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

Yilin Deng,1 Oliver G. McDonald,2,3,4 Anna L. Means,3,6 Richard M. Peek Jr.,7 M. Kay Washington,2 Sari A. Acra,1 D. Brent Polk,8,9,10 and Fang Yan1

1Department of Pediatrics, 2Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee; 3Department of Cell and Developmental Biology, Vanderbilt University, Nashville, Tennessee; 4Epithelial Biology Center, 5Vanderbilt-Ingram Cancer Center, 6Department of Surgery, 7Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee; 8Department of Pediatrics, 9Department of Biochemistry and Molecular Medicine, Keck School of Medicine of University of Southern California, Los Angeles, California; 10Division of Gastroenterology, Hepatology and Nutrition, Children’s Hospital Los Angeles, Los Angeles, California

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 & AIDS: 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 Setd1β 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 Setd1β 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)
Probiotics, which originally were defined as living microorganisms with low or no pathogenicity that exert beneficial effects on the health of the host,1–4 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.4,5 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. Phylogenetic 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 colonization15 and transient exposure to p40 in the neonatal period16 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 through 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 IECs17 and immune cells.18 Su(var)3-9, enhancer-of-zeste and trithorax domain containing 1 (Setd1β) 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 condition 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 1A) 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 1B). 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 1C). 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
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 1D). 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 1D). 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 were not observed with p40 treatment.

**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 10⁶ 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.
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. 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 2A).

Activation of TGFβ signaling plays roles in preserving the intestinal barrier function and inhibiting proinflammatory cytokines and chemokine production in IECs. 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. H2O2 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 2B), an effect that was abolished by co-treatment with TGFβ-neutralizing antibodies (Figure 2B). Immortalized mouse colonocytes with floxed Smad4 alleles (IMC5fl/fl) were generated from mice on the Immortomouse background. IMCfl/fl cells were transduced with adenoviral-Cre to delete Smad4 (IMC5fl/null) (Figure 2C). p40 inhibited the H2O2-induced disruption of tight junctions in IMC5fl/fl cells, but not in IMC5fl/null cells (Figure 2D). In addition, p40 was able to block lipopolysaccharide-induced expression of proinflammatory chemokines, C-C motif chemokine ligand 20, and keratinocyte chemooattractant in IMC5fl/fl cells. p40 failed to exert these effects in IMC5null cells (Figure 2E). Collectively, these data suggest that a p40-stimulated increase in TGFβ production supports intestinal barrier function and attenuates inflammatory cytokine release from IECs.

Epigenetic Regulation by p40 Promotes TGFβ Production in IECs

The long-lasting effects of 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. In particular, the gut microbiota can regulate epigenetic modifications in the intestine, including H3K4me3, which is permissive for gene activation. We therefore asked if p40 might regulate TGFβ production through H3K4 methyltransferase, Setd1b. We found that p40 significantly up-regulated Setd1b gene expression and protein production (Figure 4B) in YAMC cells. The increase in Setd1b gene expression was observed in enteroids and colonoids with p40 treatment (Figure 4C). Furthermore, up-regulation of Setd1b protein also was coupled to a global increase in H3K4me1 and H3K4me3 in YAMC cells (Figure 4D). We did not find significant effects of p40 on the regulation of other methyltransferases, such as Setd1a, mixed lineage leukemia (MLL)1, or MLL2 in YAMC (Figure 4E).

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. These hydrogels can protect proteins, such as p40, from damage by proteases, acid, and other intestinal contents in the intestinal tract. 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 3A). 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 3A).

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 3B). 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 3B). 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 3C), 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.
short-term effects on stimulating *Setd1b* gene expression in colonic tissues (Figure 5A). However, neither neonatal nor adult p40 supplementation induced long-term consequences on *Setd1b* gene expression (Figure 5B). 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 5C 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 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) Caco2 cells were treated with H2O2 (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 IMCS4fl/fl and IMCS4null cells were prepared for Western blot analysis. (D) IMCS4fl/fl and IMCS4null cells were treated with H2O2 (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. (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 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.
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 6A and B). As expected, p40 treatment increased H3K4me3 levels in YAMC cells transduced with nontarget shRNA, but not in Setd1β-deficient cells (Figure 6C). 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 6D and E). p40 failed to stimulate TGFβ gene expression (Figure 6F) and protein production.
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. 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 7A). 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...
TGFβ-neutralizing antibody, but not by the isotype control antibody (Figure 7B and C).

We then asked if the sustained increase in TGFβ production by neonatal p40 supplementation prevents colitis in adult mice (Figure 8A). 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 factor α (Figure 8B). 

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 Setd1b. Nontargeting shRNA was used as a control. Knockdown efficiency of Setd1b 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 Tgfβ1 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.
factor (TNF) and interferon-γ (IFN-γ). TNBS-induced inflammation in adult mice with neonatal p40 supplementation (inflammation score, 2.0 ± 1.22) was significantly lower than the inflammation in mice without neonatal p40 supplementation (inflammation score, 4.0 ± 0.93; \( P = .0169 \)) (Figure 8B 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 ± 1.14; \( P = .013 \)), but not by the isotype control IgG treatment (inflammation score, 1.8 ± 1.10; \( P > .05 \)) (Figure 8B 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 8D). These effects were inhibited by the treatment of TGFβ-neutralizing antibody (\( P < .05 \)), but not IgG treatment (\( P > .05 \)) (Figure 8D). 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 8E).

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 9A), which is well characterized by increased intestinal epithelial injury and production of inflammatory cytokines. Consistent with the previous report, adult mice receiving neonatal p40 supplementation showed lower levels of injury and acute colitis and TNF production upon DSS treatment (Figure 9B–D). Similar to TNBS colitis, these effects were inhibited by TGFβ-neutralizing antibody but not isotype IgG control treatment in adult mice (Figure 9B–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. 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 8. 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 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 TNBS or TNBS and IgG co-treatment. Ab, antibody; Sac, sacrifice.

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 μg/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.
influencing health and disease susceptibility in adulthood. This work contributes to further understanding this relationship by discovering an epigenetic program modulated by p40-stimulated Setd1b 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. 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. 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. 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. 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 (Lgr5)-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 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 Setd1b 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.
programs at different developmental stages.\textsuperscript{35} The immature human intestinal epithelium has the intrinsic capability of establishing stable host–microbe symbiosis.\textsuperscript{36} Therefore, it is possible that in addition to Setd1b, 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.\textsuperscript{37} TGFβ signaling has multiple roles in suppressing inflammation, such as controlling immune responses through Treg induction and inducing protective cellular responses.\textsuperscript{24–26,38} Clinical studies have shown that the locus encoding SMAD3, an effector of TGFβ signaling, is associated with IBD susceptibility,\textsuperscript{39} and enhancing TGFβ signaling benefits in patients with Crohn’s disease.\textsuperscript{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,\textsuperscript{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.\textsuperscript{8–11} We have now shown that p40 up-regulates Setd1b gene expression and H3K4me3 in IECs in the absence of EGFR expression (Figure 11A and B). Furthermore, p40 was able to stimulate transactivation of the EGFR in IECs without Setd1b gene expression (Figure 11C). 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

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.
pathways to prevent and treat colitis. p40 ameliorated DSS-induced colitis in WT mice. This effect was diminished in Egfr
fl
/Villin (Vil)-Cre mice with the EGFR specifically deleted in IECs. 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
/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, 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. 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.

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 CaCl2 (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 7A and 8A.

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: 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. Cells were labeled with R-phycocerythrin (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. Enteroids and colonoids were cultured in Matrigel (Corning, Bedford, MA) and overlaid
with IntestiCult Organoid Growth Medium (06005; STEM-
CELL 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, tsAS8.21 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 nonper-
missive temperature (37°C) for 3 passages. An EGFR knock
out (EGFRfl/fl) mouse colonic epithelial cell line was gener-
ated from the colonic epithelium of EGFR-null mice crossed
with IntestiCult Organoid Growth Medium (06005; STEM-
CELL 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, tsAS8.21 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 nonper-
missive temperature (37°C) for 3 passages. An EGFR knock
out (EGFRfl/fl) mouse colonic epithelial cell line was gener-
ated from the colonic epithelium of EGFR-null mice crossed
with IntestiCult Organoid Growth Medium (06005; STEM-
CELL 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, tsAS8.21 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 nonper-
missive temperature (37°C) for 3 passages. An EGFR knock
out (EGFRfl/fl) mouse colonic epithelial cell line was gener-
ated from the colonic epithelium of EGFR-null mice crossed
with IntestiCult Organoid Growth Medium (06005; STEM-
CELL Technologies, Vancouver, Canada) in the absence or
presence of p40 (100 ng/mL) in Matrigel and in medium.

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 chro-
matin was diluted in ChIP dilution buffer and immunopre-
cipitated 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’-
GACACTGCGCTGTCGCAAGG-3’ and reverse: 5’-GGGATGC-
GAGGGATCAAGAGG-3’. Results were expressed as a per-
centage 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 TGFβ1 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
TGFβ1 was used to generate the standard concentration
curve. Cell numbers were counted at the end of experi-
ments. The TGFβ1 concentration was representative as of ng/
106 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
with that in the control group. The density fold change was obtained by comparing it with the same sample. The relative band density in the control group was set as 1. The density fold change 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 mean ± 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.

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-Setd1α (44922; Cell Signaling Technology), anti-Setd1β (44922; Cell Signaling Technology), anti-MML1 (14689; Cell Signaling Technology), anti-MML2 (63735; Cell Signaling Technology), anti–WD55 (13105; Cell Signaling Technology), anti–WD55 (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 mean ± SEM.

**References**

1. Lilly DM, Stillwell RH. Probiotics: growth-promoting factors produced by microorganisms. Science 1965; 147:747–748.
2. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 2014;11:506–514.
3. Thomas CM, Versalovic J. Probiotics-host communication: regulation of gut microbiota. Gut Microbes 2010;1:148–163.
4. Vanderpool C, Yan F, Polk DB. Mechanisms of probiotic action: implications for therapeutic applications in inflammatory bowel diseases. Inflamm Bowel Dis 2008; 14:1585–1596.
5. Lichtenstein L, Avni-Biron I, Ben-Bassat O. Probiotics and prebiotics in Crohn’s disease therapies. Best Pract Res Clin Gastroenterol 2016;30:81–88.
6. Yan F, Cao H, Cover TL, Whitehead R, Washington MK, Polk DB. Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. Gastroenterology 2007;132:562–575.
7. Bauerl C, Abitayeva G, Sosa-Carrillo S, Mencher-Beltran A, Navarro-Lieo N, Coll-Marques JM, Zuniga-Cabrera M, Shaikhin S, Perez-Martinez G, P40 and P75 are singular functional muramidases present in the Lactobacillus casei/paracasei/rhamnosus taxon. Front Microbiol 2019:10:1420.
8. Wang L, Cao H, Liu L, Wang B, Walker WA, Acra SA, Yan F. Activation of epidermal growth factor receptor mediates mucin production stimulated by p40, a Lactobacillus rhamnosus GG-derived protein. J Biol Chem 2014;289:20234–20244.
9. Wang Y, Liu L, Moore DJ, Shen X, Peek RM, Acra SA, Li H, Ren X, Polk DB, Yan F. An LGG-derived protein promotes IgA production through upregulation of APRIL expression in intestinal epithelial cells. Mucosal Immunol 2017;10:373–384.
10. Yan F, Cao H, Cover TL, Washington MK, Shi Y, Liu L, Chaturvedi R, Peek RM Jr, Wilson KT, Polk DB. Colon-specific delivery of a probiotic-derived soluble protein ameliorates intestinal inflammation in mice through an
EGFR-dependent mechanism. J Clin Invest 2011; 121:2242–2253.

11. Yan F, Liu L, Dempsey PJ, Tsai YH, Raines EW, Wilson CL, Cao H, Cao Z, Liu L, Polk DB. A Lactobacillus rhamnosus GG-derived soluble protein, p40, stimulates ligand release from intestinal epithelial cells to trans-activate epidermal growth factor receptor. J Biol Chem 2013;288:30742–30751.

12. Allaire JM, Crowley SM, Law HT, Chang SY, Ko HJ, Valance BA. The intestinal epithelium: central coordinator of mucosal immunity. Trends Immunol 2018; 39:677–696.

13. Tamburini S, Shen N, Wu HC, Clemente JC. The microbiome in early life: implications for health outcomes. Nat Med 2016;22:713–722.

14. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, Lee JC, Schumm LP, Sharma Y, Anderson CA, Essers J, Mitrovic M, Ning K, Cleynen I, Theatre E, Spain SL, Raychaudhuri S, Goyette P, Wei Z, Abraham C, Achkar JP, Ahmad T, Aminiejad L, Ananthakrishnan AN, Andersen V, Andrews JM, Baidoo L, Balschun T, Bampton PA, Bitton A, Boucher G, Brand S, Buning C, Cohain A, Cichon S, D’Amato M, De Jong D, Devaney KL, Dubinsky M, Edwards C, Ellinghaus D, Ferguson LR, Franchimont D, Fransen K, Gorelik L, Flavell RA. Transforming growth factor-beta family signalling. Nature 2003;425:577–584.

15. Shen X, Liu L, Cao H, Moore DJ, Wang B, Peek RM, Acra SA, Polk DB. Neonatal colonization of mice with LGG promotes intestinal development and decreases susceptibility to colitis in adulthood. Mucosal Immunol 2017;10:117–127.

16. Shen X, Liu L, Peek RM, Acra SA, Moore DJ, Wilson KT, He F, Polk DB, Yan F. Supplementation of p40, a Lactobacillus rhamnosus GG-derived protein, in early life promotes epidermal growth factor receptor-dependent intestinal development and long-term health outcomes. Mucosal Immunol 2018;11:1316–1328.

17. Takahashi K, Sugi Y, Nakano K, Tsuda M, Kurihara K, Hosono A, Kaminogawa S. Epigenetic control of the host gene by commensal bacteria in large intestinal epithelial cells. J Biol Chem 2011; 286:35755–35762.

18. Ganal SC, Sanos SL, Kallfass C, Oberle K, Johner C, Kirschning C, Lienenklaus S, Weiss S, Staeheli P, Aichele P, Diefenbach A. Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. Immunity 2012; 37:171–186.

19. Shilatifard A. The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. Annu Rev Biochem 2012; 81:65–95.

20. Tanoue T, Atarashi K, Honda K. Development and maintenance of intestinal regulatory T cells. Nat Rev Immunol 2016;16:295–309.

21. Whitehead RH, VanEeden PE, Noble MD, Atalipiotis P, Jat PS. Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. Proc Natl Acad Sci U S A 1993;90:587–591.

22. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003;425:577–584.

23. Lee PS, Chang C, Liu D, Derynck R. Sumoylation of Smad4, the common Smad mediator of transforming growth factor-beta family signaling. J Biol Chem 2003; 278:27853–27863.

24. Chen W, Ten Dijke P. Immune-regulation by members of the TGFbeta superfamily. Nat Rev Immunol 2016; 16:723–740.

25. Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. Nat Rev Immunol 2002;2:46–53.

26. Ihara S, Hirata Y, Koike K. TGF-beta in inflammatory bowel disease: a key regulator of immune cells, epithelium, and the intestinal microbiota. J Gastroenterol 2017; 52:777–787.

27. Means AL, Freeman TJ, Zhu J, Woodbury LG, Marincola-Smith P, Wu C, Meyer AR, Weaver CJ, Padmanabhan C, An H, Zi J, Westinger BC, Chaturvedi R, Brown TD, Deane NG, Coffey RJ, Wilson KT, Smith JJ, Sawyers CL, Goldenring JR, Novitskiy SV, Washington MK, Shi C, Beauchamp RD. Epithelial Smad4 deletion up-regulates inflammation and promotes inflammation-associated cancer. Cell Mol Gastroenterol Hepatol 2018;6:257–276.

28. Freeman TJ, Smith JJ, Chen X, Washington MK, Roland JT, Means AL, Eschrich SA, Yeatman TJ, Deane NG, Beauchamp RD. Smad4-mediated signaling inhibits intestinal neoplasia by inhibiting expression of beta-catenin. Gastroenterology 2012;142:562–571, e562.

29. Lillycrop KA, Burdge GC. Epigenetic mechanisms linking early nutrition to long term health. Best Pract Res Clin Endocrinol Metab 2012;26:667–676.

30. Strober W, Fuss JJ, Blumberg RS. The immunology of inflammatory bowel disease. Annu Rev Immunol 2002;20:495–549.

31. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 1990;98:694–702.

32. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, Glickman JN, Siebert R, Baron RM, Kasper DL, Blumberg RS. Microbial exposure during early life has
persistent effects on natural killer T cell function. Science 2012;336:489–493.

33. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. Science 2016;352:539–544.

34. Clevers H. The intestinal crypt, a prototype stem cell compartment. Cell 2013;154:274–284.

35. Kabiri Z, Greicius G, Zaribafzadeh H, Hemmerich A, Counter CM, Virshup DM. Wnt signaling suppresses MAPK-driven proliferation of intestinal stem cells. J Clin Invest 2018;128:3806–3812.

36. Hill DR, Huang S, Nagy MS, Yadagiri VK, Fields C, Mukherjee Bons B, Dedhia PH, Chin AM, Tsai YH, Thodla S, Schmidt TM, Walk S, Young VB, Spence JR. Bacterial colonization stimulates a complex physiological response in the immature human intestinal epithelium. Elife 2017;6.

37. Clark M, Colombel JF, Feagan BC, Fedorak RN, Hanauer SB, Kamm MA, Mayer L, Regueiro C, Rutgeerts P, Sandborn WJ, Sands BE, Schreiber S, Targan S, Travis S, Vermeire S. American gastroenterological association consensus development conference on the use of biologics in the treatment of inflammatory bowel disease. Gastroenterology 2007;133:312–339.

38. Sedda S, Marafini I, Dinallo V, Di Fusco D, Monteleone G. The TGF-beta/Smad system in IBD pathogenesis. Inflamm Bowel Dis 2015;21:2921–2925.

39. Lees CW, Barrett JC, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. Gut 2011;60:1739–1753.

40. Feagan BG, Sands BE, Rossiter G, Li X, Usiskin K, Zhan X, Colombel JF. Effects of Mongersen (GED-0301) on endoscopic and clinical outcomes in patients with active Crohn’s disease. Gastroenterology 2018;154:61–64, e66.

41. Monteleone G, Kumberova A, Croft NM, McKenzie C, Steer HW, MacDonald TT. Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. J Clin Invest 2001;108:609–619.

42. Fuss IJ, Marth T, Neurath MF, Pearlstein GR, Jain A, Streber W. Anti-interleukin 12 treatment regulates apoptosis of Th1 T cells in experimental colitis in mice. Gastroenterology 1999;117:1078–1088.

43. Neurath MF, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, Mizoguchi A, Mizoguchi E, Muder J, Galle PR, Bhan A, Autschbach F, Sullivan BM, Szabo SJ, Glimcher LH, Blumberg RS. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn’s disease. J Exp Med 2002;195:1129–1143.

44. Lu N, Wang L, Cao H, Liu L, Van Kaer L, Washington MK, Rosen MJ, Dube PE, Wilson KT, Ren X, Hao X, Polk DB, Yan F. Activation of the epidermal growth factor receptor in macrophages regulates cytokine production and experimental colitis. J Immunol 2014;192:1013–1023.

45. Disse RS, Frey MR, Whitehead RH, Polk DB. Epidermal growth factor stimulates Rac activation through Src and phosphatidylinositol 3-kinase to promote colonic epithelial cell migration. Am J Physiol Gastrointest Liver Physiol 2008;294:G276–G285.