reduction and IBD pathogenesis.

16

17 ***Setd2*^{Vil-KO} Mice Are More Susceptible to DSS-Induced Colitis**

18 To assess a role of SETD2 in colonic inflammation, we crossed *Setd2*-*flox* mice (*Setd2*^{f/f}
19 mice) with *Villin-Cre* mice to obtain an intestinal epithelium-specific *Setd2* knockout
20 mouse strain (*Villin-Cre*; *Setd2* *flox/flox* mice, hereinafter referred to as *Setd2*^{Vil-KO}
21 mice). *Setd2*^{Vil-KO} mice were born with the correct Mendelian frequency. As expected,
22 *Setd2* was efficiently ablated and H3K36me3 was substantially reduced in the intestinal

1 epithelium of *Setd2^{Vil-KO}* mice by immunohistochemical staining and immunoblot
 2 validation (Figure 2A-2B). Histological examination of 8-week-old *Setd2^{Vil-KO}* mice
 3 revealed no obvious abnormalities in the colon crypt (Supplementary Figure 1A).
 4 However, as compared to the *Setd2^{ff}* mice, the levels of several proinflammatory
 5 cytokines and chemokines (IL-1 α , CXCL1) were significantly higher in the colonic
 6 sections of *Setd2^{Vil-KO}* mice (Supplementary Figure 1B). Meanwhile, *Setd2^{Vil-KO}* mice
 7 exhibited abnormal decreases of mucus-producing goblet cells and AMP-producing
 8 Paneth cells (Supplementary Figure 1C). These observations were further confirmed by
 9 RT-qPCR analysis (Supplementary Figure 1D), suggesting that *Setd2* deficiency in the
 10 intestine specifically causes a decrease in the number of secretory cells, which in turn
 11 leads to a disturbance in the homeostasis of the intestinal epithelium.

12
 13 To further define the role of SETD2 in colitis, we assessed the consequence of *Setd2*
 14 loss in acute colitis by challenging *Setd2^{ff}* and *Setd2^{Vil-KO}* mice with 2% DSS and then
 15 monitored their susceptibility by histological and morphological characteristics (Figure
 16 2C). After 5 days of DSS administration, *Setd2^{Vil-KO}* mice lost more body weight than
 17 *Setd2^{ff}* mice (Figure 2D), suggesting that *Setd2^{Vil-KO}* mice probably had enhanced
 18 inflammation and intestinal damage, as body weight loss is one of the characteristics
 19 for the severity of DSS-induced colitis^{44, 45}. In addition, macroscopic dissection
 20 revealed significantly shorter colons in the *Setd2^{Vil-KO}* mice compared with the *Setd2^{ff}*
 21 mice (Figure 2E). On further histopathological examination, the *Setd2^{Vil-KO}* mice
 22 exhibited widespread damages in the colon, with more ulcerations as compared with

1 *Setd2^{fl/fl}* mice. At both 5 and 10 days post DSS treatment, the *Setd2^{fl/fl}* colon showed
 2 minimal to mild inflammation, while colon from *Setd2^{Vil-KO}* mice displayed moderate
 3 to severe inflammation, with many areas of complete crypt loss and erosions (Figure
 4 2F). The excess inflammatory response observed in the *Setd2^{Vil-KO}* mice was also
 5 associated with a loss of goblet cells and Paneth cells (Figure 2G). Since *Setd2^{Vil-KO}*
 6 mice showed higher epithelial damage and inflammation, we then analyzed immune
 7 cell infiltration by performing flow cytometry. At 5 days post DSS treatment, we
 8 observed a higher number of CD4 + T cells, neutrophils, and macrophages in *Setd2^{Vil-}*
 9 *KO* mice compared with control mice (Figure 2H). Notably, the immune cell infiltration
 10 also extended to the entire colon, as evidenced by the accumulation of F4/80-positive
 11 cells in relatively non-inflamed areas of the *Setd2^{Vil-KO}* colons (Figure 2I). It is well-
 12 known that infiltrating immune cells produce cytokines and chemokines to resolve the
 13 inflammation process^{46, 47}. As expected, the DSS-treated *Setd2^{Vil-KO}* colons produced
 14 prominently more proinflammatory cytokines and chemokines than the colons from the
 15 *Setd2^{fl/fl}* littermates (Figure 2J). Collectively, these results demonstrate that depletion of
 16 *Setd2* leads to exacerbated colitis, suggesting that SETD2 plays a pivotal role in
 17 resolving inflammatory damages in the colonic epithelium.

18

19 ***Setd2* Deficiency Drives Inflammation-Associated CRC**

20 The observation that *Setd2^{Vil-KO}* mice suffered from the sustained inflammation,
 21 prompted us to investigate a possible role of SETD2 in colitis-associated tumorigenesis.
 22 We induced CRC by injecting the DNA-methylating agent azoxymethane (AOM),

1 followed by three cycles of 2% DSS treatments, and each cycle consisted of 5 d of
 2 2%DSS water followed by 14 d of water alone. We recorded the changes in body weight
 3 throughout the duration of the DSS treatment, and determined the tumor burden after
 4 90 days of AOM treatment. We found that *Setd2*^{Vil-KO} mice suffered from chronic
 5 inflammation and lost noticeably more body weight compared with the control mice
 6 (Figure 3A). Compared with the *Setd2*^{fl/fl} mice, *Setd2*-deficient mice had more
 7 macroscopic polypoid lesions, which were on average threefold larger in size (Figure
 8 3B). Histological analysis revealed that around 50% of polyps in *Setd2*^{Vil-KO} mice were
 9 classified as high-grade dysplasia. However, the lesions developed in *Setd2*^{fl/fl} mice were
 10 mainly graded as low-grade dysplasia or hyperplasia (Figure 3C). Accordingly, the
 11 colons of *Setd2*^{Vil-KO} mice exhibited higher proliferative rates, indicated by Ki67
 12 staining, than those in the control mice (Figure 3D). Thus, *Setd2* ablation strongly
 13 facilitates the development and progression of inflammation associated CRC.

14

15 **Disruption of *Setd2* Induces Apoptosis and Barrier Dysfunction in the Intestinal** 16 **Epithelium**

17 Given that intestinal epithelial barrier dysfunctions could frequently contribute to gut
 18 inflammation^{6, 8, 9}, and that there were increased epithelial damages in the absence of
 19 *Setd2* (Figure 2F), we investigated whether the loss of *Setd2* compromised the barrier
 20 integrity by assessing the distribution of junction proteins and permeability of intestinal
 21 epithelial cells. We examined the distribution of the tight junction protein 1 (ZO-1) and
 22 E-cadherin in *Setd2*^{fl/fl} and *Setd2*^{Vil-KO} mice, two markers to reveal the integrity of

1 junction structure and epithelial barriers. In contrast to the controls, the *Setd2*-deficient
2 colons showed partially disrupted or discontinuous ZO-1 and E-cadherin staining
3 (Figure 4A), indicating that an intact mucosal barrier was compromised in *Setd2^{Vil-KO}*
4 mice. This observation was further strengthened by Western blot assays showing
5 reduced expression of ZO-1, Claudin-1 and E-cadherin proteins in *Setd2^{Vil-KO}* mice
6 compared with control mice (Figure 4B). In accordance with the severe ulceration
7 (Figure 2F), intestinal permeability was markedly increased in *Setd2^{Vil-KO}* mice,
8 evidenced by the enhanced fluorescence in the serum of DSS-treated *Setd2^{Vil-KO}* mice
9 fed with FITC-labeled dextran (Figure 4C). Thus, these results indicate that *Setd2*
10 silencing in colons leads to epithelial barrier dysfunction and colonic leakage.

11
12 Considering that epithelial apoptosis is one of the mechanisms by which DSS can
13 induce intestinal inflammation or colitis, and that the epithelial barrier is disrupted in
14 the absence of *Setd2* (Figure 4C), we examined possible defects in epithelial cell
15 survival in the *Setd2^{Vil-KO}* mice. On the histopathological examination, the numbers of
16 TUNEL- and cleaved caspase-3-positive cells were significantly higher in the colon
17 sections from the *Setd2^{Vil-KO}* mice than in those from the *Setd2^{fl/fl}* mice (Figure 4D). This
18 observation was further strengthened by western blotting showing markedly elevated
19 signaling intensities of cleaved caspase-3 in *Setd2^{Vil-KO}* mice compared with *Setd2^{fl/fl}*
20 mice (Figure 4E). We next established intestinal organoid cultures to assess whether
21 loss of *Setd2* induces apoptosis in a cell-intrinsic manner. 7-aminoactinomycin D (7-
22 AAD) whole-mount staining and cleaved caspase-3 immunostaining displayed a

1 substantial increase in apoptosis in *Setd2*-depleted cells relative to the controls (Figure
2 4F). These data emphasize that SETD2 is intrinsically required for the colonic epithelial
3 homeostasis, and the loss of *Setd2* results in the barrier dysfunction and the subsequent
4 inflammation.

6 **SETD2 Modulates ROS Homeostasis to Regulate Colon Inflammation**

7 To further investigate the molecular mechanisms in which SETD2 regulates intestinal
8 epithelial homeostasis, we performed RNA-seq analysis. Next-generation sequencing
9 using RNA from *Setd2*^{Vil-KO} and *Setd2*^{ff} IECs after 4 d of DSS treatment respectively
10 revealed that the global transcriptome was changed dramatically in *Setd2*^{Vil-KO} IECs
11 compared to the *Setd2*^{ff} IECs, indicating a significant function of SETD2 in IECs
12 (Figure 5A). Among a total of 18168 genes expressed, 644 genes were up-regulated,
13 and 605 genes were down-regulated (Fold change > 1.25) in *Setd2*^{Vil-KO} IECs. Gene
14 Ontology (GO) term analysis of the expression profile indicated that there was a
15 significant enrichment of genes linked to apoptotic regulation, inflammatory response
16 and oxidation activity (Figure 5A). These results were validated by RT-qPCR
17 (Supplementary Figure 2A). Notably, the mRNA expression levels of some antioxidant
18 genes were significantly down-regulated, suggesting increased oxidative stress in the
19 absence of *Setd2*. To confirm this finding, we performed 8-oxo-2'-deoxyguanosine (8-
20 OHdG) staining to examine oxidative stress in colon. We detected that 8-OHdG level
21 was significantly increased in the colons of *Setd2*^{Vil-KO} mice after DSS treatment (Figure
22 5B). Oxidative stress is associated with intestinal inflammation and CRC pathogenesis,

1 and ROS plays an important role in apoptosis induction and inflammation damage
 2 under both physiologic and pathologic conditions^{12, 13}. To study the role of ROS in
 3 SETD2-mediated inflammation, we isolated the IECs from *Setd2^{Vil-KO}* and *Setd2^{ff}* mice
 4 after DSS treatment. Flow cytometry analysis by staining 2',7'-
 5 dichlorodihydrofluorescein diacetate (H2DCFDA, a fluorescent probe that reacts with
 6 ROS) indicated that *Setd2*-deficiency IECs produced significantly more amount of ROS
 7 relative to that in the control IEC cells (Figure 5C). These results indicate that ROS is
 8 a necessary player in SETD2-mediated colitis.

9

10 To provide evidence that ROS indeed contributes to the barrier defects and colitis, we
 11 performed rescue assays to determine whether blocking ROS could reverse the
 12 inflammatory phenotype caused by the *Setd2* loss. Two-month-old *Setd2^{Vil-KO}* and
 13 *Setd2^{ff}* mice were pretreated with NAC (100 mg/kg) every day from 4 days before
 14 treated with 2% DSS to the end of the experiment, except for the 5 days during which
 15 DSS was administered^{12, 48}. According to colon length and histopathological staining
 16 (Figure 5D-5E), the blockade of ROS production via the administration of NAC in the
 17 *Setd2^{Vil-KO}* mice completely eased the severity of inflammation, and the colonic
 18 morphology score was restored to the normal value. As judged by the expression levels
 19 of proinflammatory cytokines and chemokines, the severe inflammatory response
 20 occurring in the *Setd2^{Vil-KO}* mice was largely mitigated by the ROS inhibition (Figure
 21 5F). Additionally, the NAC treatment restored the numbers of Paneth and goblet cells
 22 to levels similar to those in the control mice (Supplementary Figure 2B-2C). Besides,

1 NAC-treated *Setd2^{Vil-KO}* mice exhibited a less barrier disruption and epithelial cell
 2 apoptosis than *Setd2^{fl/fl}* mice (Figure 5G-5J). In addition, the relieved epithelial apoptosis
 3 was also repeated by intestinal organoid culture experiment, indicated by cleaved
 4 caspase 3 staining (Figure 5K). Altogether, these results suggest that the blockade of
 5 ROS could reverse the phenotype of epithelial barrier disruption and colon
 6 inflammation in *Setd2^{Vil-KO}* mice. Thus, ROS plays an important role in SETD2-
 7 mediated colitis.

8

9 **SETD2-Mediated H3K36me3 Facilitates the Transcriptions of Antioxidant Genes** 10 **to Maintain ROS Homeostasis**

11 To elucidate the molecular basis by which SETD2 modulated ROS, we
 12 immunoprecipitated H3K36me3-bound chromatin in *Setd2^{Vil-KO}* and *Setd2^{fl/fl}* IECs after
 13 4 d of DSS treatment and analyzed the precipitated DNA by deep sequencing. 178919
 14 and 217927 H3K36me3 peaks were identified in *Setd2^{Vil-KO}* and *Setd2^{fl/fl}* IECs,
 15 respectively. The altered density of H3K36me3 intervals (*Setd2^{Vil-KO}* versus *Setd2^{fl/fl}*)
 16 were widely localized within whole genomic regions, including gene bodies, promoters,
 17 and intergenic zones (Figure 6A-6B), indicating that SETD2 is responsible for the
 18 H3K36 trimethylaiton on these regions. To correlate the chromatin binding with the
 19 transcriptional regulation, we integrated the ChIP-seq data with the expression profile
 20 and the Venn diagrams indicated that 906 (including downregulated and upregulated)
 21 genes showed direct H3K36me3 occupancies and expression changes upon the *Setd2*
 22 ablation (Figure 6C). GO term analysis revealed that these overlapping 906 genes were

1 still closely related to oxidative stress pathway (Figure 6C). As shown in figure 5,
2 ablation of *Setd2* increased ROS production and down-regulated the expression levels
3 of antioxidant genes, suggesting that Setd2/H3K36me3 might positively regulate some
4 antioxidant-coding genes related to ROS production. As expected, antioxidant genes
5 including *Prdx3*, *Prdx6*, *Gclm*, and *Srxn1* were listed among the 906 genes described
6 above, and their downregulation was validated in colon epithelial cells collected from
7 *Setd2^{Vil-KO}* mice, comparing with cells from *Setd2^{fl/fl}* mice (Figure 6D). Direct
8 H3K36me3 occupancies within these candidate gene loci were also seen in Genome
9 Browser tracks (Figure 6E). We further validated the existence of H3K36me3 bindings
10 at the gene bodies of *Prdx3*, *Prdx6*, *Gclm* and *Srxn1* by the ChIP-qPCR assays, and
11 found that the intensity of H3K36me3 bindings within these gene loci decreased along
12 with the loss of *Setd2* (Figure 6F). Consistent with these results, there were significant
13 positive correlations between mRNA levels of SETD2 and that of PRDX3, PRDX6,
14 GCLM and SRXN1 respectively based on GSE57945 database (Figure 6G). Thus,
15 *Setd2* loss could repress the transcription of antioxidant genes, which were important
16 for antioxidant defenses. The peroxiredoxin [PRDX] family contains the most of
17 important antioxidant proteins. PRDX6, a bifunctional 25-kDa protein, is one member
18 of PRDXs⁴⁹. Next, we sought to investigate whether restoration of PRDX6 in *Setd2*-
19 depleted IECs would attenuate the overproduction of ROS and improve the cell survival
20 as compared with *Setd2*-deleted IECs. As expected, overexpression of PRDX6 in *Setd2*-
21 depleted IECs could significantly reduce the ROS production and the cleaved caspase-
22 3 signals (Figure 6H-6I). Thus, ectopic *Prdx6* could alleviate the overproduction of

1 ROS and improve the cellular survival in the *Setd2*-deficient intestinal epithelium.

2 Together, these results indicate that SETD2 prevents colon inflammation and epithelial

3 barrier defects via the regulation of antioxidant to restrain ROS over reaction.

4

5 Gut microbes also play an important role in driving colonic inflammatory responses⁵,

6 ⁵⁰. To rule out the possibility that the intestinal microbiota contributes to epithelial

7 barrier damage caused by *Setd2* deficiency, we generated gut microbiota-depleted mice

8 by treating *Setd2*^{Vil-KO} and *Setd2*^{ff/ff} mice with drinking water containing an antibiotic

9 cocktail for 4 weeks and then treated them with 2%DSS^{51, 52}. As compared with the

10 *Setd2*^{ff/ff} mice, there was less bacterial richness but unchanged gut microbiota

11 composition in untreated *Setd2*^{Vil-KO} mice (Supplementary Figure 3A-B). Interestingly,

12 depletion of the gut microbiota by antibiotics eased the severity of colitis and reduced

13 expression of proinflammatory cytokines and chemokines of *Setd2*^{Vil-KO} mice

14 comparable to those observed in the *Setd2*^{ff/ff} mice (Supplementary Figure 3C-D).

15 However, antibiotic-treated *Setd2*^{Vil-KO} mice still exhibited more severe barrier

16 disruption and epithelial cell apoptosis than *Setd2*^{ff/ff} mice (Supplementary Figure 3E-J).

17 These results suggested that depletion of the gut microbiota did not facilitate the

18 recovery of intestinal barrier in *Setd2*^{Vil-KO} mice. Thus, the gut microbiota does not

19 contribute to intestinal barrier disruption caused by *Setd2* deficiency. Considered

20 together, SETD2 protects the colon epithelial barrier from inflammatory insults through

21 a mechanism dependent on oxidative stress (Figure 7).

Discussion

Although epigenetic dysregulation is recently believed to contribute to development of intestinal inflammation^{24, 25, 53} and epithelial barrier disruption is one of the major causes of IBD, whether epigenetic elements are drivers during the regulation of epithelial barrier integrity and colonic inflammation and tumorigenesis has not been genetically determined. In this study, we established mice with epithelium-specific deletion of *Setd2* (*Setd2*^{Vil-KO} mice), and then challenged with various inducing agents such as DSS and AOM to generate different types of models of colonic inflammation and tumorigenesis. We demonstrated clearly that deletion of *Setd2* that is responsible for one of the major histone modifications, disturbs intestinal epithelial homeostasis and worsens barrier defects by modulating oxidative stress, which promotes colonic inflammatory response and subsequently tumorigenesis (Figure 7). Moreover, the excess inflammatory damage occurring in the *Setd2*-depleted mice was largely alleviated by the ROS inhibition through antioxidant NAC treatment. Our results highlight the importance that SETD2 functions as a homeostatic regulator that integrates the intact epithelial barrier by modulating oxidative stress in the colon. Support for our findings comes from a recent report indicating that mutations of the *Setd2* gene occur in up to 17% in UC samples with a high risk of developing colorectal carcinoma²⁸. Thus, our study provides the first direct causal evidence that SETD2 plays a pivotal role in IBD and colitis-associated CRC.

The present study reveals a mechanism by which SETD2 inhibits ROS overreaction to

1 maintain intestinal epithelial homeostasis against intestinal inflammation and
 2 tumorigenesis. Ulcerative colitis is usually characterized by mucosal barrier
 3 dysfunction, and intestinal barrier integrity is essential for preventing microbial-driven
 4 inflammatory response. Our study found that *Setd2* loss results in epithelial barrier
 5 dysfunction and colonic leakage, which promotes the subsequent inflammation
 6 response. ROS plays an important role in cell apoptosis induction and inflammation
 7 under both physiologic and pathologic conditions^{12, 13, 19}. Mammalian cells are always
 8 exposed to ROS produced by environmental oxidants or endogenous metabolism^{54, 55}.
 9 We found that *Setd2* deficiency in the colon epithelium leads to ROS accumulations,
 10 and then enhances epithelial cell death and barrier disruption. And this finding is further
 11 supported by the experiment of antioxidant NAC treatment. The NAC treatment in
 12 *Setd2^{Vil-KO}* mice attenuates the ROS reactions and relieves the inflammation symptoms.
 13 Therefore, excess ROS accumulation is mainly responsible for the colon inflammation
 14 observed in the *Setd2^{Vil-KO}* mice.

15
 16 The notion that SETD2 regulates colon inflammation is via modulation of ROS
 17 homeostasis is further supported by our more detailed analysis that SETD2 facilitates
 18 the transcriptions of antioxidant genes. It is well known that SETD2 exerts epigenetic
 19 regulation via promoting the transcriptions of its downstream genes by marking gene
 20 bodies with H3K36me3 and facilitating the elongation of their pre-mRNA^{29, 56}. At
 21 molecular level, we show that *Setd2* deficiency down-regulates the mRNA levels of the
 22 members of antioxidant genes including *Prdx3*, *Prdx6*, *Gclm* and *Srxn1* in the analysis

1 of sequencing results. The current findings demonstrate that SETD2 attenuates colonic
 2 inflammation through maintaining the expression of antioxidant genes to suppress
 3 oxidative stress. Our work is further corroborated by the rescue experiment showing
 4 that overexpression of PRDX6 in *Setd2*-depleted IECs could reduce the ROS
 5 production and improve epithelial cell survival. Clinically, we also found that
 6 expression level of SETD2 in IBD samples is positively correlated with the expressions
 7 of PRDX3, PRDX6, GCLM and SRXN1, respectively. Therefore, our findings reveal
 8 for the first time the important role of SETD2-mediated H3K36me3 modification
 9 involved in the oxidative stress pathway in the maintenance of intestinal epithelial
 10 homeostasis, and provide insights into the molecular mechanisms underlying the
 11 intestinal inflammation and colitis-associated CRC with epigenetic disorders.

12
 13 It is worth mentioning that SETD2 appears to interact with various proteins to influence
 14 transcription. Notably, our previous work reported that SETD2-mediated H3K36me3
 15 could participate in cross-talks with other chromatin markers in oocytes, including
 16 H3K4me3 and H3K27me3 in transcribing regions³⁸. In the present study, we
 17 demonstrate that expression of some antioxidant genes is regulated by SETD2-
 18 mediated H3K36me3. However, little is known about the epigenetic functions of other
 19 chromatin markers on regulating these genes in IBD. It would be interesting to study
 20 the physiological role of the cross-talk between H3K36me3 and other chromatin
 21 markers in IBD and colitis-associated CRC in the future. Moreover, recent studies have
 22 also revealed that SETD2 not only exerts its classical role in controlling gene

transcription by mediating modifications of histone proteins, but also has the capacity to regulate cellular signaling through modification of non-histone substrates. For example, it has been reported that SETD2 could tri-methylate α -tubulin on K40 and mediate K525me1 of STAT1, which implicates a vital role in mitosis and antiviral immunity respectively^{57, 58}. In the present study, we show that SETD2-mediated H3K36me3 promotes the expression of some antioxidant genes. However, it remains to be determined whether SETD2 can interact with these proteins and methylate them to affect the progression of IBD and tumorigenesis.

9

In summary, our findings highlight that SETD2 plays a critical role in the intestinal homeostasis and regulates barrier function and colon inflammation by modulating oxidative stress in the colon. We also established a SETD2/H3K36me3-deficient IBD mouse model that could be used for pre-clinical researches on colitis with epigenetic disorders. Given that SETD2 mutation accounts for 17% IBD patients with a high risk of developing colorectal carcinoma, our results may provide insight into our understanding of pharmaceutical investigation of these diseases associated with SETD2/H3K36me3 mutation.

18

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1 **Figure Legends**

2 **Figure 1. SETD2 expression is downregulated in IBD patients and DSS treated** 3 **mice.**

4 **(A)** Boxplot of SETD2 expression levels in healthy controls and UC patients (using
5 dataset GSE9452; n = 26).

6 **(B)** RT-qPCR analysis of SETD2 transcripts in IBD specimens and healthy subjects (n
7 = 23).

8 **(C)** Schematic representation of the DSS protocol used to induce acute colitis.

9 **(D-H)** Samples are all derived from 8-week-old wild-type mice with or without DSS

10 treatment. **(D)** Representative images of H&E-stained colon sections as indicated. Scale

11 Bars: 100um. **(E)** Alcian blue-Periodic acid Schiff (AB-PAS; goblet cells) staining and

12 lysozyme (Lys; Paneth cells) staining in the colon as indicated, and quantitation results

13 are shown in the right (n=3). Scale Bars: 100um. **(F)** RT-qPCR analysis of whole colon

14 homogenates to assess cytokine and chemokine production (n = 4). **(G)** RT-qPCR

15 analysis of SETD2 mRNA in control and DSS-treated wild-type mice(n=4). **(H)**

16 Immunoblot analyses of SETD2 expression in control and DSS-treated wild-type mice

17 are shown.

18 The data represent the mean $\pm$ S.E.M, and statistical significance was determined by a

19 two-tailed Student's t-test. * p < 0.05.

20

21 **Figure 2. Loss of *Setd2* in IECs aggravates DSS-induced colitis in mice.**

1 **(A-B)** Immunohistochemical **(A)** and immunoblot analyses **(B)** of SETD2 and
2 H3K36me3 expressions are shown. Scale Bars: 50um.

3 **(C)** Schematic representation of the DSS protocol used to induce acute colitis.

4 **(D, E)** *Setd2*^{Vil-KO} and *Setd2*^{ff} mice were fed 2% DSS in drinking water and loss of body
5 weights **(D)** and colon length **(E)** are recorded (n=5). Scale Bars: 1cm.

6 **(F)** H&E stained sections of colon tissues collected on day 0, 5 and 10 from 2% DSS-
7 treated mice, and the quantitation of DSS-induced epithelial damage and immune cell
8 infiltration are shown in the right. Scale Bars: 100um.

9 **(G)** Alcian blue-Periodic acid Schiff (AB-PAS; goblet cells) staining and lysozyme (Lys;
10 Paneth cells) staining in the colon as indicated. Scale Bars: 100um.

11 **(H)** After 5 d of DSS treatment, colonic lamina propria cells from *Setd2*^{Vil-KO} and *Setd2*^{ff}
12 mice are analyzed by flow cytometry for CD4 + T cells, CD11b +; F4/80 + macrophages,
13 and CD11b + ; Gr-1 + neutrophils (n = 3).

14 **(I)** Colon sections from mice treated with DSS were stained for F4/80 to detect
15 macrophages. Scale Bars: 100um.

16 **(J)** Relative mRNA expression levels of inflammatory mediators in whole colon of
17 *Setd2*^{Vil-KO} and *Setd2*^{ff} mice were determined by RT-qPCR (n=7).

18 The data represent the mean ± S.E.M, and statistical significance was determined by a
19 two-tailed Student's t-test. * p < 0.05, ** p < 0.01 and *** p < 0.001. N.S., Not
20 Significant.

21

22 **Figure 3. *Setd2* loss promotes the inflammation-associated CRC.**

1 (A) *Setd2*^{Vil-KO} and *Setd2*^{ff} mice were injected with AOM on day 0 (2 months of age)
2 and treated with 2% DSS during three 4-day cycles as indicated. Body weight changes
3 are recorded as indicated (n = 4 per genotype).

4 (B) At day 90 after the AOM injection, the mice were sacrificed to examine the tumor
5 burden (n = 5 per genotype). Scale Bars: 1cm.

6 (C) Representative images of colons and the overall grading of the tumors in each
7 genotype (χ^2 test) are shown (n = 5 per genotype). Scale Bars: upper, 200um; bottom,
8 100um.

9 (D) Representative images of Ki67 staining in the tumors from AOM/DSS-treated
10 *Setd2*^{Vil-KO} and *Setd2*^{ff} mice are shown (n=5). Scale Bars: 50um.

11 The data represent the mean $\pm$ S.E.M, and statistical significance was determined by a
12 two-tailed Student's t-test. * p < 0.05, ** p < 0.01.

13

14 **Figure 4. Loss of *Setd2* in IECs induces cell apoptosis and barrier dysfunction.**

15 (A) Representative ZO-1 and E-cadherin staining in colon sections from DSS-treated
16 (5 d) *Setd2*^{Vil-KO} and *Setd2*^{ff} mice. Scale Bars: 100um.

17 (B) Immunoblotting analysis of the indicated proteins in IECs isolated from *Setd2*^{Vil-KO}
18 and *Setd2*^{ff} mice.

19 (C) Colonic permeability was measured by the concentration of FITC-dextran in the
20 blood serum (n=3).

(D) TUNEL (Upper) and cleaved caspase 3 (Lower) staining of colon sections from DSS-treated (5 d) *Setd2^{Vil-KO}* and *Setd2^{ff}* mice, and quantitation results are shown in the right (n=5). Scale Bars: 50um.

(E) Western blot analysis of the indicated proteins in IECs isolated from DSS treated (5 d) *Setd2^{Vil-KO}* and *Setd2^{ff}* mice.

(F) Intestinal organoids derived from *Setd2^{Vil-KO}* and *Setd2^{ff}* mice. After 5 d of differentiation, 7-AAD-stained organoids were imaged, and quantitations of the fluorescence density are shown in the right (n=4). Scale Bars: 20um.

The data represent the mean $\pm$ S.E.M, and statistical significance was determined by a two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N.S., Not Significant.

11

Figure 5. SETD2 mediates ROS reaction to control cell apoptosis and colon inflammation.

(A) Heat map of RNA-seq data to compare the gene expression of colons from *Setd2^{Vil-KO}* and *Setd2^{ff}* mice. Go term analysis of gene expression changes in the right.

(B) 8-OHdG staining from DSS-treated *Setd2^{Vil-KO}* and *Setd2^{ff}* mice. Scale Bars: 50um.

(C) Histograms and quantifications of ROS in IECs isolated from DSS-treated *Setd2^{Vil-KO}* and *Setd2^{ff}* mice.

(D-J) 8-week-old *Setd2^{Vil-KO}* and *Setd2^{ff}* mice were pretreated with NAC (100 mg/kg) every day from 4 days before the treatment with 2% DSS to the end of the experiment.

Colon length (D), H&E staining (E), RT-qPCR analysis (F), ZO-1 staining (G), E-

1 cadherin staining **(H)**, Western Blotting analysis **(I)** and cleaved caspase-3 staining **(J)**
2 as indicated. Scale Bars: 100um (e, g, h); 50um (j).

3 **(K)** Organoids were isolated from 8-week-old *Setd2^{Vil-KO}* and *Setd2^{ff}* mice with or
4 without NAC(5mM) treatment, and cleaved caspase-3 staining. Scale Bars: 20um.

5 The data represent the mean $\pm$ S.E.M, and statistical significance was determined by a
6 two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N.S., Not Significant.

7

8 **Figure 6. SETD2 regulates antioxidant protein via H3K36me3.**

9 **(A)** Normalized read density of H3K36me3 ChIP-seq signals of colons from *Setd2^{Vil-}*
10 *KO* and *Setd2^{ff}* mice from 3 kb upstream of the TSS to 3 kb downstream of the TES.

11 **(B)** Analysis of the occupancy of H3K36me3 ChIP-seq peaks in gene bodies and
12 intergenic regions.

13 **(C)** Venn diagram showing the number of genes harboring H3K36me3 binding and
14 displaying expression changes in *Setd2^{Vil-KO}* IECs. Right panel shows the Go term
15 analysis of the overlapping genes.

16 **(D)** RT-qPCR analysis of antioxidant genes expression in IECs as indicated.

17 **(E)** Snapshot of H3K36me3 ChIP-Seq signals at the *Prdx3*, *Prdx6*, *Gclm* and *Srxn1*
18 gene loci in IECs isolated from DSS-treated (4 d) *Setd2^{Vil-KO}* and *Setd2^{ff}* mice.

19 **(F)** ChIP-qPCR analysis of H3K36me3 binding for *Prdx3*, *Prdx6*, *Gclm* and *Srxn1* loci
20 in IECs from DSS-treated (4 d) *Setd2^{Vil-KO}* and *Setd2^{ff}* mice, and IgG was used as the
21 control.

1 **(G)** Correlation between SETD2 and PRDX3, PRDX6, GCLM and SRXN1 expression
2 levels in IBD specimens (GSE 57945). Statistical significance was determined using
3 the Pearson correlation coefficient.

4 **(H)** Western blot analysis of the indicated proteins in *IEC^{Setd2-ff}* and *IEC^{Setd2;VIL-KO}* with
5 or without PRDX6 overexpression.

6 **(I)** Histograms and quantifications of ROS in *IEC^{Setd2-ff}* and *IEC^{Setd2;VIL-KO}* with or
7 without PRDX6 overexpression.

8 The data represent the mean $\pm$ S.E.M, and statistical significance was determined by a
9 two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

10

11 **Figure 7. Loss of *Setd2* promotes oxidative stress-induced colonic inflammation**
12 **and tumorigenesis.**

13 SETD2 directly controls multifaceted antioxidant genes including *Prdx3*, *Prdx6*, *Gclm*
14 and *Srxn1* in intestinal epithelial cells (IECs). Thus, *Setd2* loss in IECs leads to
15 insufficient antioxidant, which results in excess reactive oxygen species (ROS), and
16 thereby compromises barrier integrity and promotes inflammation. Compensatory
17 regeneration coupled with oxidative stress-induced epithelial damage promotes the
18 malignant progression of CRC.

19

Figure 1.

Figure 2.

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Figure 6.