Microbiota derived short chain fatty acids promote histone crotonylation in the colon through histone deacetylases

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The recently discovered histone post-translational modification crotonylation connects cellular metabolism to gene regulation. Its regulation and tissue-specific functions are poorly understood. We characterize histone crotonylation in intestinal epithelia and find that histone H3 crotonylation at lysine 18 is a surprisingly abundant modification in the small intestine crypt and colon, and is linked to gene regulation. We show that this modification is highly dynamic and regulated during the cell cycle. We identify class I histone deacetylases, HDAC1, HDAC2, and HDAC3, as major executors of histone decrotonylation. We show that known HDAC inhibitors, including the gut microbiota-derived butyrate, affect histone decrotonylation. Consistent with this, we find that depletion of the gut microbiota leads to a global change in histone crotonylation in the colon. Our results suggest that histone crotonylation connects chromatin to the gut microbiota, at least in part, via short-chain fatty acids and HDACs.

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Histone post-translational modifications (HPTMs) are fundamental regulators of gene expression and are tightly controlled by enzymes that respond to the availability of metabolic precursors. Histone acetylation is a well-studied HPTM usually linked to active genes and is added to various lysine groups of histones by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). More recently, various longer chain acylations of histones have been characterized, including crotonylation, butyrylation, and hydroxybutyrylation. These acylations have been linked to cellular metabolism, because they reflect the availability of the short-chain fatty acids (SCFAs) and their coenzyme A adducts in the cell (reviewed in refs. ). This has been demonstrated by introducing crotonate (2-butenoate), an SCFA moiety produced intracellularly as an intermediate of metabolic processes, to the cell culture media which affects histone crotonylation levels. Histone crotonylation reprograms the functionality of nucleosomes, setting it apart from histone acetylation, by favoring interactions with a specific set of chromatin modifiers.

A link between cellular metabolism, SCFAs, and transcriptional regulation is particularly relevant in the intestine where microorganisms break down complex carbohydrates to SCFAs such as acetate, propionate, and butyrate. SCFAs are an important component of normal gut physiology by providing a major energy source for the colon epithelial cells. They also affect cellular functions and modulate immune responses, in part by affecting gene expression and the epigenome through inhibiting HDACs. Here, we explore histone crotonylation in intestinal...
epithelial cells and show that histone H3 lysine 18 crotonylation (H3K18cr) is readily detectable in this tissue and that histone crotonylation is regulated by class I HDACs. Our findings suggest that histone crotonylation connects chromatin structure to the gut microbiota via HDACs and SCFAs.

Results

Histone crotonylation abundance in the intestine. Western blot analysis of the level of histone crotonylation in several tissues (colon, brain, liver, spleen, kidney) using the antibodies against crotonyl-lysine and H3K18cr indicates that the greatest levels of histone crotonylation are in colon and, interestingly, brain among the tissues analyzed (Fig. 1a). An approximately 70 kDa protein in the brain extract is recognized by the antibody against crotonyl-lysine, indicating the presence of a crotonylated non-histone protein in the brain.

To characterize the pattern of histone H3 PTMs in intestinal cells, we analyzed small intestine epithelium, colon epithelium, and small intestine crypt-enriched fractions by LC-MS/MS (Supplementary Fig. 1, LC-MS: liquid chromatography-mass spectrometry). This analysis allowed the site-specific identification of 38 differentially modified peptides (Supplementary Table 1). Among them, we identified lysine methylation and acetylation combinations at several H3 peptides and increased levels of monomethylated H3K4 in both small intestinal crypt and colon fraction compared to the fraction from whole small intestine epithelium. Di- and tri-methylated H3K4 were below the detection limit, likely due to their low abundance (Supplementary Fig. 1 and Supplementary Table 1). Most interestingly, we identified histone lysine crotonylation at several histone H3 residues: K9, K14, K18, K27, and K56 (Table 1, Supplementary Fig. 2, and Supplementary Table 1). H3K18 crotonylation (H3K18cr), in association with H3K23 acetylation, was the most abundant histone H3 crotonylation mark in the intestine, characterized it further by chromatin immunoprecipitation-sequencing (ChiP-seq). This analysis showed that H3K18cr is associated with transcription start sites (TSS) (Figs. 2a–d), similar to H3K4me3 (Fig. 2c), as has been shown before in macrophages. To investigate the link between H3K18cr and transcription, we performed RNA sequencing (RNA-seq) on colon epithelial crypts and found higher gene expression levels associated with increased H3K18cr enrichment over TSS (Fig. 2c). KEGG pathway analysis of genes with high levels of H3K18cr over their TSS highlights various pathways, in particular several involved in cancer, suggesting that deregulation of histone crotonylation may be linked to cancer (Fig. 2f, Supplementary Fig. 6).

Microbiota and histone crotonylation in the mouse colon. As histone crotonylation has been linked to cellular metabolism and we found it to be relatively abundant in the gut, we hypothesized that this modification may be linked to the SCFAs that are generated by intestinal microbiota, especially in the colon. Treatment of mice for 3 days with a cocktail of antibiotics led to a reduction of the bacterial load (Suppl. Figure 7a) and a reduction of SCFAs in colon luminal content and serum (Fig. 3a). This was linked to a noticeable global decrease of histone crotonylation in the colon tissue, which was particularly clear with histone H4K8 crotonylation but also for H3K18 and H4 crotonylation (as detected by the anti-Kcr antibody) (Figs. 3b, c). Interestingly, we found that the treatment with antibiotics was also linked to increased amounts of HDAC2 (Figs. 3d, e; changes in HDAC1 and HDAC3 levels were not consistent, Supplementary Fig. 7b).

SCFAs promote histone crotonylation. Next we investigated if the drop in SCFA concentration in the colon lumen and serum could account for the observed global reduction in histone crotonylation. When added to the media of human colon carcinoma cells (HCT116) and mouse small intestinal organoids, the SCFA crotonate promoted H3 and H4 crotonylation, thereby confirming previous findings (Fig. 4a, Supplementary Fig. 8a). We found that histone crotonylation is highly dynamic, being increased within 2 h of crotonate addition to the medium and lost within 1 h of wash out (Supplementary Fig. 8b). The chemically related SCFA butyrate, which is naturally present in the intestine, also promoted histone crotonylation at physiologically relevant concentrations, both in gut organoids and HCT116 cells (Fig. 4a, Supplementary Fig. 8c). Therefore, these in vitro observations are consistent with the idea that depletion of the microbiota leads to a decrease in histone crotonylation of the colon epithelium because of the drop in SCFAs.

HDAC inhibitors upregulate histone crotonylation. Since butyrate is a well-known HDAC inhibitor, we hypothesized that HDACs might regulate histone crotonylation and their inhibition explains the increase in histone crotonylation upon butyrate treatment. To test this hypothesis, we treated colon organoids with butyrate and found an increase in H3K18cr, as detected by the anti-Kcr antibody (Figs. 5a, b). However, this increase was not observed after treatment with pan-HDAC inhibitors such as vorinostat and valproic acid

| Table 1 List of K-crotonyl histone-modified peptides |
|-----------------|----------------|----------------|------|----------------|---|------|
| Modified sequences | Modified sites | Gene names | Charge | m/z | RT | Score |
| K(cr)STGKK(*)APR | K9 | HIST2H3 | 2+ | 507.7921 | 71.3 | 60.3 |
| K(*)STGKK(cr)APR | K14 | HIST2H3 | 3+ | 338.8639 | 72.9 | 33.4 |
| K(cr)QLATKAAR | K18 | HIST2H3 | 2+ | 550.3367 | 90.8 | 93.1 |
| K(cr)QLATK(ac)AAR | K18 | HIST2H3 | 2+ | 548.8273 | 91.0 | 89.1 |
| K(cr)SAPATGGVKKPHR | K27 | HIST2H3 | 3+ | 531.3110 | 85.4 | 39.7 |
| YQK(*)SYELLIR | K56 | HIST2H3 | 2+ | 659.8710 | 129.0 | 47.3 |

Summary of identified crotonylation sites on histone H3 from the crypt fraction of the small intestine using MS/MS analysis

S: small intestine, cr: crotonylation, ac: acetylation, (*) chemical alkylation
We found that other HDAC inhibitors are also able to promote histone crotonylation in HCT116 cells, including trichostatin A (TSA), the clinically relevant suberanilohydroxamic acid (SAHA), and valproic acid (VPA, Supplementary Fig. 9). MS275 (entinostat), a selective inhibitor of class I HDACs, which has been tested in various clinical trials, significantly promoted histone crotonylation at sub-micromolar concentrations in HCT116 cells (Fig. 4b). This outcome cannot be explained by cross-reactivity of the anti-crotonyl antibodies to acetylated histones (Supplementary Fig. 5). A ChIP-qPCR for H3K18cr observed a significant increase in enrichment upon MS275 treatment in different genomic loci including repetitive regions, suggesting that class I HDAC inhibition leads to a spread of histone crotonylation into intergenic areas (Fig. 4c).

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To investigate how HDAC inhibitors change the distribution of histone crotonylation over the genome, we performed ChIP-seq analysis of H3K18cr and H3K18ac. This showed a relative decrease of read counts over TSS when HCT116 cells were treated with MS275 (Fig. 5a, b). This could be explained with an overall increase of these marks over the genome outside of the TSS regions. ChIP-seq measures the relative proportional distribution of a mark over the genome. If the proportional increase of H3K18cr over TSS is lower than the one outside of TSS, an increase of histone crotonylation over regions outside of TSS (which cover a much greater proportion of the genome than all TSS combined) will lead to an apparent proportional drop of this mark in TSS regions.

Interestingly, H3K18cr and H3K18ac peaks correlate highly and occur at the same sites genome-wide in HCT116 cells, with or without MS275 treatment (Figs. 3c–g). MS275 treatment of HCT116 cells caused changes in gene expression, which are linked to changes in H3K18 acetylation and crotonylation (Fig. 5f, g). Thus, gene silencing and chromosome segregation defects occurring upon HDAC inhibitor treatments may not only be due to aberrant histone acetylation, but could also be linked to histone crotonylation.

Histone crotonylation is linked to the cell cycle. We performed cell cycle arrest and release experiments in presence or absence of the class I HDAC specific inhibitor MS275. The results indicated that histone crotonylation, including H3K18cr, is linked to cell cycle progression, showing an increase in S and G2-M phase over G1 arrested cells (Fig. 6, lanes 1–8). This experiment also suggests that class I HDACs may be involved in this cell cycle-mediated modulation of histone crotonylation levels, as the G1-arrest-linked downregulation of histone crotonylation is inhibited in the presence of MS275 compared to untreated cells (Fig. 6, lanes 1, 2, 9, 10). In addition, in MS275-treated cells, histone crotonylation was modulated during the cell cycle, but levels remained generally higher compared to untreated cells (Fig. 6, lanes 9–16).

Class I HDACs are histone decrotonylases. To test further if we could link class I HDACs to decrotonylation, we transiently over-expressed a fusion protein of HDAC1 with green fluorescent protein (GFP), an N-terminal deletion mutant version of this or GFP in HCT116 cells, sorted the transfected cells, and monitored histone crotonylation by western blot analysis. This showed that the over-expression of HDAC1 but not its mutant version with an N-terminal truncation (or GFP alone) caused some reduction of global histone H3K18 and H4K8 crotonylation (Supplementary Fig. 10).

To validate the ability of HDACs to decrotonylate histones, we tested this activity with recombinant, purified HDACs in vitro experiments and found that the class I HDAC enzymes HDAC1, HDAC2, and the HDAC3/Ncor1 complex efficiently removed the crotonyl moiety of in vitro crotonylated histones (Fig. 7a). Using this in vitro approach, we estimated enzyme kinetic values for the decrotonylation of H3K18cr and deacetylation of H3K18ac and found that HDAC1 has a similar capacity to perform each reaction. HDAC1 has lower V$_{\text{max}}$ and K$_{\text{cat}}$ values for the decrotonylation compared to the deacetylation reactions indicating that HDAC1 has a higher maximum turnover with the acetyl substrate than the crotonyl substrate (Fig. 7b, Supplementary Fig. 11). We found that the K$_{\text{m}}$ with acetylated histones of 1.47 μM was similar to a published K$_{\text{m}}$ of HDAC with a BOC-lys (acetyl)-AMC of 3.7 μM. Published K$_{\text{m}}$ values with different fluorescent or tritiated acetyl substrates vary from 0.68 to 78 μM. Relative to this, the K$_{\text{m}}$ of the crotonyl substrate was lower at 0.42 μM suggesting that HDAC1 can efficiently bind crotonylated histones at low concentrations of substrate. This may be relevant in the context of the cell where HDAC1 could respond to minor fluctuations in the availability of the crotonyl substrate.

In vitro, HDAC inhibitors TSA and butyrate reduced decrotonylation by HDAC1 significantly, demonstrating that the HDAC inhibitors, including SCFAs, affect decrotonylation by HDACs directly (Fig. 7c). TSA inhibited deacetylation at lower doses (IC50 ~5 nM) than decrotonylation (IC50 ~50 nM) highlighting the differential impact of inhibitors on HDAC activity and potential for specific drug development. We also found that crotonate inhibited decrotonylation and deacetylation in vitro (Fig. 7c). Remarkably, at crotonate concentrations of 6 mM or higher, HDAC1 catalyzed the addition of the crotonyl moiety to lysine groups of histones (Fig. 7d). This did not occur with acetate or butyrate, possibly due to their lower chemical reactivity compared to crotonate. While this finding may not be directly biologically relevant, as it is unlikely that such high concentrations of crotonate are found intracellularly, it highlights the reversible nature of the decrotonylation reaction by HDAC1.

A previous study dismissed HDACs 1–11 as decrotonylases based on an in vitro fluorometric assay using a peptide analog. We also did not find decrotonylase activity for HDAC1 using the same assay, but found that the BOC-Lys(crotonyl)-AMC compound used is inhibitory for the deacetylation step of the BOC-Lys(acetyl)-AMC. This suggests that the HDACs interact with BOC-Lys(crotonyl)-AMC, but potentially cannot release the compound (Fig. 8). Future studies will examine to what extent HDACs require the peptide context of histone H3 for efficient decrotonylation activity.

Discussion

A key finding of our study is that histone crotonylation is surprisingly abundant in the intestinal epithelium, especially the crypt fraction of the small intestine and the colon. This supports the notion that histone crotonylation does not simply reflect ‘metabolic noise’, as in the ability of p300 to use crotonyl-CoA to modify histones, but has specific functions, which might vary between tissues. We also find high levels of histone crotonylation...
Fig. 3 Microbiota depletion affects colonic histone crotonylation and HDAC2. Antibiotic treatment led to a decrease in luminal and serum SCFA levels in mice (n ≥ 3, from experiment 2). A acetate, propionate, and butyrate concentrations were measured in the colon lumen and serum by gas chromatography. Unpaired t-tests were conducted, *p-value < 0.05 and ***p-value < 0.001. Values of zero were below detectable levels. B Quantifications of western blot analysis of colon extracts from untreated and treated mice, n ≥ 3. Experiments 1 and 2 are repeat experiments. Center values (small bar) are the average of the treatment group relative to the untreated group. Two-way ANOVA (two-tailed) was performed on quantified bands to compare the effect of treatment for both experiments together; * corresponds to a p-value of < 0.05 and *** corresponds to < 0.001. The quantification showed a statistically significant decrease in H4 crotonylation as detected by the anti-Kcr antibody and in H4K8cr, H4K8ac, and H3K18cr levels upon antibiotics treatment. C Global changes in various colon histone crotonylation and acetylation marks and HDAC2 as seen in representative western blots of colon extracts, from experiment 1. D Two-way ANOVA was performed on quantified bands from western blotting analysis with anti-HDAC2. A statistically significant increase was observed (p-value < 0.05)
in the brain. As SCFAs are taken up from the colon into the blood stream (as supported by our own data, Fig. 3a), SCFAs might be taken up by brain cells, where they may affect histone modifications. Future studies will determine if there are brain-specific functions for histone crotonylation.

We found that the SCFA butyrate, a well-known HDAC inhibitor, promotes histone crotonylation in intestinal cell and organoid culture. This led us to test other HDAC inhibitors, which showed that class I specific HDAC inhibitors strongly promoted histone crotonylation at concentrations at which they affected histone acetylation and implicated class I HDACs in histone deacetylation. Our in vitro assays of recombinant, purified class I HDAC1-3 show that they are deacetylases and demonstrates that HDAC inhibitors, including butyrate, reduce deacetylation. Furthermore, the low fixed value of HDAC1 with crotonylated histones suggests that it can efficiently deacetylolate this relatively less abundant histone modification. Together, these findings strongly suggest that SCFAs such as butyrate promote histone crotonylation by inhibiting the deacetylase activity of HDACs in colon epithelial cells. Future studies will address to what extent SCFAs also promote crotonylation by acting as substrates for the generation of intracellular crotonyl-CoA.

Our finding that class I specific HDAC inhibitors promoted global histone crotonylation led us to explore where this increased histone crotonylation (H3K18cr) occurs in the genome in cultured colon carcinoma cells. We show that the increase is not because of a greater number of crotonylation peaks but a diffuse spread of crotonylation genome-wide, including within repetitive regions. Therefore, while H3K18cr ‘peaks’ do exist over TSS, these findings suggest that pervasive crotonylation also occurs across the genome and that it is controlled by class I HDACs.

Additionally, we show that histone crotonylation is modulated during the cell cycle and that class I HDACs play a role in this regulation. In synchronized cells, we find an increase of histone crotonylation during S phase which is enhanced when cells are treated with the HDAC inhibitor MS275. Newly synthesized histones are acetylated on several specific lysine residues (e.g., K5 and K12 on histone H4; K14, K18 on histone H3) prior to their deposition onto nascent chromatin. These acetylations are then globally removed following nucleosome assembly, which is important for the maintenance of repressive chromatin, such as pericentromeric heterochromatin. Class I HDACs are targeted to replicating chromatin and mediate this deacetylation. Therefore, the levels of these histone acetylations are modulated in a cell cycle and HDAC-dependent manner. In this study, we provide evidence that histone crotonylation marks behave similarly to the pre-deposition acetylation marks: they are low in G1 arrested cells and increase as cells progress through S phase and this modulation depends on class I HDACs. Therefore, histone crotonylation is not simply modulated through dilution with non-crotonylated histones through the cell cycle, but appears to be actively regulated by HDACs. Future studies will address if histone crotonylation is also mediated prior to histone deposition onto nascent chromatin and will address how HDACs and other factors regulate histone crotonylation concomitant with chromatin replication.

Importantly, consistent with our finding that microbiota-generated SCFAs such as butyrate promote histone crotonylation in vitro, we show that depletion of the microbiota led to a loss of histone crotonylation. Our data suggest that the levels of histone crotonylation in the gut reflect the generation of SCFAs by the microbiota. Given the important role of microbiota-generated SCFAs in modulating immunity and metabolism, our work...
proposes that the signaling between the microbiota and chromatin might be mediated through histone crotonylation. Future work will explore further the roles of histone crotonylation in normal gut physiology including host–microbiome interaction, inflammation, and disease.

A surprising finding of our work is that depletion of the microbiota of mice with antibiotics not only led to a drop in luminal and serum SCFAs, but also an increased expression of HDAC2 in colon. A reduction in histone crotonylation is consistent with both changes. A loss of SCFAs upon microbiota...
depletion may lead to stabilization or increased expression of HDAC2. In this context, it is interesting to note that a previous study has shown that the stability of HDAC2 is selectively reduced by the HDAC inhibitor valproic acid (a branched SCFA) or butyrate and this is mediated by Ubc8-RLL1 targeted proteasomal degradation.22 Remarkably, HDAC2 expression has been linked to colorectal tumorigenesis33, 34. It will be interesting to examine how this links to our finding that H3K18cr peaks occur over a significant number of genes linked to cancer pathways. Future studies will examine how HDAC2 expression, microbiota, and SCFAs are linked and how this affects histone cromotylation over specific genes and cancer progression in the colon.

While our study was in revision, a study by Yu, Wong, and collaborators also demonstrated histone deacromylation activity of class I HDACs using transfection experiments in cell culture.23 Our study complements and extends these and related findings36,37 as we explore the effect of HDAC inhibitors on the distribution of histone cromotylation, the link between histone cromotylation and the cromycylic acid and investigate how the microbiota influence these processes. This expands our understanding of the functional spectrum of these exciting drug targets in cancer therapy and other diseases38, 39 and provides a basis for the development of specific inhibitors of the deacromylation versus deacetylation activity of these enzymes.

Methods

Mice. Mice were C57BL/6 background, details available on request. Male mice were used for all experiments. All mice were kept in specific pathogen-free conditions and fed ad lib. Mice for the ChIP and RNA-seq experiments were housed at the Babraham Institute Biological Service Unit. All experimental protocols at Babraham Institute were approved by the Babraham Research Campus local ethical review committee and the Home Office (PPL 80/2488 and 70/8994). The antibiotic treatment experiments were performed at the University of Campinas. Male C57BL/6 mice at age 8–12 weeks were provided by the Multidisciplinary Centre for Biological Investigation (CEIMB) and all the experimental procedures were approved by the Ethics Committee on Animal Use of the Institute of Biology, University of Campinas (protocol number 3742-1). No sample size calculation was performed, and the number of samples were excluded from the analysis. No blinding was conducted.

Antibiotic treatment of mice. Mice received 200 µl of a mixture of antibiotics (5 mg/ml of neomycin, 5 mg/ml of gentamicin, 5 mg/ml of ampicillin, 5 mg/ml of metronidazole, and 2.5 mg/ml of vancomycin, Sigma Aldrich) daily for 3 days by gavage. The weight of the animals was monitored throughout the experiment. At the end of the treatment period, feces were collected and snap-frozen in liquid nitrogen. After that, the animals were anesthetized using a mix of ketamine and xylazine (300 and 30 mg/kg, respectively) and the blood was collected by cardiac puncture. The blood was maintained at room temperature (RT) for 30 min and then centrifuged (3000 g for 8 min). The serum was collected and frozen at −80 °C. After euthanizing the animals by cervical dislocation, the entire intestine was opened longitudinally and washed three times with ice cold Hank’s balanced salt solution without Ca2+/Mg2+ (HBSS). Intestinal and colon epithelium were collected with 30 mM EDTA/HBSS on ice with shaking for 30 min for small intestine and 1 h for colon; 50 ml Falcon tubes containing the tissue were then shaken vigorously by hand (2–3 shakes/second) for 5 min. The colon epithelium was incubated for an additional 10 min on ice in 30 mM EDTA/HBSS and further shaken for 5 min. Mucus and sub-mucosa were removed by dripping the material through a 100 µm followed by a 75 µm cell strainer. The extracted cells were pelleted at 475g at 4 °C for 10 min. The cells were washed with ice cold HBSS and re-pelleted as above for further use. For the H3K18cr ChIP, three colonies were combined.

Small intestinal and colon epithelium extraction. Animals were sacrificed by cervical dislocation or exposure to CO2. Dissected small intestines and colons were opened longitudinally and washed three times with ice cold Hank’s balanced salt solution without Ca2+/Mg2+ (HBSS). Intestinal and colon epithelium were collected with 30 mM EDTA/HBSS on ice with shaking for 30 min for small intestine and 1 h for colon; 50 ml Falcon tubes containing the tissue were then shaken vigorously by hand (2–3 shakes/second) for 5 min. The colon epithelium was incubated for an additional 10 min on ice in 30 mM EDTA/HBSS and further shaken for 5 min. Mucus and sub-mucosa were removed by dripping the material through a 100 µm followed by a 75 µm cell strainer. The extracted cells were pelleted at 475g at 4 °C for 10 min. The cells were washed with ice cold HBSS and re-pelleted as above for further use. For the H3K18cr ChIP, three colonies were combined.

Purification and enzymatic digestion of histones. For isolating a crypt-encephrinated fraction, villi were removed by shaking the small intestine pieces for 10 s after incubation for 5 min on ice in HBSS–30 mM EDTA and before the additional incubation in HBSS–30 mM EDTA for 15 min. Histones were then acid extracted following a protocol published in ref.42: one small intestine/colon was used per extraction followed by M5 analysis. Small intestine epithelium, colon epithelium, and a crypt-encephrinated fraction were homogenized in lysis buffer (10% sucrose; 0.5% Triton, pH 7.4) and centrifuged at 3000 g for 10 min. The supernatant was transferred to microtubes, and 1 µl of 1 M hydrochloric acid, and 100 µl of butanol were added. The tubes were vortexed for 2 min and centrifuged at 18,000g for 1 min. The supernatant was transferred to microtubes, and 1 µl was injected into the gas chromatograph. For serum measurements, 20 mg of sodium chloride, 10 mg of sodium chloride, 20 µl of 1 M hydrochloric acid, and 100 µl of butanol were added to 100 µl of serum samples. Tubes were vortexed and centrifuged as previously described and 1 µl was injected into the gas chromatograph. To quantify SCFAs, a calibration curve for the concentration range of 0.15–1 mg/ml was constructed. SCFAs measurements were performed following a recently published protocol41: chromatographic analyses were performed using an Agilent 6890 system with ExChrom software, equipped with a 7683B automatic liquid sampler, a flame ionization detector (FID) (Agilent Technologies, USA), and a fused-silica capillary RTX-WAX (Restec Corporation, U.S.) with dimensions of 60 m × 0.25 mm internal diameter (i.d.) coated with a 0.15-µm thick layer of polyethylene glycol. The initial oven temperature was 100 °C (hold 2 min), which was increased to 200 °C at a rate of 15 °C/min. The FID temperature was maintained at 260 °C, and the flow rates of H2, air, and the make-up gas N2 were 35, 350, and 25 ml/min, respectively. Sample volumes of 1 µl were injected at 260 °C using a split ratio of approximately 25:1. Nitrogen was used as the carrier gas at 25 ml/min. The runtime for each analysis was 12.95 min.

Purification and enzymatic digestion of histones. For isolating a crypt-encephrinated fraction, villi were removed by shaking the small intestine pieces for 10 s after incubation for 5 min on ice in HBSS–30 mM EDTA and before the additional incubation in HBSS–30 mM EDTA for 15 min. Histones were then acid extracted following a protocol published in ref.42: one small intestine/colon was used per extraction followed by M5 analysis. Small intestine epithelium, colon epithelium, and a crypt-encephrinated fraction were homogenized in lysis buffer (10% sucrose; 0.5 mM EGTA, pH 8.0; 15 mM NaCl; 60 mM KCl; 15 mM HEPES; 0.5% Triton; 0.5 mM PMSF; 1 mM DTT; 5 mM NaF; 5 mM Na3VO4; 5 mM Na-butyrate, cocktail of protease inhibitors (Sigma)). Nuclei were separated from the cytoplasm by centrifugation on sucrose cushions, washed in cold PBS, and then extracted in 0.4 N HCl overnight (o.n.) at 4 °C. Core histones, together with linker histones protein, were dialyzed against 100 mM ice-cold acetic acid. The concentration of purified samples was measured using the Bradford protein assay. Approximately 10 µg were separated on SDS-PAGE and bands corresponding to the histones H3 were excised and in-gel digested. Briefly, gel bands were cut in pieces and destained with repeated washes in 50% acetonitrile (ACN) in H2O, alternated with dehydration steps in 100% ACN. Gel pieces were then in-gel chemically alkylated by incubation with D6-acetic anhydride (Sigma 175641) in 1 M NH4HCO3 and CH3COONa solution as catalyst. After 3 h at 37 °C with high shaking in a thermomixer, chemically modified gel slices were washed with NH4HCO3, alternated with ACN.
Histone crotonylation is cell cycle regulated by class I HDACs. Cell cycle block and release experiment on HCT116 cells using CDK4/6 inhibitor abemaciclib with and without MS275. Lanes 1–9: asynchronous cells, lane 2: G1 arrested cells, lanes 3–8: increase in histone crotonylation (Kcr), H3K18cr and H3K18ac upon release into S phase. Lanes 10: abemaciclib with and without MS275. Lanes 1 and 9: asynchronous cells, lane 2: G1 arrested cells, lanes 3–10: H3K18cr and H3K18ac are upregulated during a G1 arrest and S phase when class I HDACs are inhibited with MS275. For the experiments in lanes 9–16, cells were blocked in G1 using 15 nM abemaciclib in the presence of 5 μM MS275 and released into S phase in the presence of 1 μM MS275. Cell cycle profiles are shown at the top, western blots in the middle, and quantifications of those, as calculated relative to H3 and normalized to the DMSO (vehicle) sample, at the bottom. Representative of two experiments is shown.

**LC-MS/MS.** The peptide mixtures were analyzed by online nano-flow LC-MS/MS using an EASY-nLC 1000 (Thermo Fisher Scientific) connected to a QExactive (Thermo Fisher Scientific) through a nano-electrospray ion source. The nano-LC system was operated in one column setup with a 23-cm analytical column (75 μm inner diameter, 350 μm outer diameter) packed with C18 resin (ReproSil, Dr. Maisch, Germany) configuration. Solvent A was 0.1% formic acid (FA) in ddH2O and solvent B was 80% ACN with 0.1% FA. Samples were injected in an aqueous 1% TFA solution at a flow rate of 500 nl/min. Peptides were separated with a gradient of 0–40% solvent B for 100 min, followed by a gradient of 40–60% in 5 min, and 60–95% over 5 min at a flow rate of 250 nl/min. The Q-Exactive instrument was operated in the data-dependent acquisition (DDA) to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300–1150) were analyzed in the Orbitrap detector with resolution R = 60,000 at m/z 200. The 10 most intense peptides were sequentially isolated to a target value of 3 × 106 and fragmented by high-energy collisional dissociation (HCD) with a normalized collision energy setting of 27%. The maximum allowed ion accumulation times were 20 ms for full scans and 50 ms for MS/MS and the target value for MS/MS was set to 1 × 106. Standard mass spectrometric conditions for all experiments were as follows: spray voltage, 2.4 kV; no sheath and auxiliary gas flow.

**MS data analysis and relative abundance profiling.** Acquired RAW data were analyzed by MaxQuant(MQ) software v1.5.2.8, using the Andromeda search engine16. Uniprot Mouse database (70,902 entries) was used for peptide identification. Enzyme specificity was set to Arg-C. Estimated false discovery rate of all peptide identifications was set at a maximum of 1% (Decoy database-based approach). Mass tolerance for searches was set to a maximum of 6 parts per million (ppm) for peptide masses and 20 ppm for HCD fragment ion masses. A maximum of three missed cleavages was allowed. In the search, we focused on lysine methylation and acylation, including as variable modifications: D3-acetylation (+45.0294 Da), D3-acetylation (+45.0294 Da) plus monomethylation (+14.016 Da), and diacetylation/tetraacetylation (+90.0588 Da). The percentage of modified peptides was used to determine the amount of Kcr, H3K18cr and H3K18ac.
dimethylation (+28.031 Da), trimethylation (+42.046 Da), acetylation (+42.010 Da), and crotonylation (+68.074 Da). The use of high-accuracy criteria for HCD fragment ions tolerance (20 ppm) guarantee the capability to discriminate among other possible forms of acylation (e.g., lysine butyrylation (70.0418 Da) and β-hydroxybutyrylation (86.0367 Da)), therefore they were not included in the search. MaxQuant search results were exported and peptides with Andromeda score <60 and localization probability score <0.75 were removed. Filtered data were subjected to manual inspection and validation using the viewer.exe module integrated in MQ software. Extracted ion chromatograms were constructed for each precursor based on the m/z value, using a mass tolerance of 10 ppm with a mass precision up to four decimals. For each histone-modified peptide, the relative abundance percentage (RA%) was estimated by dividing the area under the curve (AUC) of each modified peptide over the sum of the areas corresponding to all observed isoforms of that peptide, including the unmodified forms. Significant changes among crypt
Chemicals) for 48 h and released by washing 2× with PBS and adding fresh HCT116 cells were blocked at G1 with 15 nM of abemaciclib (LY2835219, Selecky DMEM media containing glucose and pyruvate, 10% FBS, 2 mM L-glutamine, 100 µl 50 mM Tris–HCl, pH 8.0, 10 mM EDTA 1% SDS (Chip lysis buffer) on ice. Sonication of the chromatin to 100–500 bp fragment size in polystyrene tubes was performed with a water-cooled Bioruptor (Diagenode), high power, 4°C, 12 cycles, 30 s on, 30 s off. The sonicated material was transferred to a 1.5-ml tube, incubated for 30–45 min on ice, and centrifuged at 15,000 g at 4°C to precipitate SDS. We pooled three colons to perform three Chip experiments, with 20–25 µg equivalent of DNA for each experiment. For immunoprecipitation, the chromatin was diluted to 1:10 with ChIP dilution buffer (16.7 mM HCl, pH 8.0, 1.2 mM EDTA, 167 mM NaCl, 1% Triton X-100), 50 µM acetylcholine (Sigma) and H3K4me3 antibody (Active Motif Cat: AT-H3K4me3). Libraries were prepared with Illumina’s TruSeq kit. After agarose gel purification, sequencing libraries were sequenced on an Illumina HiSeq2500 sequencer according to manufacturer’s instructions. RNA samples were processed with the KAPA Library Preparation Kit for Illumina®. cDNA fragments were purified using the KAPA PureMag PCR purification kit and sequenced on the Illumina MiSeq (2×150 bp) platform. All reads were mapped to the hg19 (Genome Build 19) human genome reference, and peaks were determined using the MACS2 tool. Genes with a fold change >2 and adjusted p value <0.05 were considered as significant.

Whole cell extract preparations. For whole cell extract preparation, cells were detached with trypsin, washed in PBS, and boiled in Laemmli sample buffer (Biorad) for 5 min. The extracts were briefly sonicated to remove high molecular weight DNA before loading on an SDS-polyacrylamide gel for electrophoresis.

Intestinal organoid seeding and cultures. Small intestinal crypts were derived from wildtype C57BL/6 mice using a slightly modified protocol from reference 12. In brief, collected small intestinal crypts were opened longitudinally and the majority of villi removed by gentle scraping with a coverslip. The tissue was cut to 3–5 mm pieces, washed five times with cold PBS, and vigorous shaking. After 30 min incubation on ice with 2 mM EDTA/PBS, the remaining villi were removed with more shaking and tissue pieces were incubated for additional 30 min in 5 mM EDTA/PBS on ice. After another short shake, crypts were passed through 40-µm cell strainer and pelleted at 425×g 1500 rpm at 4°C for 10 min. The pellet was washed with cold PBS and 100–200 crypts were suspended in 50 µl Red-phenol-free Matrigel (BD Biosciences) droplets. After polymerization, complete medium containing advanced DMEM/F12 (Sigma), 2 mM Glutamine (Invitrogen), 10% (v/v) HTF (Gibco), 100 U/mL pen/strep (Invitrogen), 1 µM apicidin (Sigma), 1X B27 supplement (Invitrogen), 1X N2 supplement (Invitrogen), 50 ng/ml mouse EGF (Peprotech), 100 μg/ml mouse Noggin (Peprotech), and 10% human R-spondin-1-conditioned medium from R-spondin-1-transfected HEK293T cells (Calibrex) was added to the cultures. Medium was changed every 3 days and organoids passed after 7–10 days.

ChIP-seq of extracted colon epithelium. The colon epithelium cell pellet was resuspended in 10 µl of PBS-1% formaldehyde and fixation was carried out for 10 min, at RT with gentle agitation. The reaction was quenched by the addition of glycine (0.125 M final concentration) and the cells were pelleted at 475×g at 4°C for 10 min, washed once with PBS, re-pelleted and either snap-frozen in liquid nitrogen for storage at –80°C or processed further immediately. The cell pellet was washed 2× with PBS and incubated for 2 h at 4°C on a rotating wheel. Following this incubation, tubes were spun briefly and bound material was separated from unbound using a magnetic stand on ice.

RNA samples were processed with the KAPA Library Preparation Kit for Illumina®. cDNA fragments were purified using the KAPA PureMag PCR purification kit and sequenced on the Illumina MiSeq (2×150 bp) platform. All reads were mapped to the hg19 (Genome Build 19) human genome reference, and peaks were determined using the MACS2 tool. Genes with a fold change >2 and adjusted p value <0.05 were considered as significant.
The publication provides a detailed protocol for the ChIP-qPCR analysis of H3K4me3 and H3K18ac, along with various bioinformatic and computational tools used to analyze the data. The methods section includes descriptions of the materials used, such as antibodies (anti-H3K18ac, anti-H3K4me3, etc.), cell lines (HCT116, NBL2), and reagents (acetyl chloride, crotonoyl chloride, etc.). The procedures for treating cells (e.g., with histone deacetylase inhibitors), preparing DNA samples, and performing qPCR are outlined. Bioinformatic analysis involves the use of tools like QIAGEN’s Quan-tumgene, which is used to quantify the expression levels of genes. The results are then analyzed using software like GraphPad Prism for statistical analysis and visualization.

Table 2: Primers used in ChIP-qPCR

| Name        | Feature          | Forward (5′−3′)                  | Reverse (5′−3′)                  |
|-------------|------------------|----------------------------------|----------------------------------|
| up PUMA     | Intergenic       | GTCGACCCAGTTACCAACCCCTT          | CCCAACTGTCCCTGTCGTGA             |
| ADAD1       | TSS of not expressed gene | ACTTCAGGGAAGGAGGGAAGGACTT        | TCACTGGCAGAGGGTTTTTT             |
| CLDN7       | TSS of expressed gene | AGCAAGGCTGTGCCTGTGTC            | CCACTGGAACCTAAAGGCGG             |
| pZ1         | TSS of expressed gene | TTGGTGTTGTCCGACGTCTCCGG         | AAATAGGCAACACGCTTCTCTTTT         |
| PUMA        | TSS of expressed gene | TCACTTCCAAACACTGGCTTT           | AAAGGGGACCCAGGGCCTTGGGG          |
| Chr1 SAT    | Repetitive region | CGACTACCTTGAAAGAGAC            | TCCACGAGGAAGGACCAAGAA            |
| Chr4 SAT    | Repetitive region | GAACCTGTGTTGCTTGGTACA          | GTATGCCCACACTCCTTTAC             |
| SAT2        | Repetitive region | TGATGATGATGCTACGTCGGA           | TACATGATCCTGGTCTGCC              |
| NBL2        | Repetitive region | TCCCACAGGATCTGGTTAA            | TGGGCAAGAACCTCCTTTGC             |

ChIP-qPCR data analysis. Prior to analysis, a logarithmic transformation of the data was carried out. Subsequently, we carried out a two-way ANOVA test followed by Holm-Sidak’s multiple comparison test. Data were analyzed using the GraphPad Prism Software.

In vitro enzymatic assays of histone deacetylation and deacetylation. Recombinant human histone H3.1 (NEB) was acetylated or crotonylated in vitro using the recombinant catalytic domain of p300 (human, ENZO) in presence of acetyl-CoA or crotonyl-CoA (Sigma-Aldrich), respectively. The reaction was carried out with 5.65 µM histone H3.1 and 0.66 µM P300 catalytic domain in 50 mM Tris-HCL, pH 8, 50 mM KCl, 0.1 mM EDTA, 0.01 % Tween-20, 10% glycerol, 1 mM DTT, and 87 µM acetyl-CoA or crotonyl-CoA at 30 °C for 2 h. The reaction was stopped by heating at 65 °C for 5 min and the histones were dialyzed with two volumes of HDAC buffer (25 mM Tris-HCL, pH 7.5, 50 mM KCl, 1 mM MgCl₂, 1 mM ZnSO₄) for either deacetylation or deacetylation reactions. Deacetylation/deacetylation was typically performed with 1.75 µM modified histones and 0.12 µM HDAC1 (recombinant, human, Active Motif) or 0.18 µM HDAC2 (recombinant, human, Active Motif) at 30 °C and stopped at 95°C for 1 min. Five different modified histone concentrations and five different time points were performed in triplicate for the deacetylation and deacetylation reactions.

Each reaction was spotted in quadruplicate and left to dry before being rinsed with transfer buffer and TBS-T. Western blot was performed using anti-H3K18ac and anti-H3K4me3 antibodies. Spots were quantified using ImageJ and spot intensity was converted to substrate concentration by multiplying each relative value by the concentration of modified histone in the reaction mix. Substrate concentration against time were plotted for each histone concentration and a linear regression was fitted for the first 30 ± 1 min of the reaction, as appropriate. The rate of each reaction set was plotted as replicates against substrate concentration. GraphPad Prism Version 7 software was used to calculate K_m, V_max, and K_cat.

Colorimetric deacetylation assay. Synthesis of BOC-Lys(acetyl)-AMC and BOC-Lys(crotonyl)-AMC and the colorimetric assay was essentially as in ref. 2, with some modifications: for the synthesis of crotonyl-N-hydroxysuccinimide, N-hydroxysuccinimide (149 mg, 6 equivalents, eq) was dissolved in 2.5 ml anhydrous dichloromethane to which disopropylethylamine (167 mg, 6 eq) was added. The solution was cooled in an ice bath and trans-crotonyl chloride (135 mg, 6 eq) was added and the reaction stirred at 20 °C for 3 h. Ethyl acetate (5 ml) was added and the solution washed with 5 ml brine, dried over magnesium sulfate and concentrated. The crude product was dissolved in 3 ml dry ACN and the appropriate amount used in the next step. Acetyl-N-hydroxysuccinimide was prepared in the same way but using acetyl chloride rather than crotonyl chloride. For the synthesis of BOC-Lys(crotonyl)-AMC, BOC-Lys-AMC acetate salt (100 mg, 1 eq) purchased from Bachem AG was dissolved in 4 ml water:ACN (1:1). Sodium bicarbonate (19.95 mg, 1.1 eq) was added in 1 ml water followed by trans-crotonyl-N-hydroxysuccinimide ester (2 eq) in 2 ml ACN. The reaction was left for 18 h and then concentrated to remove the ACN. The product was extracted into ethyl acetate, dried over anhydrous magnesium sulfate and concentrated. The product was purified on silica in dichloromethane:methanol (15:1). BOC-Lys(acetyl)-AMC

Bioinformatic analysis. RNA-seq: Sequencing reads were adaptor trimmed using Trim Galore! (version 0.4.2) and mapped to the mouse genome (GRCm38/mm10) reference genome with HISat2 (version 2.0.5). Analysis of RNA-seq data was performed with SEQUONK version 3.0 on filtered reads with a MAPQ score of >60 for uniquely mapped reads. Read counts were quantified using the RNA-seq quantitation pipeline implemented in Sequonk, quantifying only probes with at least one read. Probe read values were corrected for transcript length and divided into percentile bins according to their average expression levels of three replicates. ChIP-qPCR analysis: For mouse colon and HCT116 H3K18ac, two replicates and for H3K4me3 three biological replicates were used. Sequencing reads were adaptor trimmed using Trim Galore! (version 0.3.8 and 0.4.2) and mapped to the mouse genome (GRCm38/mm10) or human (GRCh38) reference genomes with Bowtie2 (version 2.0.4). Analysis of ChIP-qPCR data was performed with SEQUONK version 3.0 on filtered reads with a MAPQ score of >42, duplicate reads were always removed. For Fig. 2b, the genome was segmented in 1000 bp non-overlapping ‘probes’ and read counts were quantified for each probe and normalized to the largest dataset. For Fig. 2c, H3K4me3 peaks were identified using the MACS peak finder, embedded in the SEQUONK program with the ChIP-qPCR data of H3K4me3 from colon source cells and INPUT as reference. Selected fragment size was 300 bp and p-value significance threshold was 10⁻². Reads were quantitated in and ±5 kbp around the MACS peaks. For Fig. 2d, read counts were quantified over the TSS (using a window of ±1 kbp upstream of genes) and probes that had more than 100 reads in input were removed in both input and ChIP. For Fig. 2e, read counts were quantified over the TSS (using a window of ±0.5 kbp upstream of transcripts) and were overlapped with RNA-seq data. KEGG pathway analysis was with DAVID 6.8 with electronic annotations excluded.
was prepared in the same way but using acetyl-N-hydroxylysucine rather than crotonyl-N-hydroxylysucine.

The enzymatic reaction was set up in a 96-well plate in triplicate in a 51-μl volume with 0.035 μM HDAC1 and 0.2 mM BOC-Lys(acetyl)-AMC/BOC-Lys (crotonyl)-AMC in HDAC buffer (see above) at 30 °C for 2 h. After this, 5 μl of a 1 mg/ml trypsin solution in 1 mM HCl was added and the mixture was incubated 1 h at 37 °C. Fluorescence was read with a Pherastar plate reader at 360 nm excitation/450 nm emission.

Data availability
Next-generation sequencing data have been submitted to GEO under accession code GSE96035. The authors declare that all other data supporting the findings of this study are within the manuscript and its supplementary files or are available from the corresponding authors upon request.

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Author contributions
P.V.-W., T.B., M.A.R.V., J.D., and R.F. conceived the research and designed the experiments. A.C. and T.B. performed mass spectrometry analysis; P.J. performed ChIP-seq in colon epithelium; A.L. performed RNA-seq of colon epithelium; R.F., R.O.C., J.L.F., and M.A.R.V. performed antibiotics treatment experiments in mice and bacterial load measurements; W.R.R., F.T.S., and C.M.F. performed SCFAs measurements; J.K. performed immunohistochemistry; R.F., S.B., Z.H., and P.V.-W. performed in vitro enzymatic assays; C.S. and E.S. performed ChIP-seq and RNA-seq of HCT116 cells; E.S. performed the cell cycle experiments; J.C. performed chemical synthesis; R.F., J.D., C.S., E.S., A.L., H.B., J.K., R.O.C., J.L.F., F.T.S., H.P., M.S., R.M., C.M., and P.V.-W. performed further experiments; R.F., R.O.C., J.G., A.L., and M.V. set up gut organoid culture; R.F., C.S., P.J., A.L., C.M.F., A.C., T.B., M.A.R.V. and P.V.-W. performed the data analysis; P.V.-W. wrote the manuscript with inputs from almost all co-authors, especially R.F.

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