thought to influence the development of the T helper cell lineage. Notably, insufficient microbial exposure results in the imbalance of immune responses, leading to allergy and IBD [10]. Previous epidemiological studies connected sanitary conditions in childhood with these immunological disorders, originating what is now known as the "hygiene hypothesis" [11]. This theory was left unverified for many years until recently, when Cahenzli et al. reported that microbial stimuli early in life are sufficient to suppress IgE production in mice [9]. Notably, exposure to microbiota in adulthood, no matter how diverse, failed to reduce serum IgE levels. These findings establish that exposure to diverse microbiota early in life is required to regulate IgE responses, exemplifying the hygiene hypothesis [11]. Early exposure to intestinal microbial stimuli may also be a key in shaping the T_H2-biased immune response, most likely by inducing the differentiation of other T_H cell subsets—including T_H1, T_H17, and Treg cells. In fact, inoculation with certain bacterial strains in GF mice incites the development of splenic T_H1, intestinal T_H17, and Treg cells [4, 6, 8, 12]. Since there are many excellent review articles describing commensal microbiota-dependent effector T_H cell induction [13–15], we will only briefly describe it here.

Colonization of GF mice with *Bacteroides fragilis*, a gut-indigenous gram-negative bacterium, polarizes splenic $CD4^+$ T cells toward a T_H1 cells. Polysaccharide A (PSA) produced by *B. fragilis* is a prerequisite for the induction of T_H1 cells [8] and serves as a TLR2 ligand for $CD4^+$ T cells [16]. Likewise, stimulation of T_H1 cells with the lipopeptide TLR2 ligand, Pam3, augments IFN-γ production through p38 MAPK and JNK activation [17]. Pam3-mediated IFN-γ induction does not require TCR stimulation, indicating the importance of an innate immunity-like signaling pathway in T_H1 cell activation.

Segmented filamentous bacteria (SFB) mainly residing in the ileum of rodents are a potent inducer of T_H17 cells. Colonization by SFB appears to facilitate small intestinal T_H17 differentiation in a TLR signaling-independent manner (Fig. 1), as the population of T_H17 in colonic lamina propria was comparable between WT and MyD88/TRIF double knockout mice [18]. This event was also found to be independent of NOD-RIP2 signaling [4]. Importantly, SFB-dependent upregulation of serum amyloid A plays a key role in the induction of T_H17 in the small intestine [4]. Goto and colleagues recently reported that SFB antigen presentation by dendritic cells (DCs) via MHC class II-dependent pathway is essential for the induction of T_H17 cells although presentation by group 3 innate lymphoid cells (ILC3) negatively regulates T_H17 differentiation [19]. While SFB is responsible for T_H17 development in the ileum, colonic T_H17 cells are induced by bacteria-derived ATP. Colonic $CD70^+$ DCs that express the ATP-sensing receptors, P2X and P2Y, preferentially induce the differentiation of naive T cells into T_H17 cells [18]. However, several questions remain concerning which bacterial strains release ATP, as well as the mechanism by which DCs recognize its presence across an epithelial barrier.

Development of Treg cells by commensal bacteria

Treg cells prevent the generation of unfavorable immune responses against commensal microbiota by producing the anti-inflammatory cytokine, IL-10, functioning as a sink for secreted IL-2 by CD25 expression, and through CTLA-4-mediated interactions with CD28 ligands (e.g., CD80 and CD86) expressed on DCs [20–22]. Treg cells are currently classified into thymus-derived Treg (tTreg) and peripherally induced Treg (pTreg) cells according to their origin [23]. Of note, these subsets were previously designated as naturally occurring Treg (nTreg) and induced Treg (iTreg) cells, respectively, and iTreg cells are now referred to as in vitro-generated Treg cells. tTreg and pTreg cells can be distinguished by the differential expression of Helios and neuropilin1 (Nrp1), with low to undetectable expression of these markers present in pTreg cells [24–26]. However, these may not be strict phenotypic markers, since pTreg cells can exhibit Nrp1 expression under inflammatory conditions, as observed in an experimental model of autoimmune encephalomyelitis (EAE) [26].

The number of colonic, but not small intestinal, Treg cells is reduced in GF as compared to conventional mice [6]. Notably, there is a striking reduction in the $Helios^-Foxp3^+$ population that can be rescued by inoculation with commensal bacteria. The same is true in mammalian newborns. Mammalian fetuses are maintained under sterile conditions and are only exposed to microbes immediately after birth. A subpopulation of bacteria colonize the mucosal surface, such as the colon, and facilitate the induction, migration, and proliferation of colonic Treg cells. As a consequence, the percentage of Treg cells among total $CD4^+$ T cells in the colon rapidly increases during infancy [6, 27]. This exposure-mediated expansion was apparently confined to the colon, and it did not expand to systemic lymphoid tissues, such as the spleen. These observations indicate that bacterial colonization is a key determinant for the development of Treg cells in the intestinal immune system. This notion is further supported by the finding that the $Nrp1^-$ Treg (pTreg) subset is abundant in the colonic lamina propria, but not in the spleen [26]. Several recent studies have identified bacterial strains responsible for the induction of functional Treg cells in the colon. For example, Round and Mazmanian identified that TLR2 stimulation on $CD4^+$ T cells with PSA of *B. fragilis* induces the development IL-10-producing Treg cells [28]. Furthermore, Atarashi et al. demonstrated that exposure to a cocktail of 46 *Clostridium* strains belonging to clusters IV and XIVa is sufficient to promote the differentiation of colonic $Helios^-$ Treg cells in GF mice [6]. Oral inoculation with feces isolated from the 46 strain-associated gnotobiotic mice into infant

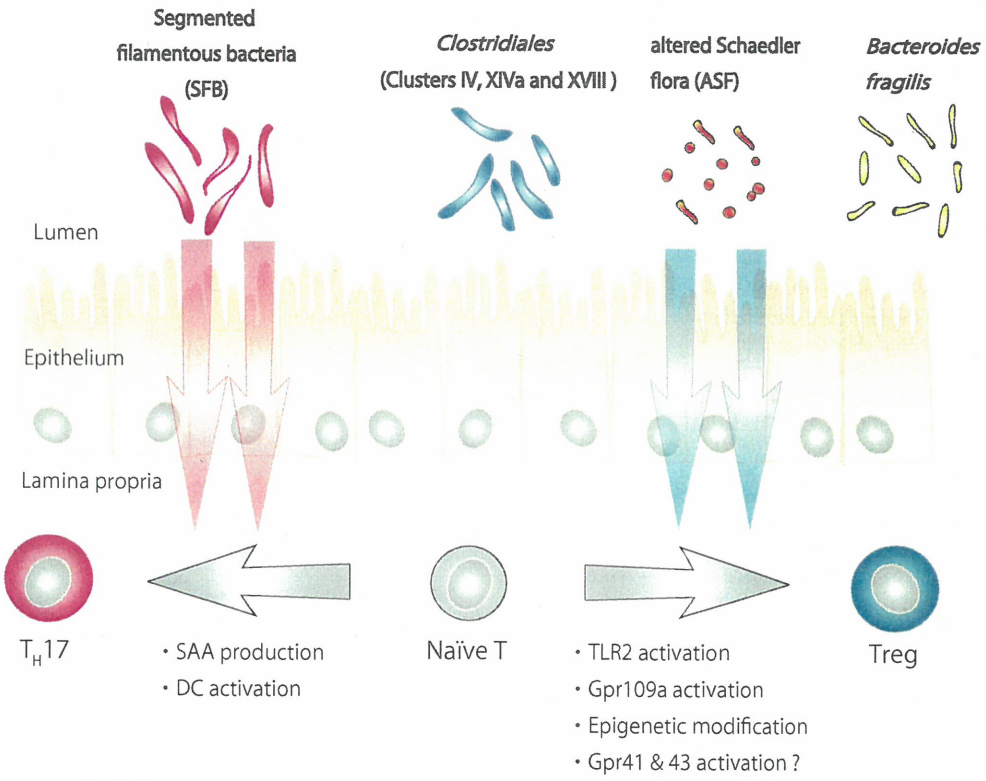

Fig. 1 T_H17 and Treg induction by commensal bacteria. SFB colonization potently induces T_H17 development in the small intestine through SAA production. Conversely, *Clostridiales* clusters IV, XIVa, and XVIII SAA facilitate Treg development in the colon through Gpr109 activation on DCs and epigenetic modifications in DCs and $CD4^+$ T cells

specific pathogen-free (SPF) mice attenuates ovalbumin (OVA)-induced IgE responses and the development of DSS-induced colitis by facilitating the differentiation of colonic Treg cells. Importantly, a decrease in the abundance of *Clostridiales* cluster IV and XIVa species (e.g., *Clostridium leptum* and *Clostridium coccoides*, respectively) has been implicated in the development of IBD [29, 30]. In accordance with these reports, a mixture of 17 strains from *Clostridiales* clusters VI, XIVa, and XVIII isolated from human feces also exhibit this Treg-inducing activity [31], raising the possibility that these species may contribute to the maintenance of intestinal immune homeostasis in humans. Unlike *B. fragilis*-dependent IL-10^+ Treg induction, the 46 *Clostridiales* strains robustly induce Treg cell development in the absence of MyD88, a key adaptor responsible for TLR-dependent signaling [6]. Furthermore, SPF mice deficient in RIP2 or CARD9, adaptor molecules for NOD or Dectin-1, respectively, possess normal numbers of colonic Treg cells. Based on these observations, pattern recognition receptors are dispensable for *Clostridiales*-dependent Treg induction. Recently, we and several other groups have demonstrated that commensal microbe-derived short-chain fatty acids (SCFAs)—particularly those from *Clostridiales* —promote the colonic migration and de novo generation of Treg cells (Fig. 2) [32–35]. These reports were consistent with studies identifying that *Clostridiales* clusters IV and XIVa can actively produce butyrate [31, 36, 37].

Geuking et al. showed that inoculation of GF mice with altered Schaedler flora (ASF) leads to activation and de novo generation of Treg cells in the colon (Fig. 1) and reciprocally diminishes T_H1 and T_H17 responses [5]. ASF is a defined set of benign intestinal commensal microbiota composed of eight bacterial species, which have been selected to normalize cecal volume of GF mice. The bacterial community in ASF-colonized mice actively undergoes microbial fermentation to produce small molecule metabolites. It is interesting to speculate whether these metabolites could mediate the induction of Treg cells in the colon, as observed in *Clostridiales*-associated mice.

Short-chain fatty acids facilitate gut homing and development of Treg cells

SCFAs are produced in the cecum (murine) and colon (human) by the bacterial fermentation of nondigestible materials, such as dietary fiber and resistant starches [38]. Among them, butyrate plays a central role in de novo generation of pTreg cells in the colon [34]. In support of this idea, administration of butyrylated high-amylose maize starch, used to deliver butyrate to the colonic lumen, significantly increased the number of colonic Treg cells. In addition, when drinking water containing a high concentration (150 mM) of SCFAs was provided to GF or antibiotic-treated mice, all of the three

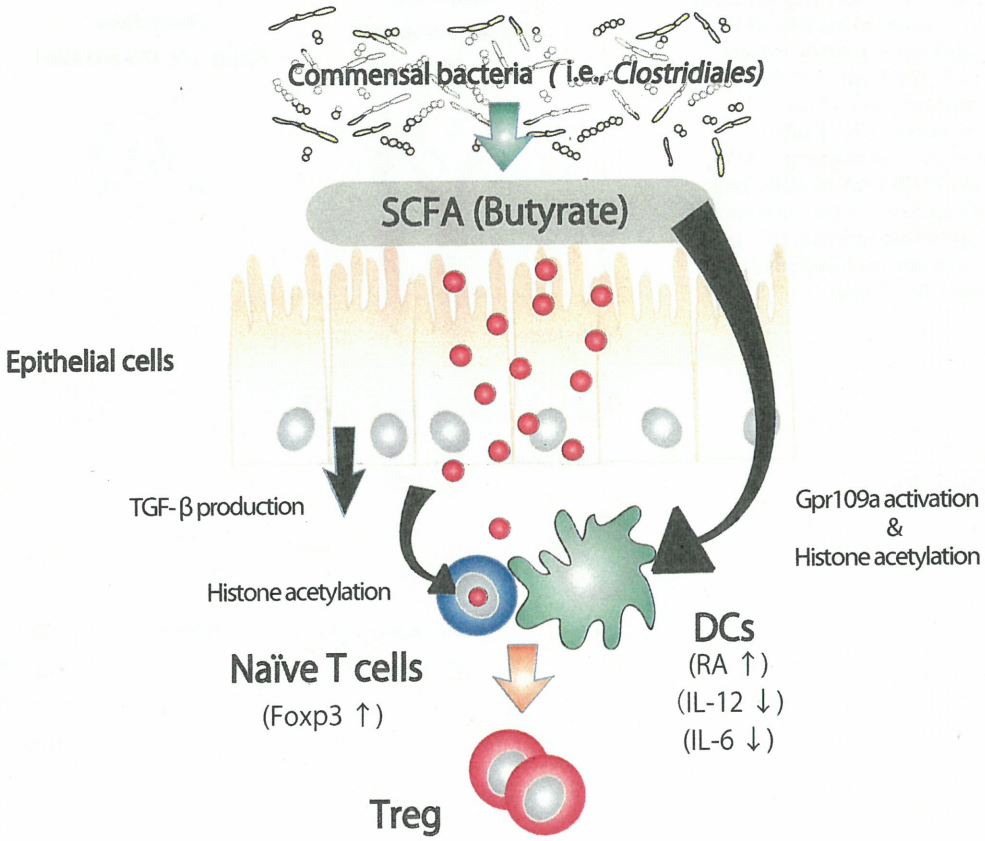

Fig. 2 Commensal microbe-derived SCFAs, in particular butyrate, induce the differentiation of colonic Treg through epigenetic modifications and Gpr109a activation. Butyrate elicits histone H3 acetylation at *Foxp3* gene regulatory regions and facilitates the differentiation of naive $CD4^+$ T cells into Treg cells. In contrast, a mixture of SCFAs triggers TGF-β secretion by epithelial cells through an unknown mechanism. Butyrate can also stimulate DCs and macrophages to produce IL-10 and retinoic acid, both of which are important for the development of IL-10-producing Tregs in the colon. Therefore, SCFAs contribute to the maintenance of intestinal homeostasis through multiple mechanisms

SCFAs—particularly acetate and propionate—were sufficient to enhance the frequency of colonic Treg cells [32, 33]. Conversely, in vitro experiments determined that butyrate could most efficiently increase the conversion of naive $CD4^+$ T cells into $Foxp3^+$ cells, whereas physiological concentrations of propionate and acetate imparted moderate or no effects, respectively [34]. The discrepancy between these in vivo and in vitro findings suggests that SCFAs increase the number of colonic Treg cells not only by promoting differentiation, but also through other mechanisms, such as regulating the colonic migration of extraintestinal Treg cells. Indeed, orally administered propionate induced the upregulation of Gpr15, a Treg-specific gut-homing molecule, in Treg cells [32, 39]. We suspect that a large portion of orally administered SCFAs are absorbed into the portal vein and/or utilized by epithelial cells in the upper part of the intestine prior to reaching the colon. Given that commensal microbiota produces SCFAs predominantly in the large intestine, the oral administration of SCFAs may be inappropriate to evaluate the physiological roles of those locally produced. Alternatively, low fiber diets containing acetylated, propionylated, or butyrylated high-amylose maize starches are suitable means to investigate the biological functions of each SCFA individually. These chemically modified starches resist small intestinal amylolysis and reach the colon in quantities where the esterified acid is released by commensal microbes, subsequently raising in levels within the colonic content [38]. Consistent with the in vitro observations, the differentiation of colonic Treg cells is significantly augmented by diets containing butyrylated starch, whereas those with propionylated or acetylated starches either slightly induced or failed to induce Treg cells in SPF mice, respectively. Collectively, these data suggest that locally produced butyrate plays a central role in de novo Treg development in the colon, whereas the oral administration of acetate and propionate seems to mediate the migration of Tregs into the colon through the upregulation of Gpr15 in a Gpr43-dependent manner.

Butyrate facilitates Treg differentiation by at least two different mechanisms. First, exposing naive $CD4^+$ T cells to butyrate enhances the acetylation status of histone H3 in the promoter and CNS3 enhancer regions of the *Foxp3* gene loci [34]. Second, butyrate alters the phenotype of DCs. Research by Singh et al. suggests that the activation of Gpr109a, a receptor for butyrate and niacin, induces Raldh1 expression in DCs to promote the production of RA production, leading to the induction of Treg differentiation (Fig. 2) [35]. Furthermore, systemic administration of propionate in mice following challenge with house dust mites dampens T_H2 responses and suppresses allergic airway inflammation in a Gpr41-dependent manner [40]. Notably, lung DCs from propionate-treated mice displayed an attenuated capacity to promote T_H2 cell development. This suggests that the propionate-Gpr41

axis in DCs is critical for containment of T_H2-dependent allergic response in the lung. Importantly, diets rich in fermentable carbohydrates can promote the outgrowth of bacteria belonging to the *Bacteroidetes* phylum, leading to increased serum levels of acetate and propionate and dampening the allergy-prone T_H2 response [40]. This observation supports the notion that gut commensal bacteria-derived metabolites may influence the development of both the local and systemic immune system.

Histone modification of $CD4^+$ T cells by commensal bacteria

Epigenetic regulations, including DNA methylation and histone tail modifications, play significant roles in regulating gene expression in a heritable manner [41]. The chemical modifications of DNA and histones incite conformational changes in chromatin structures toward one of two fundamental states: open chromatin permissive for gene transcription and closed chromatin to induce gene silencing [42]. Modifications on the amino-terminal tails of histones include acetylation, methylation, phosphorylation, and ubiquitylation [43, 44]. Among them, acetylation neutralizes the cationic charge of histones, which is important to tightly bind DNA. As such, histone tail acetylation eventually generates an open chromatin state [45]. Accumulating studies have demonstrated that DNA methylation and histone H3 acetylation/trimethylation within promoters and/or regulatory elements of genes encoding key transcription factors and effector molecules play critical roles in the cell fate decision and function of T cells [46–48]. Moreover, memory T cells also maintain the epigenetic marks similar to those in effector T cells, enabling them to rapidly exert a secondary response upon re-encounter with antigens by recruiting RNA polymerase II to open chromatin surrounding cytokine-encoding genes. Thus, the effector genes in memory T cells are kept under a "poised transcriptional state" [46].

In addition, epigenetic regulation is also closely associated with cell fate decisions and the stabilization of cell lineages during the development of Treg cells. The differentiation and function of Treg cells is primarily controlled by the master transcription factor, Foxp3. *Foxp3* expression is regulated by its promoter and by intragenic enhancer elements, termed conserved noncoding sequence 1–3 (CNS1–3) (Fig. 3). Among them, CNS1 and CNS3 are involved in de novo Foxp3 expression, whereas CNS2 is essential for the maintenance of Foxp3 expression [49, 50]. Notably, the CNS1 region, known as the "TGF-β sensor," harbors binding elements for Smad3, RAR, and NFAT and can be activated by TGF-β, retinoic acid, and TCR stimulation, respectively. These stimuli are required for *Foxp3* induction. In addition, CNS3 harbors a c-Rel-binding element that is responsive to signals emanating from the TCR and costimulatory receptors [50]. Furthermore, the histone acetylation status of the promoter and CNS regions is positively correlated with expression of *Foxp3*. Commensal microbe-derived butyrate is well-documented to inhibit class I and IIa histone deacetylases (HDACs) among four classes of HDACs [51, 52]. Exposure of naive $CD4^+$ T cells to butyrate enhances the acetylation status of histone H3 in the promoter, CNS1, and CNS3 regions of the *Foxp3* gene loci to promote its expression (Fig. 3) [34]. These observations demonstrate that butyrate facilitates the accessibility of the Foxp3 promoter and enhancer regions to transcriptional regulators to the enhancer elements, as well as the promoter region through acetylation of *Foxp3* gene locus. T-bet, GATA3, and RORγt are key transcription factors for T_H1, T_H2, and T_H17 cells, respectively, and butyrate affects neither the acetylation nor the expression levels of these transcription factors [34]. Likewise, butyrate inhibits T_H1 cytokine IFN-γ in rat lymph node lymphocytes stimulated with T cell mitogen concanavalin A, whereas butyrate enhances the production of IL-10 [53]. Of note, butyrate suppresses pro-inflammatory cytokine production even in innate immune cells such as macrophages and DCs most likely through HDAC inhibition [33, 54]. However, further investigation is necessary to clarify the detailed mechanisms by which butyrate facilitates anti-inflammatory response through HDAC inhibition.

Posttranscriptional modification of Foxp3 and the other transcription factors by HDACs

Eighteen HDAC genes—comprising 11 classical HDAC and seven sirtuin isoforms—have been identified in humans. These HDACs are classified into five groups: class I (HDAC1, 2, 3, and 8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC6 and 10), class III (sirtuins 1–7), and class IV (HDAC11) [55, 56]. HDACs contribute to posttranscriptional modifications on lysine residues of histone proteins, but also those of transcription factors (TFs), including STAT5 and Foxp3. Importantly, STAT5 and Foxp3 activation and/or stabilization are regulated by lysine acetylation. Extensive studies from the Hancock group revealed that HDAC6, HDAC9, and sirtuin-1 negatively regulate the induction and stability of Foxp3 through the posttranscriptional modification of STAT5, NF-κB, and Foxp3 itself [57]. Based on the observation that $HDAC6^{-/-}$ Treg cells display a greater increase in the acetylation and expression of Foxp3 compared to $Sirt1^{-/-}$ or $HDAC9^{-/-}$ Treg cells [57, 58], HDAC6 appears to play a prominent role in Foxp3 instability through its deacetylation. Conversely, HDAC9 ablation in Treg cells resulted in the increased acetylation and subsequent phosphorylation of STAT5 to mediate IL-2-dependent signal transduction. Phosphorylated STAT5 binds to the *Foxp3* promoter [59] to upregulate *Foxp3* expression. On the other hand, ablation of Sirt1

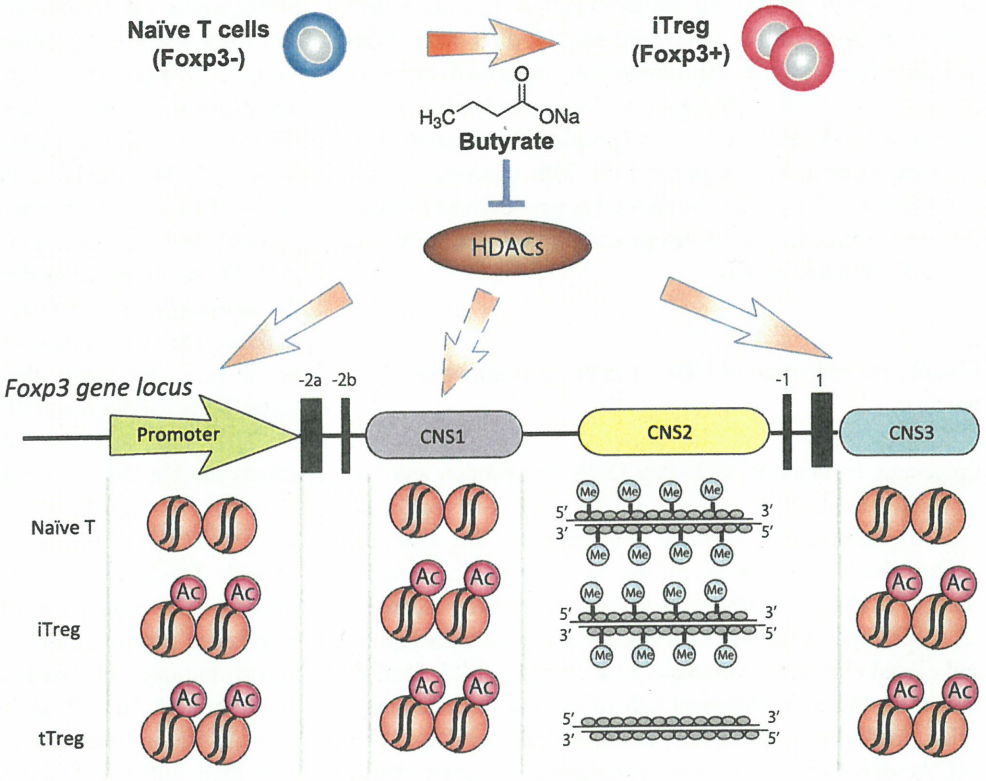

Fig. 3 Butyrate facilitates histone H3 acetylation preferentially in the promoter, CNS1, and CNS3 regions of the *Foxp3* gene locus. Histone acetylation and DNA methylation status among naive $CD4^+$ T, iTreg, and tTreg cells are schematically shown. *Ac* acetylation, *Me* methylation

enhances p65 acetylation [60], an important subunit of classical NF-κB pathway, that can also bind the *Foxp3* promoter region. As described above, butyrate is well-known to inhibit most HDACs, with the exception of classes III and IIb, namely HDAC6 and 10 [56]. Strikingly, butyrate treatment in naive T cells facilitated the histone H3 acetylation in the *Foxp3* promoter regions and its expression at the mRNA and protein levels (Fig. 3). Therefore, we speculated that HDAC9 inhibition and the resulting STAT5 acetylation might comprise a part of the mechanism underlying the butyrate-dependent selective induction of *Foxp3* independent of T-bet, Gata3, and RORγt—although this speculation is yet to been proven. An alternative, but not mutually exclusive possibility, is that certain class I HDAC isoform(s) may contribute to the selective induction of Foxp3 by butyrate. However, butyrate-mediated Gpr109a activation is also known to induce Foxp3 expression following RA production in DCs [35]. As such, further experimentation should be performed to clarify the detailed mechanisms by which butyrate induces Foxp3 expression, even in the absence of DCs (such as in naive CD4+ T cells stimulated with agonistic antibodies to TCR).

DNA methylation and the maintenance of Treg homeostasis

DNA methylation has been associated with alterations in chromatin state and transcription. In particular, cytosine methylation within CpG clusters in promoter regions correlates with gene silencing and thus utilized as a repressive marker. CNS2 is defined as the Treg-specific demethylated region (TSDR), and it harbors CpG islands. Importantly, TSDR demethylation secures the stability of Foxp3 expression [61, 62]. DNA methylation is mediated by the DNA methyltransferase (Dnmt) family of enzymes, of which Dnmt1 functions to accurately maintain DNA methylation during the S phase of the cell cycle [63]. Dnmt1-deficient T cells can express Foxp3 upon TCR and IL-2 stimulation even in the absence of TGF-β essential for its induction in normal $CD4^+$ T cells [64]. Treatment of $CD4^+$ T cells with 5-azacytidine (5-AzaC), an inhibitor of DNA methylation, also incites Foxp3 upregulation in the presence or absence of TGF-β [64, 65]. These in vitro studies imply that TCR and IL-2 signals are sufficient to induce Foxp3 expression under the condition of DNA hypomethylation.

Apart from these in vitro observations, the DNA methylation machinery is critical for Treg homeostasis in the colon, as demonstrated by the reduction of proliferating Treg cells in the mice with the T cell-specific deletion of *Uhrf1* (ubiquitin-like, with PHD and RING finger domains 1) (Uhrf1 cKO mice) [27]. Uhrf1, also known as Np95 in mouse and ICBP90 in human, plays a nonredundant role in the maintenance of DNA methylation by recognizing and recruiting Dnmt1 to hemimethylated DNA in proliferating cells [66]. Colonization of GF mice with gut microbiota induced the vigorous proliferation of colonic Treg, but not conventional $CD4^+$ T cells [27].