

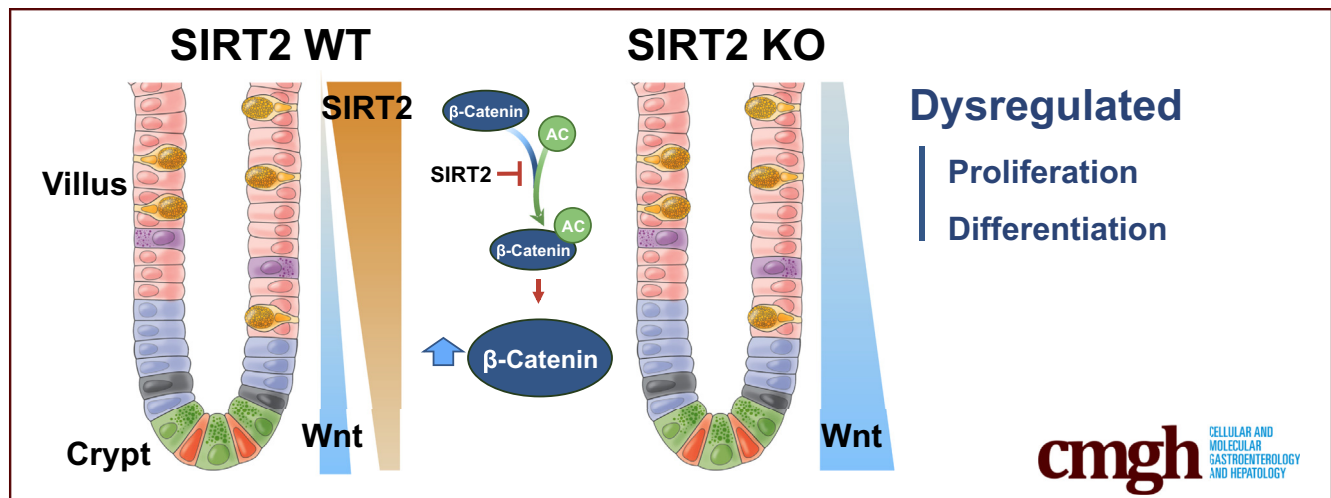
ORIGINAL RESEARCH

SIRT2 Contributes to the Regulation of Intestinal Cell Proliferation and Differentiation



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SUMMARY

Using complementary approaches (intestinal epithelial cell lines, intestinal organoids and SIRT2 knockout mice), we demonstrated that SIRT2, decreased in intestinal tissues from patients with inflammatory bowel diseases, contributes to the maintenance of intestinal epithelium homeostasis through regulation of Wnt- β -catenin signaling.

BACKGROUND AND AIMS: Intestinal mucosa undergoes a continual process of proliferation, differentiation, and apoptosis. Disruption of this homeostasis is associated with disorders such as inflammatory bowel disease (IBD). We investigated the role of Sirtuin 2 (SIRT2), a NAD-dependent protein deacetylase, in intestinal epithelial cell (IEC) proliferation and differentiation and the mechanism by which SIRT2 contributes to maintenance of intestinal cell homeostasis.

METHODS: IECs were collected from SIRT2-deficient mice and patients with IBD. Expression of SIRT2, differentiation markers (mucin2, intestinal alkaline phosphatase, villin, Na,K-ATPase, and lysozyme) and Wnt target genes (EPHB2, AXIN2, and cyclin D1) was determined by western blot, real-time RT-PCR, or immunohistochemical (IHC) staining. IECs were treated with TNF or transfected with siRNA

targeting SIRT2. Proliferation was determined by villus height and crypt depth, and Ki67 and cyclin D1 IHC staining. For studies using organoids, intestinal crypts were isolated.

RESULTS: Increased SIRT2 expression was localized to the more differentiated region of the intestine. In contrast, SIRT2 deficiency impaired proliferation and differentiation and altered stemness in the small intestinal epithelium *ex vivo* and *in vivo*. SIRT2-deficient mice showed decreased intestinal enterocyte and goblet cell differentiation but increased the Paneth cell lineage and increased proliferation of IECs. Moreover, we found that SIRT2 inhibits Wnt/ β -catenin signaling, which critically regulates IEC proliferation and differentiation. Consistent with a distinct role for SIRT2 in maintenance of gut homeostasis, intestinal mucosa from IBD patients exhibited decreased SIRT2 expression.

CONCLUSION: We demonstrate that SIRT2, which is decreased in intestinal tissues from IBD patients, regulates Wnt- β -catenin signaling and is important for maintenance of IEC proliferation and differentiation. (*Cell Mol Gastroenterol Hepatol* 2020;10:43–57; <https://doi.org/10.1016/j.jcmgh.2020.01.004>)

Keywords: Sirtuin; IEC; Intestinal Epithelial Cells; Wnt/ β -Catenin; Intestinal Homeostasis; Mouse Model.

Mammalian intestinal epithelial cells (IECs) exhibit a multitude of physiological functions, including absorption of nutrients, secretion of hormones and antimicrobial peptides, response to infectious agents, and regeneration after physical damage.^{1,2} The intestinal mucosa undergoes a process of continual renewal characterized by active proliferation of crypt-based stem cells localized near the base of the crypts.³ As these cells progress up the crypt-villus axis, there is cessation of proliferation and subsequent differentiation into 1 of the 4 primary cell types (ie, enterocytes, goblet, Paneth, and enteroendocrine cells).^{4,5} The mechanisms that regulate stem cell maintenance, proliferation, differentiation, and apoptosis are precisely orchestrated to ensure proper organ maintenance. However, the underlying molecular mechanisms regulating intestinal cell proliferation and differentiation are not entirely known.

The Wnt/ β -catenin signaling pathway has a central role for intestinal homeostasis. Impaired Wnt/ β -catenin signaling leads to crypt death,^{6,7} while pathway hyperactivation drives cancer.⁸ High Wnt signaling is found at the crypt base,⁹ where intestinal stem cells (ISCs) exhibit nuclear β -catenin and transcriptional signatures associated with Wnt/ β -catenin pathway activation. Wnt/ β -catenin signaling is a critical driver of stem cell self-renewal and cell specialization during development as well as adult tissue homeostasis.¹⁰ Wnt/ β -catenin signaling is essential for maintaining the epithelial proliferative compartment and controlling differentiation.^{11–14} Wnt binding leads to the stabilization of β -catenin, which enters the nucleus to regulate Wnt pathway-target genes. Activation of Wnt/ β -catenin signaling contributes to development of colitis-associated intestinal cancers.¹⁵ Elucidating the extent and mechanisms of Wnt signaling in the intestine has potential to broaden our understanding of epithelial homeostasis and may be of particular relevance for disorders such as inflammatory bowel disease (IBD) and colitis-associated cancers.¹⁵

Human sirtuin proteins (SIRT1–7) possess a unique nicotinic adenine dinucleotide (NAD)-dependent protein deacetylase activity.¹⁶ Sirtuin proteins are involved in regulation of metabolism, proliferation, differentiation and cell survival.¹⁷ SIRT1 protein prevents intestinal inflammation by regulating the gut microbiota.¹⁸ SIRT1 deficiency in the intestine specifically activates secretory cells, as is evident by the elevated number of intestinal Paneth and goblet cells without alteration of other cell types or the proliferation rate of IECs.¹⁸ SIRT2 regulates cell differentiation, growth, autophagy, and cell cycle¹⁹; deficiency of SIRT2 induces chromosome alterations and subsequent tumor development in the liver.^{20,21} Moreover, SIRT2 is required for pluripotent and hematopoietic stem cell maintenance and regenerative capacity. It also protects against development of inflammatory processes in the intestine.^{22–24} However, unlike SIRT1, the role of SIRT2 in intestinal cells is largely undefined.

In the present study, we report a critical role for SIRT2 in the maintenance of intestinal homeostasis. We show that intestinal epithelial SIRT2, mainly expressed in the villus, is

critically involved in regulation of intestinal proliferation, differentiation, and stemness through modulation of the Wnt/ β -catenin signaling. Importantly, SIRT2 deficiency in the intestinal epithelium results in increased proliferation and impaired differentiation. From a clinical perspective, patients with IBD exhibit markedly reduced SIRT2 expression in their intestinal mucosa. A reduction in SIRT2 levels in the intestinal epithelium results in activated Wnt/ β -catenin signaling, leading to altered regulation of proliferation and differentiation.

Results

Upregulation of SIRT2 Is Associated With Intestinal Cell Differentiation

To determine the expression of sirtuin proteins along the crypt-villus axis, IECs were isolated from the villus and crypt (Figure 1A). Purity of the cell fractions was confirmed by expression of villin (marker of IEC differentiation) localized to the villus fraction and expression of proliferating cell nuclear antigen (marker of DNA synthesis) localized to the crypt. A differential expression pattern for the SIRT1 and SIRT2 proteins was noted, with SIRT2 mainly expressed in the villus (containing differentiated, non-proliferating cells) and expression of SIRT1 identified mainly in the crypt (containing undifferentiated, proliferating cells). SIRT2 is expressed as 2 functionally similar isoforms (isoform 1 and 2).²⁵ Our results indicate that SIRT1 and SIRT2 may contribute to the processes of intestinal cell proliferation and differentiation. Consistent with our findings, SIRT1 is highly enriched in the small intestine crypts and is an important mediator of host-microbiome interactions.^{18,26} Based on our findings of localized expression of SIRT2 to the more differentiated region of the intestine, we hypothesized that SIRT2 may play a more critical role in intestinal cell differentiation.

To correlate the expression pattern of SIRT2 protein in the human intestine, sections of normal human small bowel and colon were analyzed from 5 adult patients. Intense staining for SIRT2 was localized to the most differentiated region of the small intestine (ie, villus) or colon (ie, upper crypt) (Figure 1B). This is consistent with an overall increase in SIRT2 expression in intestinal cell differentiation.

We next examined SIRT2 expression during induced in vitro differentiation. As shown in Figure 1C, treatment

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Abbreviations used in this paper: AB, Alcian blue; IAP, intestinal alkaline phosphatase; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IHC, immunohistochemistry; ISC, intestinal stem cell; LYZ, lysozyme; mRNA, messenger RNA; MUC2, mucin2; NaBT, sodium butyrate; NAD, nicotinamide adenine dinucleotide; PBS, phosphate-buffered saline; RT-PCR, reverse-transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; SIRT, sirtuin; TNF, tumor necrosis factor; WT, wild-type.



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2352-345X

<https://doi.org/10.1016/j.jcmgh.2020.01.004>

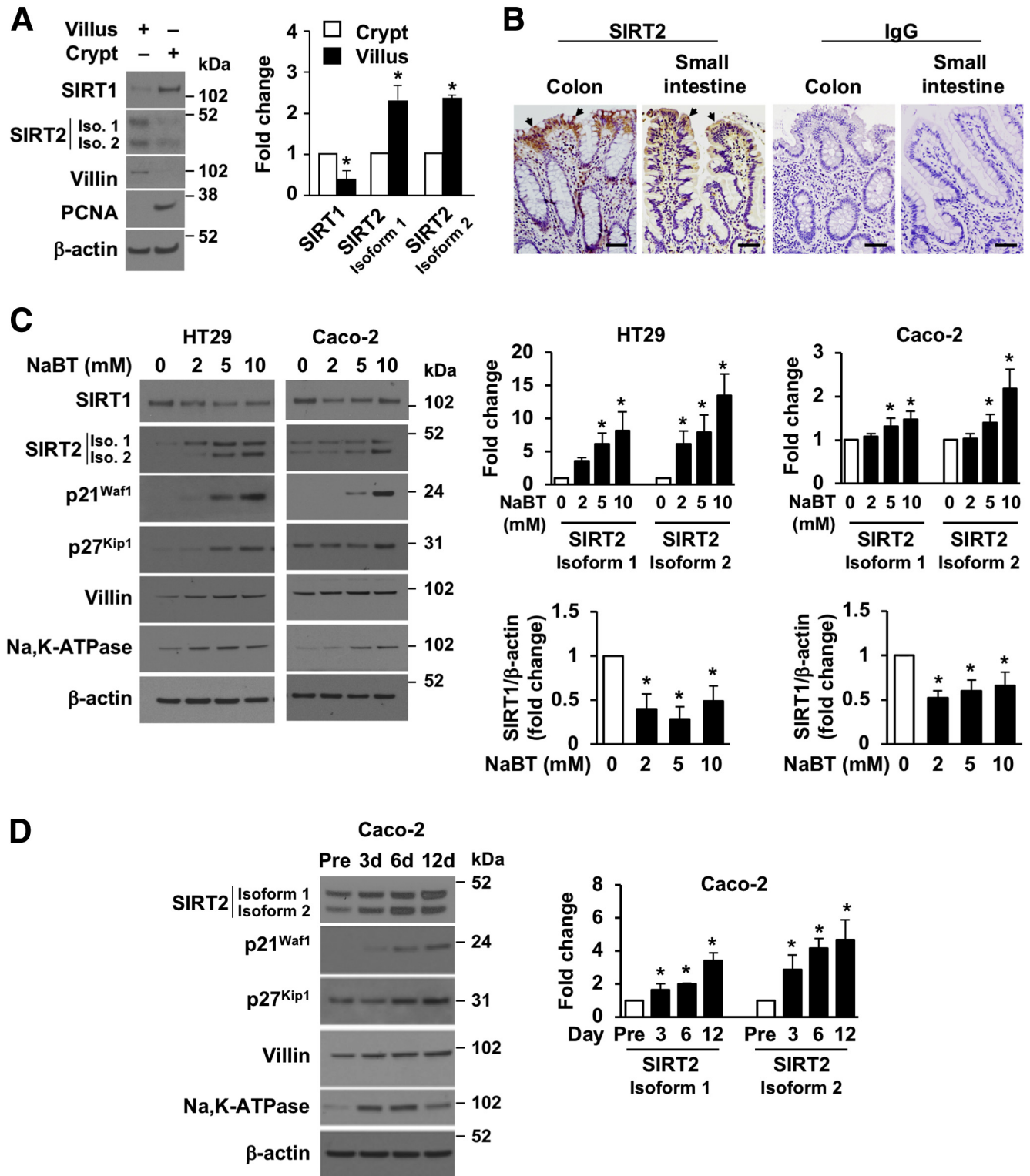


Figure 1. Induction of SIRT2 is associated with intestinal cell differentiation. (A) Villus and crypt proteins were extracted from mouse small intestine. SIRT1, SIRT2, villin, proliferating cell nuclear antigen, and β -actin was detected by Western blotting. (B) Immunohistochemical analysis of SIRT2 protein expression in normal human colon and small intestine. Human normal colon and small intestine sections were fixed and stained with primary anti-human SIRT2 antibody. SIRT2 is specifically expressed in the more differentiated region of the small intestine (ie, villus) or colon (ie, upper crypt). Scale bars = 50 μ m. The images are representative of 5 cases. (C) HT29 or Caco-2 cells were treated with NaBT at various dosages for 48 hours. (D) Caco-2 were incubated 3, 6 and 12 days after confluency to differentiation. Cells were lysed and Western blot analysis was performed using antibodies as indicated. The images are representative of 3 independent experiments. SIRT2 isoform 1, SIRT2 isoform 2, and SIRT1 signals from 3 separate experiments were quantitated densitometrically and expressed as fold change with respect to β -actin ($n = 3$, data represent mean \pm SD; * $P < .05$ vs control).

with sodium butyrate (NaBT) induced enterocyte differentiation as demonstrated by dramatic increases in expression of cell cycle-dependent kinase inhibitors p21^{Waf1}, p27^{Kip1}, and enterocyte markers villin and Na,K-ATPase expression in HT29 and Caco-2 cells. Similar to the results noted in the crypt and villus fractions, NaBT-induced differentiation is associated with decreased SIRT1 expression and increased SIRT2 expression. In agreement with these findings, SIRT2 expression was increased, along with villin and Na,K-ATPase, during Caco-2 spontaneous differentiation (Figure 1D). These in vitro results correlate with our in vivo findings by linking increased SIRT2 expression with the most highly differentiated region of the intestinal mucosa.

Loss of SIRT2 Results in Impaired Intestinal Cell Differentiation

As further confirmation of the effect of SIRT2 on intestinal cell differentiation, we next determined whether SIRT2 deficiency impairs differentiation. We analyzed the small intestine of mice with SIRT2 knockout (SIRT2^{-/-}) at 3 months of age. Interestingly, SIRT2^{-/-} mice showed decreased intestinal expression of mucin2 (MUC2), villin, and Na,K-ATPase proteins and decreased intestinal alkaline phosphatase (IAP) activity (Figure 2A, B), suggesting that SIRT2 contributes to intestinal goblet cell and enterocyte differentiation. In contrast, knockout of SIRT2 showed increased expression of lysozyme (LYZ) (Figure 2A), suggesting an actual increase in Paneth cell differentiation. The decreased messenger RNA (mRNA) levels of IAP and MUC2 as well as increased mRNA level of LYZ in SIRT2^{-/-} mice were also demonstrated by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) (Figure 2C). In addition, Fast Red staining revealed a marked decrease in IAP activity in the small bowel of SIRT2^{-/-} mice (Figure 2D), further demonstrating decreased enterocyte differentiation. In agreement with the decreased MUC2 expression detected by Western blot and RT-PCR, a reduction in the number of goblet cells was found in the small bowel of SIRT2^{-/-} mice as noted by Alcian blue (AB) staining (Figure 2E, F). Moreover, staining the intestinal sections from SIRT2^{-/-} mice for LYZ revealed an obvious increase in Paneth cells (Figure 2G, H). Paneth cells are found at the bottom portion of small intestinal crypts of both SIRT2^{-/-} and wild-type (WT) mice, and no abnormal morphology was noted in SIRT2^{-/-} mice compared with WT mice. Therefore, consistent with the expression pattern of SIRT2, our studies indicate that SIRT2 contributes to intestinal enterocyte and goblet cell differentiation but appears to attenuate Paneth cell differentiation.

We next utilized an intestinal organoid model to examine whether knockout of SIRT2 expression reflects decreased differentiation. As shown in Figure 3A, an intestinal organoid, initiated from a single crypt, forms the organoid structure consisting of multiple cells after 5 days in culture. We tested whether knockout of SIRT2 affects enterocyte differentiation induced by incubation of organoids with NaBT. Incubation of mouse organoids with NaBT (2.5 mM) for 48 hours resulted in a more differentiated enterocyte

phenotype, as noted by increased IAP activity and IAP mRNA expression (Figure 3B). Importantly, this induction of differentiation was attenuated or blocked by SIRT2 knockout. Moreover, consistent with the observation from mouse tissue, organoids from SIRT2^{-/-} mice showed elevated LYZ mRNA (Figure 3C) and decreased MUC2 mRNA expression (Figure 3D) compared with WT mice. Treatment with NaBT decreased MUC2 expression and had only a small effect on LYZ expression. These results further demonstrate that SIRT2 knockout impairs enterocyte and goblet cell maturation but, in contrast, increases Paneth cell differentiation.

SIRT2 Deficiency Results in Increased Proliferation in Intestinal Epithelium

As SIRT2 deficiency results in impaired intestinal cell differentiation, we next determined whether SIRT2 functions in the control of intestinal epithelium renewal. We analyzed the intestine of SIRT2^{-/-} mice at 3 months of age and found that the small intestine and colon were significantly longer (Figure 4A, B) with increased villus length and crypt depth in SIRT2^{-/-} mice compared with that of WT mice (Figure 4C, D). We observed increased proliferation in the intestine of SIRT2^{-/-} mice as noted by increased expression of Ki67 (Figure 4E, F) and cyclin D1 (Figure 4G, H), indicating that SIRT2 deficiency results in an increase in crypt cell proliferation.

As SIRT2 deletion promotes crypt cell proliferation, we postulated that SIRT2 might also play a role in regulating ISC activity. To investigate this hypothesis, we next determined the effects of SIRT2 on growth of intestinal organoids. The activity of ISCs was assessed based on their ability to drive the formation of organoids.^{27,28} We assayed the organoid-forming capacity of crypts that were isolated from the small intestine of either WT or SIRT2^{-/-} mice. Notably, SIRT2 deficiency resulted in an increase in crypt organoid-forming capacity after 3 days in culture (Figure 5A, B). In addition, the number of lobes per organoid, another indicator of stem cell function,²⁹ was higher in organoids from SIRT2^{-/-} mice (Figure 5C). Consistent with these findings, the mRNA levels of ISC markers, LGR5 and OLFM4, were upregulated in SIRT2-deficient organoids as compared with WT control animals (Figure 5D). Similar increases in these markers (Figure 5E) and the numbers of OLFM4-positive cells (Figure 5F) were found in small intestine crypts of SIRT2^{-/-} mice. These results suggest that SIRT2 deficiency promotes the activity of ISCs.

SIRT2 Deficiency Results in Enhanced Wnt/ β -Catenin Signaling in IECs

Wnt/ β -catenin is critical for intestinal proliferation and differentiation.¹⁵ Therefore, we next determined whether SIRT2 alters Wnt/ β -catenin signaling in the intestine. We found that β -catenin protein and its well-established target genes EPHB2, AXIN2, and cyclin D1, were significantly upregulated in organoids (Figure 6A, B) and IECs (Figure 6D-F) from SIRT2^{-/-} mice as compared with WT control animals. Consistent with SIRT2 knockout, inhibition of SIRT2 by

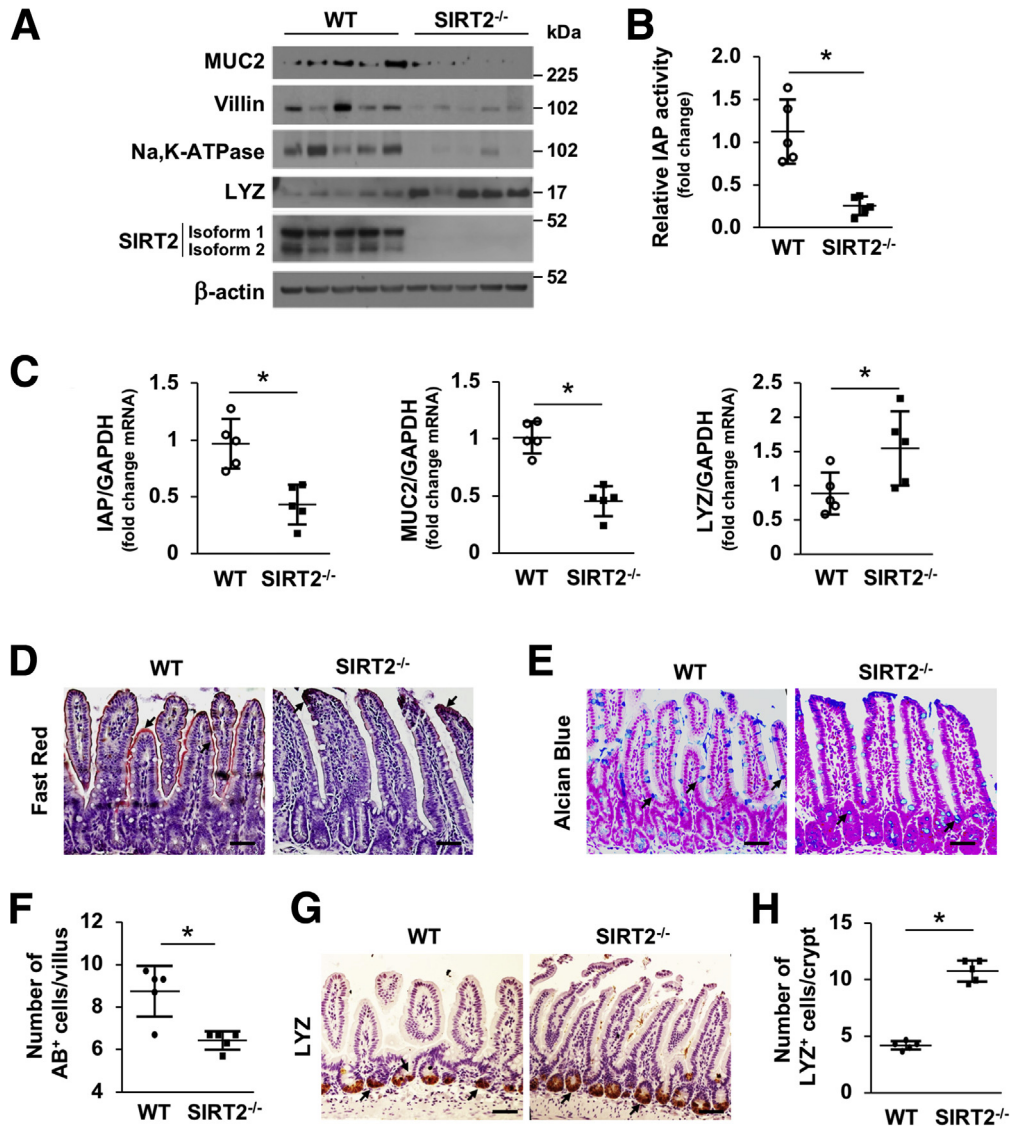


Figure 2. Knockout of SIRT2 disturbs intestinal cell differentiation in vivo. (A, B) Small intestinal mucosal protein lysates were extracted for Western blot detection of MUC2, villin, Na,K-ATPase, LYZ, SIRT2 and β -actin protein expression. (A) Each well represents a different mouse from the relevant group. (B) Small intestinal mucosal protein lysates were extracted and IAP activity determined ($n = 5$, data represent mean \pm SD; $*P < .05$ vs WT). (C) Total RNA was extracted from mucosa and IAP, MUC2, and LYZ mRNA expression was assessed by real-time RT-PCR ($n = 5$, data represent mean \pm SD; $*P < .05$ vs WT). (D) Representative Fast Red staining of the small intestine revealed a decrease in IAP expression (arrows). (E) Representative AB staining of the small intestine revealed a decrease in mucinous goblet cells in SIRT2^{-/-} mice compared with WT mice (arrows). (F) Quantification of AB-positive cells in WT and SIRT2^{-/-} mice ($n = 5$, 3 villi per mouse, data represent mean \pm SD; $*P < .05$ vs WT). (G) Representative IHC staining of the small intestine for LYZ showed the increase in Paneth cells (arrows) in SIRT2^{-/-} mice compared with WT mice. (H) Quantification of LYZ-positive cells in control and SIRT2^{-/-} mice ($n = 5$, 3 crypts per mouse, data represent mean \pm SD; $*P < .05$ vs WT). Scale bars = 50 μ m.

treatment with SIRT2 inhibitor AGK2 increased expression of EPHB2, AXIN2 and cyclin D1 in organoids (Figure 6C). Notably, knockout of SIRT2 increased β -catenin acetylation (Figure 6D, bottom panel), which stabilizes β -catenin and enhances β -catenin signaling,³⁰ suggesting that SIRT2 inhibits β -catenin through regulation of β -catenin deacetylation. To further determine the effects of SIRT2 on β -catenin signaling, the human colorectal cancer cell line, RKO, was used. Treatment of RKO cells with SIRT2 inhibitor AGK2 (Figure 6G) or transfection with small interfering RNA targeting SIRT2

(Figure 6H) increased the expression of β -catenin protein and its target proteins, c-Myc and cyclin D1. The inhibition of SIRT2 was noted by the increased acetylation of tubulin, a target of SIRT2.¹⁹ Consistent with SIRT2 knockout, inhibition or knockdown of SIRT2 increased β -catenin acetylation in RKO cells (Figure 6G, H, bottom panels). To determine if SIRT2 directly deacetylates β -catenin in vitro, acetylated Flag- β -catenin was immunoprecipitated from 293T cells over-expressing Flag- β -catenin and incubated with recombinant human SIRT2 in the presence or absence of NAD⁺, a cofactor

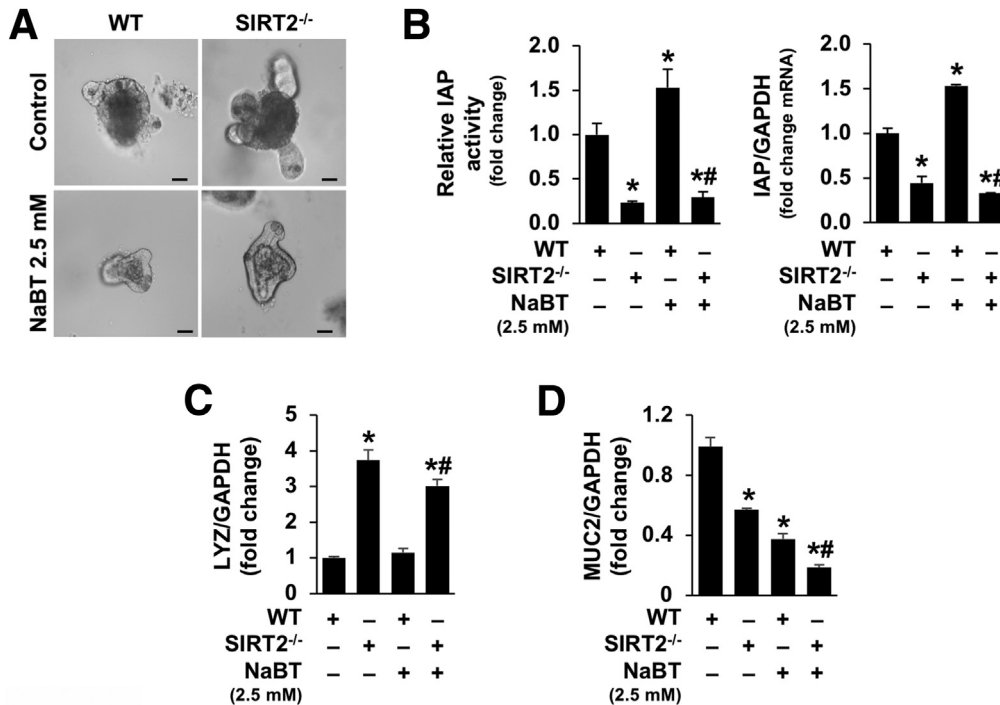


Figure 3. SIRT2 deficiency impairs intestinal enterocyte and goblet cell differentiation in an ex vivo model. Organoids were isolated from proximal small intestine and incubated for 3 days, followed by incubation with or without NaBT for 48 hours. (A) Microscopy analysis of organoids. Scale bars = 100 μ m. (B) IAP activity and mRNA levels determined by real-time RT-PCR (n = 3). Expression of (C) LYZ and (D) MUC2 determined by real time RT-PCR (n = 3). Data represent mean \pm SD; * P < .05 vs WT; ** P < .05 vs NaBT plus WT. Data are from 1 of 3 independent experiments with similar results.

required for sirtuin deacetylase activity.³¹ Active SIRT2 protein directly deacetylated β -catenin in vitro (Figure 6F). These results demonstrate that SIRT2 deacetylates β -catenin and decreases β -catenin protein levels in IECs. Together, these results strongly suggest that SIRT2 regulates intestinal cell proliferation and differentiation through negative regulation of Wnt/ β -catenin signaling (Figure 6J).

Expression of SIRT2 Is Decreased in Inflamed Colitis From Patients With IBD

The deregulation of differentiation and proliferation is associated with IBD.³² To investigate the possible impact of SIRT2 on human intestinal pathology, we examined changes in the levels of SIRT2 in human colonic mucosa from patients with IBD. Colonic mucosal tissues from patients with IBD and patients without mucosal inflammation or injury (control subjects) were collected, and colonic epithelial cells purified by cell sorting using immunomagnetic beads as described previously.^{33,34} SIRT2 mRNA (Figure 7A) and protein levels (Figure 7B) were significantly reduced in the intestinal epithelium of IBD patients as compared with control (ie, non-IBD) samples, indicating a possible role of SIRT2 in regulating human intestinal epithelial homeostasis.

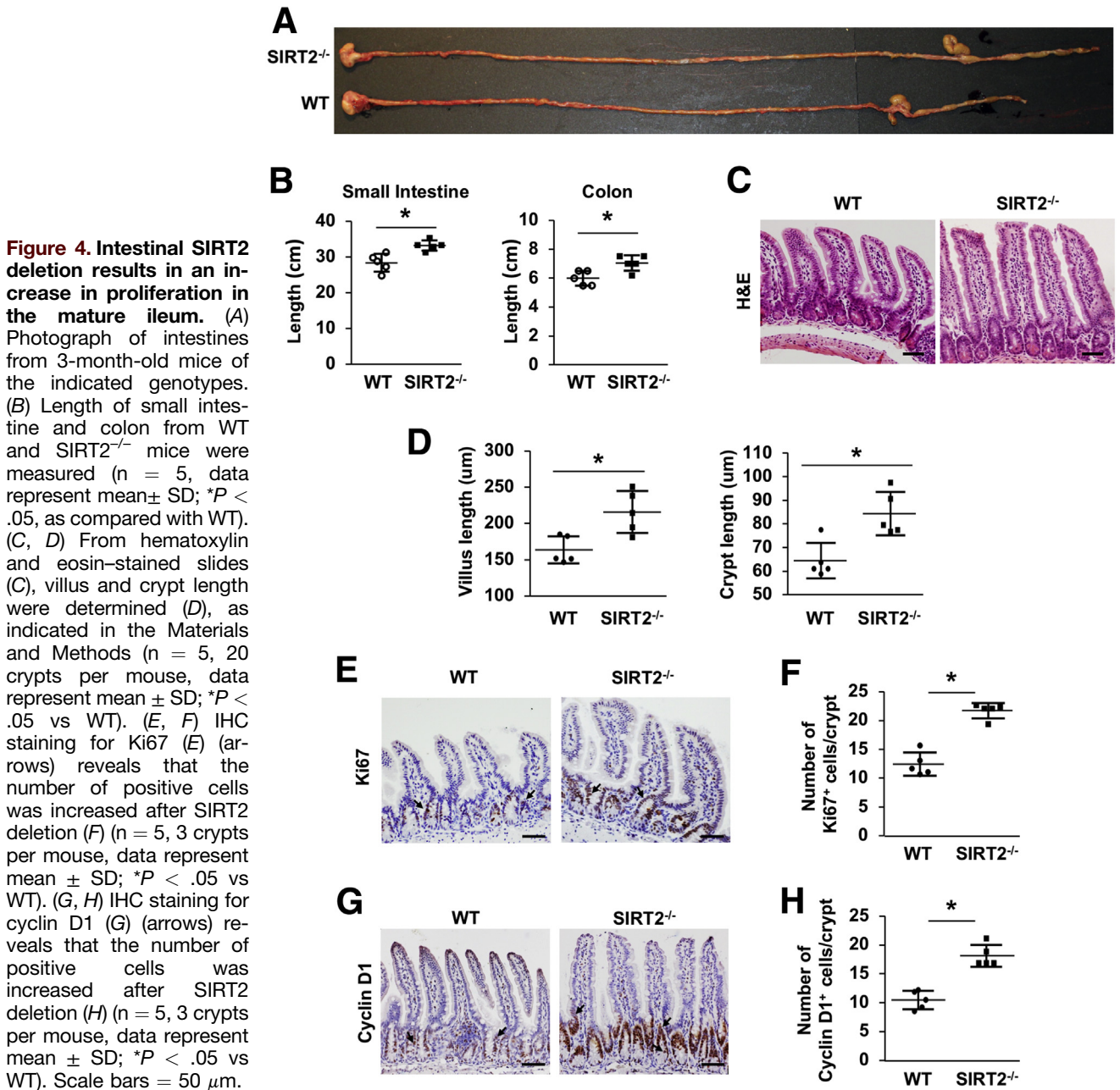
Tumor necrosis factor (TNF) plays an important role in mediating the inflammation of inflammatory bowel disease. Increased expression of TNF, a critical proinflammatory cytokine, is noted in the inflamed mucosa of patients with IBD.³⁵ Anti-TNF therapies are effective for treatment of Crohn's disease and ulcerative colitis.³⁶ To further investigate the possible effect of SIRT2 in IBD, we next determined whether TNF can regulate SIRT2 expression in IECs. To this end, HIEC6, HT29, and cultured mouse small intestinal

organoids were treated with TNF for 24 hours and SIRT2 protein levels were determined by Western blot. As shown in Figure 7C-E, TNF decreased SIRT2 protein expression in HIEC6 (Figure 7C), HT29 (Figure 7D), and organoids (Figure 7E), indicating that TNF is able to repress the expression of SIRT2 in IECs. Collectively, our findings indicate that decreased levels of SIRT2 may contribute to the pathogenesis of human IBD.

Discussion

Defining the cellular factors and signaling pathways that maintain intestinal epithelial proliferation and differentiation is crucial to our understanding of normal gut adaptation and aberrant intestinal growth; however, the precise mechanisms for these processes remain to be clearly elucidated. Here, we find SIRT2 is upregulated in the process of intestinal cell differentiation. In addition, we show that SIRT2 deletion in intestinal cells promotes proliferation, inhibits enterocyte and goblet cell differentiation, and increases Paneth cell lineage. SIRT2 deficiency leads to greater levels of stem cell activity ex vivo and in vivo. Moreover, SIRT2 regulates growth and differentiation of the intestinal mucosa by inhibiting Wnt/ β -catenin signaling (Figure 6J), which regulates intestinal cell proliferation and differentiation. These findings represent a major conceptual advance linking SIRT2 with intestinal mucosal renewal and highlights the impact of SIRT2 on normal intestinal mucosal adaptation and aberrant intestinal growth.

Our findings indicate that SIRT2 levels are dramatically increased in differentiated intestinal cells compared with undifferentiated cells. Moreover, our results, obtained from multiple complementary models (ie, cultured IECs, ex



in vivo-prepared organoids, and in vivo), strongly support the notion that SIRT2 is essential for maintenance of normal intestinal homeostasis. In agreement with our findings, upregulation of SIRT2 contributes to human pluripotent stem cell differentiation.²² Moreover, previous studies showed that SIRT2 is upregulated during in vitro differentiation of mouse embryonic stem cells; however, SIRT2 knockdown promotes mesoderm and endoderm lineages while compromising ectoderm differentiation,³⁷ suggesting a select role of SIRT2 in various cell types. We showed that deletion of SIRT2 resulted in activation of Wnt/ β -catenin signaling. Wnt activation blocks differentiation and drives hyperproliferation in the intestine.³⁸ In contrast, inhibition

of Wnt/ β -catenin signaling blocked proliferation and increased enterocyte differentiation.³⁹ Activation of Wnt signaling inhibits goblet and enteroendocrine cell differentiation¹⁴ and promotes differentiation of progenitors into Paneth cells.⁴⁰ In agreement with activation of Wnt/ β -catenin, deletion of SIRT2 results in decreased enterocyte and goblet cell differentiation, whereas SIRT2 deficiency increases the Paneth cell lineage. Moreover, SIRT2 stained prominently in small intestinal villi, the most differentiated region of the small bowel mucosa. Additionally, activation of Wnt/ β -catenin signaling is highest in the bottom of the crypts and gradually decreases along the crypt-villus axis.⁴¹ Together, these results suggest that SIRT2 regulates intestinal cell

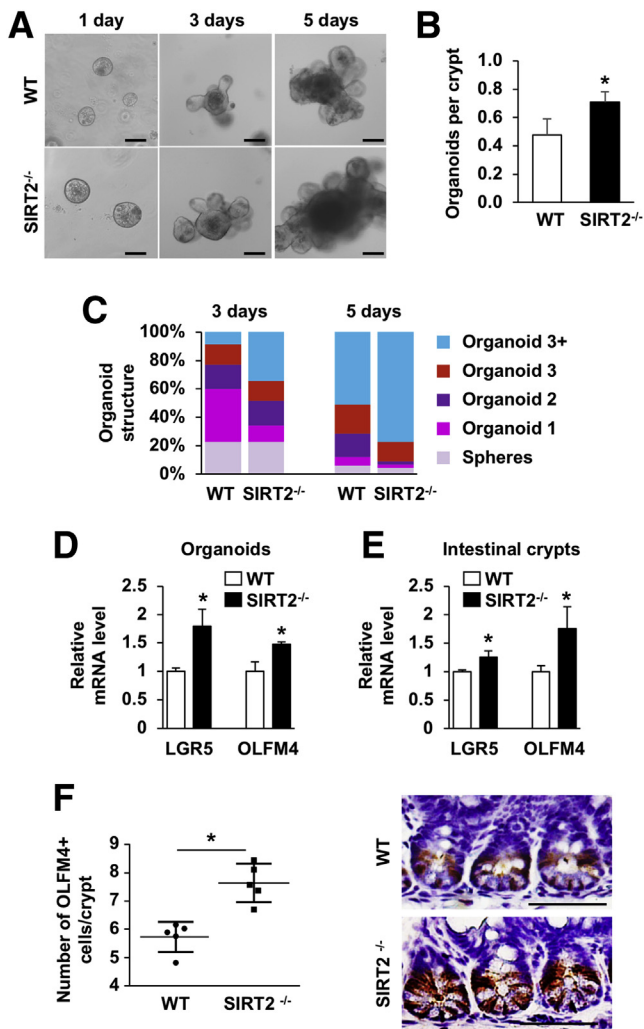


Figure 5. Intestinal epithelial SIRT2 deficiency results in the increased stemness. (A) Small intestinal organoids from WT and SIRT2^{-/-} mice during the first 5 days of culture. Representative images of crypt culture from each condition. (B) Colony-forming efficiency from WT and SIRT2^{-/-} mice after 3 days in culture; n = 3 mice per group. (C) Organoid structural complexity of experiment shown in panel A (n = 3). Expression of ISC markers in (D) organoids and (E) small intestinal crypts from WT and SIRT2^{-/-} mice detected by real-time RT-PCR; n = 3 (data represent mean ± SD; *P < .05 vs WT). (F) Quantification (left) and representative images (right) of OLFM4⁺ stem cells by IHC. (n = 5, 15 crypts per mouse, data represent mean ± SD; *P < .05 vs WT). Scale bars = 50 μm.

differentiation through regulation of Wnt/ β -catenin signaling.

We showed that SIRT2 negatively regulates Wnt/ β -catenin signaling in intestinal cells. SIRT2 can either inhibit or activate Wnt/ β -catenin signaling depending on the cell type. For example, SIRT2 activates Wnt/ β -catenin through Akt/GSK-3 in hepatocellular carcinomas.^{42,43} In contrast, SIRT2 inhibition of Wnt/ β -catenin has been shown in certain types of cells.^{44–46} SIRT2 is a deacetylase of E-cadherin, and acetylation of nuclear E-cadherin

attenuates its interaction with β -catenin, which releases β -catenin from the complex, resulting in increased expression of its downstream genes and accelerated tumor growth and migration in colorectal cancer cells.⁴⁴ SIRT2 binds to, deacetylates, and activates GSK3 β , thereby decreasing β -catenin expression in cardiomyocytes.⁴⁵ Moreover, SIRT2 knockout results in increased acetylation of β -catenin and activation of the Wnt signaling pathway in mouse embryonic fibroblasts.⁴⁶ Consistent with this finding, we showed that inhibition or knockout of SIRT2 results in increased β -catenin acetylation and expression. Although phosphorylation promotes β -catenin ubiquitination and degradation, acetylation masks the ubiquitination sites, thus preventing β -catenin ubiquitination and degradation and promoting its activity.³⁰ Stabilized β -catenin accumulates in the cytosol and then translocates to the nucleus, where it binds with transcriptional coactivators and regulates expression of the canonical Wnt target genes. Our results show that inhibition of SIRT2 activates Wnt/ β -catenin through increased β -catenin acetylation and stabilization in intestinal cells. In contrast, knockout of SIRT1, which we and others have shown is highly enriched in the small intestine crypts,^{18,26} results in the inhibition of Wnt/ β -catenin signaling in intestinal cells, suggesting a differential effect of sirtuins on the Wnt/ β -catenin pathway.

The intestinal epithelium of IBD patients undergoes repeated cycles of injury and repair in response to chronic, recurring inflammation.⁴⁷ We found decreased SIRT2 expression in the intestinal epithelium from patients with IBD. In addition, we show that SIRT2 deficiency exhibits enhanced epithelial proliferation in mice. These findings are of particular importance from a clinical perspective, since the mucosa of IBD patients exhibits enhanced epithelial proliferation, which decreases with the resolution of inflammation.⁴⁸ Moreover, we show that SIRT2 inhibition results in Wnt/ β -catenin pathway activation, which is a feature of chronic IBD in animal models.⁴⁹ These results suggest that decreased SIRT2 expression may contribute to Wnt/ β -catenin pathway activation and intestinal epithelial proliferation during IBD. TNF, a major cytokine expressed in the inflamed mucosa of IBD patients, promotes intestinal epithelial proliferation.¹⁵ TNF directly activates β -catenin-dependent transcription in the intestine and promotes epithelial regeneration during colitis.⁵⁰ We found that TNF represses SIRT2 expression, suggesting that TNF induces Wnt/ β -catenin signaling in IECs through, at least in part, repression of SIRT2 expression. Our findings provide a direct link between SIRT2 and intestinal inflammation.

Although we demonstrated impaired intestinal epithelial differentiation in the SIRT2 mice (at 3 months of age), spontaneous colitis is not noted in SIRT2 knockout mice. SIRT2 deficiency increases the inflammatory phenotype during cerulein-induced acute pancreatitis and strongly impairs the recovery from pancreatic tissue injury.⁵¹ Importantly, loss of SIRT2 shows enhanced inflammatory responses during aging, even without exposing an agent that induces an inflammation.⁵¹ Consistent with our findings, Lo

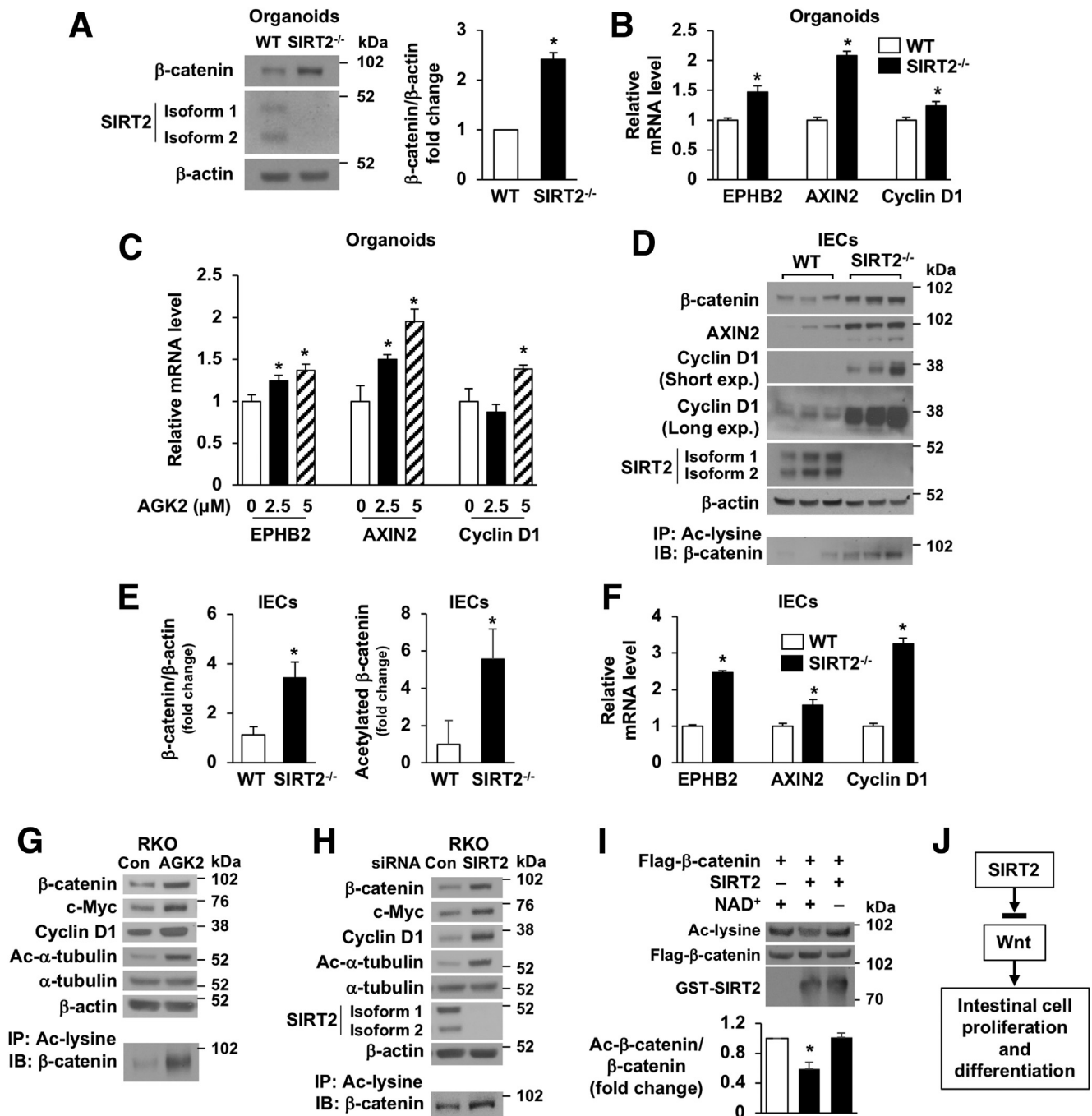


Figure 6. SIRT2 deficiency results in enhanced Wnt/ β -catenin signaling. (A) Loss of SIRT2 results in the increased β -catenin protein expression in crypt organoids (left panel). β -catenin protein signals from 3 separate experiments were quantitated densitometrically and expressed as fold change with respect to β -actin (right panel). (B) mRNA levels of Wnt/ β -catenin target genes in crypt organoids ($n = 3$) from WT and SIRT2^{-/-} mice after 5 days in culture. (C) Crypt organoids were isolated from WT mice and treated with SIRT2 inhibitor AGK2 for 48 hours. mRNA levels of Wnt/ β -catenin target genes were detected ($n = 3$). Levels of β -catenin protein, acetylated β -catenin, and its (D) targets and (E) quantification of β -catenin protein and acetylated β -catenin in IECs ($n = 3$). (F) mRNA levels of Wnt/ β -catenin target genes in IECs ($n = 3$) from WT and SIRT2^{-/-} mice. (G) RKO cells were treated with SIRT2 inhibitor AGK2 (5 μ M) for 48 hours. (H) RKO cells were transfected with nontargeting control small interfering RNA (siRNA) or SIRT2 siRNA. Inhibition or knockdown of SIRT2 increased acetylation of β -catenin and activation of β -catenin signaling in RKO cells. (I) Flag- β -catenin was isolated from 293T cells treated with trichostatin A/nicotinamide and then incubated with recombinant human GST-SIRT2 protein, followed by analysis of Flag- β -catenin (upper panel). Acetylated β -catenin signals from 3 separate experiments were quantitated densitometrically and expressed as fold change with respect to total β -catenin (lower panel). The images are representative of 3 independent experiments. Results are representative of 3 experiments ($n = 3$, data represent mean \pm SD; * $P < .05$). (J) Model proposed to explain the role of SIRT2 in intestinal cell proliferation and differentiation.

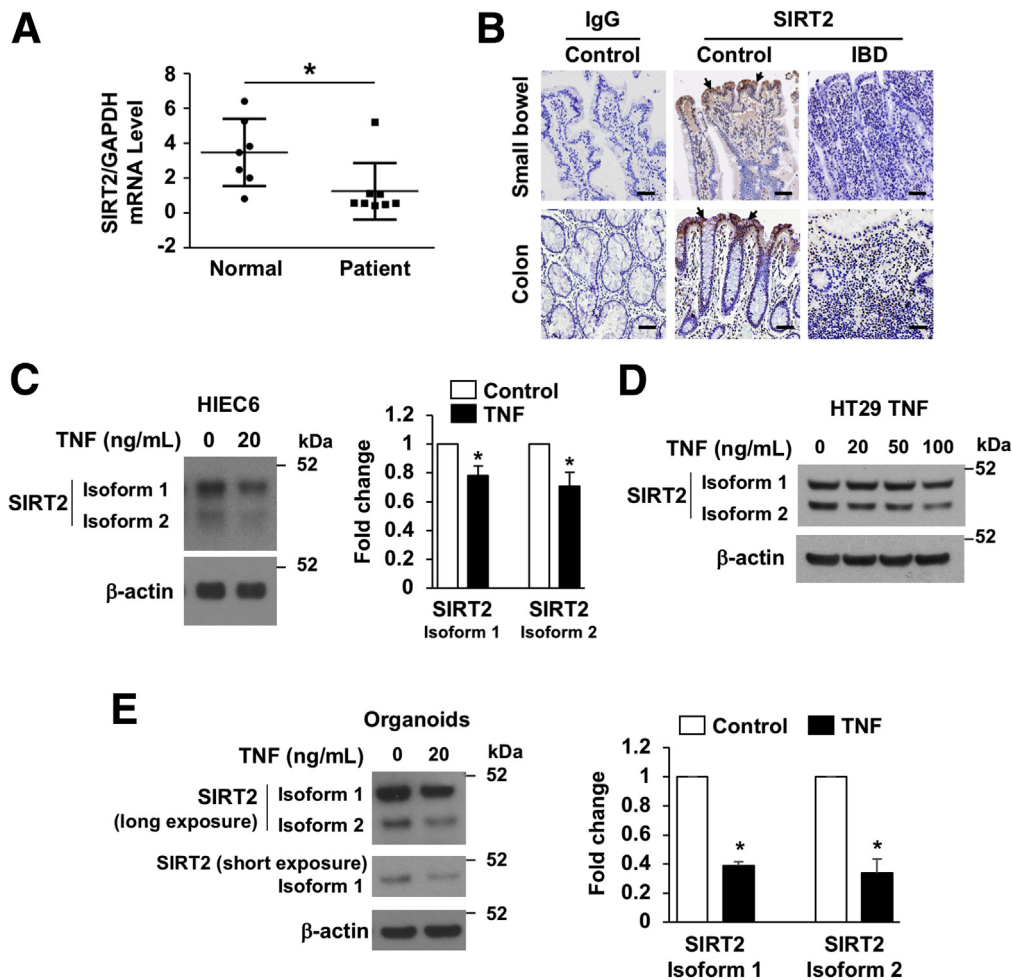


Figure 7. Intestinal epithelial SIRT2 deficiency is associated with colitis in humans. (A) The mRNA levels of *SIRT2* are reduced in human IBD patients. mRNA from purified colonic epithelium of human IBD patients and controls were analyzed by real RT-PCR ($n = 7-8$, $*P < .05$). (B) IHC staining of SIRT2 protein in the colon and small intestine from control individuals and IBD patients. All these experiments were repeated in human tissue samples obtained from 5 control individuals or patients with IBD and showed similar results. Treatment with TNF repressed SIRT2 protein expression in (C) HIEC6, (D) HT29, and (E) mouse small intestinal organoids. Cells were treated with TNF for 24 hours. SIRT2 expression was determined by Western blot. The images are representative of 3 independent experiments. SIRT2 signal from 3 separate experiments were quantitated densitometrically and expressed as fold change with respect to β -actin ($n = 3$, data represent mean \pm SD; $*P < .05$ vs control).

Sasso et al²⁴ showed that SIRT2 knockout mice do not demonstrate intestinal inflammation, whereas SIRT2 deficiency promotes dextran sodium sulfate-induced inflammatory responses. Similarly, dysregulated intestinal cell differentiation was noted in mice with intestinal deletion of SIRT1.^{18,26} However, SIRT1 deletion induced spontaneous intestinal inflammation only in aged mice (ie, 22–24 months of age).¹⁸ These results suggest that the sensitivity to an inflammatory response is age-dependent. It is likely that mice with SIRT2 deficiency may gradually develop spontaneous intestinal colitis with aging.

The key event in the onset of colitis is a breakdown of the intestinal epithelial barrier.⁵² To maintain barrier integrity, the intestinal epithelium undergoes constant renewal driven by stem cells at the base of the intestinal crypts.⁵³ Stem cells differentiate into functionally distinct epithelial cells and are ultimately shed into the intestinal

lumen. Wnt/ β -catenin signaling acts as the central organizer of epithelial stem cell identity and maintenance. Recent observations suggest that Wnt ligands may play a role in the early stages of crypt regeneration.⁴⁹ Moreover, sustained Wnt activation inhibits enterocyte and goblet differentiation and drives hyperproliferation in the intestine.^{14,38,39} Reduced SIRT2, which drives β -catenin stabilization, would be helpful for promoting crypt regeneration but possibly at the expense of barrier function due to the inhibitory effects on enterocyte and goblet cell differentiation in chronic inflammation.

The findings presented in our current study are of significant biological and clinical relevance. Disruption of intestinal epithelial homeostasis due to abnormal gene-environment interaction leads to various intestinal disorders including IBD and small intestinal or colonic cancers.^{54,55} As enhanced epithelial proliferation and

dysregulated epithelial differentiation have been characterized as important features of chronic bowel inflammation,^{48,56} it is likely that SIRT2 deficiency contributes to the enhanced intestinal inflammation due to altered IEC proliferation and differentiation. Moreover, decreased protein expression of SIRT2 was also found in colorectal cancer cells compared with the adjacent noncancerous tissues.⁵⁷ In addition, sustained activation of the Wnt pathway likely contributes to colitis-associated cancer development.¹⁵ As SIRT2 deficiency results in the activation of the Wnt pathway, aged mice with SIRT2 knockout may also have an increased risk for development of colitis-associated cancer. Our study defines a novel role for SIRT2 as a critical regulator of intestinal homeostasis, thus providing a better mechanistic understanding of SIRT2 and its potential beneficial effects in a variety of intestinal diseases.

Materials and Methods

Cell Culture, Transfection, and Treatment

Human colon cancer cell lines, HT29, Caco-2, and RKO, and the normal human IEC line, HIEC6, purchased from ATCC (Manassas, VA), were maintained in standard culture conditions.⁵⁸ Cells were treated with NaBT (Sigma-Aldrich, St. Louis, MO) or TNF at various dosages, then were harvested and extracted for RNA and protein. Differentiation markers for enterocytes (the brush-border enzyme IAP, villin, and Na,K-ATPase), goblet cells (characterized by increased MUC2 expression), and Paneth cells (induction of LYZ) were determined.

Mice

C57BL/6 and homozygous SIRT2^{-/-} mice [Strain Name B6.129-SIRT2^{tm1.1Fwa}/J, Stock Number 012772] were purchased from the Jackson Laboratory (Sacramento, CA), maintained on a 12-hour light/dark schedule in filter top isolators with autoclaved water under specific pathogen-free conditions, and fed autoclaved standard laboratory chow ad libitum. Age- and gender-matched mice were used for all experiments. The ileum from WT and SIRT2^{-/-} mice was harvested, opened, and washed with ice-cold phosphate-buffered saline (PBS). Half of the sample was used for immunohistochemistry (IHC); the mucosa from the other portion were scraped with a glass slide and scraped mucosa was immediately snap frozen in liquid N₂ for total protein and RNA extraction. All animal procedures were conducted in accordance with National Institutes of Health guidelines and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Mouse Intestinal Crypt Isolation and Organoid Culture

Intestinal crypts and villi were isolated as previously reported.^{59,60} Briefly, mouse small intestine was opened longitudinally and washed with ice-cold PBS to remove the luminal contents. Villi were removed by scraping the

luminal surface into ice-cold PBS. Villi were collected from the supernatant by centrifugation at 300 *g* for 5 minutes. Crypt fractions were prepared by rinsing the intestines with ice-cold PBS and cutting them into 2- to 4-mm pieces. The fragments were washed in 20-mL ice-cold PBS with gentle pipetting until the supernatant was almost clear (5–10 washes). Fragments were incubated in ice-cold PBS containing 10-mM EDTA for 30 minutes at 4°C. Crypts were released by pipetting with ice-cold PBS. Washing in ice-cold PBS was repeated until most of the crypts were released, as determined by microscopic analysis. Crypt suspensions were passed through a 70- μ m cell strainer and centrifuged at 300 *g* for 5 minutes. Isolated crypts were mixed with Matrigel and cultured in Advanced Dulbecco's modified Eagle medium/F12 medium as described previously.⁶⁰ Colony-forming efficiency was calculated by plating 50–300 crypts and assessing organoid formation 3 days after initiation of cultures as described.⁶¹ The levels of differentiation markers were measured by real time RT-PCR or Western blotting.

Mouse IEC Isolation

IECs were isolated as described.²⁸ Briefly, mouse small intestine was opened longitudinally and minced. Samples were washed in 150-mM NaCl containing 1-mM DTT and were then resuspended in dissociation buffer (130-mM NaCl, 10-mM EDTA, 10-mM HEPES [pH 7.4], 10% fetal calf serum, and 1-mM DTT) and incubated at 37°C for 30 minutes. The tubes were then shaken vigorously to liberate epithelial cells from the lamina propria. The epithelial cell suspension was carefully aspirated and washed in ice-cold PBS, and cell pellets were collected by centrifugation.

Human Biopsy Samples and Human Colonic Epithelial Cell Isolation

Human intestine biopsy specimens were obtained from patients at the University of Kentucky undergoing diagnostic or surveillance colonoscopy for known or suspected Crohn's disease or ulcerative colitis. For comparison, biopsy specimens were obtained from healthy patients undergoing routine colon cancer surveillance. Collection of all patient materials for this study was approved by the University of Kentucky Institutional Review Board (#48678). Human colonic epithelial cell isolation was performed as described.³³ Briefly, human colon epithelial samples were delivered from the endoscopy room in ice-cold PBS. Whole tissue biopsies, placed in collagenase IV with Dulbecco's modified Eagle medium/10% fetal bovine serum in a 1:10 dilution, chopped with scissors and agitated gently with a 1-mL pipette tip, remained in this room-temperature solution for 20–30 minutes. The tissue was then passed through a 40- μ m sieve, spun down at 300 *g* at 4°C. The resulting pellet was used for positive epithelial cell selection by magnetic separation using anti-Epcam antibody and microbeads. Cells were frozen and stored at –80°C until analysis.

Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted and treated with DNase (Promega, Madison, WI). Synthesis of complementary DNA was performed with 1 μ g of total RNA using reagents in the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA). TaqMan probe and primers were purchased from Applied Biosystems. Quantitative real-time RT-PCR analysis was performed as we have described previously.⁵⁸

Histology

Sections from WT and SIRT2^{-/-} mice were stained with hematoxylin and eosin as previously described.⁶² Morphology was assessed in high-quality sections containing nonfragmented villi. Villus height and crypt depth were measured with Aperio ImageScope - Pathology Slide Viewing Software (Leica Biosystems Imaging, Inc, Vista, CA). Crypt depth was measured as the length between the bottom of the crypts and the crypt-villus junction; villus height was determined by the length between the crypt-villus junction and the top of the villus. At least 20 well-oriented crypts and villi were counted per mouse by a single blinded observer.

Immunohistochemistry, AB Staining, and IAP Staining

IHC and AB staining were performed as we have described previously.⁶² Tissue was processed for routine IHC staining using the following antibodies: rabbit anti-LYZ (Diagnostic BioSystems, Pleasanton, CA), anti-cyclin D1 (Abcam, Cambridge, MA), anti-Olfm4 (Cell Signaling, Danvers, MA), and anti-Ki67 (Novus Biologicals, Centennial, CO). Negative controls (including no primary antibody or isotype-matched mouse immunoglobulin G) were used in each assessment. AB staining was performed according to standard protocol using AB pH 2.5 Stain Kit (Dako, Carpinteria, CA). IAP staining was performed using Vulcan Fast Red Chromogen kit (Biocare Medical, Concord, CA), following the manufacturer's recommendations. Formalin-fixed, paraffin-embedded tissue samples of normal human small bowel and colon were utilized for SIRT2 staining using anti-SIRT2 antibody (Novus Biologicals, Centennial, CO).

In Vitro Deacetylation Assay

293T cells were transiently transfected with Flag-tagged β -catenin plasmid, as we have described,⁶³ and treated with 0.5- μ M trichostatin A and 25-mM nicotinamide for 8 hours. The acetylated, Flag-tagged β -catenin protein was immunoprecipitated using agarose-conjugated anti-Flag antibody. Deacetylation assay was performed as described.⁶⁴ Briefly, the acetylated, Flag-tagged β -catenin protein on agarose beads was incubated with recombinant human glutathione S-transferase-tagged SIRT2 protein (2 μ g) (Active Motif, Carlsbad, CA) in deacetylation buffer (50-mM Tris-HCL pH 8.0, 150-mM NaCl, 1-mM MgCl₂, 5-mM NAD⁺) at 30°C for 3 hours. The reaction was stopped with sodium dodecyl sulfate (SDS) sample buffer, subjected to SDS-

polyacrylamide gel electrophoresis, and analyzed by Western blot with acetylated-lysine antibody.

Western Blot Analysis

Western blotting was performed as we have described.⁵⁸ Antibodies to SIRT1, SIRT2, p21^{Waf1}, α -tubulin, ac- α -tubulin, Na,K-ATPase, AXIN2, cyclin D1, β -catenin, c-Myc, acetylated-lysine (all from Cell Signaling), proliferating cell nuclear antigen (Santa Cruz, Dallas, TX), and villin and p27^{Kip1} (BD, San Jose, CA), and LYZ, MUC2 (Abcam), and β -actin (Sigma, St. Louis, MO) were used.

Immunoprecipitation Assay

Immunoprecipitation assay was performed as we have described.⁶⁵ For immunoprecipitation, 0.5–1 mg of protein from lysate was incubated with 2 μ g of appropriate antibody or control IgG antibody overnight. Protein G-conjugated agarose beads were used to capture the immune complexes. After a brief centrifugation, supernatant was discarded, and beads were washed thrice with ice-cold PBS (1 \times) 1000 rpm at 4°C for 1 minutes. The immunoprecipitated protein was resolved by SDS-PAGE and detected by Western blotting.

Alkaline Phosphatase Activity Assay

Protein was extracted from cells with lysis buffer and concentrations were determined. Cell lysates (20 μ L) were used to determine IAP activity by a commercially available kit (Sigma-Aldrich) as we have described previously.⁵⁸

Statistical Analysis

For in vitro experiments and in vivo studies, pairwise comparisons between 2 groups were performed using 2-sample *t* test or analysis of variance for multiple groups with contrast statements. Adjustment in *P* values due to multiple pairwise testing between groups was performed using the Holm's step-down procedure. Bar graphs represent mean \pm SD levels in each group. Comparisons of the number of LYZ⁺, AB⁺, OLFM4⁺, Ki67⁺, and Cyclin D1⁺ in the intestine were performed between WT and SIRT2^{-/-} mice using the linear mixed model to account for multiple observations from multiple crypts per mouse. For the in vivo animal studies, sufficient sample size was utilized to provide 80% power to detect a 2.0 mean difference in SD units between groups based on a 2-sided, 2-sample *t* test with 5% significance level. For in vitro studies, 3 replicates were utilized for each cell culture condition and each experiment was repeated at least 3 times. Representative data from the repeat experiments are presented. All data from animal samples with measurement of study endpoints were included in the analysis. All authors had access to the study data and have reviewed and approved the final manuscript.

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Received September 6, 2019. Accepted January 8, 2020.

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Acknowledgments

The authors thank Donna Gilbreath for manuscript preparation; Dana Napier for tissue sectioning and staining; the Biospecimen Procurement and Translational Pathology, and Biostatistics and Bioinformatics Shared Resource Facilities of the University of Kentucky Markey Cancer Center (supported by National Cancer Institute grant P30 CA177558 to BME).

Conflicts of interest

The authors disclose no conflicts.

Funding

This work was supported by National Institutes of Health grants R01 DK48498 (to B. Mark Evers), R01 DK095662 (to Terrence A. Barrett), P30 CA177558 (to B. Mark Evers), and a VA Merit grant I01CX001353 (to Terrence A. Barrett).