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ORIGINAL ARTICLE

Sodium butyrate induces autophagy in colorectal cancer cells through LKB1/AMPK signaling

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Abstract

Butyrate is produced by the fermentation of undigested dietary fibers and acts as the promising candidate for cancer treatment. However, the mechanism underlying sodium butyrate (NaB)-induced autophagy in colorectal cancer is not yet completely understood. The expressions of LC3-II protein and mRNA were detected by western blot and quantitative RT-PCR in colorectal cancer (CRC) cell lines HCT-116 and HT-29, respectively. Autolysosome formation was observed by transmission electron microscope. AMPK and LKB1 were inhibited by chemical inhibitor or siRNAs and confirmed by western blot. NaB elevated the protein and mRNA expressions of LC3 in a dose-dependent manner. NaB treatment increased the formation of autolysosome and expression of phosphorylated liver kinase B1 (LKB1), AMP-activated protein kinase (AMPK), and acetyl-CoA carboxylase (ACC). Treatment with compound C (an inhibitor of AMPK) and siRNA-mediated knockdown of AMPK and LKB1 significantly attenuated NaB-induced autophagy in CRC cells. Collectively, these findings indicated that LKB1 and AMPK are critical for NaB-mediated autophagy and may act as the novel targets for colorectal cancer therapy in the future.

Keywords Sodium butyrate · Autophagy · Colorectal cancer · Liver kinase B1 (LKB1) · AMP-activated protein kinase (AMPK)

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide, with an estimate of 1.4 million cases and 693,900 deaths in 2012 [36]. Despite the advancement of screening, surgical techniques, radiotherapy, and chemotherapy for CRC, the five-year survival of CRC remains unsatisfied [2]. Therefore, it is urgently required to further investigate the molecular network underlying CRC progression to develop novel therapeutic approaches.

Butyrate, one of the by-products of bacterial fermentation of undigested dietary fibers, is implicated in maintaining colon epithelium homeostasis, which is functionally regulated by a number of factors, including the concentration of butyrate, cell type, or presence of additional dietary compounds or endogenous factors [25]. Butyrate serves as an important energy source and survival factor for normal cell, whereas shows promising anti-cancer effects in various types of cancer cells, including CRC, lymphoma, and breast cancer cells [6, 32, 34]. Butyrate prevents the deacetylation of histone by inhibiting the enzyme activities of histone deacetylases (HDACs), thus modulating expression of a number of functional genes involved in regulation of cell cycle progression, differentiation, apoptosis, and autophagy. It has also been shown that butyrate treatment reduces the metastatic ability of cancer cells through inhibition of CD44 and pro-MMP-2 [1, 40]. However, the molecular mechanisms involved in sodium butyrate (NaB)-mediated autophagy in CRC have not been fully explored.

Autophagy is a highly conserved evolutionary intracellular degradative process contributing to metabolic homeostasis, degradation or removal of proteins and organelles, cell

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survival, differentiation, and tissue development [38]. There are many biological and environmental factors which could induce or boost autophagy, such as starvation, hypoxia, low energy, acidity, and other mechanisms. Formation of double-membrane vesicles is required for autophagy to carry cargo for lysosomal degradation [23]. Autophagy is a double-edged sword; it can be utilized to meet the heightened nutrient demand found in proliferative cancer cells [5]. On the other hand, autophagy has been reported to protect cancer cells from apoptosis under radiation or chemotherapy [23, 44]. These seemingly contradictory findings suggest that the role of autophagy in tumorigenesis remains to be further investigated.

AMP-activated protein kinase (AMPK) consisting of catalytic α subunits and regulatory β and γ subunits is regulated by AMP/ATP ratio and plays a critical role in energy balance. The α-subunit of AMPK could be phosphorylated at the conserved threonine residue (Thr172) by upstream kinases, such as liver kinase B1 (LKB1), calcium/calmodulin-dependent protein kinase (CaMKK), and transforming growth factor β (TGF-β)-activated kinase (TAK1). Several studies have reported that AMPK is important for anti-cancer and autophagy by regulation of cell cycle arrest and tumor growth inhibition. LKB1, also termed as serine/threonine kinase 11(STK11), is a tumor suppressor and acts as the upstream kinase of AMPK in cancers [9]. The LKB1 mutation was first discovered in the Peutz-Jeghers syndrome, correlated with the risk of gastrointestinal hamartomatous polyposis and cancers [35]. Loss of LKB1 has been shown to be involved in tumorigenesis and cancer progression in many types of cancer, such as renal cell carcinoma [8], prometastatic tumor [24], pancreatic cancer [27], and hepatocellular carcinoma [15]. To date, the role of LKB1 and AMPK in NaB-induced CRC autophagy is unclear. Based on the above observations, this study aimed to investigate whether NaB induces autophagy in CRC cells and the underlying molecular mechanisms.

Materials and methods

Reagents and antibodies

Small interfering RNA (siRNA) duplexes specific for AMPKα, LKB1, and control siRNA were purchased from GenePharma (Shanghai, China). Lysis buffer, protease, and phosphatase inhibitor cocktail were purchased from Bestbio (Shanghai, China). Lipo6000™Transfection reagent was purchased from Beyotime (Shanghai, China). Trypsin, fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM) containing a high concentration of glucose, Opti-MEM, and RPMI-1640 medium were purchased from Gibco-Invitrogen (Carlsbad, CA, USA). Phosphate-buffered saline (PBS) was purchased from HyClone (Logan, Utah, USA). Sodium butyrate (NaB), compound C, dimethyl sulfoxide (DMSO), non-fat milk, monodansylcadaverin (MDC), acridine orange (AO), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRIzol was purchased from Invitrogen (Carlsbad, CA, USA). Anti-ACC, anti-p-ACC, anti-AMPKα, anti-p-AMPKα, anti-LKB1, anti-p-LKB1, anti-LC3, and anti-GAPDH antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). SYBER Green PCR kits were obtained from Takara (Dalian, China).

Cell culture

The human colorectal carcinoma HCT-116 and the colorectal adenocarcinoma HT-29 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). HCT-116 cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). HT-29 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

Transmission electron microscopy

HCT-116 cells were harvested by trypsinization, washed twice with PBS, and fixed with ice-cold glutaraldehyde (2.5% glutaraldehyde in PBS (pH 7. 4)) for 30 min. The cells were washed with PBS, fixed in osmium tetroxide (OsO4), and embedded in Spurr's Epon. Representative areas were chosen for ultrathin sectioning and viewed with a Hitachi 7500 electron microscope (Japan).

RNA isolation and quantitative real-time PCR

Total cellular RNA was extracted from HCT-116 and HT-29 cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Only the RNA samples with 260/280 nm absorbance ratios of 1.8–2.0 were considered of acceptable purity. RNA (450 ng) was reverse-transcribed to cDNA. The real-time RT-PCR mixture consisted of SYBR®Premix Ex Taq™II (Tli RNaseH Plus) (TAKARA, RR820A), 1 μL of a forward primer, 1 μL of a reverse primer, 90 ng of a cDNA sample, and DEPC water (final volume = 25 μL). Forty-five cycles of PCR were conducted under the following conditions: pre-incubation stage, 30 s at 95 °C; 2 step amplication, 5 s at 95 °C and 1 min at 60 °C; melt curve stage, 60 s at 95 °C, 55 s at 30 °C, and 1 s at 97 °C; and cooling stage, 30 s at 37 °C. PCR products were measured with LightCycler® 96 System (Roche Diagnostics, Mannheim, Germany). GAPDH was used as an internal control to correct for sample variation.

The PCR primer sequence is as follows:

For STK11,

forward: CTGATGTCGGTGGGTATGGA, reverse: GTACTTGCCGATGAGCTTGG;

For LC3,

forward: AAGTGGCTGAGTACCGACC, reverse: GATCTCCAGCTGCCACAAAC;

For GAPDH,

forward: CAAATTCCATGGCACCGTCA, reverse: ATCTCGCTCCTGGAAGATGG.

Western blot

HCT-116 and HT-29 cells were lysed with lysis buffer (Bestbio, Shanghai, China) containing a commercial protease and phosphatase inhibitor cocktail (Bestbio, Shanghai, China). Protein samples (40 μg) were subjected to 10% SDS-PAGE, then transferred to the PVDF membranes (Bio-Rad Laboratories), and probed with primary antibody. Secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences) goat anti-rabbit IgG was then applied. After washing, the membranes were visualized by chemiluminescence detection kit (Pierce Biotechnology) according to manufacturer's instructions. Target protein signals were normalized to GAPDH as the loading control. Densitometry analysis was carried out using Image J software (National Institutes of Health, USA).

Transfection with small interfering RNA

Small interfering RNAs (siRNAs) specific for LKB1 and AMPKα were purchased from GenePharma (Shanghai, China). Transfection of siRNA was performed using Lipo6000™ transfection reagent according to the supplier's protocol. HCT-116 and HT-29 cells were transfected with indicated siRNAs at 30% confluence. Prior to each of the following transfection steps, the wells of the cultured cells were replaced with 2 mL of Opti-MEM® medium. One hundred picomoles of siRNA was added to one tube and mixed gently; the other tube was added with 5 μL Lipo6000™ transfection reagent and gently mixed. After standing for 5 min at room temperature, the culture medium containing the siRNA was gently added to the culture solution containing the Lipo6000™ transfection reagent, and the tube was gently inverted mix under room temperature for 20 min. A mixture of Lipo6000™ transfection reagent and siRNA was added to each well, and the culture medium was changed after 5 h. The specific siRNA sequences are as follows:

LKB1 siRNA-1sense: 5′-CCAACGUGAAGAAGGAAAUTT-3′,
Antisense: 5′-AUUUCCUUCUUCACGUUGGTT-3′;
LKB1 siRNA-2sense: 5′-GGAUGACAUCAUCUACACUTT-3′,
Antisense: 5′-AGUGUAGAUGAUGUCAUCCTT-3′;
AMPK siRNA-1sense: 5′-GGGAACAUGAAUGGUUUAATT-3′,
Antisense: 5′-UUAAACCAUUCAUGUUCCCTT-3′;
AMPK siRNA-2sense: 5′-CGGGAUCAGUUAGCAACUATT-3′,
Antisense: 5′-UAGUUGCUAACUGAUCCCGTT-3′;

The scrambled control siRNA sequences were as follows:

Sense: 5′-UUCUCCGAACGUGUCACGUTT-3′ and
Antisense: 5′-ACGUGACACGUUCGGAGAATT-3′.

Statistical analysis

All experiments were repeated at least three times independently. Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed by one-way analysis of variance coupled with a Student-Newman-Keuls (SNK) post hoc test or Dunnett T3 test using SPSS 21.0 statistical software (SPSS, Inc., Chicago, IL, USA). A P value smaller than 0.05 was considered as statistical significance.

Results

NaB induced autophagy in CRC cells

To verify whether NaB induced the autophagic response in CRC cells, we analyzed the expression of free LC3-I and lipid-bound LC3-II by western blot. LC3 is an important constituent of the autophagosome and widely used as a biochemical marker of autophagy [33]. After NaB treatment for 24 h, a marked induction of LC3 was detected in HCT-116 (Fig. 1a) and HT-29 (Fig. 1b). We found that the expression of the LC3-II protein was significantly increased starting from 0.1 mM of NaB treatment in HCT-116 cells, but at 1 mM NaB in HT-29 cells for LC3-II protein induction (Fig. 1a, b), indicating that HCT-116 cells were more sensitive to NaB on autophagic response than HT-29 cells. The accumulation of LC3-II was positively correlated with the concentration of NaB, suggesting that NaB induced autophagy in a dose-dependent manner. Likewise, NaB also significantly upregulated LC3B expression at mRNA levels in a dose-dependent manner (Fig. 1c, d).

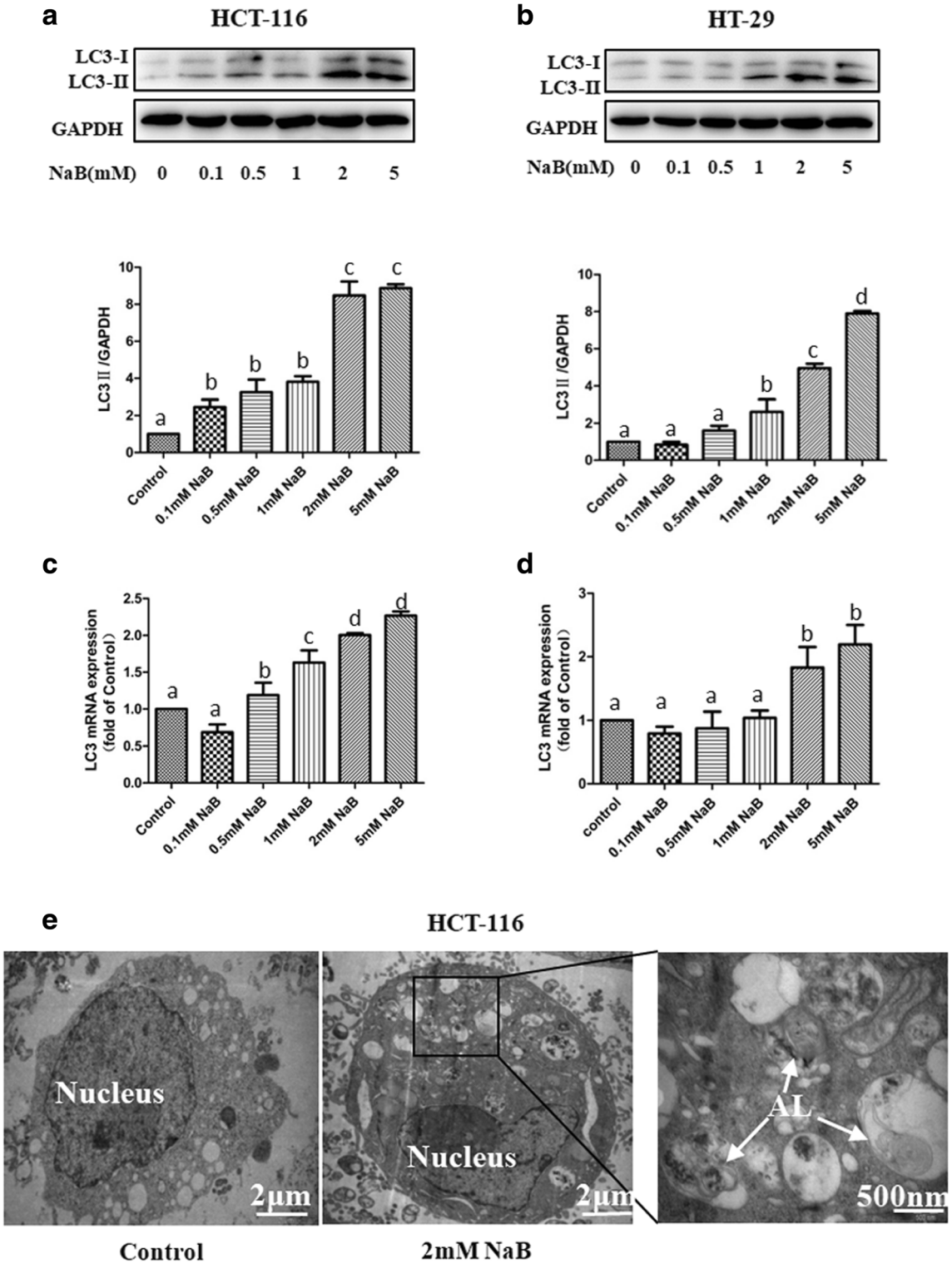


Fig. 1 Sodium butyrate induced autophagy in colorectal cancer cells. HCT-116 (**a**) and HT-29 (**b**) cells were treated with the indicated concentrations of sodium butyrate (NaB) for 24 h. Representative blots of LC3-II expression were quantified by densitometry and normalized to GAPDH (ratio of LC3-II: GAPDH). mRNA expressions of LC3 in HCT-116 (**c**) and HT-29 (**d**) cells were determined by real-time PCR analysis. Data are shown as the mean ± SD of three independent experiments. Different letters indicate statistically significant differences between groups ($P < 0.05$). **e** Electronic microscopy images of control and NaB (2 mM)-treated HCT-116 cells. AL, autolysosome

To further confirm whether NaB induces CRC cell autophagic morphology changes, transmission electron microscope was used to observe the formation of autophagosomes. The accumulation of double-membrane vesicles was dramatically

increased in HCT-116 cells treated with 2 mM NaB for 24 h than that in the control group (Fig. 1e). These data clearly indicated autophagosome formation in response to NaB treatment in CRC cells.

AMPKα/ACC signaling was activated by NaB in colorectal cells

AMPK has been extensively studied as an important metabolic regulator of energy homeostasis, and AMPK is involved in the modulation of autophagy [10]. To confirm whether the autophagic effect of NaB is related to AMPK signaling, western blot was performed in NaB-treated CRC cells. As shown in Fig. 2a, treatment with NaB dose-dependently increased the expression of Thr172 phosphorylated AMPKα (p-AMPKα) in HCT-116 cells. To further define whether NaB-mediated phosphorylation of AMPKα functionally activates downstream signaling, we detected the phosphorylation status of acetyl-CoA carboxylase (ACC), the critical downstream molecule of AMPK. We found that the expression of Ser79 phosphorylated ACC (p-ACC) was increased after treatment with NaB in HCT-116 (Fig. 2a). Consistently, we also found the induction of p-AMPKα and p-ACC resulted from NaB treatment in HT-29 cells (Fig. 2b), indicating that this phenomenon was not cell line specific. However, this AMPK/ACC activation effect of NaB was increased to the maximum at 1 mM and then slightly decreased in HT-29 cells (Fig. 2b). Compared with control group, NaB (5 mM for HCT-116 cells, 1 mM for HT-29 cells) caused a drastic induction of phosphorylated AMPKα (Thr172) and ACC (Ser79). These data indicated that NaB activates the AMPK/ACC signaling cascade in CRC cells.

AMPK is required for NaB-mediated autophagy in CRC cells

To further investigate whether AMPK is required for NaB-induced autophagy, we used the AMPK inhibitor compound C (2 μM) to pretreat HCT-116 and HT-29 cells for 1 h before NaB exposure. Com. C effectively blocked NaB-stimulated phosphorylation of AMPKα and ACC (Fig. 3a, b). More important, NaB-induced LC3 expression was also suppressed by compound C in protein (Fig. 3a, b) and mRNA (Fig. 3c, d) levels. These results indicated that AMPK is critical for NaB-mediated autophagy in CRC cells. To further confirm the role of AMPK in NaB-induced autophagic response, we used the AMPKα-specific siRNAs to knockdown the expression of AMPKα. AMPKα-specific siRNAs effectively reduced the expression of AMPKα both in protein and RNA levels compared with control siRNA in HCT-116 and HT-29 cells (Supplementary Fig. 1). The AMPKα siRNAs also decreased the expressions of AMPKα, p-AMPKα, ACC, and p-ACC (Fig. 4a, b). Additionally, the expression of LC3-II was significantly downregulated by AMPKα siRNA in the protein (Fig. 4a, b) and mRNA levels (Fig. 4c, d). These results, including the specific chemical inhibitor and genetic inhibition of AMPKα, indicated that AMPK was required for NaB-induced autophagy in CRC cells.

LKB1 signaling was involved in NaB-induced autophagy in CRC cells

LKB1 is a tumor suppressor and acts as the upstream serine/threonine kinase that phosphorylates and activates AMPK signaling [13, 33]. Therefore, we detected the effects of NaB on LKB1 expression and activation in CRC cells. NaB treatment induced the phosphorylation of LKB1 in HCT-116 (Fig. 5a) and HT-29 cells (Fig. 5b). LKB1 phosphorylation significantly elevated at 2 and 5 mM NaB in HCT-116 (Fig. 5a) but at 0.1, 0.5, 1, 2, and 5 mM NaB in HT-29 cells (Fig. 5b). To determine whether LKB1 is functionally involved in the regulation of NaB-induced autophagy in CRC cells, we knockdowned the expression of LKB1 by LKB1-specific siRNAs. LKB1-specific siRNAs dramatically suppressed the expression of LKB1 in both protein and mRNA level in HCT-116 and HT-29 cells (Supplementary Fig. 2). Moreover, LKB1 siRNAs significantly suppressed the downstream signaling of LKB1, including the phosphorylation of AMPKα and ACC in HCT-116 (Fig. 6a, e) and HT-29 cells (Fig. 6b, f). In addition, the expression of LC3-II was also downregulated by LKB1α siRNA in the protein (Fig. 6a, b) and mRNA levels (Fig. 6c, d). These results indicated that LKB1 is required for NaB-induced AMPK and ACC phosphorylation and autophagy in CRC cells.

Discussion

Autophagy is a lysosomal degradation pathway for the breakdown of intracellular proteins and organelles. Mounting evidence reveals that autophagy is essential for the progression or prevention of cancer. NaB exhibits the anti-tumor activity by regulation of several cancer-related genes [28], but whether autophagy involves in NaB-mediated biological effects in colon cancer cells is still not fully understood. Here, we demonstrated that NaB induces autophagy in CRC cells, at least partially, through activation of the LKB1-AMPK signaling pathway for the first time. Our previous study has shown that chloroquine treatment alone increases the accumulation of LC3-II in both HCT-116 and HT-29 cells, indicating inhibition of autophagy successfully blocked LC3 degradation [43]. In addition, chloroquine and NaB co-treatment considerably elevates the LC3-II level as compared with the NaB treatment alone [43]. These findings suggest that NaB treatment increased autophagic flux in our model.

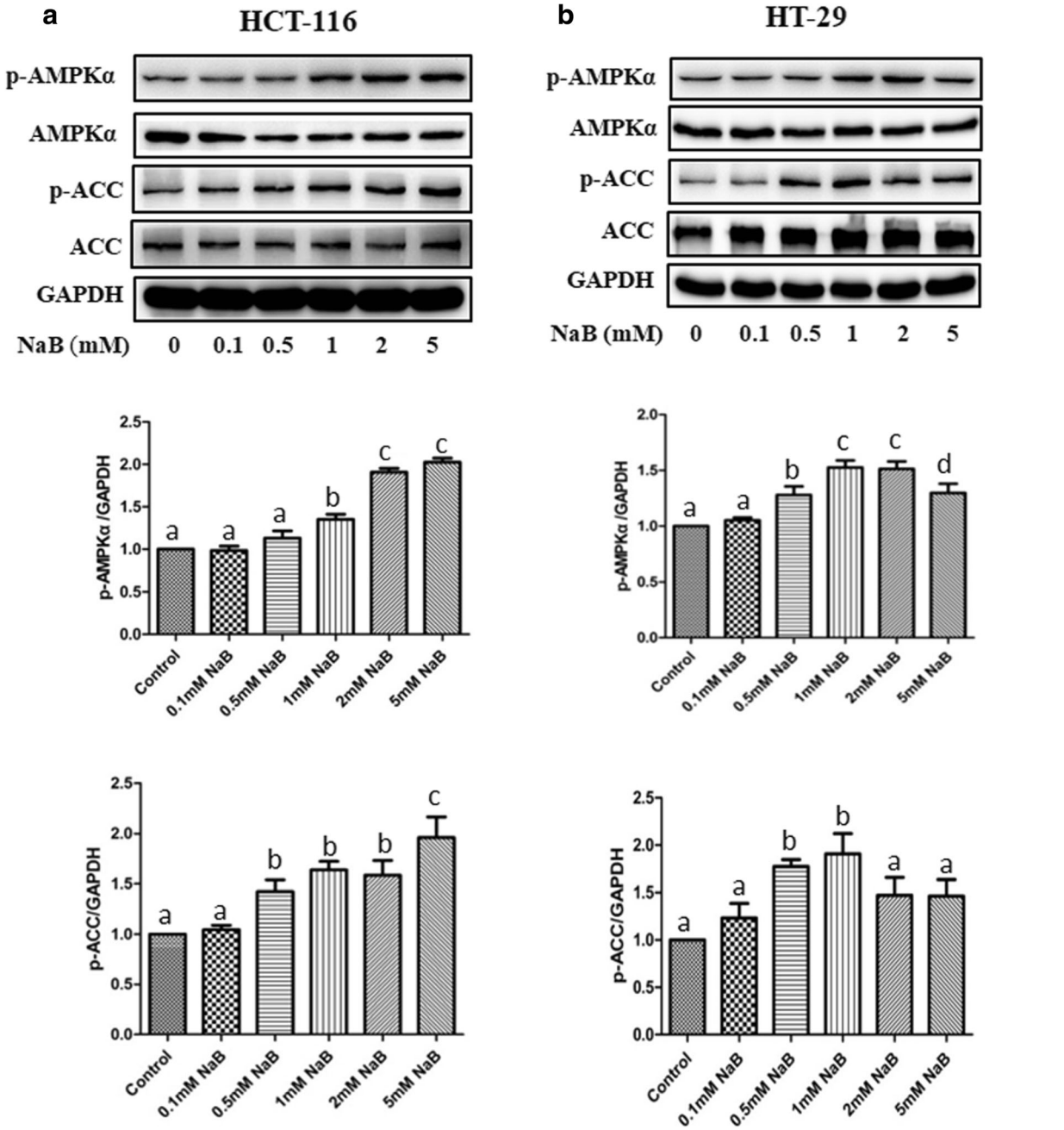


Fig. 2 Sodium butyrate induced AMPK and ACC activation in colorectal cancer cells. HCT-116 (**a**) and HT-29 (**b**) cells were treated with the indicated concentrations of sodium butyrate (NaB) for 2 h. Protein expression of AMPKα, p-AMPKα, ACC, and p-ACC was determined by western blot. Representative blots of p-AMPKα and p-ACC expression were quantified by densitometry and normalized to GAPDH. The fold change compared to control group is indicated as the mean ± SD of three independent experiments. Different letters indicate statistically significant differences between groups ($P < 0.05$)

NaB exhibits the anti-cancer activities, such as decreasing tumor proliferation and inducing apoptosis via regulation of histone deacetylase (HDAC), SIRT-1, caspase 3, NFκB, and ROS in various types of cancer [14, 26, 28]. Our previous study showed that the cell viability was significantly inhibited at 0.5, 1, 2, and 5 mM of NaB in HCT-116 cells but at 2 and 5 mM of NaB in HT-29 cells [43]. HDAC has been studied as an attractive target for anti-cancer drug development by targeting the cancer epigenome for decades [39]. Since NaB is a natural product with the inhibitory activity of HDAC, there are many clinical trials aiming to evaluate NaB as a potential anti-cancer drug for treating human cancers [17]. Beside the HDAC inhibitory effect, NaB shows anti-cancer ability in different cancers through various pathways. In hepatocellular carcinoma cells, NaB increased the ROS level and then inhibited Akt and mTOR signaling, which in turn

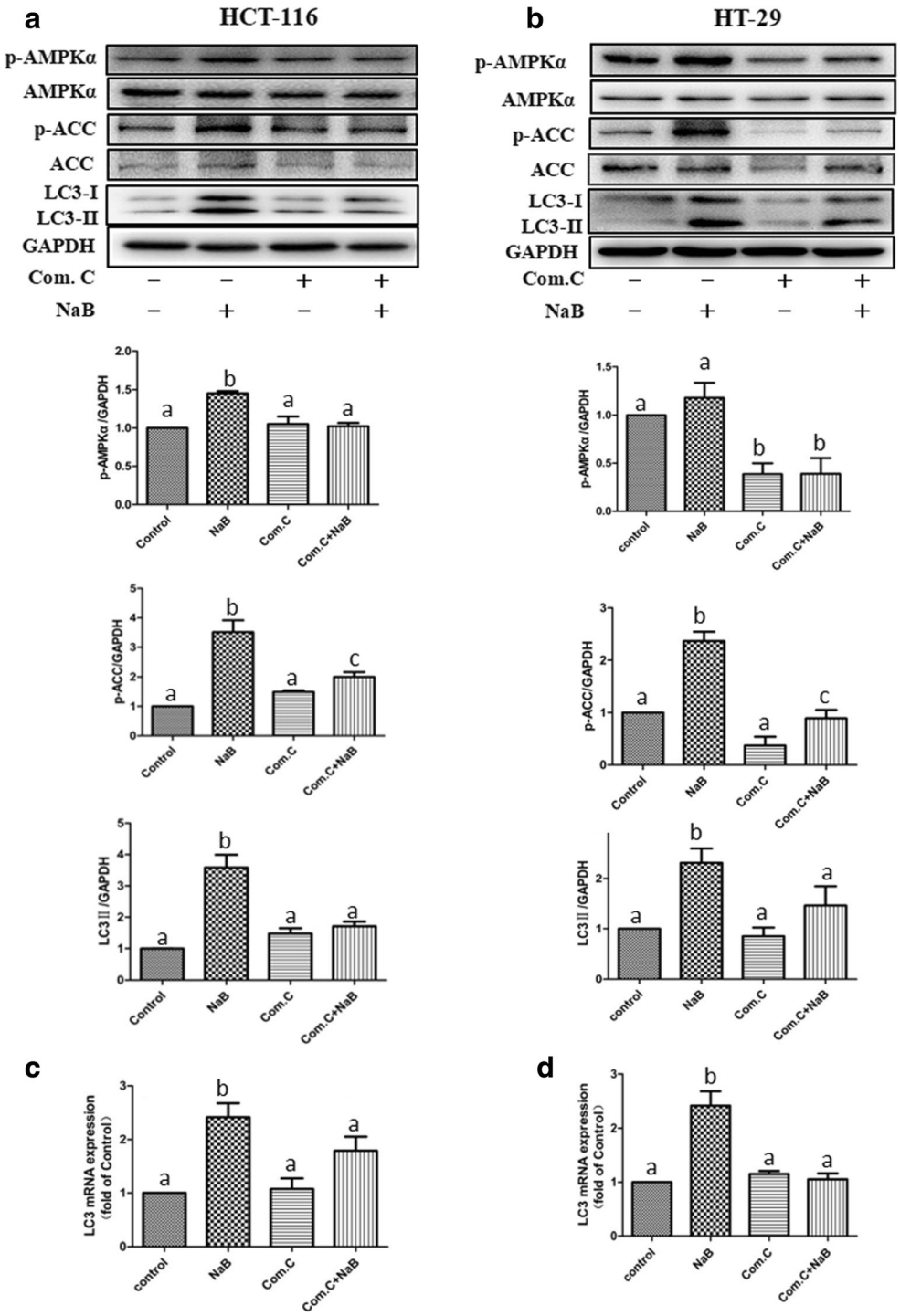


Fig. 3 Inhibition of AMPK activity suppressed sodium butyrate-induced autophagy in colorectal cancer cells. HCT-116 (**a**) and HT-29 (**b**) cells were pretreated with or without 2 μM compound C (Com. C) for 1 h, followed by NaB (2 mM) treatment for 24 h. The expression of AMPKα, p-AMPKα, ACC, p-ACC, and LC3 was performed by western blot analysis. Representative blots of p-AMPKα, p-ACC, and LC3-II expression were quantified by densitometry and normalized to GAPDH. The fold change is indicated as the mean ± SD of three independent experiments. mRNA levels of LC3 in HCT-116 (**c**) and HT-29 (**d**) cells were determined by quantitative real-time RT-PCR. Different letters indicate statistically significant differences between groups ($P < 0.05$)

resulted in autophagic response [29]. Tributyrin (a butyric acid prodrug) shows chemopreventive effects by activation of p53-mediated tumor suppressive pathway [4]. In combination with calpain inhibitor, NaB increased the expression of tumor suppressors, including ARHI, p21, and RARβ2 to inhibited cell cycle progression and induced autophagy in ovarian cancer cells [18]. These studies indicate that NaB may modulate different pathways to illustrate multiple biological functions. To date, accumulated evidence suggests the involvement of LKB1-AMPK energy-sensing pathway in the process of autophagy, such as vascular smooth muscle cell (VSMC) senescence, non-small-cell lung cancer, hepatocellular carcinoma, and atherosclerosis [12, 21, 22, 42]. In the present study, we also found that NaB induced autophagy through LKB1/AMPK/ACC axis in CRC cells, but the final fates of NaB-treated CRC cells need further investigations.

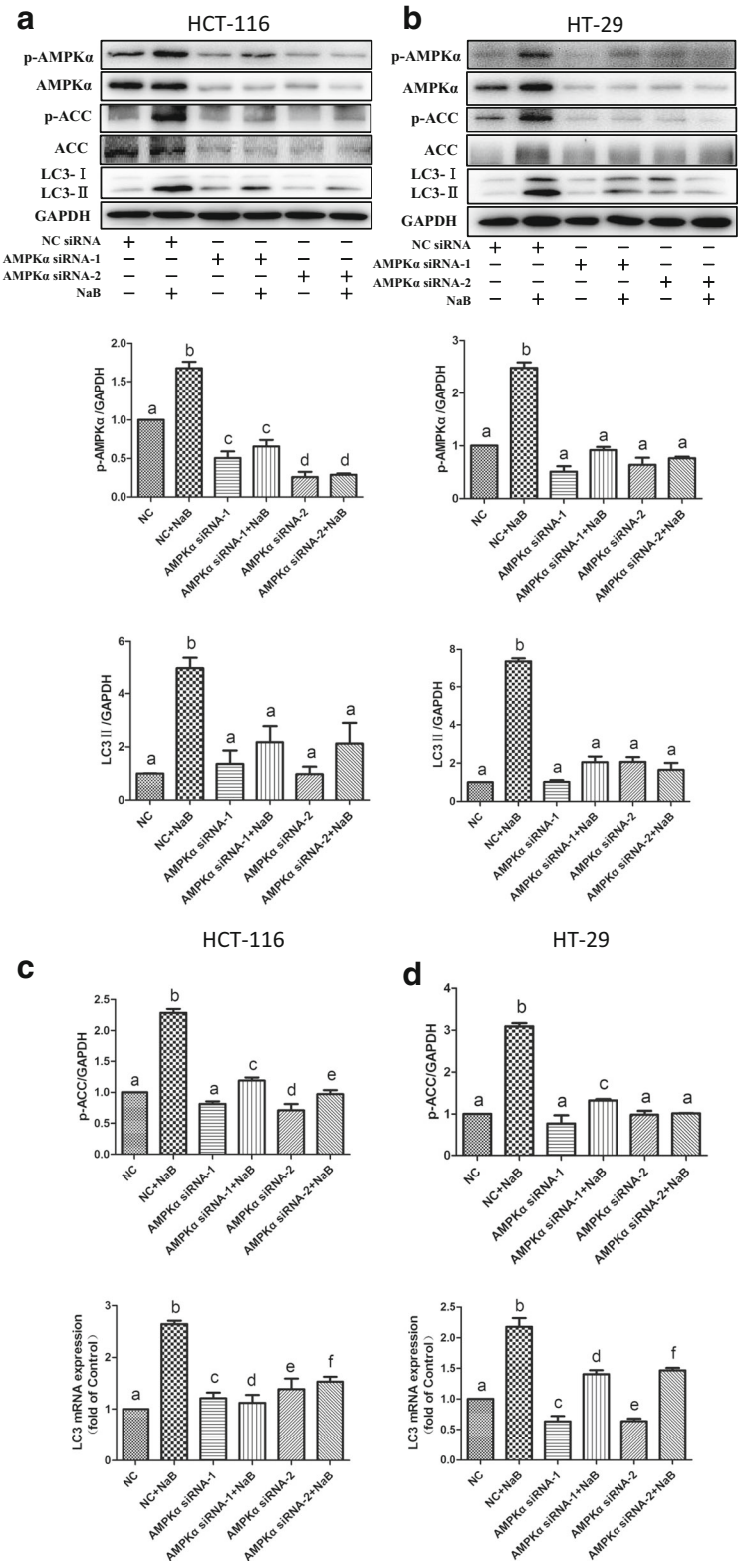


Fig. 4 RNA interference targeting AMPKα blocked sodium butyrate-induced autophagy in colorectal cancer cells. HCT-116 (**a**) and HT-29 (**b**) cells were transfected with negative control (NC) or AMPKα-specific siRNAs for 48 h and then treated with or without 2 mM sodium butyrate (NaB) for 24 h. AMPKα, p-AMPKα, ACC, p-ACC, and LC3 protein expressions were examined by western blot. The expression level of each protein was determined by densitometry and normalized to GAPDH. mRNA levels of LC3 in HCT-116 (**c**) and HT-29 (**d**) cells were determined by real-time RT-qPCR. Means and SD of three independent experiments are shown. Different letters indicate statistically significant differences between groups ($P < 0.05$)

A study screening 10 human CRC cell lines by Lazarova et al. has demonstrated that human CRC cell lines can be divided into two categories according to their response to NaB treatment: those respond to NaB treatment with a high fold induction of canonical Wnt activity and apoptosis (e.g., COLO201, LS174T, DLD-1, HCT-116) and those exhibit a relatively lower fold induction of canonical Wnt activity and apoptosis in response to NaB treatment (e.g., LoVo, SW48,

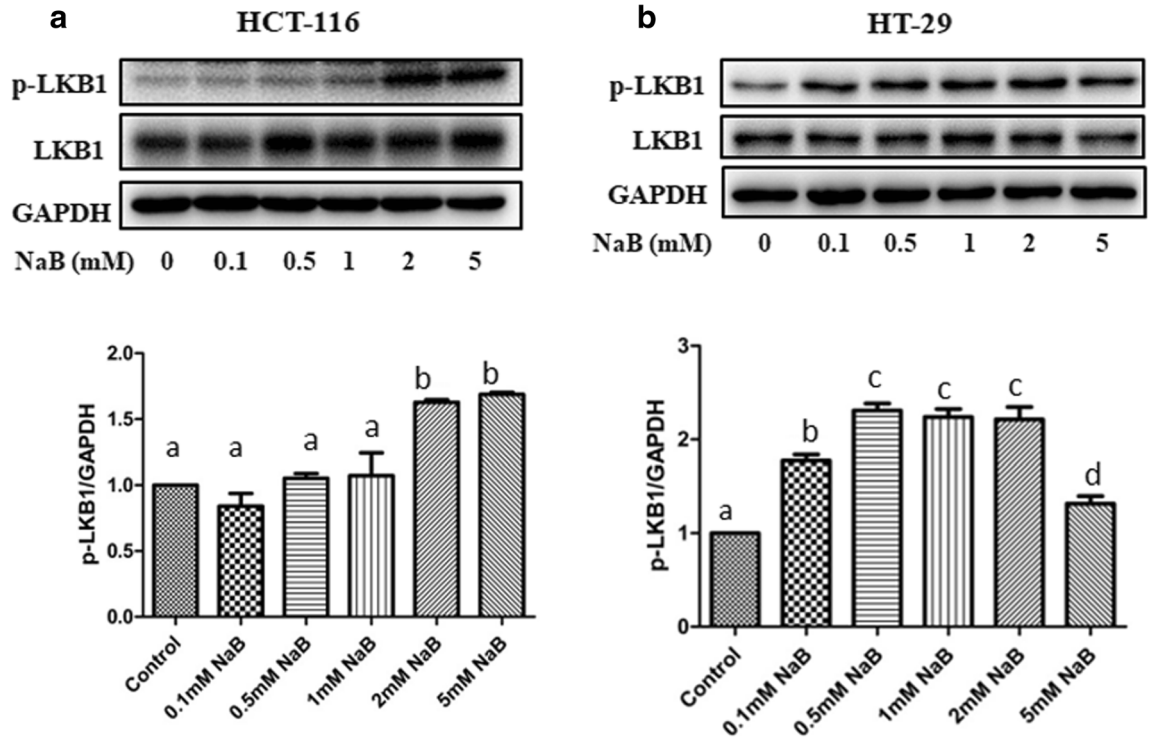
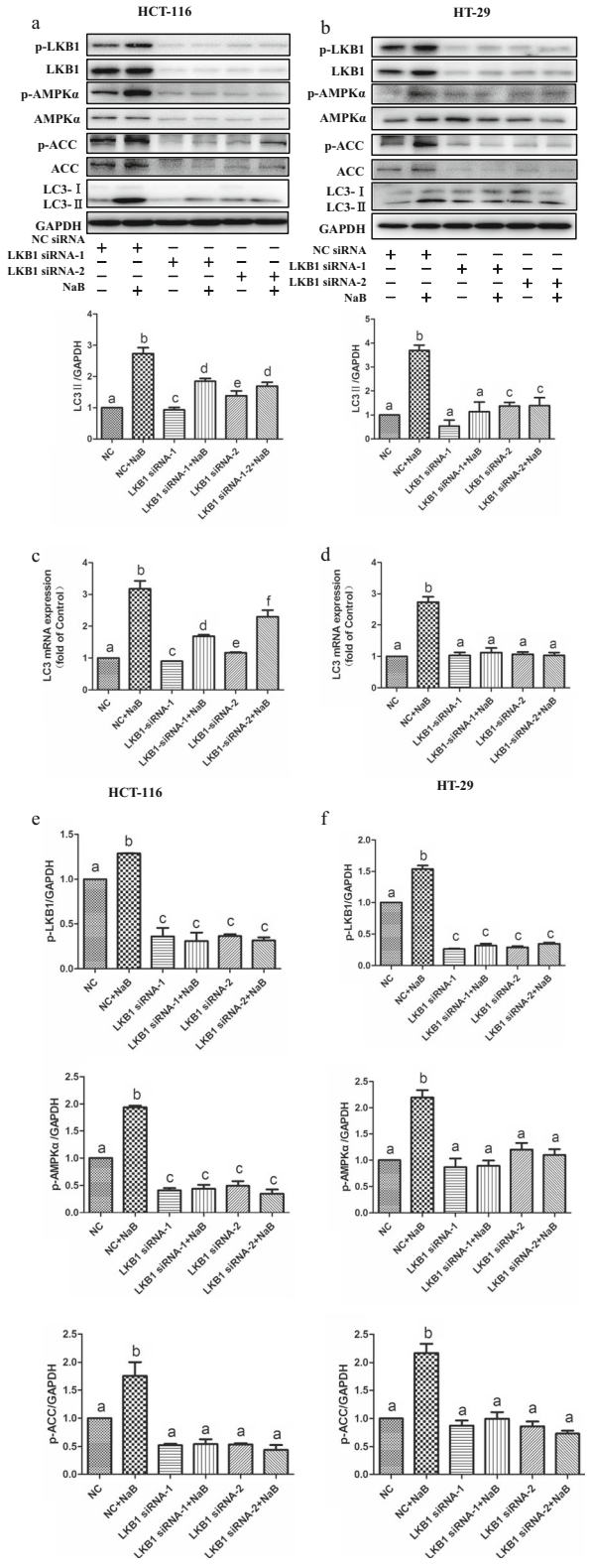


Fig. 5 Sodium butyrate induced LKB1 activation in CRC cells. HCT-116 (**a**) and HT-29 (**b**) cells were treated with the indicated concentrations of sodium butyrate (NaB) for 2 h. Representative blots of p-LKB1 expression were quantified by densitometry and normalized to GAPDH. The fold change is indicated as the mean ± SD of three independent experiments. Different letters indicate statistically significant differences between groups ($P < 0.05$)

RKO, HT-29) [19]. Thus, we selected two CRC cell lines (HCT-116 and HT-29) with different responses to NaB treatment in this study. Donohoe et al. have detected 3.5 mM, 0.8 mM, and 0.5 mM concentration of NaB in the proximal, medial, and distal segments of mouse colon lumen, and suggested that the concentration of NaB in colonic epithelial cells generally ranges between 0.5 and 5 mM [7]. In this study, we used 0.1 mM to 5 mM concentration of NaB, which is within the physiological range. We observed subtle differences in the responses to NaB treatment under concentration gradient (0–5 mM) between HCT-116 and HT-29 cells. For example, NaB upregulated p-LKB1, p-AMPK, and p-ACC expression at protein levels in a dose-dependent manner in HCT-116 cells but not HT-29 cells. The expression of p-LKB1, p-AMPK, and p-ACC was firstly increased to the maximum at 1 mM, and then this induction was minor reduced at 2 and 5 mM in HT-29 cells. LKB1 phosphorylation significantly elevated at 2 and 5 mM of NaB in HCT-116 but at 0.1, 0.5, 1, 2, and 5 mM concentration of NaB in HT-29 cells. Our previous study also showed that cell viability was significantly inhibited at 0.5, 1, 2, and 5 mM concentration of NaB in HCT-116 cells but at 2 and 5 mM of NaB in HT-29 cells [43]. Since Lazarova et al. have shown that HCT-116 cells are more sensitive to NaB than HT-29 cells in the activation of Wnt signaling [19], therefore, the differences in intracellular signaling thresholds between HCT-116 and HT-29 cells may contribute to the differences in the responses to NaB treatment between HCT-116 and HT-29 cells in our study. Additionally, it has been reported that treatment with 3 mM NaB for 48 h mediated autophagy to support HT-29 cells survival, but induced autophagic cell death and apoptosis in HCT-116 cells [37]. The different responses in the two CRC cell lines may result from the differentiating ability of cells. These findings implied that different CRC subtypes might respond to butyrate therapy differently in a clinical setting.

Autophagy is an evolutionarily conserved degradative process for removing organelles and proteins under harmful conditions [11]. There are three subtypes of autophagy, including macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) [30]. In our study, we did not exactly distinguish which type of autophagy was induced by NaB; although we mainly referred to macroautophagy, we did not exclude other two types of autophagy, the microautophagy and CMA, which may also be involve in NaB's functions. Autophagy interaction with apoptosis decides the fate of cancer cells in response to anti-cancer treatments, including chemotherapy, target therapy, and radiation therapy [31]. Autophagy may parallel with apoptosis or sequentially leads to or follow by an apoptotic process in CRC cells upon the stimulations [3, 20]. Otherwise, lines of evidence also reported the protective function of autophagy by antagonized apoptosis in CRC cells under anti-cancer drugs treatment [31]. Our previous study has already shown that preventing autophagy enhanced NaB-induced apoptosis in CRC cells [43].



◀ **Fig. 6** LKB1 siRNA suppressed NaB-induced AMPK/ACC activation and autophagy in colorectal cancer cells. HCT-116 (**a**) and HT-29 (**b**) cells were transfected with negative control (NC) or LKB1-specific siRNAs for 48 h and then treated with or without 2 mM of NaB for 24 h. Negative control (NC) scramble siRNA acts as the negative control for the transfection. The expressions of LKB1, p-LKB1, AMPKα, p-AMPKα, ACC, p-ACC, and LC3 proteins were investigated by western blot. The expression level of each protein was determined by densitometry and normalized to GAPDH (**e**, **f**). mRNA levels of LC3 in HCT-116 (**c**) and HT-29 (**d**) cells were determined using real-time PCR. Means and SD of three independent experiments are shown. Different letters indicate statistically significant differences between groups ($P < 0.05$)

The destiny of cancer cells is decided by the balance between numerous autophagy-related or apoptosis-related proteins, such as Bcl-2 family, p53, mTOR, Atg5, and Beclin-1 [31].

Cancer cells may have different ways in response to the anti-cancer therapy, such as apoptosis, necrosis, necroptosis, and autophagy [16, 41]. In summary, the present study demonstrates that the LKB1-AMPKα signaling pathway plays a critical role in NaB-induced autophagy in CRC cells. Further study on the molecular mechanisms involved in the cancer progression and drug response of CRC may provide novel therapeutic strategy and clinical values.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

1. Barshishat M, Levi I, Benharroch D, Schwartz B (2002) Butyrate down-regulates CD44 transcription and liver colonisation in a highly metastatic human colon carcinoma cell line. Br J Cancer 87: 1314–1320
2. Breugom AJ, Swets M, Bosset JF, Collette L, Sainato A, Cionini L, Glynne-Jones R, Counsell N, Bastiaannet E, van den Broek CBM, Liefers GJ, Putter H, van de Velde CJH (2015) Adjuvant chemotherapy after preoperative (chemo) radiotherapy and surgery for patients with rectal cancer: a systematic review and meta-analysis of individual patient data. Lancet Oncol 16:200–207
3. Coker-Gürkan A, Arisan ED, Obakan P, Akalın K, Özbey U, Palavan-Unsal N (2015) Purvalanol induces endoplasmic reticulum stress-mediated apoptosis and autophagy in a time-dependent manner in HCT116 colon cancer cells. Oncol Rep 33:2761–2770
4. de Conti A, Tryndyak V, Koturbash I, Heidor R, Kuroiwa-Trzmielina J, Ong TP, Beland FA, Moreno FS, Pogribny IP (2013) The chemopreventive activity of the butyric acid prodrug tributyrin in experimental rat hepatocarcinogenesis is associated with p53 acetylation and activation of the p53 apoptotic signaling pathway. Carcinogenesis 34:1900–1906

5. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, Nelson DA, Jin S, White E (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10:51–64
6. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, Bultman SJ (2011) The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab 13:517–526
7. Donohoe DR, Collins LB, Wali A, Bigler R, Sun W, Bultman SJ (2012) The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. Mol Cell 48: 612–626
8. Duivenvoorden WC, Beatty LK, Lhotak S, Hill B, Mak I, Paulin G, Gallino D, Popovic S, Austin RC, Pinthus JH (2013) Underexpression of tumour suppressor LKB1 in clear cell renal cell carcinoma is common and confers growth advantage in vitro and in vivo. Br J Cancer 108:327–333
9. Gan R, Li H (2014) Recent progress on liver kinase B1 (LKB1): expression, regulation, downstream signaling and cancer suppressive function. Int J Mol Sci 15:16698–16718
10. Ha J, Guan KL, Kim J (2015) AMPK and autophagy in glucose/glycogen metabolism. Mol Asp Med 46:46–62
11. Hale AN, Ledbetter DJ, Gawriluk TR, Rucker EB III (2013) Autophagy: regulation and role in development. Autophagy 9: 951–972
12. Han D, Li SJ, Zhu YT, Liu L, Li MX (2013) LKB1/AMPK/mTOR signaling pathway in non-small-cell lung cancer. Asian Pac J Cancer Prev 14:4033–4039
13. Hardie DG (2013) The LKB1-AMPK pathway-friend or foe in cancer? Cancer Cell 23:131–132
14. Hofmanova J, Hyrslova Vaculova A, Kozubik A (2013) Regulation of the metabolism of polyunsaturated fatty acids and butyrate in colon cancer cells. Curr Pharm Biotechnol 14:274–288
15. Huang YH, Chen ZK, Huang KT, Li P, He B, Guo X, Zhong JQ, Zhang QY, Shi HQ, Song QT, Yu ZP, Shan YF (2013) Decreased expression of LKB1 correlates with poor prognosis in hepatocellular carcinoma patients undergoing hepatectomy. Asian Pac J Cancer Prev 14:1985–1988
16. Koehler BC, Jäger D, Schulze-Bergkamen H (2014) Targeting cell death signaling in colorectal cancer: current strategies and future perspectives. World J Gastroenterol 20:1923–1934
17. Lane AA, Chabner BA (2009) Histone deacetylase inhibitors in cancer therapy. J Clin Oncol 27:5459–5468
18. Lapinska K, Housman G, Byler S et al (2016) The effects of histone deacetylase inhibitor and calpain inhibitor combination therapies on ovarian cancer cells. Anticancer Res 36:5731–5742
19. Lazarova DL, Bordonaro M, Carbone R, Sartorelli AC (2004) Linear relationship between Wnt activity levels and apoptosis in colorectal carcinoma cells exposed to butyrate. Int J Cancer 110: 523–531
20. Lee HY, Chung KJ, Hwang IH, Gwak J, Park S, Ju B, Yun E, Kim DE, Chung YH, Na MK, Song GY, Oh S (2015) Activation of p53 with ilimaquinone and ethylsmenoquinone, marine sponge metabolites, induces apoptosis and autophagy in colon cancer cells. Mar Drugs 13:543–557
21. Lee KY, Kim JR, Choi HC (2016) Genistein-induced LKB1-AMPK activation inhibits senescence of VSMC through autophagy induction. Vasc Pharmacol 81:75–82
22. Li GH, Lin XL, Zhang H et al (2004) Ox-Lp(a) transiently induces HUVEC autophagy via an ROS-dependent PAPR-1-LKB1-AMPK-mTOR pathway. Atherosclerosis 243:223–235
23. Liu EY, Ryan KM (2012) Autophagy and cancer - issues we need to digest. J Cell Sci 125:2349–2358
24. Liu W, Monahan KB, Pfefferle AD, Shimamura T, Sorrentino J, Chan KT, Roadcap DW, Ollila DW, Thomas NE, Castrillon DH, Miller CR, Perou CM, Wong KK, Bear JE, Sharpless NE (2012) LKB1/STK11 inactivation leads to expansion of a prometastatic tumor subpopulation in melanoma. Cancer Cell 21:751–764
25. Lupton JR (2004) Microbial degradation products influence colon cancer risk: the butyrate controversy. J Nutr 134:479–482
26. Medina V, Edmonds B, Young GP, James R, Appleton S, Zalewski PD (1997) Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. Cancer Res 57:3697–3707
27. Morton JP, Jamieson NB, Karim SA et al (2010) LKB1 haploinsufficiency cooperates with Kras to promote pancreatic cancer through suppression of p21-dependent growth arrest. Gastroenterology139 586-597(597):e1–e6
28. Pant K, Yadav AK, Gupta P, Islam R, Saraya A, Venugopal SK (2017) Butyrate induces ROS-mediated apoptosis by modulating miR-22/SIRT-1 pathway in hepatic cancer cells. Redox Biol 12: 340–349
29. Pant K, Saraya A, Venugopal SK (2017) Oxidative stress plays a key role in butyrate-mediated autophagy via Akt/mTOR pathway in hepatoma cells. Chem Biol Interact 273:99–106
30. Qian HR, Yang Y (2016) Functional role of autophagy in gastric cancer. Oncotarget 7:17641–17651
31. Qian HR, Shi ZQ, Zhu HP, Gu LH, Wang XF, Yang Y (2017) Interplay between apoptosis and autophagy in colorectal cancer. Oncotarget 8:62759–62768
32. Ruemmele FM, Schwartz S, Seidman EG, Dionne S, Levy E, Lentze MJ (2003) Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway. Gut 52:94–100
33. Shackelford DB, Shaw RJ (2009) The LKB1–AMPK pathway: metabolism and growth control in tumour suppression. Nat Rev Cancer 9:563–575
34. Suman S, Das TP, Reddy R, Nyakeriga AM, Luevano JE, Konwar D, Pahari P, Damodaran C (2014) The pro-apoptotic role of autophagy in breast cancer. Br J Cancer 111:309–317
35. Swart C, Du Toit A, Loos B (2016) Autophagy and the invisible line between life and death. Eur J Cell Biol 95:598–610
36. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A (2015) Global cancer statistics, 2012. CA Cancer J Clin 65:87–108
37. Tylichová Z, Straková N, Vondráček J, Vaculová AH, Kozubík A, Hofmanová J (2017) Activation of autophagy and PPARγ protect colon cancer cells against apoptosis induced by interactive effects of butyrate and DHA in a cell type-dependent manner: the role of cell differentiation. J Nutr Biochem 39:145–155
38. Vikram A, Anish R, Kumar A, Tripathi DN, Kaundal RK (2017) Oxidative stress and autophagy in metabolism and longevity. Oxidative Med Cell Longev 2017:3451528
39. West AC, Johnstone RW (2014) New and emerging HDAC inhibitors for cancer treatment. J Clin Invest 124:30–39
40. Zeng H, Briske-Anderson M (2005) Prolonged butyrate treatment inhibits the migration and invasion potential of HT1080 tumor cells. J Nutr 135:291–295
41. Zhang X, Chen L (2016) The recent progress of the mechanism and regulation of tumor necrosis in colorectal cancer. J Cancer Res Clin Oncol 142:453–463
42. Zhang H, Li N, Wu J, Su L, Chen X, Lin B, Luo H (2013) Galangin inhibits proliferation of HepG2 cells by activating AMPK via increasing the AMP/TAN ratio in a LKB1-independent manner. Eur J Pharmacol 718:235–244
43. Zhang J, Yi M, Zha L, Chen S, Li Z, Li C, Gong M, Deng H, Chu X, Chen J, Zhang Z, Mao L, Sun S (2016) Sodium butyrate induces endoplasmic reticulum stress and autophagy in colorectal cells: implications for apoptosis. PLoS One 11:e0147218
44. Zhao Z, Li J, Ye R, Wu X, Gao L, Niu B (2017) A phase II clinical study of combining FOLFIRI and bevacizumab plus erlotinib in 2nd-line chemotherapy for patients with metastatic CRC. Medicine (Baltimore) 96:e7182