



RESEARCH PAPER

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Fatty acids produced by the gut microbiota dampen host inflammatory responses by modulating intestinal SUMOylation

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ABSTRACT

The gut microbiota produces a wide variety of metabolites, which interact with intestinal cells and contribute to host physiology. The effect of gut commensal bacteria on host protein SUMOylation, an essential ubiquitin-like modification involved in various intestinal functions, remains, however, unknown. Here, we show that short chain fatty acids (SCFAs) and branched chain fatty acids (BCFAs) produced by the gut microbiota increase protein SUMOylation in intestinal cells in a pH-dependent manner. We demonstrate that these metabolites inactivate intestinal deSUMOylases and promote the hyperSUMOylation of nuclear matrix-associated proteins. We further show that BCFAs inhibit the NF- κ B pathway, decrease pro-inflammatory cytokine expression, and promote intestinal epithelial integrity. Together, our results reveal that fatty acids produced by gut commensal bacteria regulate intestinal physiology by modulating SUMOylation and illustrate a new mechanism of dampening of host inflammatory responses triggered by the gut microbiota.

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Introduction


The gut microbiota produces a wide variety of metabolites diffusing to the intestinal mucosa and modulating intestinal cell activities.¹ Some of these metabolites may even cross the intestinal barrier and reach distant organs via the bloodstream or via nerve communications. Fatty acids constitute a major class of metabolites produced by intestinal bacteria. They include the so-called Short Chain Fatty Acids (SCFAs), which are carboxylic acids with aliphatic tails of one to six carbons.² Acetate, butyrate, and propionate are the main SCFAs produced in the human colon and derive mostly from the anaerobic catabolism of dietary fibers by intestinal bacteria.^{3,4} Branched Chain Fatty Acids (BCFAs), such as isobutyrate, isovalerate, or 2-methylbutyrate, constitute another class of fatty acids produced by bacteria with one or more methyl branches on the carbon chain. BCFA mostly derives from the breakdown of proteins by

intestinal bacteria and more particularly from the catabolism of branched-chain amino-acids (valine, leucine, and isoleucine, producing isobutyrate, isovalerate, or 2-methylbutyrate, respectively).⁵

Fatty acids regulate intestinal cell activities by various mechanisms. They may bind to specific receptors expressed on intestinal cells, such as GPR41/FFAR3, GPR43/FFAR2, and GPR109A, and activate various signaling pathways.⁶ Fatty acids may also directly enter into intestinal cells by passive diffusion or by facilitated transport. Fatty acids are weak organic acids, which exist in solution either as acidic or basic forms. Only the acidic (uncharged) forms may passively diffuse across the plasma membrane, whereas the basic (negatively charged) forms are uptaken via specific transporters such as MCT1, MCT4, SMCT1, or SMCT2.⁷ Once in intestinal cells, fatty acids participate to the cell metabolism. For example, colonocytes were shown to use butyrate as a major energy source or, alternatively, isobutyrate when butyrate availability is low.^{8,9} Finally, fatty

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acids may regulate intestinal cell activities by interfering with post-translational modifications such as neddylation.^{10,11} The impact of fatty acids on other ubiquitin-like modifications in intestinal cells has not been described yet.

SUMOylation is a ubiquitin-like modification consisting in the covalent addition of SUMO (Small Ubiquitin-like Modifier) peptides to target proteins. Five SUMO paralogs have been identified in humans that share 45–97% sequence identity. SUMO1, SUMO2, and SUMO3, which are the most studied paralogs, can be conjugated to both overlapping and distinct sets of proteins.¹² The conjugation of SUMO to lysine residues of target proteins is catalyzed by an enzymatic machinery composed of one E1 enzyme (SAE1/SAE2), one E2 enzyme (UBC9), and several E3 enzymes.¹³ SUMOylation is a reversible modification as the isopeptide bond between SUMO and its target can be cleaved by specific proteases called deSUMOylases.¹⁴ The consequences of SUMO conjugation on target proteins are very diverse and include changes in protein localization, stability, activity, or interactions with other cellular components.^{12,15,16}

SUMOylation plays essential roles in intestinal physiology as it limits detrimental inflammation while participating to tissue integrity maintenance.^{17,18} Interestingly, several intestinal bacterial pathogens were shown to interfere with epithelial cell SUMOylation.¹⁹ *Listeria monocytogenes*, for example, secretes a pore-forming toxin triggering the degradation of the host cell E2 SUMO enzyme and the rapid loss of SUMO-conjugated proteins.^{20,21} *Salmonella enterica* serovar Typhimurium also targets the host E2 SUMO enzymes during infection by inhibiting its translation via miRNA-based mechanisms.²² *Shigella flexneri*, finally, similarly switches off the SUMOylation machinery by triggering a calpain-dependent cleavage of the SUMO E1 enzyme SAE2 in infected cells.²³ In contrast to these examples of pathogens dampening intestinal cell SUMOylation, the impact of gut commensal bacteria on the SUMOylation of intestinal proteins remains unknown. We investigate here whether bacterial metabolites derived from the gut microbiota regulate intestinal cell activities by modulating host protein SUMOylation. We demonstrate

that bacterial fatty acids induce an hyperSUMOylation in intestinal cells, dampen inflammatory responses, and promote intestinal epithelial integrity.

Material and methods

Animals

Animal care and experimentation were approved by a regional Animal Experimentation Ethics Committee (APAFIS#21102–2019061810387832 v2) and complied with the guidelines of the European Commission for the handling of laboratory animals (Directive 2010/63/EU). All efforts were made to minimize suffering of animals.

Eight-weeks-old C57Bl/6JrJ male mice (Janvier Labs, Le-Genest-Saint-Isle, France) were housed at 23°C (5 animals/cage) with a 12-h light–dark cycle in regular open cages. All animals were fed with a non-sterilized standard rodent diet (3430.PM. S10, Serlab, France). Drinking water was not sterilized. After 1 week of acclimatization to the animal facility, animals were split into two groups (5–10 animals/group): one group received antibiotics by oral gavage once a day, while the other group had no antibiotic treatment and were gavaged once a day with drinking water. For oral gavages, mice received a volume of 10 µL/g body weight of drinking water supplemented with 0.1 mg/mL Amphotericin-B (Sigma-Aldrich), 10 mg/mL Ampicillin (Sigma-Aldrich), 10 mg/mL Neomycin trisulfate salt hydrate (Sigma-Aldrich), 10 mg/mL Metronidazole (Sigma-Aldrich), and 5 mg/mL Vancomycin hydrochloride (Sigma-Aldrich).²⁴ This solution was delivered with a stainless steel tube without prior sedation of the mice. To prevent fungal overgrowth in the antibiotic-treated animals, mice were pre-treated with Amphotericin-B for 3 days before the beginning of the protocol.²⁴ As for antibiotic treatment, Amphotericin-B was delivered by oral gavage (10 µL/g body weight of drinking water supplemented with 0.1 mg/mL Amphotericin-B).²⁴ All displayed results are representative of at least two independent experiments. At the end of the study, all animals were euthanized by an intraperitoneal injection of an overdose of ketamine (200 mg/kg body weight) and xylazine (20 mg/kg body

weight). Segments from the jejunum, the cecum and the colon were then removed, as well as cecal contents, frozen in liquid nitrogen and stored at -80°C .

Quantification of cecal microorganisms by quantitative PCR

Quantitative real-time polymerase chain reaction (qPCR) was performed on DNA samples extracted from mice cecal contents to monitor the efficiency of bacterial depletion in mice treated with antibiotics, as described in ref. 24. To quantify total eubacteria, qPCR was performed using primers targeting the bacterial 16S rRNA gene (Eub-338 F, 5'-ACTCCTACGGGAGGCAGCAG-3' and Eub-518 R, 5'-ATTACCGCGGCTGCTGG-3').²⁵ The C_q determined in each sample were compared with a standard curve made by diluting genomic DNA extracted from a pure culture of *E. coli*, for which cell counts were determined prior to DNA isolation.

Protein extraction from mouse intestinal tissues

Intestinal tissues were mechanically lysed using bead beating in a buffer containing 50 mM HEPES pH 8.0, 8 M urea buffer, supplemented with 10 mM N-ethyl-maleimide (NEM). Tissue lysates were then centrifugated for 15 min at 13,000xg at 4°C . Supernatants were collected, mixed 1:1 with Laemmli buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 100 mM dithiothreitol [DTT], 0.02% bromophenol blue), and analyzed by immunoblotting.

Cell culture

CACO2 (American Type Culture Collection (ATCC)-HTB-37), HeLa (ATCC-CCL2), and T84 (ATCC CCL-248) cells were cultivated at 37°C in a 5% CO_2 atmosphere. CACO2 and HeLa cells were cultivated in Minimum Essential Medium (MEM) (Eurobio) supplemented with 2 mM L-Glutamine (Invitrogen), 10% Fetal Bovine Serum (FBS, Eurobio), non-essential aminoacids (Sigma-Aldrich), 1 mM sodium pyruvate (Gibco), and a mixture of penicillin (10000 U/mL), and streptomycin (10 mg/mL). T84 cells were cultivated in

DMEM/F12 (Dulbecco's Modified Eagle Medium F-12) (Eurobio) supplemented with 10% FBS and 2.5 mM L-Glutamine.

CACO2 and T84 cells were seeded in wells at a density of 1.0×10^5 cells/cm² and 1.5×10^5 cells/cm², respectively, the day before treatments.

Before treatments, cell culture medium was replaced by HBSS (Hanks' Balanced Salt Solution; Sigma-Aldrich). Cells were then treated as indicated in the text. For BCFA and SCFA treatments, 100 mM stock solutions in water were first prepared from the corresponding acidic form (e.g. isobutyric acid) (Sigma-Aldrich) or from the sodium salt of the corresponding basic form (e.g. sodium isobutyrate) (Sigma-Aldrich) and then further diluted in cell culture media (HBSS). When needed, the pH of cell culture medium was shifted using either 0.1 M NaOH or 0.1 M HCl solution. For treatments with ROS inhibitors, CACO2 cells were pre-incubated for 30 min with 5 mM N-acetyl-cysteine (NAC) or 10 μM Diphenyleneiodonium (DPI) and then incubated for 1 h with 5 mM isobutyric acid or isovaleric acid. For immunoblotting experiments, cells were lysed directly in Laemmli buffer. For TNF α treatments, CACO2 cells were first incubated with BCFAs or SCFAs for 1 hour and then incubated with 100 ng/mL recombinant human TNF α (PeproTech). For immunoblotting and qRT-PCR analyses, cells were lysed after 30 min or 1 h of incubation with TNF α , respectively. For transepithelial electrical resistance (TEER) measurements, cells were incubated for 24 h with TNF α . Viability of cells incubated with HBSS at various pH or with BCFAs was assessed using the CellTiter-Glo[®] luminescent cell viability assay (Promega), according to the manufacturer's protocol.

Immunoblot analyses

Cell lysates and protein extracts from intestinal tissues in Laemmli buffer were boiled for 5 min, sonicated and protein content was analyzed by electrophoresis on TGX Stain-free pre-cast SDS-polyacrylamide gel (Bio-rad). Proteins were then transferred on PVDF membranes (GE Healthcare) and detected after incubation with specific antibodies using ECL Clarity Western blotting Substrate (Bio-Rad). Primary and secondary antibodies used

for immunoblot analyses are described in Supplementary Table S1. All displayed immunoblots are representative of at least three independent experiments. Quantifications of proteins were performed on a ChemiDoc Imaging System (Bio-rad). SUMO2/3-conjugated protein levels (above 50 kDa), SUMO1-conjugated protein levels (above 50 kDa), and other specific protein levels were normalized either by the level of total proteins above 50 kDa (determined using the TGX-stain free imaging technology; Bio-rad) or by the level of actin in each lysate.

Detection of reactive oxygen species

Detection of ROS was adapted from ref.²⁶ Luminol was dissolved in NaOH 0.1 M to obtain a 50 mM stock solution. A stock solution of 1000 U/mL HRP (HorseRadish Peroxidase) was prepared in parallel in PBS (Phosphate-Buffered Saline). Culture media from CACO2 cells treated with BCFAs or SCFAs or incubated in HBSS at pH 5.2 were collected and centrifugated for 5 min at 13,000xg at room temperature to eliminate cell remnants. The pH of the obtained supernatants was then buffered to 7.5 to avoid pH-dependent interferences with luminol activity. Luminol (1 mM final concentration) and HRP (4 U/mL) were finally added to each culture media and luminescence was quantified immediately on a luminometer (Tecan).

Evaluation of deSUMOylase activity

DeSUMOylase activity assays were adapted from ref.²⁷ For *in vitro* cell lines, CACO2 and T84 cells, grown in 12-well plates and treated or not with BCFAs, were scraped in 100 μ L lysis buffer (Tris HCl pH 8.0 50 mM, EDTA 5 mM, NaCl 200 mM, Glycerol 10%, NP40 0.5%). For *in vivo* tissues, cecal segments were resuspended in lysis buffer (800 μ L for 100 mg of tissues), mechanically lysed using bead beating and further diluted 25 times in lysis buffer. Negative controls were prepared by adding 10 mM N-ethylmaleimide (NEM; Sigma-Aldrich) to lysates. Recombinant human SUMO1-AMC and SUMO2-AMC proteins (R&D Systems) were diluted in parallel to 500 nM in Assay buffer (Tris

HCl pH 8.0 50 mM, Bovine Serum Albumin (BSA) 100 μ g/mL, Dithiothreitol (DTT) 10 mM). For each measurement, 10 μ L of cell or tissue lysates were mixed with 40 μ L of SUMO-AMC containing Assay buffer and fluorescence (λ_{Ex} = 380 nm; λ_{Em} = 460 nm) was recorded for 30 min at 37°C on a Flexstation 3 microplate reader (Molecular Devices). DeSUMOylase activities were determined by calculating the initial speed of fluorescence emission in each lysate, normalized by the quantity of proteins in the corresponding sample, determined in parallel using BCA assays (PierceTM BCA Protein Assay Kit).

Cell fractionation

Fractionation of CACO2 cells incubated or not with 5 mM isobutyric, isovaleric, or butyric acids for 5 h was performed with the Subcellular Protein Fractionation Kit (Thermo Scientific), according to the manufacturer's protocol. Extracts corresponding to cytosolic, nuclear soluble, and chromatin-bound fractions were collected and mixed 1:1 with Laemmli buffer. The remaining insoluble pellets, corresponding to the nuclear matrix, were resuspended directly in Laemmli buffer. All fractions were then boiled for 5 min and sonicated before immunoblotting analyses.

Evaluation of intracellular pH

CACO2 and T84 cells, grown on 96-well plates, were loaded with 2 μ M BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester; Invitrogen) for 30 min in HBSS at 37°C. BCECF is a fluorescent dye exhibiting a pH-dependent excitation profile.²⁸ Cells were then washed twice and incubated in HBSS with or without BCFAs or with HBSS at definite pH. Cell fluorescence ($\lambda_{Emission}$ = 535 nm) was recorded at two different excitation wavelengths: 440 nm and 490 nm. Comparison of the ratios of fluorescence intensities (490/440 nm) was performed to detect changes in intracellular pH. Calibration solutions (with pH ranging from 4.5 to 7.5) were used as controls (Intracellular pH Calibration Buffer Kit; Molecular Probes).

Quantification of proinflammatory cytokine expression

Total RNAs were extracted from CACO2 cells using the RNeasy Plus Mini kit (Qiagen), following manufacturer's instructions. For each condition, 1 µg of total RNAs was reverse transcribed using random hexamers and M-MLV reverse transcriptase (Invitrogen). Specific cDNAs were then quantified by qPCR using Itaq Universal SYBR Green Supermix (Bio-Rad). GAPDH was used as an internal reference for normalization. Primers used in this study are hGAPDH_F (5'-TGCCATCAATGACCCCTTCA-3'), hGAPDH_R (5'-TGACCTTGCCCACAGCCTTG-3'), hIL8_F (5'-TGGCAGCCTTCCTGATTT-3'), hIL8_R (5'-AACTTCTCCACAACCCTCTG-3'), hCCL20_F (5'-TTTGCTCCTGGCTGCTTTGA-3') and hCCL20_R (5'-GCAAGTGAAACCTCCAACCC-3'). Serial dilution of target cDNAs was included on each plate to generate a relative curve and to integrate primer efficiency in the calculations of mRNA quantities.

Evaluation of intestinal epithelial permeability

CACO2 cells were seeded in Transwell inserts and cultivated for 21 days. Monolayer formation and differentiation was monitored by daily evaluation of transepithelial electrical resistance (TEER) measurement, performed with an EVOM epithelial volt ohm meter equipped with "chopstick" electrodes. After three weeks, cell culture media were replaced by HBSS. Cells were preincubated or not with isobutyric or isovaleric acids for 1 hour. 100 ng/mL TNFα was then added to both apical and basolateral compartments. TEER was evaluated after 24 h of incubation.

Results

Gut microbiota depletion decreases SUMO2/3 protein conjugation in the cecum

To determine whether the gut microbiota affects intestinal SUMOylation, we compared the global SUMOylation patterns of intestinal segments from conventional mice or from mice with a depleted gut microbiota. Depletion of mice intestinal bacteria was performed via the oral gavage of a cocktail of antibiotics during 7 days.²⁴ We quantified by qPCR the total

amount of Eubacteria in the cecal content of mice treated with antibiotics to ensure that the efficiency of bacterial depletion was above 75%. The SUMOylation patterns of segments from the jejunum, the cecum, and the colon were then analyzed by immunoblotting experiments using anti-SUMO1 and anti-SUMO2/3 antibodies (Figure 1(a)). The level of SUMO-conjugated proteins (above 50 kDa) was quantified in each sample (Figure 1(b)). Interestingly, we observed that mice with a depleted gut microbiota exhibit a significant decrease in the level of SUMO2/3-conjugated proteins in the cecum (Figure 1). This decrease is specific to the SUMO2/3 isoform as the cecal level of SUMO1-conjugated proteins is not modified in response to antibiotic treatment. This decrease in SUMO2/3-conjugated protein levels is furthermore specific to the cecum as we did not observe any significant modification of the SUMOylation patterns in the jejunum or colon of mice treated with antibiotics (Figure 1). Together, these results suggest that the gut microbiota regulates the level of protein conjugated to SUMO2/3 in specific intestinal segments.

BCFAs trigger an hyperSUMOylation of intestinal proteins

As fatty acids such as SCFAs and BCFAs are important mediators of the interactions between gut bacteria and host cells, we assessed if these metabolites may modulate intestinal cell SUMOylation. We first monitored the effect of BCFAs on intestinal cell SUMOylation *in vitro* by incubating CACO2 or T84 cells for 1 h or 5 h with isobutyric, isovaleric, or 2-methyl-butyric acids (1 mM or 5 mM final concentrations) (Figure 2, S1 and S2). Interestingly, we observed that all BCFAs induce a significant increase in the level of proteins conjugated to SUMO2/3 after 1 h or 5 h of incubation (at 5 mM concentration) in CACO2 cells. This hyperSUMOylation is similarly observed in T84 cells, after 1 h of incubation with 5 mM isobutyric, isovaleric acids or 2-methyl-butyric acids (Figure S1 and S2). In contrast to SUMO2/3-conjugated proteins, the pattern of proteins conjugated to SUMO1 is not affected by BCFAs in CACO2 cells (Figure 2). Of note, the concentrations of BCFAs used here do not decrease cell viability (Figure S3).

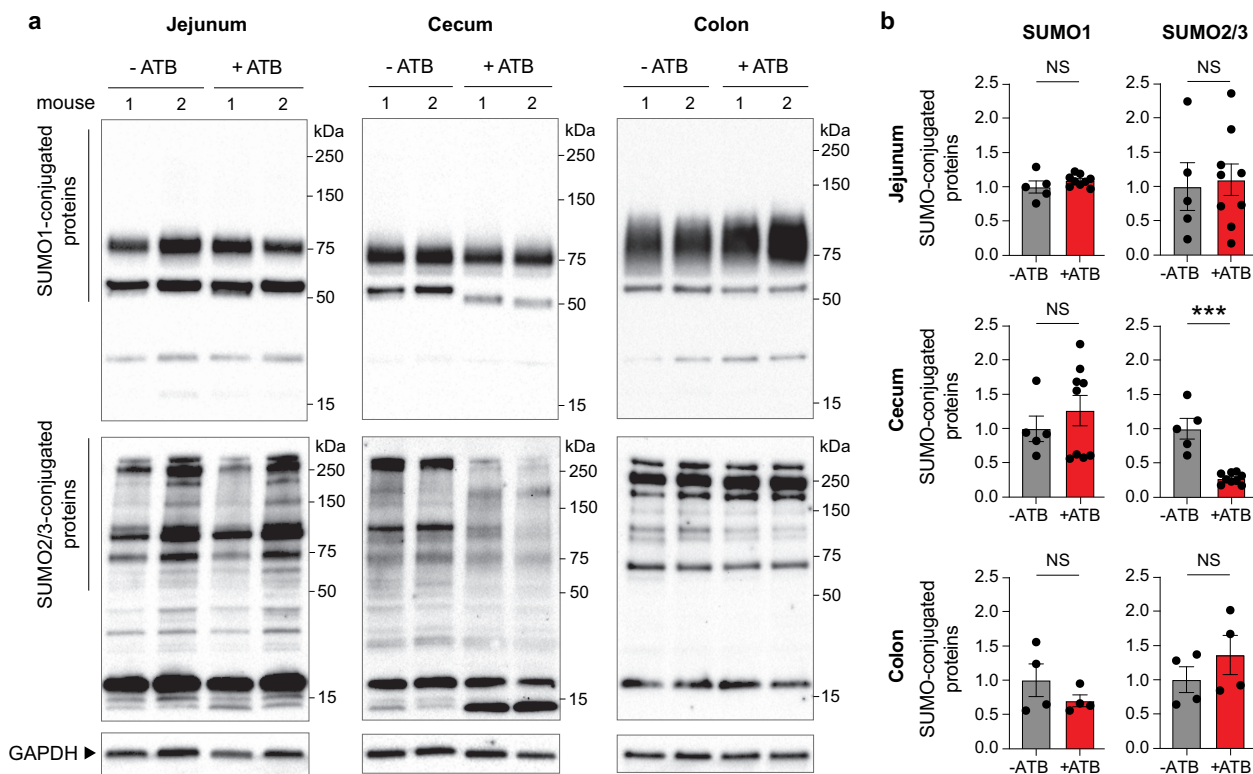


Figure 1. Gut microbiota depletion decreases protein SUMOylation in the cecum. a, Immunoblot analysis of SUMO1- and SUMO2/3-conjugated proteins in the jejunum, cecum, and colon of mice treated or not with antibiotics (ATB) (two representative mice are shown for each tissue). b, Quantification of SUMO-conjugated proteins (above 50 kDa). Values are expressed as fold-change versus untreated mice (mean \pm s.e.m.; $n = 4-9$; ***, $P < .001$; NS, not significant; two-tailed Student's t-test).

To determine whether the hyperSUMOylation induced by BCFAs is reversible, CACO2 cells were incubated with 5 mM isovaleric acid for 1 h and then washed and allowed to recover in BCFA-free culture medium for 1 or 4 h. We observed that the initial hyperSUMOylation triggered by isovaleric acid rapidly disappears after the removal of this BCFA (Figure S4).

Together, these results demonstrate that BCFAs specifically increase SUMO2/3 conjugation in intestinal cell lines. This is consistent with our previous observations *in vivo* in which microbiota depletion leads to a specific decrease in SUMO2/3 conjugation in the cecum of mice (Figure 1).

The effect of BCFAs on intestinal SUMOylation is pH dependent

BCFAs are weak organic acids, which exist in solution either as acidic (R-COOH) or basic (R-COO⁻) forms. For example, addition of 5 mM isovaleric acid in the HBSS medium leads to a solution with a pH of

~5.2 containing approximately 28% (*i.e.* ~1.4 mM) of the acidic form (isovaleric acid) and 72% (*i.e.* ~3.6 mM) of the basic form (isovalerate). In contrast, addition of 5 mM sodium isovalerate in the HBSS medium leads to a solution with a pH of ~7.5 containing approximately 0.2% (*i.e.* ~0.01 mM) of isovaleric acid and 99.8% (*i.e.* ~4.99 mM) of isovalerate. To decipher whether both acidic and basic forms of BCFAs trigger hyperSUMOylation in intestinal cells, we added 5 mM isovaleric acid to CACO2 cell culture medium and increased gradually the pH of this medium from 5.2 to 7.0 (thereby decreasing the isovaleric acid/isovalerate ratio) (Figure 3(a)). We did not observe any significant hyperSUMOylation when cells were incubated in these conditions at pH 7.0, in contrast to cells incubated at pH 5.2. This shows that only isovaleric acid (and not isovalerate) promotes SUMO-conjugation of intestinal proteins.

We then added increasing amounts of isovaleric acid to the CACO2 cell culture medium and set in parallel the pH of this medium between 5.2 and 7.0. For each pH, the amount of isovaleric acid added to

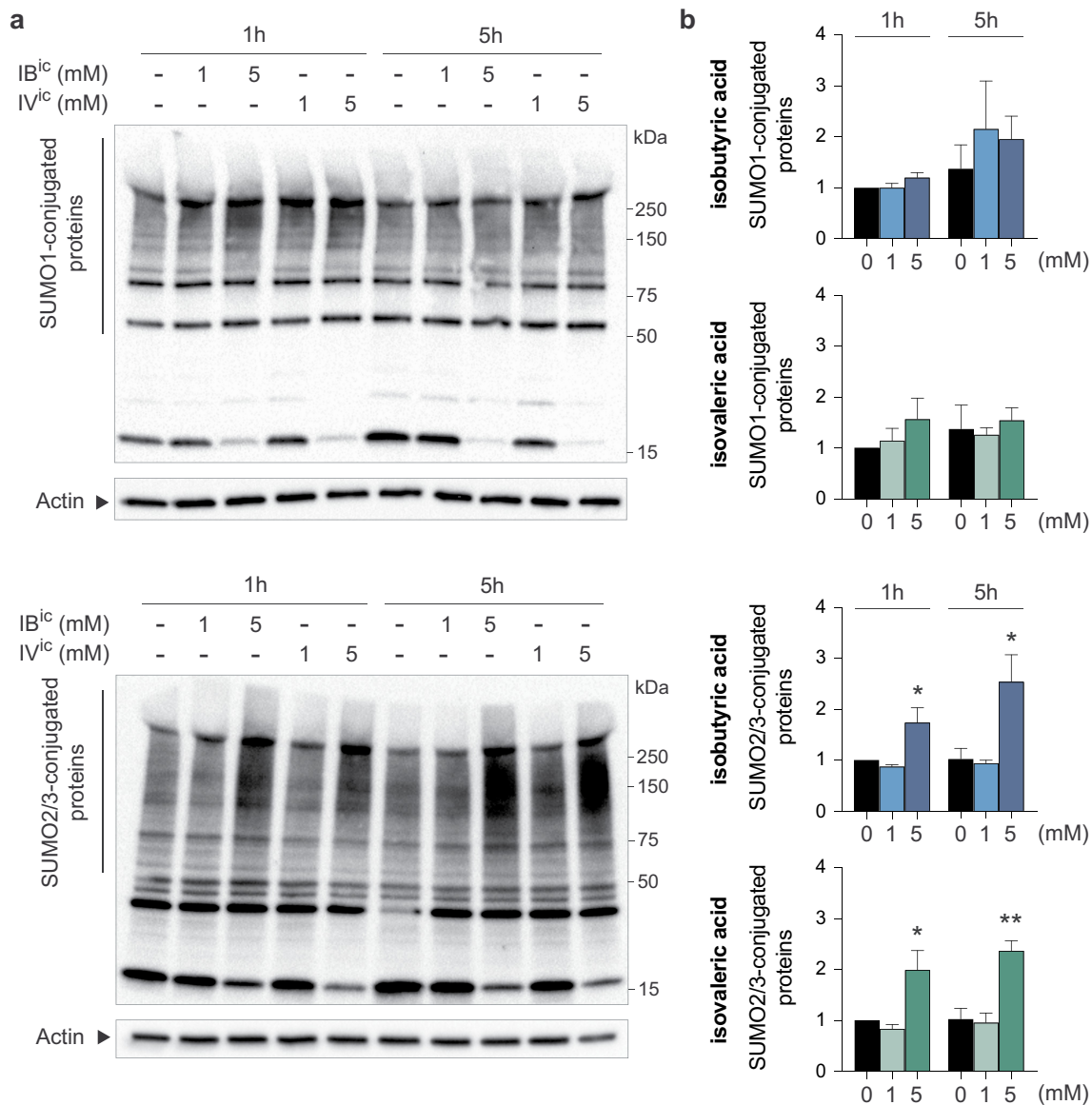


Figure 2. BCFA trigger hyperSUMOylation of intestinal proteins in CACO2 cells. a, Immunoblot analysis of SUMO1-conjugated proteins (top) or SUMO2/3-conjugated proteins (bottom) in CACO2 cells incubated with isobutyric acid (IB^{ic}) or isovaleric acid (IV^{ic}) for 1 or 5 h. b, Quantification of SUMO-conjugated proteins (above 50 kDa). Values are expressed as fold-change versus untreated cells (mean \pm s.e.m.; $n = 3-4$; *, $P < .05$; **, $P < .01$ versus untreated cells; One-way ANOVA, with Dunnett's correction).

cells was calculated to maintain a final concentration of isovaleric acid in the cell culture medium of ~ 1.4 mM. We observed that this increase in BCFA concentration restores the hyperSUMOylation observed in CACO2 cells at pH 6 and 7 (Figure 3(a) and Figure 3(c)). This result demonstrates that BCFA, when present in high concentration, trigger hyperSUMOylation even at neutral or weakly acidic pH.

To decipher if the hyperSUMOylation induced by isovaleric acid is only due to the associated acidification of the extracellular milieu, we

compared the SUMOylation pattern of CACO2 cells incubated for 1, 2, or 5 h with 5 mM isovaleric acid (pH 5.2) or with culture medium without isovaleric acid and with a pH set to 5.2 (Figure 3(b)). We observed that the hyperSUMOylation triggered by isovaleric acid cannot be recapitulated by an equivalent acidic pH (Figure 3(b) and Figure 3(c)).

Of note, the acidic forms of fatty acids are uncharged and freely diffusible across cellular membranes, in contrast to the basic forms, which are negatively charged and only cross

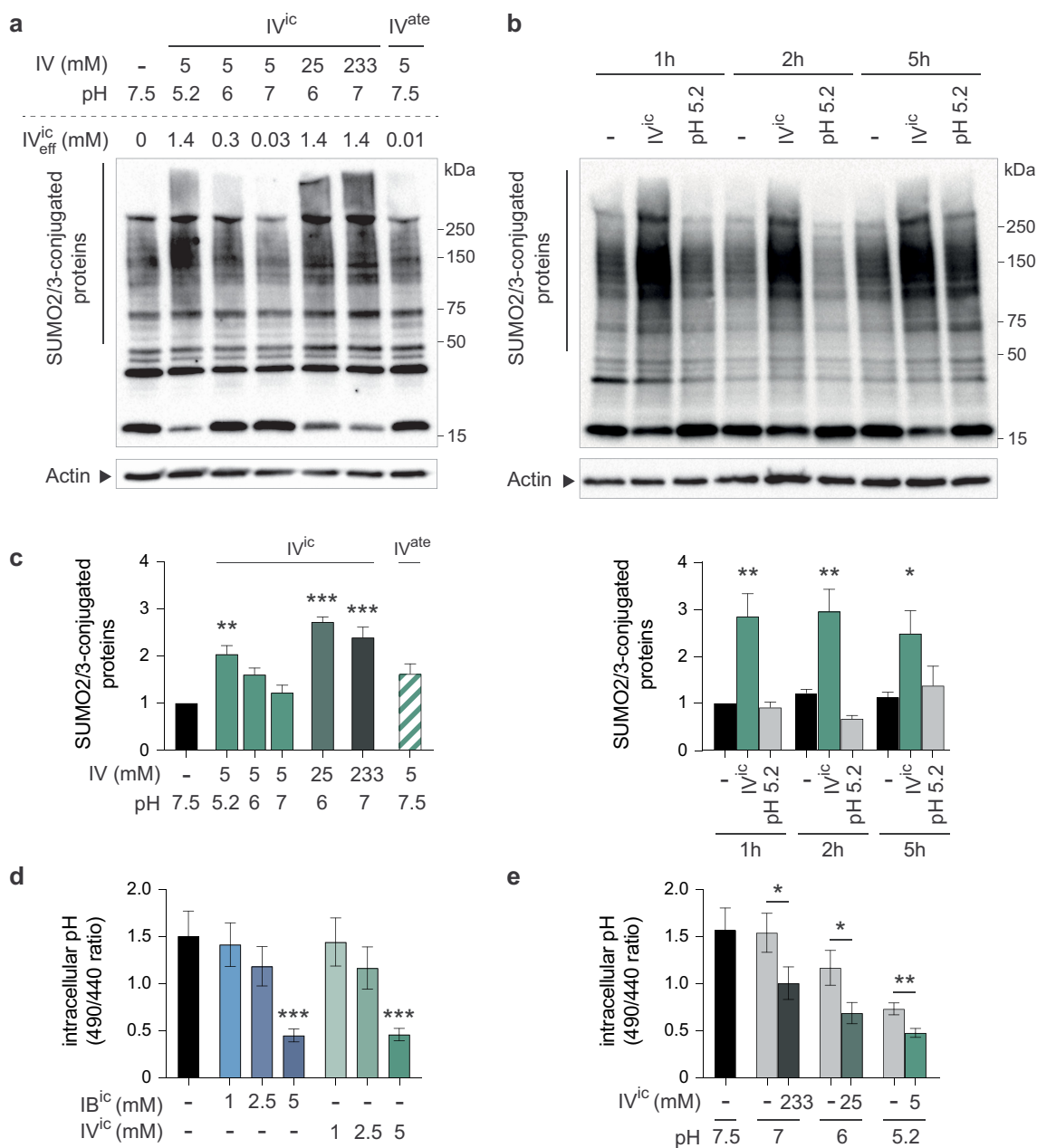


Figure 3. BCFAs-triggered hyperSUMOylation of intestinal proteins is pH-dependent. **a**, Immunoblot analysis of SUMO2/3-conjugated proteins in CACO2 cells incubated with various concentrations of isovaleric acid (IV^{ic}) or sodium isovalerate (IV^{ate}) at definite pH. The pH and the corresponding effective concentrations of isovaleric acid (IV_{eff}^{ic}) are indicated for each condition. **b**, Immunoblot analysis of SUMO2/3-conjugated proteins in CACO2 cells incubated with 5 mM isovaleric acid (IV^{ic}) or in culture medium at pH 5.2. **c**, Quantification of SUMO2/3-conjugated proteins. Values are expressed as fold-change versus untreated cells (mean ± s.e.m.; $n = 3-4$; *, $P < .05$, **, $P < .01$, ***, $P < .001$ versus untreated cells; One-way ANOVA, with Dunnett's correction). **d**, Evaluation of intracellular pH in CACO2 cells loaded with BCECF-AM and incubated with various concentrations of isobutyric acid (IB^{ic}) or isovaleric acid (IV^{ic}). Values are expressed as ratio of fluorescence intensities after excitation at 490 or 440 nm (mean ± s.d.; $n = 4$; ***, $P < .001$ versus untreated cells; one-way ANOVA, with Dunnett's correction). **e**, Evaluation of intracellular pH in CACO2 cells loaded with BCECF-AM and incubated with various concentrations of isovaleric acid (IV^{ic}) or in culture medium at definite pH (mean ± s.d.; $n = 3$; *, $P < .05$; **, $P < .01$; two-tailed Student's t-test).

membranes thanks to specific transporters. Thus, as only the acidic forms of BCFA induce an hyperSUMOylation of intestinal proteins, we

can hypothesize that these forms diffuse passively across the cell membrane, and then act intracellularly on intestinal cell SUMOylation.

To decipher whether the entry of the acidic forms of BCFAs inside cells may alter the intracellular pH (pHi) (as these acidic forms may release protons once inside cells), we monitored pHi in CACO2 and T84 cells using a pH-sensitive fluorescent dye (BCECF-AM).²⁶ Interestingly, we observed a significant decrease in pHi in both CACO2 and T84 cells incubated with 5 mM isobutyric and isovaleric acids (Figure 3(d) and S5). As an acidic extracellular milieu may also decrease pHi, we compared the pHi measured in CACO2 cells incubated with various concentrations of isovaleric acid (at definite pH) with the one obtained for cells incubated in a culture medium at similar pH (Figure 3(e)). In all conditions, the decrease in pHi triggered by isovaleric acid is greater than the one triggered by the corresponding acidic condition (Figure 3(e)). Similar results were obtained in T84 cells (Figure S5). These results strongly suggest that the acidic forms of BCFAs diffuse inside intestinal cells where they trigger a decrease in the intracellular pH. This decrease is more efficient than the one induced by an acidic extracellular milieu, probably because the plasma membrane is more permeable to the acidic forms of BCFAs than to protons alone.

We finally monitor the level of SUMO2/3-conjugation in CACO2 cells incubated for 5 h in culture medium with acidic pH. We did not observe any significant increase in SUMOylation for cells incubated in pH ranging from 7.5 down to 5.2, thereby confirming our previous observations (Figure 3(b), Figure 3(c) and Figure S5). Further decreasing the extracellular pH eventually leads to an increase in CACO2 cell SUMOylation, which is probably linked to a stronger decrease of CACO2 cell intracellular pH, as in the case of incubation with isovaleric acid (Figure S5).

Of note, incubating CACO2 and T84 cells in a culture medium with pH ranging from 7.5 to 5.0 does not decrease cell viability, nor the expression level of E1, E2, or E3 SUMO enzymes (Figure S3 and S6).

SCFAs also affect intestinal SUMOylation

To complete our results obtained with BCFAs, we determined whether SCFAs similarly impact intestinal cell SUMOylation. We incubated CACO2 cells with acetic, butyric, and propionic acid for 5 h (5 mM final concentration; pH~5.2). Interestingly,

we observed that SCFAs induce a significant increase in the level of SUMO2/3-conjugated proteins (Figure 4). In contrast, incubation of cells with sodium acetate, butyrate, or propionate (5 mM final concentration; pH~7.5) does not trigger any change in the SUMOylation pattern of CACO2 cells (Figure 4). Together, these results indicate that SCFAs, as BCFAs, modulate intestinal protein SUMOylation in a pH-dependent manner.

BCFAs-induced hyperSUMOylation is dependent of ROS production

Butyric acid was previously reported to induce ROS (Reactive Oxygen Species) production in both IEC-6 intestinal epithelial cells and HeLa cells.¹¹ We thus tested whether SCFAs and BCFAs similarly induce

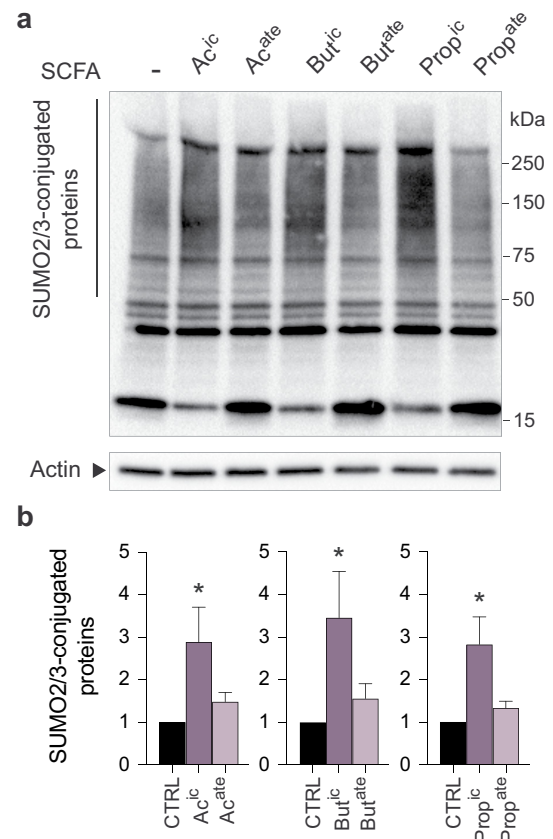


Figure 4. SCFAs trigger hyperSUMOylation of intestinal proteins in a pH-dependent manner. a, Immunoblot analysis of SUMO2/3-conjugated proteins in CACO2 cells incubated for 5 h with 5 mM SCFAs. b, Quantification of SUMO2/3-conjugated proteins. Values are expressed as fold-change versus untreated cells (CTRL) (mean \pm s.e.m.; $n = 4$; *, $P < .05$ versus CTRL; one-way ANOVA, with Dunnett's correction) (Ac^{ic}, acetic acid; Ac^{ate}, sodium acetate; But^{ic}, butyric acid; But^{ate}, sodium butyrate; Prop^{ic}, propionic acid; Prop^{ate}, sodium propionate).

ROS production in CACO2 cells. For this, we used a sensitive luminol-based ROS detection assay.²⁷ We observed that the addition of isobutyric, isovaleric, or butyric acid induces ROS production in CACO2 cells after 1 h of incubation (Figure 5(a)). This oxidative stress is transient as the level of ROS decreased between 1 and 5 h of incubation (Figure 5(a)). Interestingly, the oxidative stress induced by BCFAs and SCFAs is pH-dependent as no ROS were detected after incubation with sodium isobutyrate, isovalerate, or butyrate (Figure 5(a)). These results suggest that ROS are produced in response to the diffusion of the acidic form of BCFAs and SCFAs inside CACO2 cells and to the associated drop in intracellular pH. We confirmed this hypothesis by showing that ROS production induced by

5 mM isovaleric acid is greater than the one observed with cells incubated in the corresponding acidic conditions (pH 5.2; Figure 5(b)).

To determine whether ROS production was responsible for the hyperSUMOylation triggered by fatty acids, we pre-incubated CACO2 cells with two ROS scavengers, N-acetyl cysteine (NAC) and Diphenyleneiodonium (DPI). These cells were then incubated with isobutyric or isovaleric acids for 1 h. We observed that preincubation with oxidative stress inhibitors significantly blocks BCFAs-induced hyperSUMOylation (Figure 5(c) and Figure 5(d)). Together, these results demonstrate that the acidic forms of SCFAs and BCFAs trigger the production of ROS in intestinal cells, which in turn promotes SUMO-conjugation of intestinal proteins.

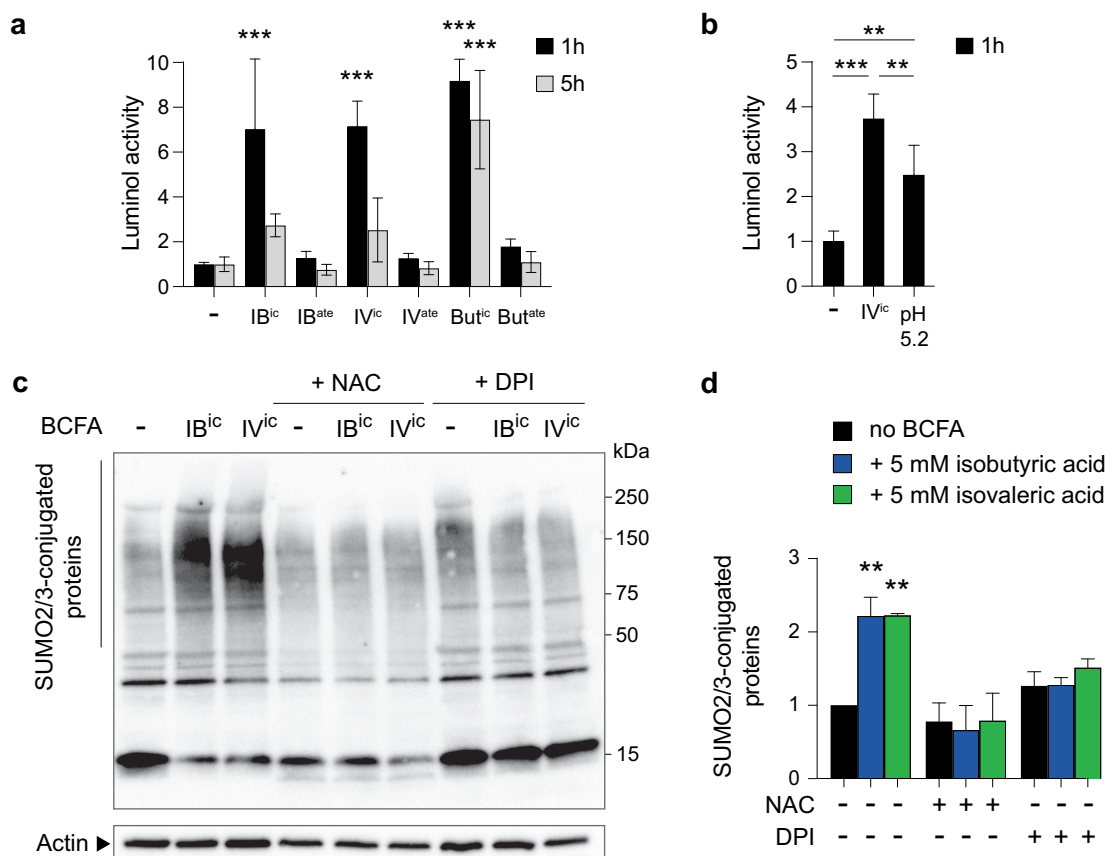


Figure 5. BCFAs and SCFAs induce hyperSUMOylation in intestinal cells via ROS production. **a**, Quantification of luminol activity in CACO2 treated with isobutyric acid (IB^{ic}), sodium isobutyrate (IB^{ate}), isovaleric acid (IV^{ic}), sodium isovalerate (IV^{ate}), butyric acid (But^{ic}) or sodium butyrate (But^{ate}) for 1 or 5 h. Values are expressed as fold-change of untreated cells (mean \pm s.d.; $n = 3$; ***, $P < .001$ versus untreated; One-way ANOVA, with Dunnett's correction). **b**, Quantification of luminol activity in CACO2 treated with isovaleric acid (IV^{ic}) or incubated in culture medium at pH 5.2 for 1 h (mean \pm s.d.; $n = 4$; **, $P < .01$; ***, $P < .001$; One-way ANOVA, with Tukey's correction). **c**, Immunoblot analysis of SUMO2/3-conjugated proteins in CACO2 cells pre-incubated or not for 30 min with 5 mM N-acetyl-cysteine (NAC) or 10 μ M Diphenyleneiodonium (DPI) and then incubated for 1 h with isobutyric acid (IB^{ic}) or isovaleric acid (IV^{ic}). **d**, Quantification of SUMO2/3-conjugated proteins. Values are expressed as fold-change versus untreated cells (mean \pm s.e.m.; $n = 3$; **, $P < .01$ versus untreated cells; One-way ANOVA, with Dunnett's correction).

BCFAs inhibit intestinal cell deSUMOylases

Global increase in SUMOylation may result either from an increase in the SUMOylation machinery's activity or from an inhibition of cellular deSUMOylases. As deSUMOylases were reported to be sensitive to oxidative stress,^{29,30} we evaluated whether BCFAs could inhibit SUMO-deconjugation in intestinal cells. For this, CACO2 was incubated with isobutyric or isovaleric acids and lysed. Cell lysates were then mixed with SUMO1 or SUMO2 peptides covalently linked to AMC (7-amino-4-methylcoumarin). The activity of deSUMOylases was then quantified in these cell lysates by measuring the fluorescence intensity of AMC released by the deSUMOylase-dependent cleavage of the amide bond between AMC and SUMO (Figure 6).³⁰ We demonstrated that incubation with 5 mM isobutyric or isovaleric acids for 5 h significantly decreases SUMO deconjugation reactions in cell lysates, both for SUMO1- and SUMO2-conjugated substrates (Figure 6). Similar results were observed in T84 cells (Figure S7). These results indicate that deSUMOylases are inhibited in response to BCFAs.

Of note, we quantified in parallel the expression levels of E1 and E2 SUMO enzymes in CACO2 cells treated with BCFAs using immunoblotting experiments. We observed that isobutyric and isovaleric acids do not alter the levels of SAE1/SAE2 or UBC9 (Figure S8).

Together, these results suggest that the hyperSUMOylation induced by BCFAs result from the inhibition of intestinal cell deSUMOylases.

To complete these results, we quantified the activity of deSUMOylases in the cecum of mice treated with antibiotics. Interestingly, we observed a significant increase in the activity of deSUMOylases in mice with a depleted gut microbiota, which nicely correlates with the observed decrease in the level of SUMO-conjugated proteins in these intestinal segments (Figure 1 and Figure 6(b)). Together, these results highlight that the activity of intestinal deSUMOylases can be regulated by gut microbiota-derived metabolites.

BCFAs/SCFAs-induced ROS do not affect cullin-1 neddylation in CACO2 cells

In addition to SUMOylation, other Ubiquitin-like proteins such as NEDD8 were reported to be sensitive to oxidative stress. Previous reports established that ROS produced in response to butyric acid exposure inactivate the NEDD8-conjugating enzyme Ubc12 and trigger the loss of cullin-1 neddylation in HeLa cells.^{10,11} We thus assessed whether BCFAs also decrease cullin-1 neddylation in CACO2 cells. Interestingly, we observed that isobutyric and isovaleric acid triggers cullin-1 deneddylation after 5 h of incubation in HeLa cells, similarly to butyric acid, but not in CACO2 cells (Figure S9). This suggests

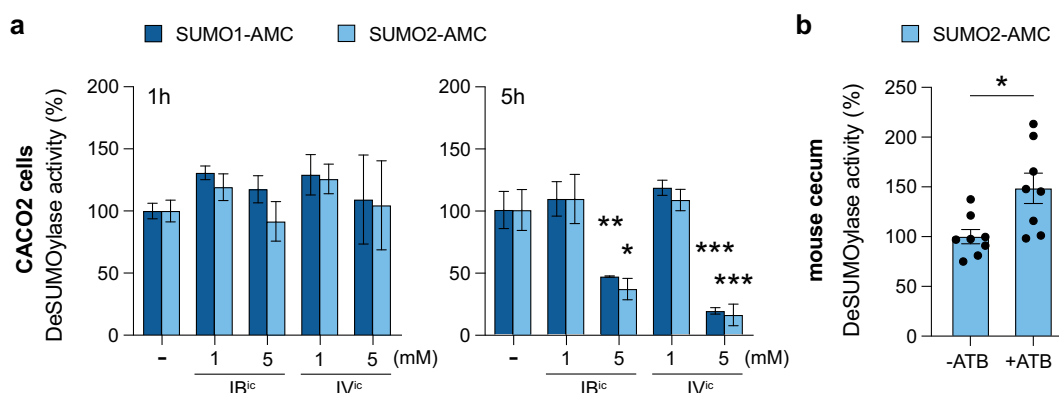


Figure 6. BCFAs inhibit intestinal cell deSUMOylases. a, DeSUMOylase activities, expressed as percentage of untreated cells, in CACO2 cells, treated or not with isobutyric acid (IB^c) or isovaleric acid (IV^c), for 1 h (left) or 5 h (right) (mean \pm s.e.m.; $n = 4-5$; *, $P < .05$; **, $P < .01$; ***, $P < .001$ versus CTRL; One-way ANOVA, with Dunnett's correction). b, DeSUMOylase activities in the cecum of mice treated or not with antibiotics (ATB), expressed as percentage of untreated (-ATB) mice (mean \pm s.e.m.; $n = 8$; *, $P < .05$; One-way ANOVA, Two-tailed Student's t-test).

that the consequences of SCFAs/BCFAs-induced ROS are cell-type dependent and that these fatty acids do not affect neddylation in CACO2 cells.

BCFAs and SCFAs promote SUMOylation of proteins associated with the nuclear matrix

In order to determine whether proteins conjugated to SUMO in response to SCFAs/BCFAs are located in specific cellular compartments, we performed cell fractionation assays. We isolated proteins from cytosolic, nuclear soluble and chromatin-associated fractions as well as proteins from the so-called nuclear matrix (a nuclear fraction characterized by its insolubility and resistance to high salt and nuclease extractions, in which several SUMO targets and enzymes, such as PML or PIASy, are accumulating^{31,32}). SUMO2/3-conjugated proteins in basal conditions (without SCFAs/BCFAs) were mainly observed in nuclear fractions (nuclear soluble and chromatin fractions), as expected since many SUMO targets are known to be nuclear.¹⁵ Very interestingly, we observed that the level of SUMO-conjugated proteins is strongly increased in the nuclear matrix fraction in response to SCFAs/BCFAs (and slightly decreased in the nuclear soluble fraction) (Figure 7). These results

highlight that SCFAs/BCFAs promote the SUMOylation of nuclear factors, which are associated with the nuclear matrix.

BCFAs/SCFAs-induced hyperSUMOylation impair NF- κ B inflammatory responses

As SUMOylation is known to regulate inflammation,^{18,33} we determined whether BCFAs/SCFAs-induced hyperSUMOylation could modulate inflammatory responses in intestinal cells. To do so, we incubated CACO2 cells with TNF α in the presence or absence of BCFAs. We then quantified the expression levels of the pro-inflammatory IL8 and CCL20 cytokines by qRT-PCR. We observed that both isobutyric and isovaleric acids downregulate the transcription of IL8 and CCL20 in response to TNF α in CACO2 cells (Figure 8(a)). We then compared the respective effect of the acidic or basic forms of BCFAs and SCFAs on the expression of these cytokines. We observed that the basic form of BCFAs and SCFAs partially decrease expression of IL8 and CCL20. Interestingly, we show that the acidic forms of BCFAs and SCFAs further decrease the expression of IL8 and CCL20 to the levels of cells unstimulated by TNF α (Figure 8(a)). As acidic forms of SCFAs/

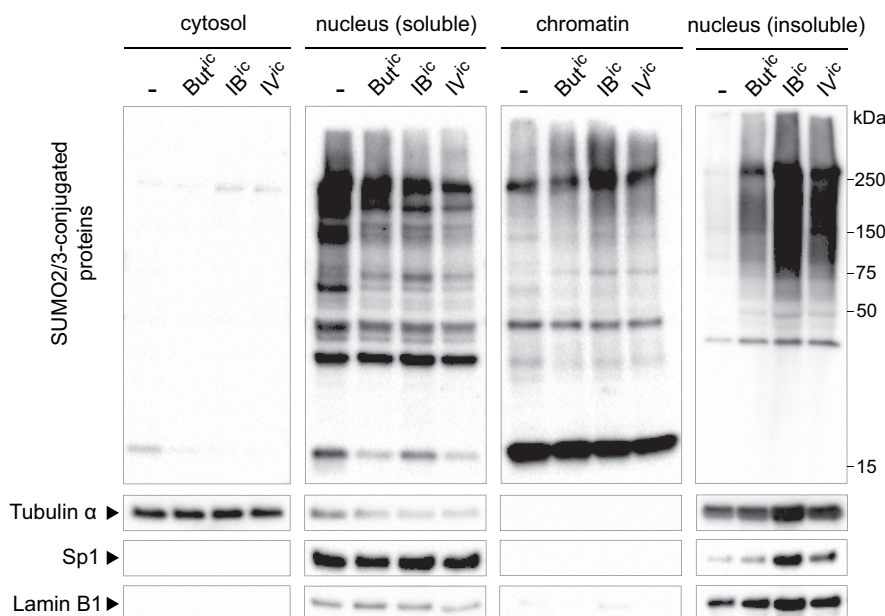


Figure 7. BCFAs and SCFAs trigger hyperSUMOylation of proteins associated with the nuclear matrix. Immunoblot analysis of SUMO2/3-conjugated proteins, Tubulin α , Sp1 and Lamin B1 in cytosolic, nuclear soluble, chromatin and nuclear insoluble fractions of CACO2 cells incubated for 5 h with 5 mM butyric acid (But^t), isobutyric acid (IB^t) or isovaleric acid (IV^t).

BCFAs trigger hyperSUMOylation in contrast to basic forms (Figures 3 and 4), these results suggest that SCFAs/BCFAs-induced hyperSUMOylation dampens pro-inflammatory cytokine expression in intestinal cells, although SCFAs/BCFAs-mediated SUMO-independent mechanisms might also be involved.

As IL8 and CCL20 expression is regulated by the NF- κ B transcription factor, we tested whether BCFAs could interfere with the NF- κ B signaling pathway. To do so, we focused on the degradation of the I κ B α inhibitor, which is a key step in the activation of NF- κ B and a pre-requisite for NF- κ B translocation into the nucleus. We quantified, using immunoblotting experiments, the level of I κ B α in CACO2 cells incubated with TNF α in the presence or absence of BCFAs and SCFAs. We observed that isobutyric, isovaleric, and butyric acids block the degradation of I κ B α triggered by TNF α (Figure 8(b) and Figure 8(c)). This inhibition was not observed with sodium isobutyrate, isovalerate, and butyrate, suggesting that the hyperSUMOylation induced by the acidic forms of SCFAs/BCFAs may block I κ B α degradation and thus dampen the NF- κ B signaling pathway (Figure 8(b) and Figure 8(c)). Again, the involvement of additional SUMO-independent mechanisms cannot be formerly ruled out here.

BCFAs promote intestinal epithelial integrity

We finally determined whether BCFAs regulate intestinal permeability. To do so, CACO2 cells were grown for 3 weeks in Transwell systems in order to reconstitute an *in vitro* model of differentiated intestinal epithelium. Cells were then incubated with TNF α and the permeability of the obtained epithelium was monitored by measuring the transepithelial electrical resistance (TEER) between the apical and basal compartments. Treatment with TNF α induces a significant decrease in TEER after 24 h, which corresponds to an increase in epithelial permeability, as previously described (Figure 8d). Interestingly, we show that incubation of CACO2 cells with isobutyric and isovaleric acids blocks this TNF α -induced increase in epithelial permeability. This result shows that BCFAs promote intestinal epithelial integrity in response to inflammatory stimuli.

Discussion

Post-translational modifications are widely used by eukaryote cells to modulate rapidly, locally and specifically the interactions or activities of key proteins. SUMOylation plays an essential role in intestinal physiology and more particularly in epithelial integrity maintenance, by controlling cell renewal and differentiation, as well as mechanical stability of the epithelium.^{17,18} Not surprisingly, several pathogens were shown to manipulate intestinal SUMOylation in order to interfere with the activity of key host factors involved in infection.¹⁹ Most of these pathogens are decreasing SUMOylation, using independent mechanisms, which illustrates a nice example of evolutive convergence. In contrast to pathogens, the potential impact of gut commensal bacteria on SUMOylation has not been investigated. Here, we show that depletion of gut bacteria in mice triggers a decrease in cecal protein SUMOylation. The level of SUMO-conjugated proteins is, in contrast, not affected in the colon. This may result either from the decrease in the levels of bacterial metabolites such as BCFAs/SCFAs along the longitudinal axis between the cecum and the rectum³⁴ or from differences in the regulation of deSUMOylase activity in these different intestinal segments.

We show that BCFAs/SCFAs upregulate intestinal SUMOylation by controlling the activity of host deSUMOylases (Figure S10). As the SUMOylation level of a given target results from the dynamic equilibrium between conjugation and deconjugation reactions, the inactivation of deSUMOylases results in an increase in protein SUMOylation levels. Interestingly, we identified that the proteins SUMOylated in response to BCFAs/SCFAs are mainly associated with the nuclear matrix. As many SUMO targets associated with the nuclear matrix, such as PML or PIASy, are involved in transcription regulation, we can hypothesize that BCFAs/SCFAs-induced SUMOylation modifies intestinal gene expression.^{15,31–33} BCFAs and SCFAs constitute the first examples of gut bacterial metabolites able to modulate intestinal SUMOylation but other classes of metabolites may regulate the level of SUMO-conjugation as well. Along the

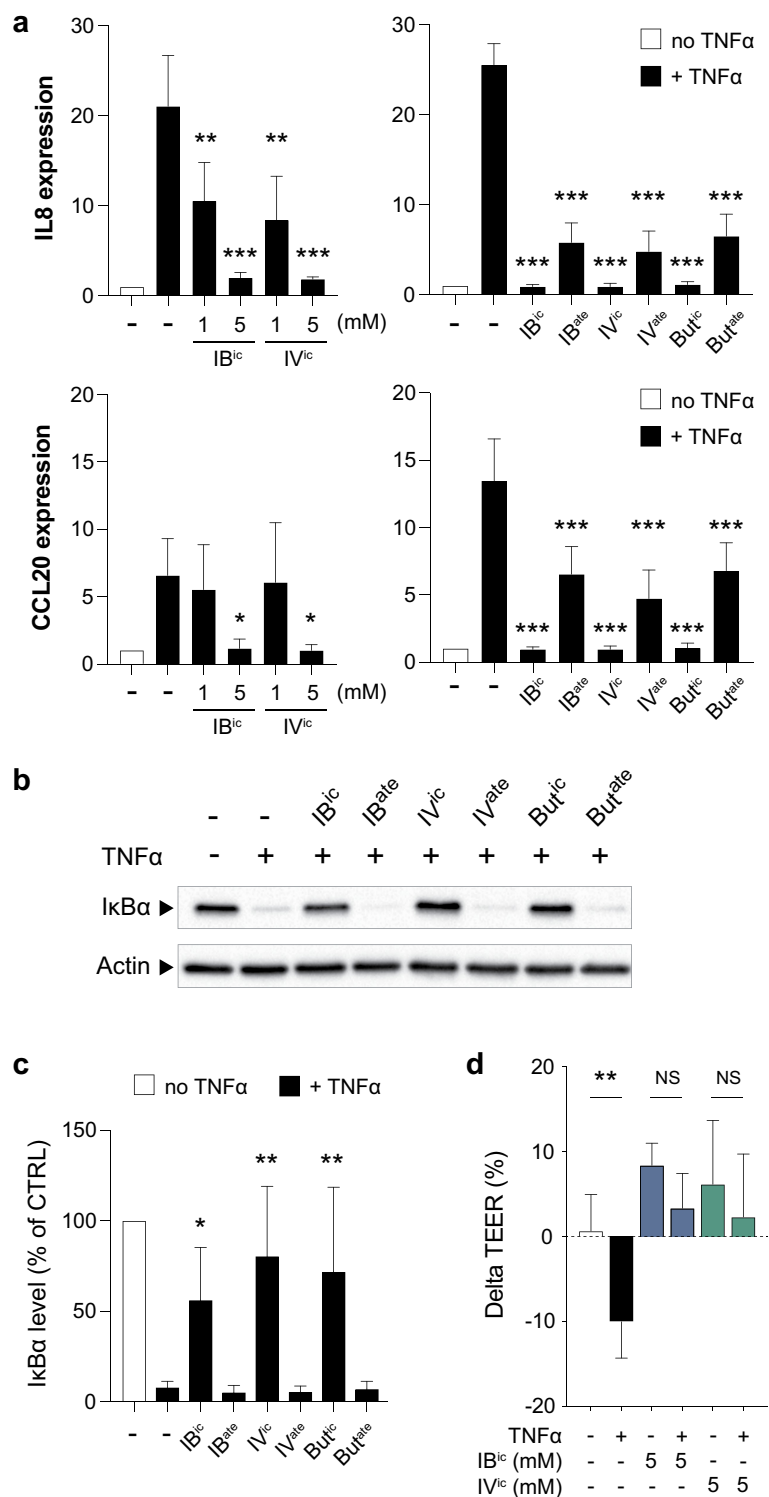


Figure 8. BCFAs and SCFAs dampen responses to TNF α in intestinal cells. a, Quantification of IL8 and CCL20 mRNA levels in CACO2 cells pre-treated or not for 1 h with BCFAs or SCFAs and then incubated for 1 h with 100 ng/mL TNF α . Values are expressed as fold change versus untreated cells (mean \pm s.d.; $n = 3-4$; *, $P < .05$; **, $P < .01$; ***, $P < .001$ vs TNF α alone; One-way ANOVA, with Dunnett's correction). b, Immunoblot analysis of I κ B α and actin levels in CACO2 cells pre-treated or not for 1 h with 5 mM BCFAs or SCFAs and then stimulated for 30 min with 100 ng/mL TNF α . c, Quantification of I κ B α levels, expressed as percentage compared to untreated cells (mean \pm s.d.; $n = 3$; *, $P < .05$; **, $P < .01$ vs TNF α alone; One-way ANOVA, with Dunnett's correction) (IB^{ic}, isobutyric acid; IB^{ate}, sodium isobutyrate; IV^{ic}, isovaleric acid; IV^{ate}, sodium isovalerate; But^{ic}, butyric acid; But^{ate}, sodium butyrate). d, TEER in CACO2 cells grown in Transwell, pre-treated or not for 1 h with BCFAs and then incubated for 24 hours with 100 ng/mL TNF α . Values are expressed as TEER percent variations compared to cells before treatment with BCFAs (mean \pm s.e.m.; $n = 4$; **, $P < .01$; NS, not significant; two-tailed Student's t -test).

same line, other sources of fatty acids, including intestinal cells, may similarly act on SUMOylation.

Our results show that BCFAs/SCFAs upregulate SUMOylation and dampen inflammatory responses of intestinal cells (Figure S10). SCFAs, and more particularly butyrate, have already been shown to modulate intestinal inflammation.³ The potential effect of BCFAs on inflammation remains in contrast poorly documented. Interestingly, long-chain BCFAs (with more than 14 carbons) were shown to decrease the expression of IL8 in response to LPS in CACO2 cells and to decrease the incidence of necrotizing enterocolitis in a neonatal rat model.^{35,36} Whether these effects are triggered by the acidic form of these long-chain BCFAs, once translocated inside intestinal cells, remains to be determined. Of note, lactic acid, which is abundantly produced by the vaginal microbiota, also elicits anti-inflammatory responses on human cervicovaginal epithelial cells.³⁷ Interestingly, only lactic acid, and not lactate, prevents pro-inflammatory cytokine expression in epithelial cells, which nicely echoes our result on the anti-inflammatory properties of the acidic forms of BCFAs/SCFAs on intestinal cells.^{38,39} The vaginal pH being naturally acid (pH <4), lactic acid is predominant in this environment compared to lactate. In the case of BCFAs and SCFAs produced by gut microbiota, the intraluminal pH is varying depending on the intestinal segment. This pH ranges from 5.5 to 7.5 in the cecum/right colon and then increases in the left colon and rectum to 6.1–7.5.³⁹ Eventhough the acidic forms of SCFAs/BCFAs are not predominant in these conditions, the physiological high concentrations of SCFAs/BCFAs (i.e. ~100 mM for SCFAs) may be high enough to have a concentration of protonated fatty acids sufficient to modulate intestinal SUMOylation.

Interestingly, SUMOylation has been involved in intestinal diseases such as Inflammatory Bowel Diseases (IBD). Indeed, patients with IBD show a downregulation of the UBC9 enzyme and a decrease in SUMOylated protein levels in the colon, which correlates with disease severity.⁴⁰ These SUMO alterations, which can also be observed in a mouse model of colitis, were proposed to contribute to intestinal immune response deregulation.⁴⁰ This hypothesis is supported by the

inhibition of gut inflammation observed in response to PIAS1 E3 ligase overexpression in the intestine and the associated increase in SUMOylation.⁴¹ Our results suggest that BCFAs/SCFAs may similarly limit inflammation in this context, by restoring SUMOylation in intestinal cells.

In conclusion, this work unveils a new mechanism used by the gut microbiota to modulate intestinal cell activities and dampen inflammation. It highlights in addition the therapeutic potential of SUMOylation targeting in the treatment of inflammatory diseases such as IBD.

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Disclosure statement

The authors declare that they have no conflict of interest.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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