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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Exposure to p40 in Early Life Prevents Intestinal Inflammation in Adulthood Through Inducing a Long-Lasting Epigenetic Imprint on TGFβ

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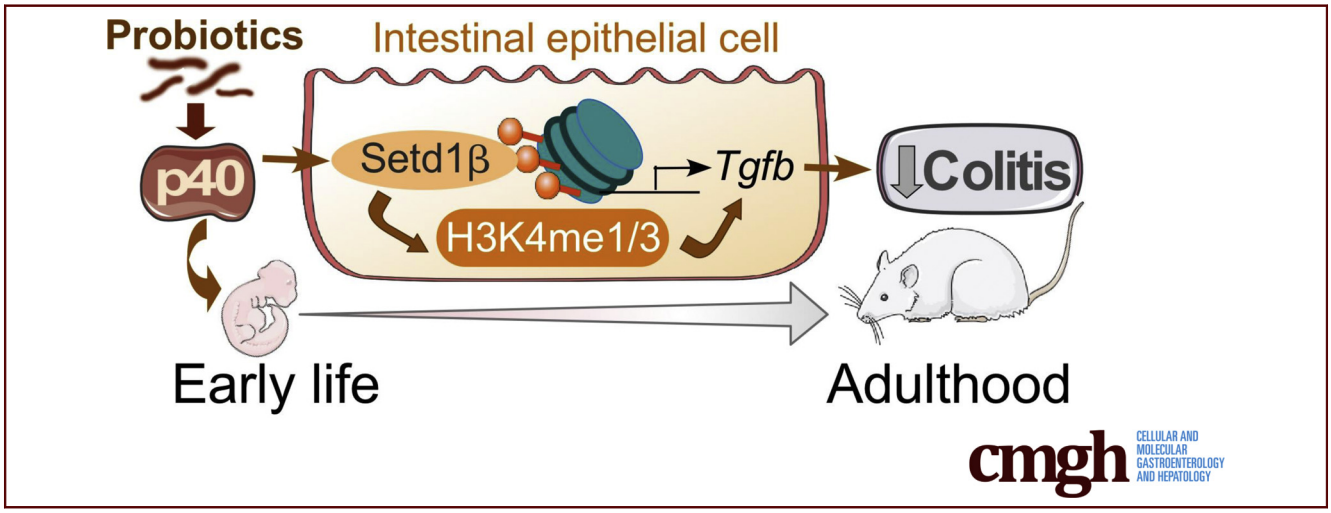


SUMMARY

A functional factor of probiotics, p40, stimulates long-lasting epigenetic imprint on transforming growth factor β through up-regulating a methyltransferase, su(var)3-9, enhancer-of-zeste and trithorax domain–containing 1β, in intestinal epithelial cells. This novel mechanism is involved in colitis prevention in adulthood by p40 supplementation in early life–induced sustained transforming growth factor β production in mice.

BACKGROUND & AIMS: Colonization by gut microbiota in early life confers beneficial effects on immunity throughout the host's lifespan. We sought to elucidate the mechanisms whereby neonatal supplementation with p40, a probiotic functional factor, reprograms intestinal epithelial cells for protection against adult-onset intestinal inflammation.

METHODS: p40 was used to treat young adult mouse colonic (YAMC) epithelial cells with and without deletion of a methyltransferase, su(var)3-9, enhancer-of-zeste and trithorax domain–containing 1β (Setd1β), and mice in early life or in adulthood. Anti–transforming growth factor β (TGFβ)-neutralizing antibodies were administered to adult mice with and without colitis induced by 2,4,6-trinitrobenzenesulfonic acid or dextran sulfate sodium. We examined *Setd1b* and *Tgfb* gene expression, TGFβ production, monomethylation and trimethylation of histone H3 on the lysine 4 residue (H3K4me1/3), H3K4me3 enrichment in *Tgfb* promoter, differentiation of regulatory T cells (Tregs), and the inflammatory status.

RESULTS: p40 up-regulated expression of *Setd1b* in YAMC cells. Accordingly, p40 enhanced H3K4me1/3 in YAMC cells in a Setd1β-dependent manner. p40-regulated Setd1β mediated programming the TGFβ locus into a transcriptionally permissive chromatin state and promoting TGFβ production in YAMC. Furthermore, transient exposure to p40 during the neonatal period and in adulthood resulted in the immediate increase in *Tgfb* gene expression. However, only neonatal p40 supplementation induced the sustained H3K4me1/3 and *Tgfb* gene expression that persisted into adulthood. Interfering with TGFβ function by neutralizing antibodies diminished the long-lasting effects of neonatal p40 supplementation on differentiation of Tregs and protection against colitis in adult mice.

CONCLUSIONS: Exposure to p40 in early life enables an epigenetic imprint on TGFβ, leading to long-lasting production of TGFβ by intestinal epithelial cells to expand Tregs and protect the gut against inflammation. *(Cell Mol Gastroenterol Hepatol 2021;11:1327–1345; https://doi.org/10.1016/j.jcmgh.2021.01.004)*

Keywords:

Histone Methyltransferase; Intestinal Epithelial Cell; Probiotic Function Factor; Regulatory T Cell.

Probiotics, which originally were defined as "living microorganisms with low or no pathogenicity that exert beneficial effects on the health of the host,"[1] are among a broad range of beneficial microbes naturally living in the human body. Evidence from current research using animal models and in vitro approaches has identified distinct cellular and molecular mechanisms through which probiotics exert health-promoting effects on the host. The beneficial effects of probiotics include regulating immunity, in terms of inhibiting proinflammatory and enhancing anti-inflammatory responses, maintaining intestinal epithelial integrity, such as preservation of barrier function and blockade of apoptosis in intestinal epithelial cells (IECs), balancing the gut microbiota profile, and blocking pathogenic bacteria.[2–4] However, probiotics in clinical trials yielded inconclusive results, thus, there is no strong evidence to support the clinical efficacy of probiotics.[2,5] The uncertain bioavailability and biopharmacology of probiotics in the gastrointestinal tract has posed challenges in assessing the beneficial effects of probiotics in prior clinical trials.

Increasing evidence suggests that components of probiotics, including probiotic-derived products and metabolites by probiotics, serve as functional factors for probiotic action. Application of these previously unrecognized functional factors could serve as therapeutic targets, bypassing clinical limitations of direct probiotic use. p40, which is a secretory protein originally isolated and cloned from culture supernatant of a probiotic bacterium, *Lactobacillus rhamnosus* GG (LGG),[6] represents one such probiotic-derived functional product. Phylogenic analysis showed that p40 is present mainly in *Lactobacillus casei*, *Lactobacillus paracasei*, and *L rhamnosus* phylogenomic groups.[7] Studies to functionally assess p40 showed that this protein transactivates epidermal growth factor receptor (EGFR) in IECs, which is required for inhibiting cytokine-induced apoptosis in IECs, preserving barrier function, up-regulating mucin production, and stimulating IgA production in the prevention and treatment of colitis in mice.[8–11] Because IECs provide the frontline response to the gut microbiota in maintaining intestinal homeostasis,[12] p40 has the potential to promote intestinal health.

Colonization of the gut microbiota during a critical window of early life confers life-long health outcomes in human beings and animals.[13] Dysbiosis in infants and children is associated with increased susceptibility to inflammatory bowel disease (IBD) in adults.[14] Therefore, elucidating the mechanisms underlying gut microbiota-regulated long-term health outcomes could provide opportunities to develop early life interventions to prevent IBD throughout a patient's lifespan. Both neonatal LGG colonization[15] and transient exposure to p40 in the neonatal period[16] in mice promoted intestinal functional maturation. Furthermore, p40 supplementation in early life was sufficient to induce sustained expansion of regulatory T cells (Tregs) in the intestinal lamina propria and durable protection against colitis that extends into adulthood.[16] Therefore, this work was focused on elucidating the mechanisms whereby exposure to p40 in early life reprograms IECs for protection against adult-onset intestinal inflammation.

Epigenetic programming serves as one of the mechanisms though which host cells recognize and translate microbial signals into long-term specific cellular responses. Epigenetic modifications have been shown to allow the gut microbiota to regulate gene expression and control cellular responses in IECs[17] and immune cells.[18] Su(var)3-9, enhancer-of-zeste and trithorax domain containing 1 (Setd1)β is a class 2 lysine methyltransferase that operates alongside other adaptor subunits within the complex proteins associated with Set1 (COMPASS) to catalyze monomethylation and trimethylation of histone H3 lysine 4 residue (H3K4me1/3) at enhancer and promoter sites to activate target gene expression.[19] Here, we show that p40 stimulates Setd1β expression, leading to the increase in H3K4me3 within the *Tgfb1* locus for up-regulating expression of transforming growth factor β (TGFβ) in IECs. Notably, p40 supplementation in early life induces sustained increase in TGFβ production in adult mice, which contributes to promoting differentiation of Tregs and protecting against colitis in adult mice. These findings support early life administration of oral p40 as a novel strategy to prevent intestinal inflammation in adulthood.

Results

p40 Up-regulates TGFβ Production in IECs, Leading to Protective Cellular Responses

The up-regulation of TGFβ by IECs for the induction of intestinal Tregs represents one of the mechanisms through which the gut microbiota contributes to maintaining intestinal homeostasis.[20] Our previous studies implied that p40 potentially may stimulate TGFβ production, leading to induction of Tregs, based on the evidence that conditioned medium from p40-treated IECs stimulated differentiation of Tregs, which was blocked by TGFβ-neutralizing antibodies.[16] We therefore tested whether and how p40 stimulates TGFβ production in IECs.

We have reported that p40 promotes growth of enteroids.[9] The effects of p40 on stimulating *Tgfb1* gene expression in IECs was first examined in the colonoid (Figure 1*A*) and enteroid models from wild-type (WT) mice in this study. Compared with untreated controls, p40 treatments significantly up-regulated *Tgfb1* gene expression in both models (Figure 1*B*). The effects of p40 on *Tgfb1* gene expression and protein production were examined further in the young adult mouse colonic (YAMC) epithelial cells.[21] p40 up-regulated *Tgfb1* gene expression in YAMC cells in a time-dependent manner (Figure 1*C*). The TGFβ protein levels in cell culture supernatants were examined by enzyme-linked immunosorbent assay (ELISA) analysis. Cell culture supernatants were collected from cells treated with p40 for different times

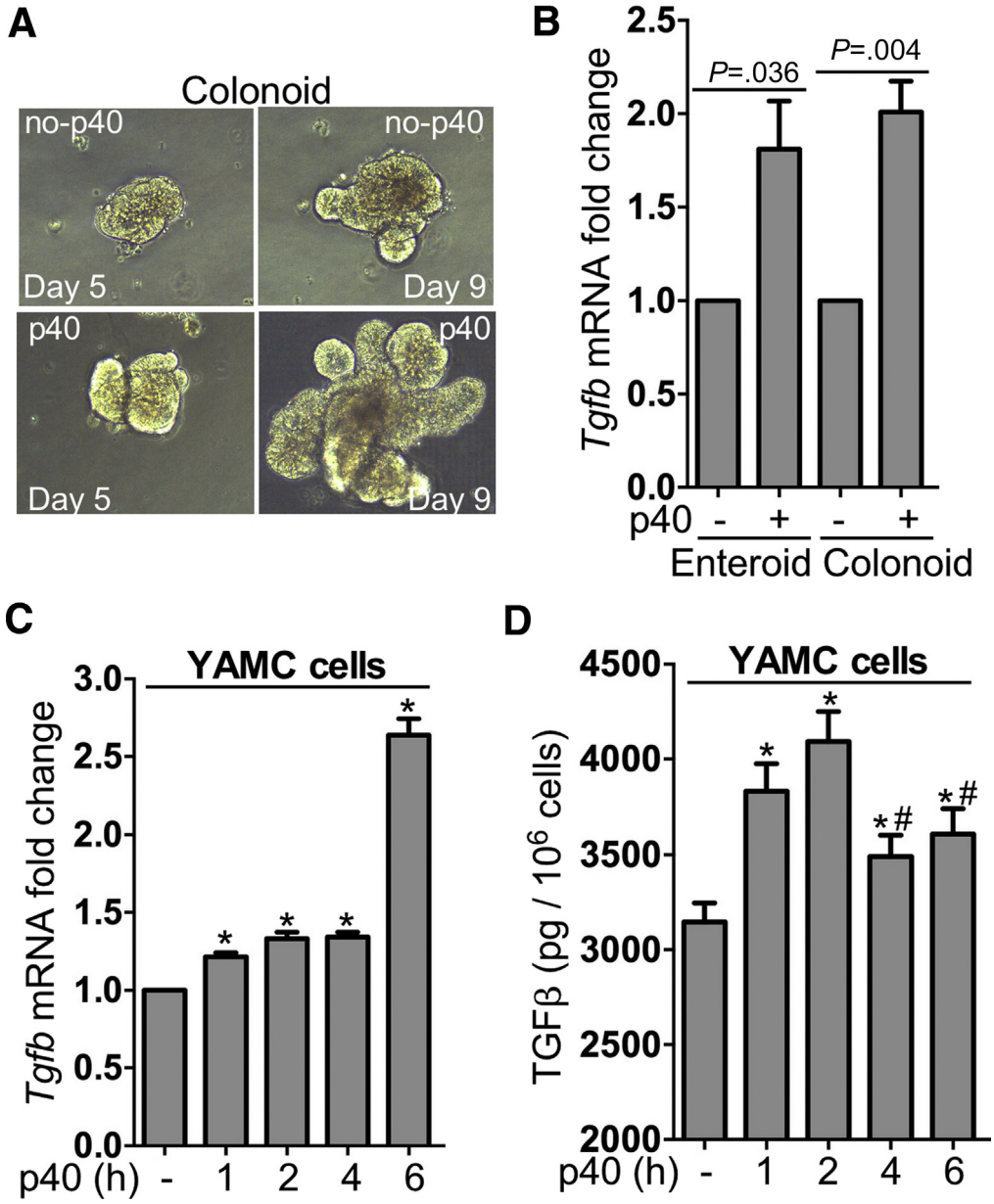


Figure 1. p40 stimulates TGFβ production in IECs. (*A* and *B*) Enteroids and colonoids from WT mice were cultured in IntestiCult organoid growth medium in the presence and absence of p40 (100 ng/mL). (*A*) Representative images of colonoids at the indicated days after culture are shown. Images were taken using a light microscope at a magnification of 10×. (*C* and *D*) YAMC cells were treated with p40 at 10 ng/mL for the indicated times. RNA was isolated from (*B*) enteroids and colonoids cultured for 9 days, and (*C*) YAMC cells for RT-PCR analysis of the expression levels of *Tgfb* mRNA. The *Tgfb* mRNA expression levels in the control groups were set as 1. The mRNA expression levels in treated groups were compared with the control group. (*D*) Supernatants from YAMC cell culture with and without p40 treatment were collected for assessing the amount of TGFβ release using enzyme-linked immunosorbent assay. The TGFβ concentration is presented as picograms per 10^6 cells. (*B–D*) Data are quantified from 3 independent experiments. (*B*) For each experiment, data represent the average of at least 5 enteroids or colonoids in each group. $^{*}P < .05$ compared with the control group. $^{\#}P < .05$ compared with the 2-hour p40 treatment group.

with treatment ending at the same time. Compared with the TGFβ levels in control YAMC cell culture supernatants, p40 treatment stimulated secretion of TGFβ by YAMC cells (Figure 1*D*). Interestingly, the levels of TGFβ in the culture supernatants did not persist over time with p40 treatment. The TGFβ levels in cell culture supernatants from cells with 2-hour p40 treatment were significantly higher than those from cells with 4- or 6-hour p40 treatment (Figure 1*D*). This evidence presumably is owing to the ligand consumption induced by TGFβ binding TGFβ-receptor I and II on the cell surface. Therefore, accumulated levels of TGFβ in culture

supernatants from cells with longer p40 treatment are lower than those from cells with shorter p40 treatment.

TGFβ binding to the TGFβ-receptor complex activates intrinsic kinase activity leading to phosphorylation of downstream targets, including SMAD2/3, which interact with SMAD4 for the regulatory effects.[22,23] We next examined if p40 might activate downstream TGFβ signaling in IECs. YAMC cells were treated with p40 for various times and the SMAD2 and 3 phosphorylation states were examined using Western blot analysis. SMAD2 and 3 phosphorylation was increased by p40 treatment for 1–4 hours (Figure 2*A*).

Activation of TGFβ signaling plays roles in preserving the intestinal barrier function[24–26] and inhibiting proinflammatory cytokines and chemokine production in IECs.[27] Therefore, the roles of p40-stimulated increase in TGFβ production in promoting protective cellular responses in IECs was tested. To this end, 2 complementary TGFβ loss-of-function approaches were used: blocking the activity of secreted TGFβ with neutralizing antibodies and interference of downstream TGFβ signaling through deletion of floxed *Smad4* alleles in IECs. H_2O_2 induced disruption of the barrier function in Caco2 cells by redistribution of a tight junctional protein, zonula occludens-1 (ZO-1), from the apical tight junctional complex to the cytoplasmic compartment observed by immunostaining, which was inhibited by p40 treatment (Figure 2*B*), an effect that was abolished by co-treatment with TGFβ-neutralizing antibodies (Figure 2*B*). Immortalized mouse colonocytes with floxed *Smad4* alleles ($IMC^{S4fl/fl}$) were generated from mice on the Immortomouse background.[21] $IMC^{S4fl/fl}$ cells were transduced with adenoviral-Cre to delete *Smad4* (IMC^{S4null})[27,28] (Figure 2*C*). p40 inhibited the H_2O_2-induced disruption of tight junctions in $IMC^{S4fl/fl}$ cells, but not in IMC^{S4null} cells (Figure 2*D*). In addition, p40 was able to block lipopolysaccharide-induced expression of proinflammatory chemokines, C-C motif chemokine ligand 20, and keratinocyte chemoattractant in $IMC^{S4fl/fl}$ cells. p40 failed to exert these effects in IMC^{S4null} cells (Figure 2*E*). Collectively, these data suggest that a p40-stimulated increase in TGFβ production supports intestinal barrier function and attenuates inflammatory cytokine release from IECs.

Sustained Increase in Tgfb Gene Expression Is Induced by p40 Supplementation in Early Life in Mice

To investigate the in vivo effects of p40 on *Tgfb1* gene expression, we used an oral encapsulation and microbial enzyme-driven release system using a food-grade pectin and zein mixture to specifically deliver p40 to the small intestine and the colon.[10] These hydrogels can protect proteins, such as p40, from damage by proteases, acid, and other intestinal contents in the intestinal tract.[10,16] The short-term effects of p40 supplementation on stimulating *Tgfb1* gene expression in mice were tested in the early stage of life and in adulthood. Neonatal p40 supplementation was performed from postnatal day 2 to day 13 (Figure 3*A*). For adult p40 supplementation, 6-week-old mice received p40 hydrogels at 20 μg/d for 2 weeks (Figure 3A). Mice were killed at the end of the p40 supplementation for testing *Tgfb1* gene expression in the colonic mucosa. Hydrogels without p40 were used as a control. *Tgfb1* messenger RNA (mRNA) levels were up-regulated significantly in colonic tissues of both neonatal and adult mice at the point immediately after p40 treatment, compared with levels in mice receiving control hydrogel treatment alone (Figure 3*A*).

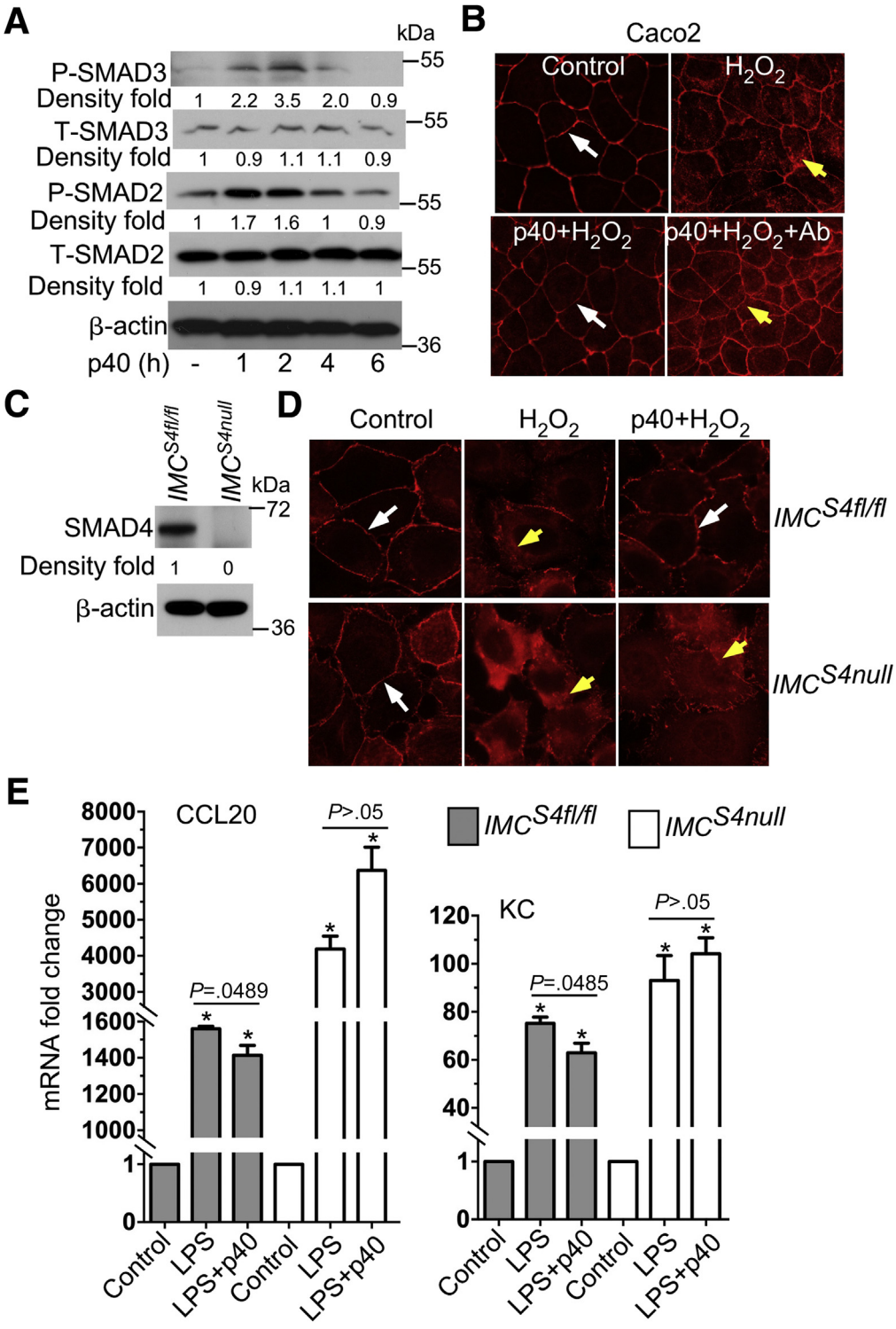
The long-term effects of p40 supplementation on stimulating *Tgfb1* gene expression were examined in mice receiving p40 treatment in either the neonatal period (days 1–21) or in adulthood (weeks 6–8). For these experiments, *Tgfb1* expression was measured 4 weeks after the final treatment (Figure 3*B*). Remarkably, neonatal p40 supplementation induced long-lasting effects on increased TGFβ gene expression in the colonic tissues of mice at least 4 weeks after p40 exposure ended, an effect not seen in adult mice treated with p40 (Figure 3*B*). We further found that *Tgfb* gene expression was up-regulated in the first and second passages of enteroids without p40 treatment from the original culture treated with p40 (Figure 3*C*), which indicates the direct effects of p40 on the intestinal epithelium. These results not only provide evidence that p40 stimulates TGFβ production in IECs, but show an important novel finding that supplementation with p40 in neonates, but not in adult mice, induces a sustained increase in mucosal *Tgfb1* mRNA expression.

Epigenetic Regulation by p40 Promotes TGFβ Production in IECs

The long-lasting effects by neonatal p40 supplementation raise the possibility that p40 exposure imprints the neonatal intestine with a stable epigenetic program that protects against inflammatory conditions. Increasing evidence indicates a significant relationship between the epigenetic modification induced by maternal and neonatal factors, including microbiota composition in early life, and health and diseases in adulthood.[29] In particular, the gut microbiota can regulate epigenetic modifications in the intestine, including H3K4me3, which is permissive for gene activation.[18] We therefore asked if p40 might regulate TGFβ production through H3K4 methyltransferase, *Setd1b*. We found that p40 significantly up-regulated *Setd1b* gene expression (Figure 4*A*) and protein production (Figure 4*B*) in YAMC cells. The increase in *Setd1b* gene expression was observed in enteroids and colonoids with p40 treatment (Figure 4*C*). Furthermore, up-regulation of Setd1β protein also was coupled to a global increase in H3K4me1 and H3K4me3 in YAMC cells (Figure 4*D*). We did not find significant effects of p40 on the regulation of other methyltransferases, such as Setd1α, mixed lineage leukemia (MLL)1, or MLL2 in YAMC (Figure 4*E*). It should be noted that p40 was shown to up-regulate the level of WD repeat-containing protein (Wdr)82 in YAMC cells, an adaptor subunit within the COMPASS complex (Figure 4*E*). Our future study will investigate whether p40–up-regulated Wdr82 mediates recruitment of methyltransferases to the TGFβ locus.

We next examined whether p40 might regulate *Setd1b* gene expression and global H3K4 methylation levels in vivo. p40 supplementation to neonatal and adult mice showed

short-term effects on stimulating *Setd1b* gene expression in colonic tissues (Figure 5*A*). However, neither neonatal nor adult p40 supplementation induced long-term consequences on *Setd1b* gene expression (Figure 5*B*). Furthermore, H3K4me1 levels in colonic epithelial cells in mice were examined by double immunostaining of H3K4me1 and E-cadherin, a marker of epithelial cells. A sustained increase in H3k4me1 in colonic epithelia cells was observed in adult mice receiving p40 supplementation in the early stage, but not in adulthood (Figure 5*C* and *D*). Thus, p40 exposure during the neonatal period transiently up-regulates *Setd1b* expression yet stably imprints H3K4 methylation in IECs that persists into adulthood.



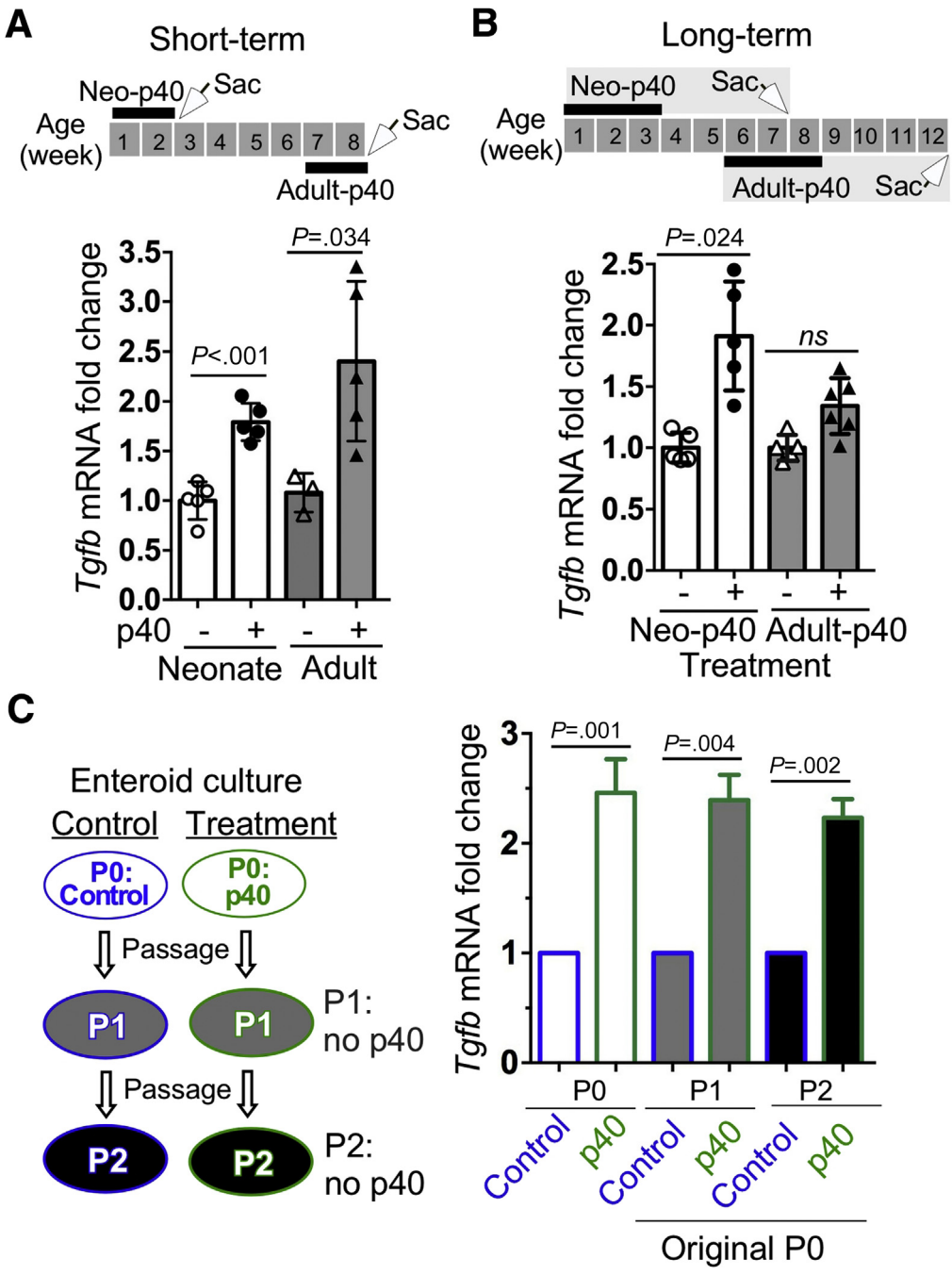


Figure 3. p40 supplementation in early stage has a long-term effect on the increase in Tgfb gene expression in mice. (*A*) For testing the short-term effects of p40 in neonate and adult mice, WT mice were supplemented with p40 from postnatal day 2 to day 14 or from week 6 to week 8. Mice were killed at the end of treatment. (*B*) For testing the long-term effects of p40 supplementation in neonatal stage and in adulthood, WT mice were supplemented with p40 from postnatal day 2 to day 21 (neo-p40) and from week 6 to week 8 (adult-p40). Mice supplemented with hydrogels without p40 were used as controls. Mice were killed 4 weeks after the end of p40 supplementation. Each symbol represents one mouse in *A* and *B*. (*C*) For testing the sustained effects of p40 on *Tgfb* gene expression in mouse enteroids, enteroids from WT mice were treated with p40 (100 ng/mL) and passaged as indicated. RNA was isolated from colonic tissues and enteroids for RT-PCR analysis of *Tgfb1* gene expression. The *Tgfb1* mRNA expression levels in the control groups were set as 1. The mRNA expression levels in treated groups were compared with the control group. (*C*) Data are quantified from 3 independent cultures.

Figure 2. (*See previous page*). p40-stimulated TGFβ production in IECs contributes to protective epithelial cellular responses. (*A*) YAMC cells were treated with p40 (10 ng/mL) for the indicated times. Total cellular proteins were prepared for Western blot analysis. β-actin blot was used as the protein loading control. (*B*) $Caco_2$ cells were treated with H_2O_2 (20 μmol/L) for 4 hours with or without 1-hour pretreatment of p40 (10 ng/mL) and TGFβ-neutralizing antibody (1 μg/mL). (*C*) Total cellular proteins from $IMC^{S4fl/fl}$ and IMC^{S4null} cells were prepared for Western blot analysis. (*D*) $IMC^{S4fl/fl}$ and IMC^{S4null} cells were treated with H_2O_2 (20 μmol/L) for 4 hours with or without 1-hour pretreatment of p40 (10 ng/mL). The cells were fixed and immunostained to localize ZO-1 using an anti–ZO-1 antibody and a Cy3-conjugated secondary antibody (red). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Membrane (*white arrows*) and intracellular (*yellow arrows*) ZO-1 distribution are shown. Images were taken using a fluorescent microscope at a magnification of 40×. (*E*) Cells were treated with lipopolysaccharide (LPS) (1 μg/mL) with and without p40 (10 ng/mL) for 24 hours. RNA was isolated for RT-PCR analysis of the expression levels of indicated genes. The mRNA expression levels in the control groups were set as 1. The mRNA expression levels in treated groups were compared with the control group of the same cell line. (*A* and *C*) The fold changes of band density are shown under the blot. (*E*) $^*P < .05$ compared with the control group of the same cell line. Data in this figure are representative of at least 3 independent experiments. Ab, antibody; CCL, CC chemokine ligands; KC, keratinocytes-derived chemokine; P-SMAD3, phosphorylated-SMAD3; T-SMAD3, total-SMAD3.

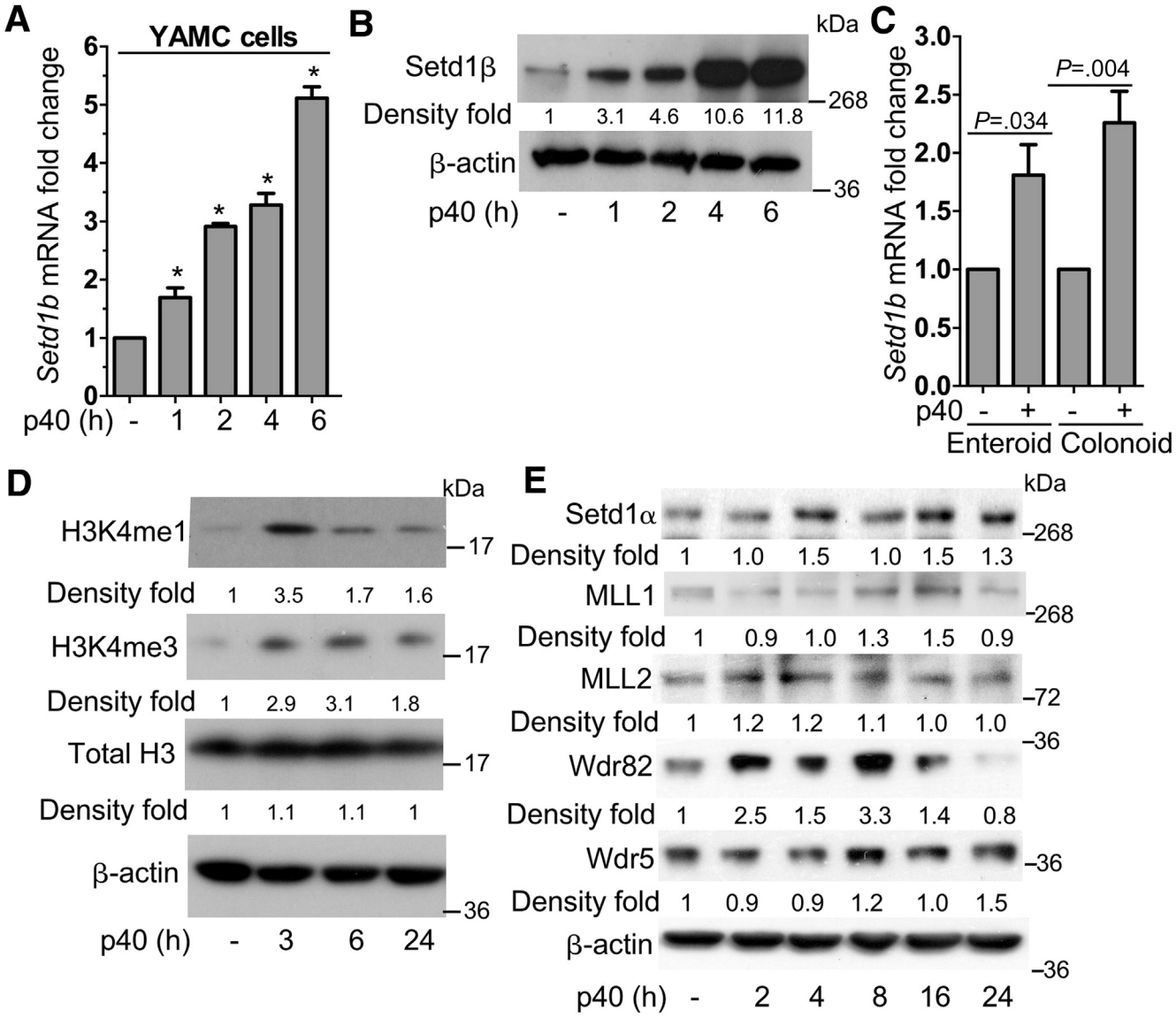


Figure 4. p40 up-regulates *Setd1β* expression in IECs. (*A*, *B*, *D*, and *E*) YAMC cells were treated with p40 at 10 ng/mL for the indicated times. (*C*) Enteroids and colonoids were generated and cultured as described in Figure 1, in the presence or absence of p40 (100 ng/mL). (*A* and *C*) RNA was isolated from (*A*) YAMC cells and (*C*) enteroids and colonoids for RT-PCR analysis of the levels of *Setd1b* mRNA. *Setd1b* mRNA expression levels in the control groups were set as 1. The mRNA expression levels in treated groups were compared with the control group. Data are quantified from 3 independent experiments. (*C*) For each experiment, data represent the average of at least 5 enteroids or colonoids in each group. (*B*, *D*, and *E*) Total cellular proteins were prepared from YAMC cells for Western blot analysis. β-actin blot was used as the protein loading control. The fold changes of band density are shown under the blot. Data are representative of at least 3 independent experiments. *$P < .05$ compared with the control group.

We further investigated more directly whether Setd1β was responsible for p40-driven production of TGFβ. A stable cell line with *Setd1b* knocked down by transducing lentiviral *Setd1b* short hairpin RNA (shRNA) into YAMC cells was generated. *Setd1b* gene expression and protein production, which were detected by reverse-transcription polymerase chain reaction (RT-PCR) analysis and Western blot analysis, respectively, were suppressed in cells transduced with a set of 3 lentiviral *Setd1b* shRNAs, but not in cells transduced with nontargeting shRNA (Figure 6*A* and *B*). As expected, p40 treatment increased H3K4me3 levels in YAMC cells transduced with nontarget shRNA, but not in Setd1β-deficient cells (Figure 6*C*). Importantly, chromatin immunoprecipitation (ChIP)–quantitative PCR experiments showed that p40 stimulated H3K4me3 enrichments at the TGFβ promoter, and this effect was partially reversed by knockdown of *Setd1b* (Figure 6*D* and *E*). p40 failed to stimulate TGFβ gene expression (Figure 6*F*) and protein production

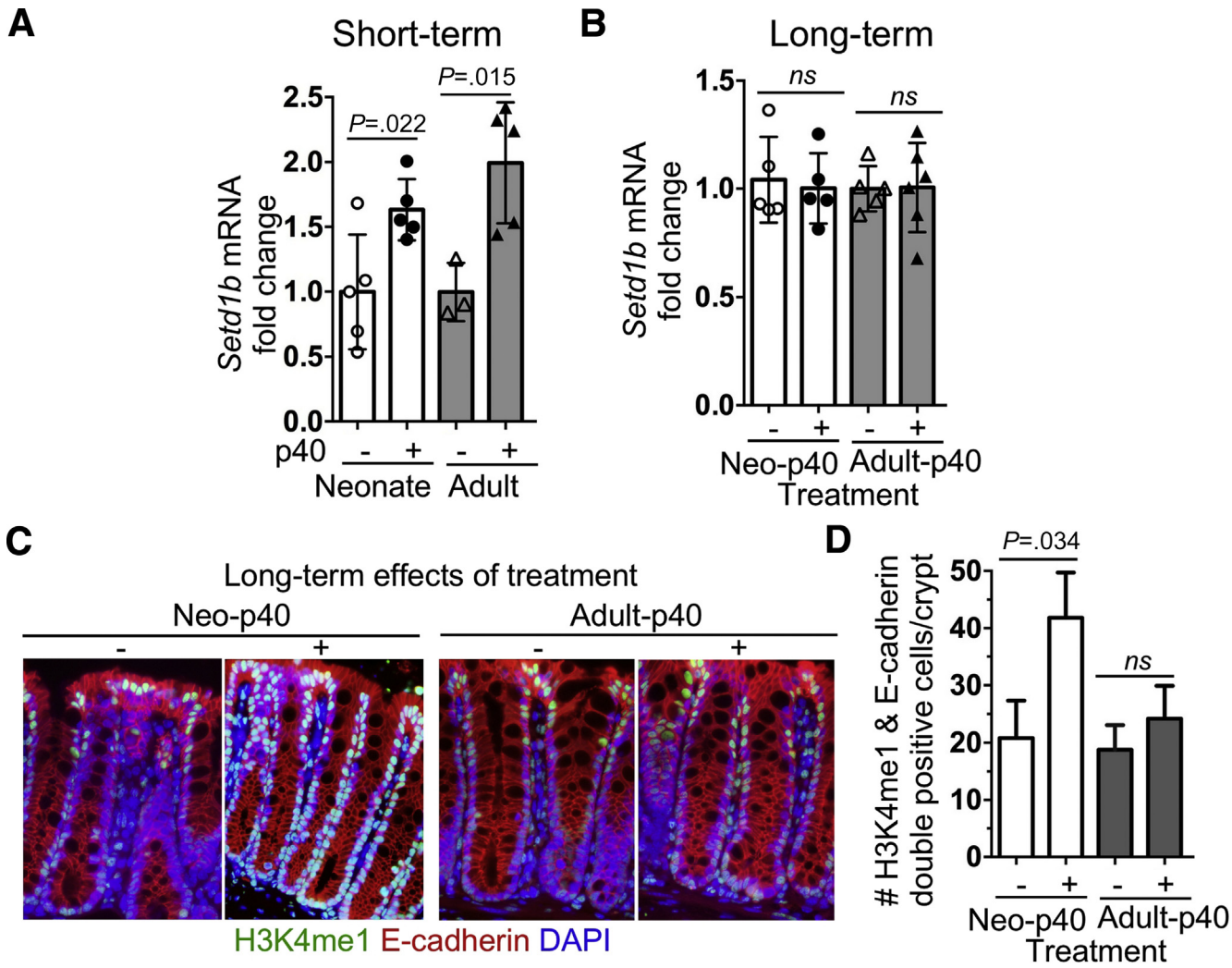


Figure 5. p40 supplementation in early life stimulates short-term up-regulation of *Setd1b* gene expression, but sustained increase in epigenetic marks in mice. The treatment plans for detecting the short-term (Figure 3*A*) and long-term effects (Figure 3*B*) are described. (*A* and *B*) RNA was isolated from colonic tissues for RT-PCR analysis of *Setd1b* gene expression. Each symbol represents one mouse in *A* and *B*. (*C*) Paraffin-embedded tissue sections were prepared for immunohistochemistry using anti-H3K4me1 antibody and fluorescein isothiocyanate–conjugated secondary antibody (green), an epithelial cell marker using anti–E-cadherin antibody and Cy3-conjugated secondary antibody (red), and nuclei using 4′,6-diamidino-2-phenylindole (DAPI) staining (blue). Images were taken using a fluorescent microscope at 40×. (*D*) The numbers of H3K4me1 and E-cadherin double-positive cells per crypt are shown. N = 5 in each group.

(Figure 6*G*) in cells with knockdown of *Setd1b* expression. Collectively, these data indicate that p40 stimulates durable TGFβ production by up-regulating *Setd1b* expression, which then programs a stable H3K4 methylation imprint into IECs that is targeted to the TGFβ locus.

Sustained Increase in TGFβ Production Induced by Supplementation With p40 in Early Life Contributes to Prevention of Colitis in Adulthood in Mice

We have previously reported on the long-lasting effects of neonatal supplementation with p40 on stimulating expansion of Tregs and prevention of colitis in adulthood in mice.[16] Therefore, we examined if a sustained increase in TGFβ production induced by p40 supplementation in early life contributes to these protective effects in adult mice. To this end, adult forkhead box P (Foxp)3-green fluorescent protein (GFP) mice previously exposed to p40 in early life were treated with either neutralizing antibodies against TGFβ or matched IgG isotype negative control antibodies (Figure 7*A*). Differentiation of Tregs was evaluated by flow cytometry analysis of populations of $CD4^+Foxp3^+$ cells in the colonic lamina propria of Foxp3-GFP mice. Adult mice receiving p40 supplementation during the first 3 weeks of life showed a significant increase in the percentage of $CD4^+Foxp3^+$ in total $CD4^+$ cells of the colon lamina propria, which was inhibited by treatment of adult mice with the

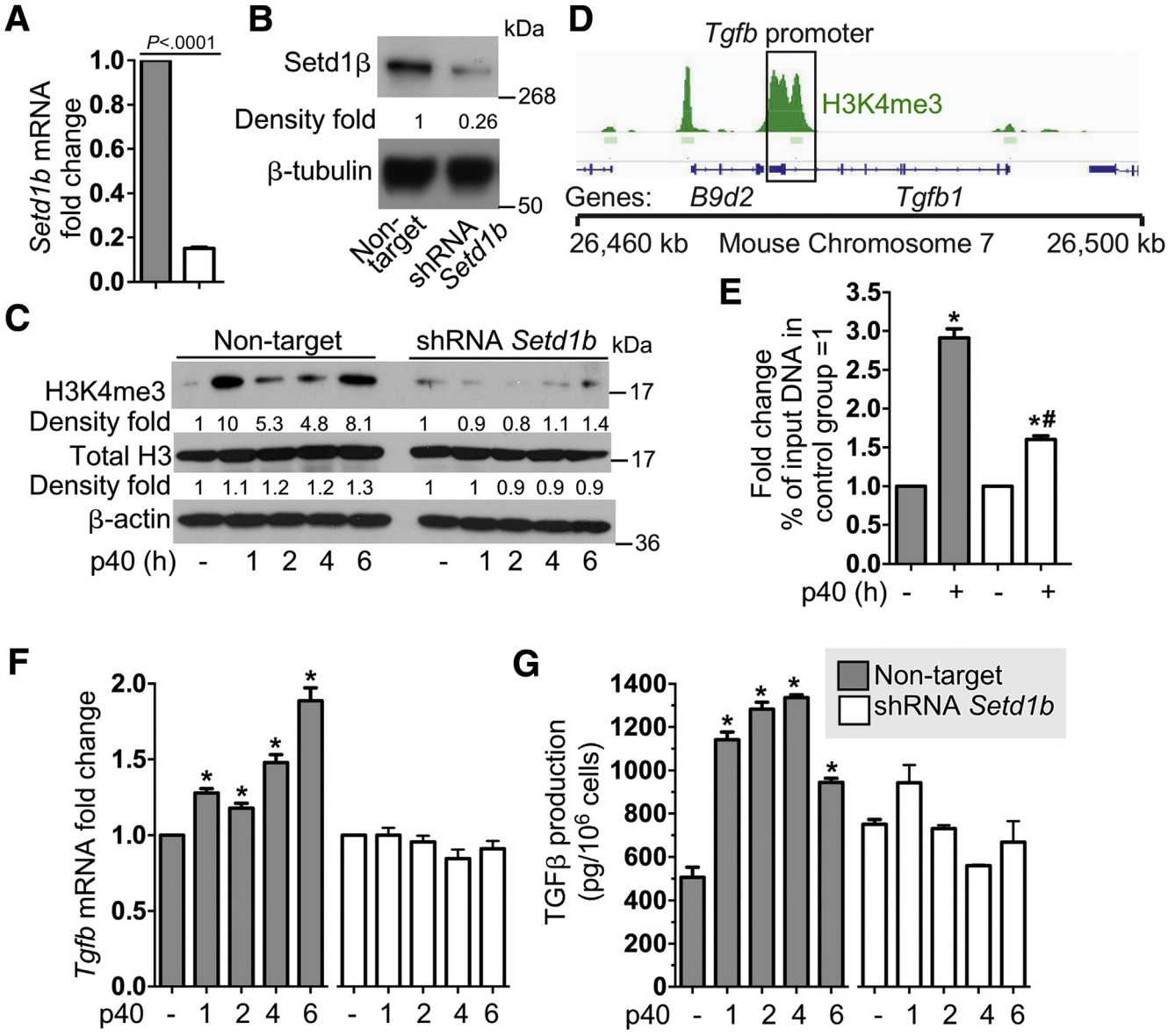


Figure 6. Increase of *Setd1b* expression in IECs mediates p40-promoted TGFβ production. (*A* and *B*) YAMC cells were transduced with lentiviral *Setd1b* shRNAs to generate a stable cell line with reduced expression of Setd1β. Nontargeting shRNA was used as a control. Knockdown efficiency of *Setd1β* was determined by assessing (*A*) mRNA expression and (*B*) protein levels using RT-PCR and Western blot analysis, respectively. The *Setd1b* mRNA expression level in the nontarget shRNA transduced cells was set as 1. (*C*) Total cellular lysates were prepared to detect p40-regulated histone methylation in the indicated cell lines. (*D* and *E*) H3K4me3 region within the *TGFβ* locus in mouse intestinal cells is shown. The enrichment of H3K4me3 in the *Tgfb1* promoter was analyzed by ChIP quantitative PCR. The percentage of relative enrichment (H3K4me3/Input) in the control group is set as 1. (*F* and *G*) Cells were treated with p40 (10 ng/mL) for the indicated times. (*F*) RNA was isolated for RT-PCR analysis of the *Tgfb1* mRNA level. The *Tgfb1* mRNA expression level in the control group with nontarget transduction was set as 1. (*G*) Supernatants from cell culture were collected for analysis of the amount of TGFβ release using enzyme-linked immunosorbent assay, as described in Figure 1. $^{*}P < .05$ compared with the control group in each cell line. $^{\#}P < .05$ compared with the p40 treatment group in nontargeting shRNA-transduced cell line. (*B* and *C*) The fold changes of band density are shown under the blot. Images shown are representative of 3 independent experiments. (*A* and *E*–*G*) Data are quantified from 3 independent experiments.

TGFβ-neutralizing antibody, but not by the isotype control antibody (Figure 7*B* and *C*).

We then asked if the sustained increase in TGFβ production by neonatal p40 supplementation prevents colitis in adult mice (Figure 8*A*). Eight-week old mice were treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS), which induces colitis by stimulating interleukin (IL)12-driven T-helper 1 immune responses, leads to disruption of the epithelial monolayer and increased inflammatory cell infiltration, with an increase in production of tumor necrosis

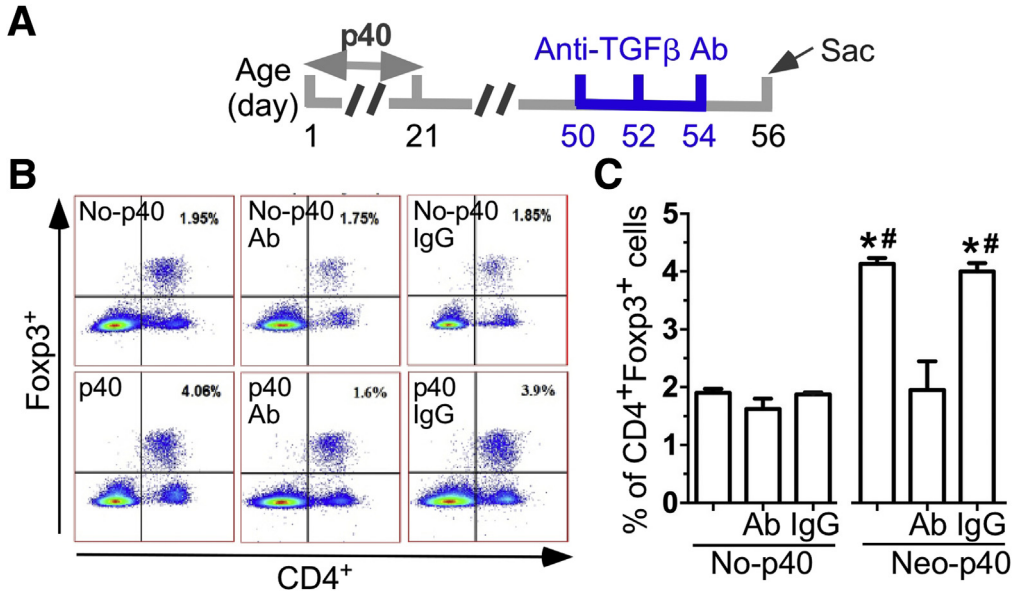


Figure 7. Neonatal p40 supplementation-promoted Treg expansion in the lamina propria of the colon in adult mice requires sustained increase in TGFβ production. (*A*) The treatment plan is shown. Foxp3-GFP mice were supplemented with p40 from postnatal day 2 to day 21 and received anti–TGFβ-neutralizing antibodies or isotype-negative control antibodies (IgG) at 50 μg/d at the indicated time points. Lymphocytes were isolated from the lamina propria of the colon. CD4- and Foxp3-expressing cells were assessed using flow cytometry analysis. (*B*) Representative contour plots of Foxp3 and CD4 are shown. Numbers in quarter 2 of contour plots represent the percentages of $CD4^+Foxp3^+$ in total lamina propria cells. (*C*) The percentages of $CD4^+Foxp3^+$ cells in total lamina propria cells are shown. N = 3 samples. Each sample contains cells from 2 to 3 mice. $^*P < .05$ compared with the counterpart in the no-p40 group. $^{\#}P < .05$ compared with the Neo-p40 group with TGFβ antibody co-treatment. Ab, anti-TGFβ antibody.

factor (TNF) and interferon-γ (IFN-γ).[30] TNBS-induced inflammation in adult mice with neonatal p40 supplementation (inflammation score, 2.0 $\pm$ 1.22) was significantly lower than the inflammation in mice without neonatal p40 supplementation (inflammation score, 4.0 $\pm$ 0.93; P = .0169) (Figure 8*B* and *C*). The significantly attenuated inflammation in adult mice receiving neonatal p40 supplementation was abolished by treatment of adult mice with the neutralizing transforming growth factor-β (TGFβ) antibody (inflammation score, 4.4 $\pm$ 1.14; P = .013), but not by the isotype control IgG treatment (inflammation score, 1.8 $\pm$ 1.10; $P > .05$) (Figure 8*B* and *C*).

Next, 2 characteristics of TNBS-induced colitis were evaluated. TNBS up-regulated levels of TNF and IFN-γ expression in the colonic mucosa were decreased significantly in mice with neonatal p40 supplementation, compared with mice without neonatal p40 supplementation ($P < .05$) (Figure 8*D*). These effects were inhibited by the treatment of TGFβ-neutralizing antibody ($P < .05$), but not IgG treatment ($P > .05$) (Figure 8*D*). A TNBS-induced colitis model also showed disruption of the intestinal epithelial integrity. The distribution of a tight junctional protein, ZO-1, was detected by immunostaining. Neonatal p40 supplementation prevented TNBS-induced redistribution of ZO-1 from apical tight junctional complexes to the cytoplasmic compartment of colon epithelial cells in adult mice with the treatment of IgG, but not TGFβ-neutralizing antibody (Figure 8*E*).

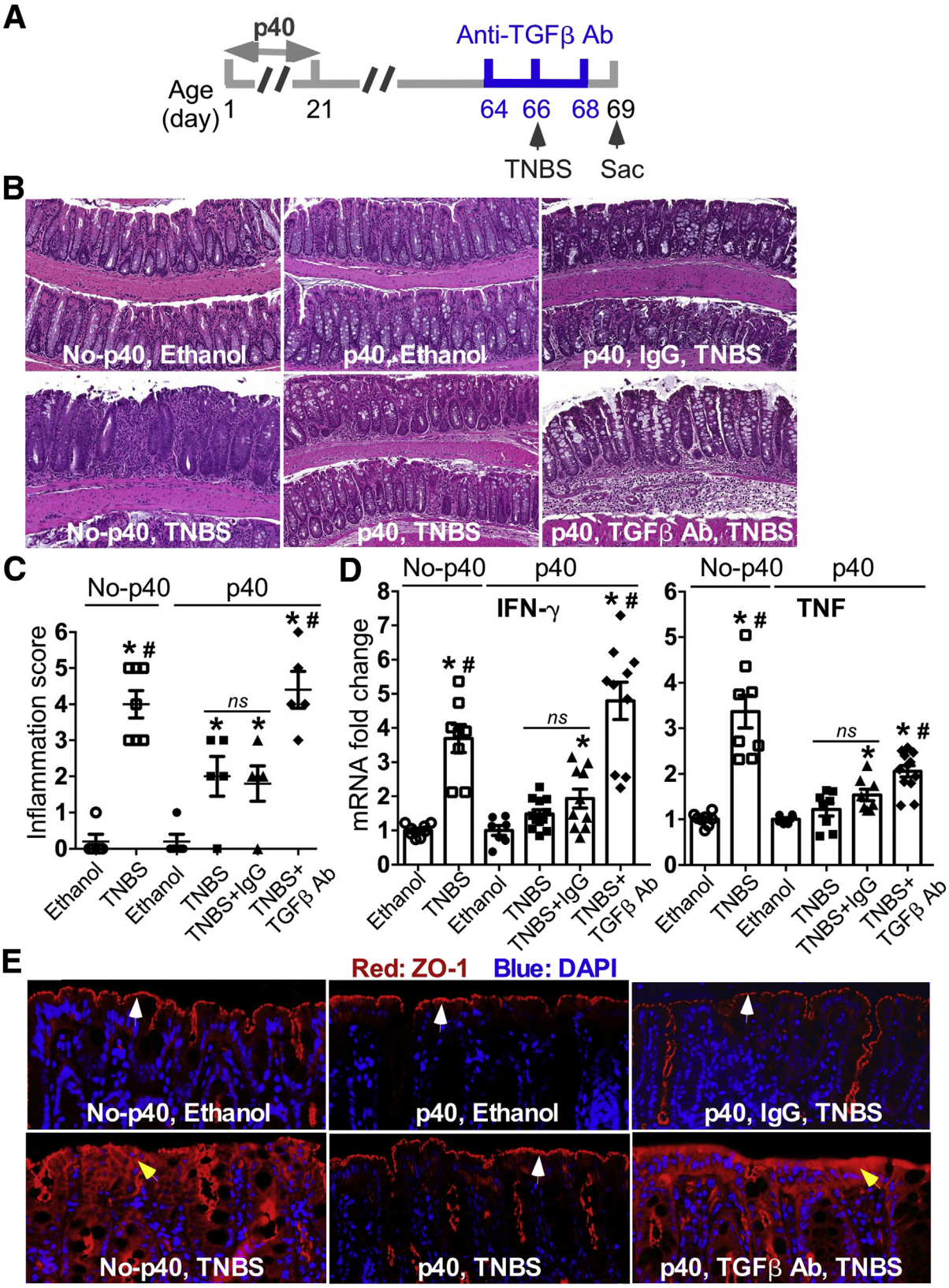
The effects of the increase in TGFβ production by neonatal p40 supplementation on adult mice were examined further in the dextran sulfate sodium (DSS) mouse model of injury and acute colitis (Figure 9*A*), which is well characterized by increased intestinal epithelial injury and production of inflammatory cytokines.[31] Consistent with the previous report,[16] adult mice receiving neonatal p40 supplementation showed lower levels of injury and acute colitis and TNF production upon DSS treatment (Figure 9*B–D*). Similar to TNBS colitis, these effects were inhibited by TGFβ-neutralizing antibody but not isotype IgG control treatment in adult mice (Figure 9*B–D*)

These data suggest that neonatal p40 supplementation stimulates a sustained increase in TGFβ production that supports protective immune responses and reduces intestinal inflammation in adult colitis models.

Discussion

Colonization by the microbiota in the neonatal gut has been recognized as an important early life event that exerts durable beneficial impact on immunity and health throughout the lifespan.[13] To advance this knowledge to clinical application, a next step forward is to elucidate the mechanisms through which the gut microbiome in early life transmits health-promoting signals and imprints them onto intestinal ecosystems in adulthood. This study showed that supplementation with probiotic-derived functional factor p40 in early life confers long-lasting effects on TGFβ production in the colon, leading to durable expansion of Tregs and prevention of colitis in adulthood. Furthermore, we identified the epigenetic effects of p40 on mouse small

intestinal epithelial (MSIE) cells in vitro and the long-lasting effects on TGFβ production in the small intestine by neonatal p40 supplementation (Figure 10). Increasing evidence indicates a significant correlation among the epigenetic changes induced by maternal and neonatal factors, including microbiota composition in early life, and



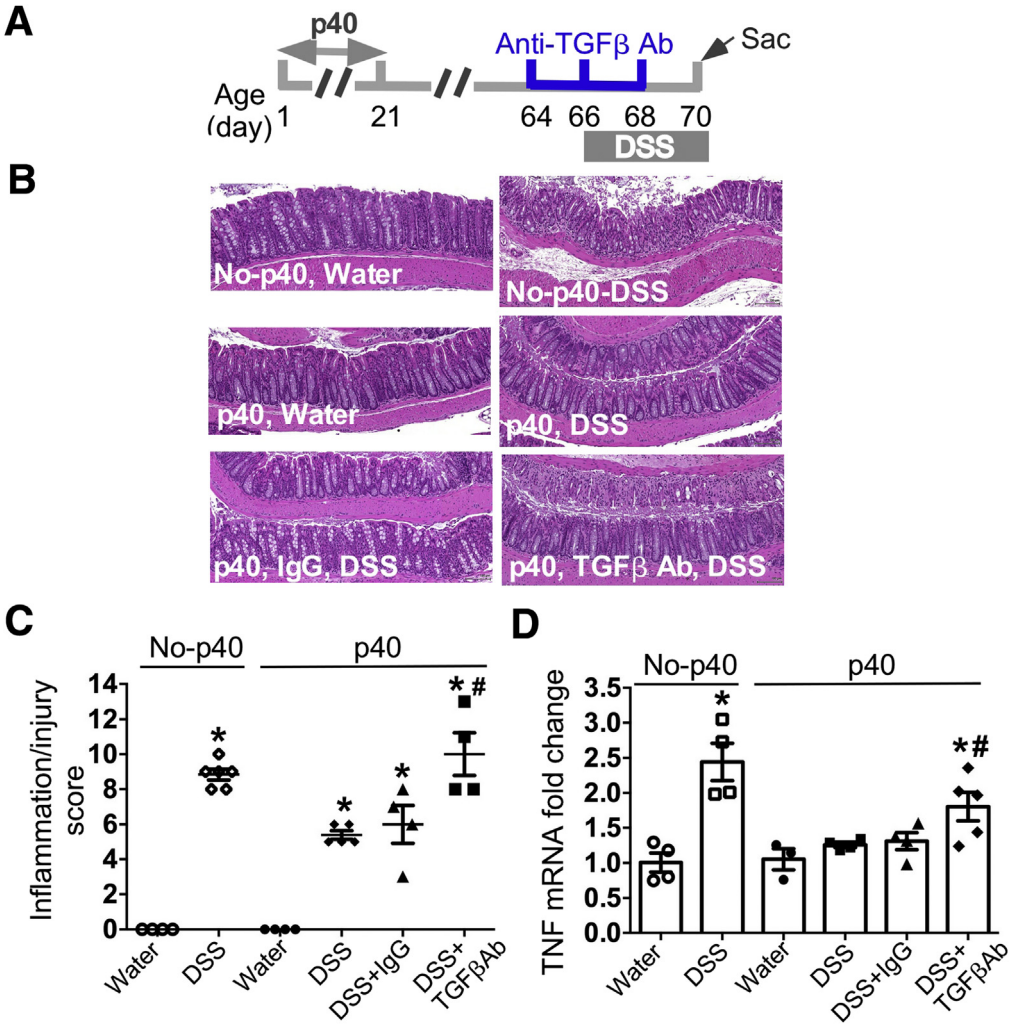


Figure 9. Sustained TGFβ production by neonatal p40 supplementation mediates. prevention of colitis in adult mice. (*A*) The treatment plan is shown. Mice were supplemented with p40 from postnatal day 2 to day 21 and received TGFβ-neutralizing antibodies or isotype control antibodies (IgG) at 50 g/d, at the indicated time points. Colitis was induced by 3% DSS in drinking water for 4 days. Mice receiving water were used as controls for DSS treatment. Mice were killed at the end of DSS treatment. (*B*) Colon sections were stained with H&E for assessment of inflammation. Slides were scanned and images were exported at 10X magnification. (*C*) The inflammation/injury scores are shown. (*D*) RNA was isolated from the colonic tissues for RT-PCR analysis of the indicated cytokine mRNA expression levels. The average cytokine mRNA expression level in the control mice of the no-p40 group was set as 1, and the mRNA expression level of each mouse was compared with this average. $^{*}P < .05$ compared with the control mice in the no-p40 group. $^{\#}P < .05$ compared with the p40 group with DSS or DSS and IgG co-treatment. Ab, antibody; Sac, sacrifice.

Figure 8. (***See previous page***). **Sustained TGFβ production by neonatal p40 supplementation mediates prevention of colitis in adult mice.** (*A*) The treatment plan is shown. Mice were supplemented with p40 from postnatal day 2 to day 21 and received TGFβ-neutralizing antibodies or isotype control antibodies (IgG) at 50 μg/d, at the indicated time points. Colitis was induced by TNBS in ethanol intrarectally. Mice receiving ethanol were used as controls for TNBS treatment. Mice were killed 4 days after TNBS treatment. (*B*) Colon sections were stained with H&E for assessment of inflammation. (*C*) The inflammation scores are shown. (*D*) RNA was isolated from the colonic tissues for RT-PCR analysis of the indicated cytokine mRNA expression levels. The average cytokine mRNA expression level in the control mice of the no-p40 group was set as 1, and the mRNA expression level of each mouse was compared with this average. $^{*}P < .05$ compared with the control mice in the no-p40 group. $^{\#}P < .05$ compared with the p40 group with TNBS or TNBS and IgG co-treatment. (*E*) Paraffin-embedded colon tissues were used to determine ZO-1 distribution by immunohistochemistry using an anti–ZO-1 antibody and a Cy3-labeled secondary antibody (red). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Slides with H&E staining and immunostaining were scanned and images were exported at 10X magnification. Membrane (*white arrowheads*) and intracellular (*yellow arrowheads*) ZO-1 distributions are shown. Ab, antibody; Sac, sacrifice.

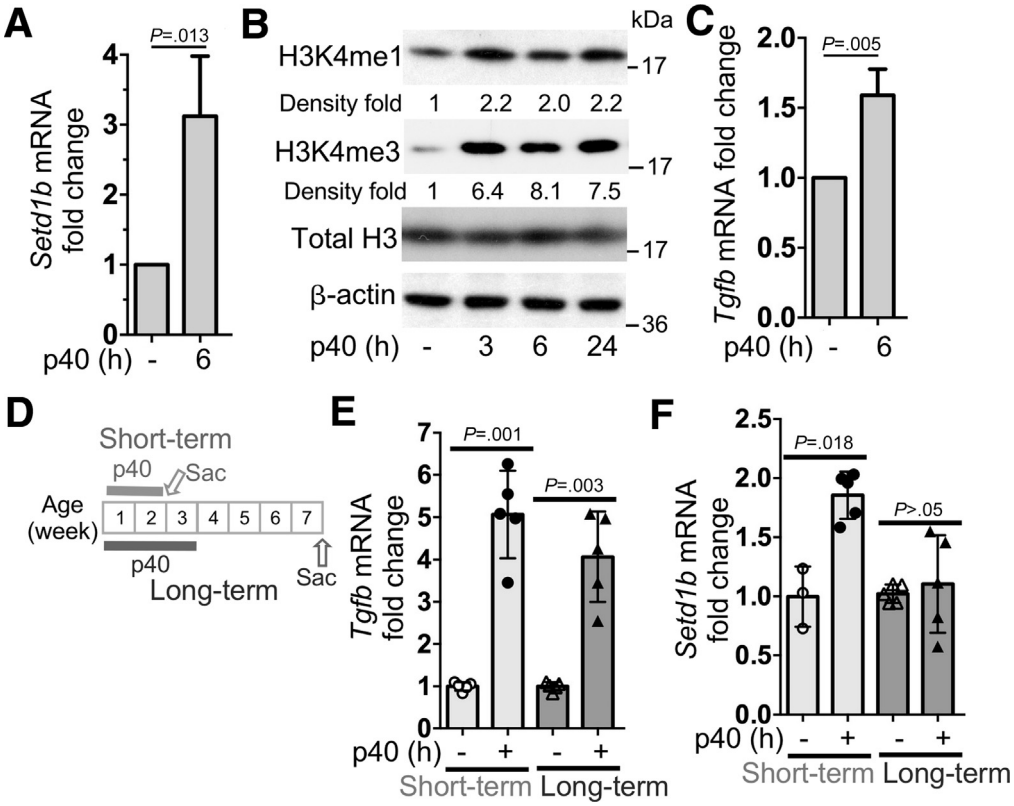


Figure 10. p40 stimulates *Tgfb* and *Setd1b* expression and histone modification in small intestinal epithelial cells in vitro and in vivo. (*A–C*) MSIE cells were treated with p40 at 10 ng/mL for the indicated times. RNA was isolated for RT-PCR analysis of the mRNA expression levels of (*A*) *Setd1b* and (*C*) *Tgfb*. The mRNA expression level in the control group was set as 1. The mRNA expression level in the treated group was compared with the control group. (*B*) Total cellular proteins were prepared from MSIE cells for Western blot analysis. β-actin blot was used as the protein loading control. The band density fold changes are shown under the bands. (*A* and *C*) Data are quantified from 3 independent experiments. (*B*) Images represent results in at least 3 independent experiments. (*D*) The treatment plan. WT mice were supplemented with p40 for testing the short-term and the long-term effects, as shown in Figure 3. Mice supplemented with hydrogels without p40 were used as controls. (*E* and *F*) RNA was isolated from small intestinal tissues for RT-PCR analysis of *Tgfb1* and *Sedt1b* gene expression. The mRNA expression level in the control groups was set as 1. The mRNA expression level in treated groups was compared with the control group. Sac, sacrifice.

influencing health and disease susceptibility in adulthood.[29] This work contributes to further understanding this relationship by discovering an epigenetic program modulated by p40-stimulated Setd1β function in the colonic epithelial cells, which mediates the increase in TGFβ production. Our novel finding provides a key mechanistic understanding of the capacity of factors produced by bacterial colonization of the gut in newborns for imprinting health in adults.[15]

Numerous studies have identified evidence that there is a limited window of opportunity for inducing stable effects by the gut microbiota on imprinting the immune system. For example, the microbial colonization in the neonatal period, not in adulthood, in germ-free mice shapes the function of natural killer T cells in adulthood.[32] Consistent with these published results, one significant finding from this study is to define the neonatal period as the time window for p40 supplementation to induce a sustained increase in TGFβ production and induction of Tregs. Previous studies have suggested that immune cells in early life could obtain memory of the microbiota influence during the development of the immune system.[33] This study identified a novel heritable mechanism: sustained induction of Tregs is through a durable increase in TGFβ production by IECs after neonatal p40 supplementation.

It should be noted that IECs must retain the memory of p40 regulation during cell division to extend the effects of early p40 exposure into adulthood. However, IECs are constantly shed into the lumen, undergoing essentially complete replacement every 5 days with continuous renewal from intestinal stem cells.[34] The long-lived characteristics of intestinal stem cells makes them potential target cells in the intestine for conferring stable cellular responses. We have found that p40 up-regulates H3K4me1 in $Lgr5^{+}$ cells in neonatal leucine-rich repeat-containing G-protein coupled receptor (Lgr)5-*iSuRe* (IRE)s-Cre enhancer trap (ERT)2 mice (data not shown). This result supports our future studies to elucidate whether p40-regulated epigenetic reprogramming in intestinal stem cells in early life is inherited by IECs in adult mice. In addition, intestinal stem cells have been found to have different developmental

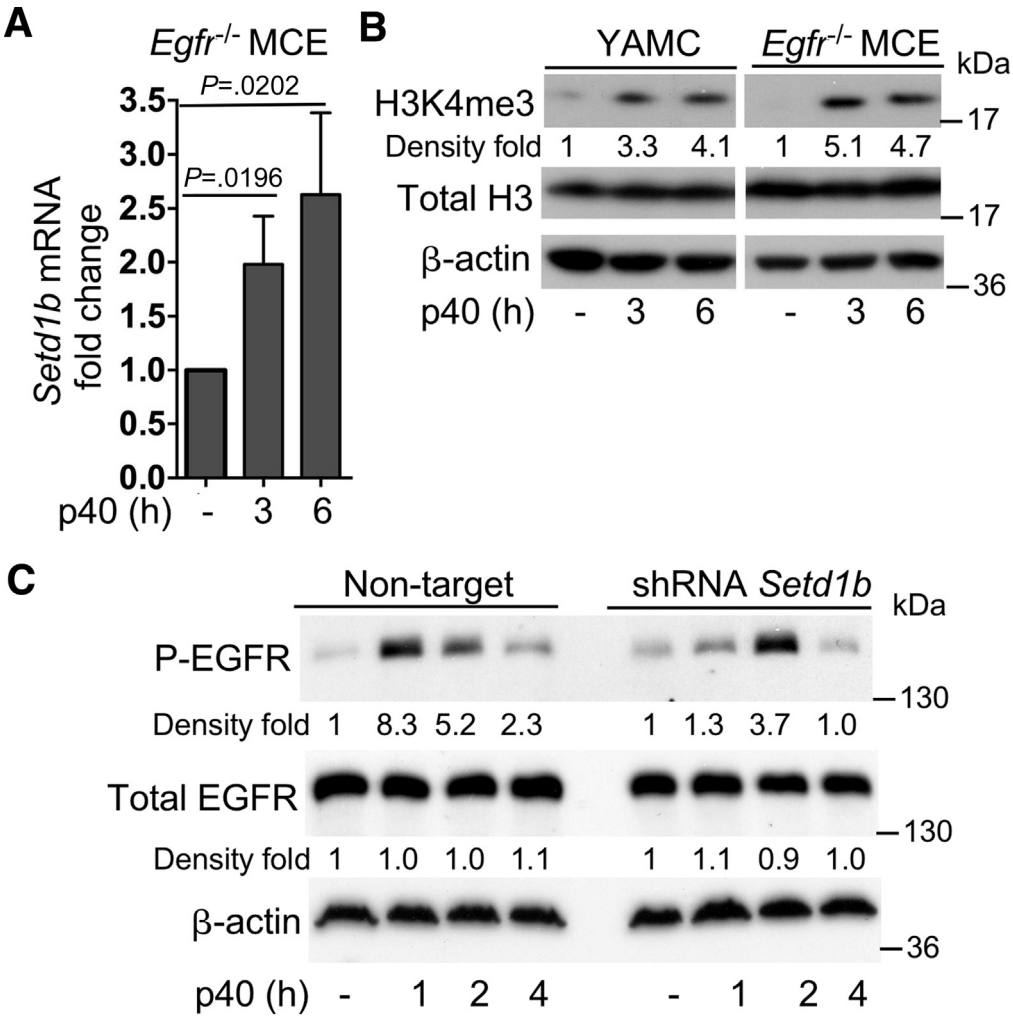


Figure 11. p40-stimulated EGFR transactivation and *Setd1b* gene expression in IECs are 2 independent functions. YAMC, *Egfr*$^{-/-}$ mouse colonic epithelial (MCE), and YAMC transduced with lentiviral *Setd1b* shRNAs or nontargeting shRNA as used in Figure 6 were treated with p40 at 10 ng/mL for the indicated times. (*A*) RNA was isolated for RT-PCR analysis of the levels of *Setd1b* mRNA. The *Setd1b* mRNA expression level in the control group was set as 1. The mRNA expression levels in treated groups were compared with the control group. (*B* and *C*) Western blot analysis of cellular lysates was performed to detect levels of H3K4me3, total H3, phospho-Tyr1068-EGFR (P-EGFR), and total EGFR. β-actin blot was used as a loading control. (*A*) Data are quantified from 3 independent experiments. (*B* and *C*) Images are representative of at least 3 independent experiments.

programs at different development stages.[35] The immature human intestinal epithelium has the intrinsic capability of establishing stable host–microbe symbiosis.[36] Therefore, it is possible that in addition to Setd1β, other factors involved in developmental programs in intestinal stem cells in early life may contribute to the persistent effects of p40. Future studies will be focused on studying p40 regulation of epigenetic memory in intestinal stem cells in early life that extends into adulthood, thus programming IECs with the ability to maintain intestinal homeostasis.

Current therapies for IBD induce sustained remission in less than half of patients; thus, new therapies are needed.[37] TGFβ signaling has multiple roles in suppressing inflammation, such as controlling immune responses through Treg induction and inducing protective cellular responses.[24–26,38] Clinical studies have shown that the locus encoding SMAD3, an effector of TGFβ signaling, is associated with IBD susceptibility,[39] and enhancing TGFβ signaling benefits in patients with Crohn's disease.[40,41] Therefore, any strategies that potentiate the function of TGFβ signaling could benefit IBD prevention and treatment. The identification of the effects of p40 on TGFβ production and its consequences for stimulating TGFβ signal targets in IECs and Treg expansion supports the significant clinical potential of p40 for IBD. In addition, although there is no strong evidence to support the clinical efficacy of supplementation with probiotics,[2,5] application of defined probiotic-derived factors, such as p40, could bypass the limitations of clinical application of probiotics, such as uncertain bioavailability and biopharmacology of probiotics in the human gastrointestinal tract. Therefore, results from this work provide a mechanistic rationale for early p40 intervention as a strategy for individuals at high risk of developing intestinal inflammation, such as IBD.

p40 has been shown to transactivate the EGFR in IECs, which induces protective cellular responses.[8–11] We have now shown that p40 up-regulates *Setd1b* gene expression and H3K4me3 in IECs in the absence of EGFR expression (Figure 11*A* and *B*). Furthermore, p40 was able to stimulate transactivation of the EGFR in IECs without *Setd1b* gene expression (Figure 11*C*). These results suggest that the epigenetic effect of p40 on TGFβ production is not associated with p40-regulated EGFR transactivation in IECs. There is evidence to support p40 regulates several signaling

pathways to prevent and treat colitis. p40 ameliorated DSS-induced colitis in WT mice. This effect was diminished in *Egfr*$^{fl/fl}$-Villin (Vil)-Cre mice with the EGFR specifically deleted in IECs.[10] However, the inhibitory effects of p40 on proinflammatory cytokine production in mice with DSS-induced colitis, such as increasing IL10 and decreasing IL6 and IL17, are similar in WT and *Egfr*$^{fl/fl}$-Vil-Cre mice. These results suggest that p40 inhibits intestinal inflammation through at least 2 independent pathways.

In summary, studies from this work further elucidate the impact of neonatal supplementation with p40 on long-term intestinal inflammation in the adult. In addition to previously reported EGFR-dependent protection from neonatal colonization with LGG or p40 treatment,[16] here we show an EGFR-independent increase in TGFβ production in IECs, also leading to protection from colitis. These results also uncover a novel epigenetic mechanism underlying the priming of IECs by p40 to enable long-lasting effects. Notably, this knowledge should provide mechanistic insights to support early intervention with p40 as a novel strategy for maintaining intestinal health in adulthood.

Materials and Methods

Purification and Encapsulation of p40

p40 was purified from LGG (53103; American Type Culture Collection, Manassas, VA) culture supernatant and saved at -80°C, as previously reported.[6] A BCA protein assay kit (23225; Pierce Thermo Scientific, Waltham, MA) was used to examine the concentration of p40 isolates. The level of endotoxin in p40 isolates was <0.03 EU/μg p40 protein examined by the Pierce LAL Chromogenic Endotoxin Quantitation Kit (88282; Pierce Thermo Scientific). p40 was encapsulated in the pectin/zein hydrogels and stored at 4°C, using methods reported previously.[10,16] Briefly, p40 was encapsulated at 0.5 and 1 μg of p40/hydrogel in pectin solution in water (2.0% w/v) for supplementation to mice at the early stage. For adult mice supplementation, p40 was encapsulated in hydrogel in pectin solution in water (6.0% w/v). Each hydrogel contained 5 μg of p40. All hydrogels were coated with zein solution (1.0% w/v) and $CaCl_2$ (0.5% w/v) in 75% ethanol solution. Hydrogels without p40 were prepared as negative controls.

Mice and Treatment

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. This study used wild-type C57BL/6J (000664; Jackson Laboratory, Bar Harbor, ME) and Balb/cJ (000651; Jackson Laboratory) mice, and Foxp3-GFP transgenic mice on a Balb/c background (006769; Jackson Laboratory). For each experiment, two 8-week-old female mice from the same litter in the same cage were mated with 1 adult male mouse and were housed until female mice were close to deliver. Mice in 1 litter were supplemented with p40-containing hydrogels. As control, mice in the other litter received hydrogels without p40. For p40 supplementation in the neonatal period, pups were supplemented with p40-containing hydrogels at 0.5 μg/d (postnatal days 2–6), 1 μg/d (postnatal days 7–13), and 1.5 μg/d (postnatal days 14–21). For p40 supplementation in adulthood, 10 μg/d of p40 was supplemented to 6-week-old mice for 3 weeks. Mice received hydrogels through oral administration. Experiments were repeated by using mice from at least 3 pairs of pregnant female mice for each treatment.

Mice with and without neonatal p40 supplementation received a monoclonal anti–TGFβ-neutralizing antibody (MA5-23795; ThermoFisher Scientific, Waltham, MA) or IgG isotype control (02-6100; ThermoFisher Scientific) at 50 μg in 100 μL of phosphate-buffered saline for each injection. This treatment was give to mice every the other day through peritoneal cavity injection. The time lines of anti-TGFβ antibody and IgG treatment are shown in Figures 7*A* and 8*A*.

Colitis was induced by TNBS in Balb/c mice with and without neonatal p40 supplementation and TGFβ antibody treatment. Mice were treated with 100 μL of 70 mmol/L TNBS in 50% ethanol intrarectally. Control mice received 100 μL of 50% ethanol intrarectally. Mice were killed 4 days after TNBS treatment.

Analysis of Colitis

Paraffin-embedded colonic tissue sections were prepared for H&E staining. Slides were scanned using the Leica SCN400 Slide Scanner (Leica, Wetzlar, Germany. Samples from the entire colon were examined by a pathologist blinded to treatment conditions for assessing inflammation. The scoring system used to assess TNBS-induced colitis was modified from a previous scoring system[42,43]: lamina propria mononuclear cell and polymorphonuclear cell infiltration, enterocyte loss, crypt inflammation, and epithelial hyperplasia were scored from 0 to 3, yielding an additive score between 0 (no colitis) and 15 (maximal colitis).

Isolation of Lymphocytes From Colonic Lamina Propria for Flow Cytometry Analysis

Lymphocytes were isolated from lamina propria of the colon of Foxp3-GFP mice with and without neonatal p40 supplementation and TGFβ antibody treatment, as described previously.[44] Cells were labeled with R-phycoerythrin (PE)–Cyanine (Cy)5–anti-cluster of differentiation (CD)4 (100410; BioLegend, San Diego, CA) by incubation for 0.5 hours at room temperature. Then, cells were analyzed using multicolor flow cytometry to determine the percentage of GFP (Foxp3 expression) and PE-Cy5.5 (CD4 expression) double-positive cells using a BD LSRII system (BD Biosciences, Franklin Lakes, NJ). Each sample contained lymphocytes from 3 to 4 mice with the same treatment.

Enteroid and Colonoid Culture

The ileum and colonic tissues were isolated from WT C57BL/6 mice for culture of enteroids and colonoids, as described previously.[9] Enteroids and colonoids were cultured in Matrigel (Cornng, Bedford, MA) and overlaid

with IntestiCult Organoid Growth Medium (06005; STEMCELL Technologies, Vancouver, Canada) in the absence or presence of p40 (100 ng/mL) in Matrigel and in medium.

Cell Culture, shRNA Transduction, and Treatment

The YAMC and MSIE cell lines were generated from immortalized mice (Immortomouse) harboring thermolabile simian virus 40 (SV40) large tumor antigen (TAg) from a SV40 strain, tsA58.[21] Cell proliferation requires the expression of SV40 TAg, which is induced by an IFN-γ-inducible H-2Kb promoter at the permissive temperature (33°C). Cells die in medium without IFN-γ at the nonpermissive temperature (37°C) for 3 passages. An EGFR knock out ($EGFR^{-/-}$) mouse colonic epithelial cell line was generated from the colonic epithelium of EGFR-null mice crossed to the Immortomouse.[45] Immortalized mouse colonocytes with floxed *Smad4* alleles ($IMC^{S4fl/fl}$) were generated from $Smad4^{fl/fl}$ mice on the Immortomouse background. IMC^{S4null} cells were generated by transducing $IMC^{S4fl/fl}$ cells with adenoviral-Cre to delete *Smad4*.[27,28]

YAMC, MSIE, $Egfr^{-/-}$ MEC (mouse epithelial cell), $IMC^{S4fl/fl}$, and IMC^{S4null} cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 5 U/mL of murine IFN-γ, 100 U/mL penicillin and streptomycin, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenous acid at 33°C with 5% CO_2. Cells were cultured in RPMI 1640 medium containing 1% FBS and 100 U/mL penicillin and streptomycin for 18 hours at 37°C before experiments.

To generate a stable cell line with *Setd1b* deletion, YAMC cells were stably transduced with 3 sets of lentiviral *Setd1b* shRNAs using the Dharmacon SMARTvector lentiviral shRNA kit, following the manufacturer's instructions (Waterbeach, UK). SMARTvector nontargeting murine cytomegalovirus (mCMV)-TurboGFP shRNA was used as a control. Transduced cells were selected by puromycin for 7–10 days.

YAMC and *shRNA*-transduced cells were treated with p40 (10 ng/mL) for Western blot analysis of activation of the TGFβ/SMAD signaling pathway. $IMC^{S4fl/fl}$ and IMC^{S4null} cells were treated with H_2O_2 (20 μmol/L) for 4 hours and lipopolysaccharide (1 μg/mL; Sigma-Aldrich, St. Louis, MO) for 24 hours in the presence or absence of p40 (10 ng/mL) co-treatment with 1-hour pretreatment for immunostaining of ZO-1 and examining proinflammatory cytokine production by RT-PCR, respectively.

The human colonic adenocarcinoma cell line, Caco-2 cells (86010202; American Type Culture Collection, Manassas, VA), were grown in Dulbecco's modified Eagle medium supplemented with 10% FBS and 100 U/mL penicillin and streptomycin at 37°C with 5% CO_2. Cells were serum-starved in Dulbecco's modified Eagle medium containing 1% FBS for 16–18 hours before experiments. For immunostaining of ZO-1, cells were treated with H_2O_2 (20 μmol/L) for 4 hours in the presence or absence of 1-hour p40 (10 ng/mL) and anti–TGFβ-neutralizing antibody (1 μg/mL) pretreatment.

ChIP Assay

Binding of H3K4me3 to the TGFβ promoter was examined using the SimpleCHIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads) (9005; Cell Signaling), according to the manufacturer's instruction. Briefly, cells were treated with 37% formaldehyde to cross-link protein and DNA. Nuclei were isolated and digested using micrococcal nuclease, and nuclear lysates were sonicated to fragment chromatin to 150–900 bp, which was verified by analyzing purified DNA using electrophoresis on 1% agarose gel. Digested chromatin was diluted in ChIP dilution buffer and immunoprecipitated using a rabbit anti-H3K4me3 antibody (9751; Cell Signaling) overnight at 4°C. The rabbit anti–histone 3 (4620; Cell Signaling, Danvers, MA) antibody and normal rabbit IgG (2729; Cell Signaling) were used as positive and negative controls, respectively. The immune complexes were captured by protein G magnetic beads and cross-linked protein and DNA was reversed by NaCl (5 mol/L) and proteinase K at 65°C for 2 hours. DNA was purified for quantification by quantitative PCR in triplicate using the TGFβ promoter specific primers: forward: 5'-GCACTGCGCTGTCTCGCAAGG-3' and reverse: 5'-GGGATGCGAGGGACTCAAGAGG-3'. Results were expressed as a percentage of control. Relative enrichment was calculated as the amount of immunoprecipitated DNA by anti-H3K4me3 antibody relative to the total amount of input (H3K4me3/Input). Relative enrichment in the control group is set as 1, for comparison by data in other groups.

ELISA of TGFβ1 Production

The level of TGFβ in supernatants of cultured YAMC cells was measured using the mouse TGFβ1 DuoSet ELISA development system (DY1679; R&D System, Minneapolis, MN), according to the manufacturer's instruction. Purified TGFβ was used to generate the standard concentration curve. Cell numbers were counted at the end of experiments. The TGFβ concentration was representative as of ng/10^6 cells.

RT-PCR Assay

Total RNA was isolated from cultured cells and enteroids and colonoids, and homogenized colon tissues, using an RNA isolation kit (Qiagen, Valencia, CA) and was treated with RNase-free DNase. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit and the 7300 RT-PCR System (Applied Biosystems, Foster City, CA). The data were analyzed using Sequence Detection System V1.4.0 software. Primers, *Setd1b* (Mm00616971), *Tgfb1* (Mm01178820), TNF (Mm00443259), and *Ifng* (Mm99999071) were purchased from Applied Biosystems. The relative abundance of β-actin mRNA was used to normalize levels of the mRNAs of interest. All complementary DNA samples were analyzed in triplicate.

Immunofluorescence Staining

Cultured cells were fixed and permeabilized with 2% Triton X-100 in phosphate-buffered saline for 5 minutes at room temperature. Paraffin-embedded tissue sections were

deparaffinized followed by antigen unmasking by boiling in sodium citrate buffer (10 mmol/L sodium citrate, 0.05% Tween 20, pH 6.0). Colonic sections and cells were blocked using 10% goat serum for 1 hour at room temperature. For ZO-1 staining, slides were incubated with a rabbit anti-mouse ZO-1 (61-7300; Invitrogen Life Technologies, Carlsbad, CA) antibody overnight at 4°C and a Cy3-labeled goat anti-rabbit IgG (111-165-003; Jackson ImmunoResearch) antibody at room temperature for 1 hour. H3K4me1 and E-cadherin double staining was performed by incubating slides with a rabbit polyclonal anti-H3K4me1 antibody (5326; Cell Signaling) overnight followed by a mouse monoclonal anti–E-cadherin antibody (610181; BD Biosciences) for 1 hour at 4°C. Then, sections were incubated sequentially with fluorescein isothiocyanate–labeled anti-rabbit (111-095-003; Jackson ImmunoResearch) for 1 hour followed by Cy3-labeled anti-mouse (115-165-003; Jackson ImmunoResearch) antibodies for 1 hour at room temperature. Sections then were mounted using Mounting Medium containing 4′,6-diamidino-2-phenylindole for nuclear counterstaining. Slides were scanned using the Apiro Versa 200 platform (Leica) or observed using a Leica DM IRB inverted microscope, and images were recorded using a Nikon DXM1200C camera (Nikon, Tokyo, Japan)

Western Blot Analysis

Cell pellets were solubilized in cell lysis buffer containing 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) 50 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 150 mmol/L NaCl, and a protease and phosphatase inhibitor mixture (Sigma-Aldrich) to obtain total cellular lysates. Protein concentrations of lysates were determined using a protein assay kit. The lysates were mixed with Laemmli sample buffer and equal amounts of protein were loaded for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blot analysis was performed using anti-total SMAD3 (9523; Cell Signaling Technology), anti–phospho-SMAD3 (9520; Cell Signaling Technology), anti–total SMAD2 (5339; Cell Signaling Technology), anti–phospho-SMAD2 (3108; Cell Signaling Technology), anti-SMAD4 (38454; Cell Signaling Technology), anti–histone 3 (Cell Signaling Technology), anti-H3K4me1 (Cell Signaling Technology), anti-H3K4me3 (Cell Signaling Technology), anti-Setd1β (44922; Cell Signaling Technology), anti-Setd1α (61702; Cell Signaling Technology), anti-MML1 (14689; Cell Signaling Technology), anti-MML2 (63735; Cell Signaling Technology), anti-WDR5 (13105; Cell Signaling Technology), anti-WDR82 (99715; Cell Signaling Technology), anti–β-actin (A2228; Sigma-Aldrich), and anti–β-tubulin (2146; Cell Signaling Technology) antibodies.

The band density was measured using the ImageJ (National Institutes of Health, Bethesda, MD) processing program. The relative band density by a specific antibody was calculated by comparing it with the β-actin band from the same sample. The relative band density in the control group was set as 1. The density fold change was obtained by comparing the relative band density in the treatment group with that in the control group.

Statistical Analysis

Statistical significance was determined by 1-way analysis of variance for multiple comparisons and the t test for comparing data from 2 samples using Prism 6.0 (GraphPad Software, Inc, San Diego, CA). A P value less than .05 was defined as statistically significant. All data are presented as means ± SEM.

Results from in vitro studies shown in this manuscript represent data from at least 3 independent experiments. Data from all mice in this study 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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