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UNRAVELING THE MECHANISMS INVOLVED IN ACETATE INDUCED APOPTOSIS IN COLORECTAL CANCER

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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O conhecimento é orgulhoso por ter aprendido tanto; a sabedoria é humilde por não saber mais. Saber o que é possível, é o começo da felicidade.

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ABBREVIATION LIST

ACF	Aberrant crypt foci
AO	Acridine Orange
APC	Adenomatous polyposis coli
AV	Annexin V
Baf A1	Bafilomicin A1
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BrdU	Bromodeoxyuridine
CatB	Cathepsin B
CatD	Cathepsin D
CatL	Cathepsin L
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CHC	α-cyano-hydroxycinnamic acid
CIN	Chromosomal instability
CRC	Colorectal Cancer
DAPI	4’,6-diamidino-2-phenylindole
DCF	Dichlorofluorescein
DHE	Dihydroethidium
DIDS	4,4’-Di-isothiocyano-2,2’-stilbenedisulfonic acid
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic acid
E-64d	(2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester
EGFR	Epidermal growth factor receptor
ERK	Extracellular-signal-regulated kinase
FAO	Food and Agricultural Organization
FAP	Familial adenomatous polyposis
FDA	United States food and drug administration
FITC	Fluorescein isothiocyanate
H_2DCF-DA	2’,7’-dihydrodichlorofluorescein diacetate
H_2O_2	Peróxido de hidrogênio
IBD	Inflammatory bowel disease

IC	Inhibitory concentration
IL	Interleukin
KRAS	Kirsten rat sarcoma viral oncogene homolog
LC3	Microtubule-associated protein 1A/1B-light chain 3
LMP	Lysosomal membrane permeabilization
LV	Leucovorin
$_{m}$CRC	Metastatic colorectal cancer
MCT	Monocarboxylate transporter
MMP	Mitochondrial membrane potential
MMR	Mismatch repair
MOMP	Mitochondrial outer membrane permeabilization
MSI	Microsatellite instability
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide
NF-kB	Nuclear factor-kappa B
PC	Phase contrast
PI	Propidium idodide
PstA	Pepstatin A
RAS	Rat sarcoma viral oncogene
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SCFA	Short-chain fatty acids
siRNA	Small interfering RNA
SMAD4	Mothers against decapentaplegic homolog 4
SMCT-1	Sodium dependent monocarboxylate transporter
SRB	Sulforhodamine B
TGF-B	Transforming growth factor β
TOM22	subunit of the outer mitochondrial membrane translocator (TOM complex)
TS	Thymidylate synthase
TSGs	Tumour suppressor genes
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labelling
UC	Ulcerative colitis

VDAC1	voltage dependent anion channel
VEGF	Vascular endothelial growth factor
VMP	Vacuolar membrane permeabilization
WHO	World Health Organization
ΔΨM	Mitochondrial membrane potential
3BP	3-Bromopyruvate
5-FU	5-fluorouracil

ABSTRACT

Colorectal cancer (CRC) is an important public health concern worldwide, especially among populations that adopt Western-style diets. Thus, it becomes important to investigate new prevention and therapeutic approaches for CRC. The use of nutraceuticals have been described as a new strategy in this context by the use of dietary Propionibacteria, found in fiber-rich food and dairy products, which produce short-chain fatty acids (SCFA). SCFA, namely butyrate, propionate and acetate have been described as anti-neoplastic agents, being recognized as an optimal strategy for prevention/treatment of CRC. Little was known about the role of acetate, but like the other SCFAs, acetate is also able to induce apoptosis in CRC cells. It has been previously demonstrated that acetic acid induces apoptosis in *Sacharomyces cerevisiae*. Thus, we aimed to understand the mechanisms underlying acetate-induced cell death in CRC cells taking into account the results obtained in the yeast model. We also aimed to study acetate transport in CRC cells, investigating the involvement of monocarboxylate transporters (MCTs) as well as regulation of MCTs expression in response to acetate. Moreover, we wanted to verify the effects of acetate in glycolytic metabolism and to explore the combined use of acetate with the glycolysis inhibitor 3-bromopyruvate (3BP) as a new strategy to potentiate the effect of acetate in CRC cells. Furthermore, we aimed to assess the effect of acetate in the main signalling pathway activated in CRC: RAS-RAF-MEK-ERK.

Three cell lines derived from human CRC, harbouring different genetic mutations namely RKO^{BRAFV600E}, HCT-15^{KRASG13D} and HCT116$^{KRASG13D;\ PI3KCA}$ were used as models. We showed that acetate treatment in CRC cells decreased cell proliferation and induced apoptosis. The apoptosis process was characterized by DNA fragmentation, caspase-3 activation and phosphatidylserine exposure to the leaflet of the plasma membrane and appearance of sub-G1 population. In addition, we found that acetate induced partial lysosome membrane permeabilization with cathepsin D (CatD) release to the cytosol and that this protease, but not CatB and CatL, has an anti-apoptotic role in acetate-induced apoptosis in CRC cells. We next showed that acetate treatment induced several mitochondrial dysfunctions as well as reactive oxygen species (ROS, total and mitochondrial) accumulation and an increase in mitochondrial mass accompanied by mitochondrial membrane

depolarization. We could also demonstrate that, like acetic acid in yeast, acetate-induced apoptosis is not associated with autophagy induction in CRC cells. Moreover, inhibition of CatD (with siRNA or pepstatin A) enhanced apoptosis associated with higher mitochondrial dysfunction and increased mitochondrial mass. This effect seems to be specific of CatD, since inhibition of CatB and CatL had no effect, nor were these proteases significantly released to the cytosol during acetate-induced apoptosis. Summing up, these results identified a novel function of CatD in the degradation of damaged mitochondria when autophagy is impaired, protecting CRC cells from acetate-induced apoptosis.

Furthermore, we showed that acetate enters CRC cells by a sodium dependent monocarboxylate transporter (SMCT-1) and passive diffusion by aquaporins. MCT-1 is also involved in acetate uptake, accompanied by an increase in expression of both MCT-1, MCT-4, as well as of the glycosylated form of CD147, the MCT1/4 chaperone in the presence of acetate. Additionally, we found that acetate promotes plasma membrane re-localization of MCT-1 and further triggers changes in glucose metabolism (decreasing both glucose consumption and lactate production). Further, we explored the combined treatment of acetate with 3BP and we demonstrated that 3BP potentiates acetate-induced apoptosis in CRC cells.

Taken together, this work demonstrates a protective role of CatD in acetate-induced apoptosis which could negatively impact the efficacy of acetate and probably of other therapeutic compounds. Thus, the use of CatD inhibitors in combination with strategies to increase acetate concentrations in the colon, namely nutraceuticals, is proposed as a new strategy to prevent/treat CRC. Our findings also show a novel approach for CRC therapy based on the elimination of CRC cells through their sensitization to acetate by association with 3BP and/or other chemotherapeutic agent which transport is mediated by MCTs.

RESUMO

O cancro colorretal (CCR) é uma importante questão de saúde pública em todo o mundo, especialmente nas populações com um estilo de dieta-ocidental. Torna-se assim importante investigar novas abordagens de prevenção e terapia para o CCR. A utilização de nutracêuticos, tem sido descrita como uma possível abordagem neste contexto, nomeadamente o uso das Propionibactérias da dieta, encontradas em alimentos ricos em fibras e laticínios, que produzem ácidos gordos de cadeia curta (AGCC). Os AGCC, nomeadamente butirato, propionato e acetato têm sido descritos como agentes anti-neoplásicos, reconhecidos na prevenção/tratamento do CCR. O papel do acetato era pouco conhecido, mas tal como os outros AGCC, também é capaz de induzir apoptose em células de CCR. Foi previamente demonstrado que o ácido acético induz apoptose em *Saccharomyces cerevisiae*. Deste modo, tivemos como objetivo compreender os mecanismos subjacentes à morte celular induzida pelo acetato em células de CCR, tendo em conta os resultados obtidos no modelo da levedura. Também tivemos como objetivo estudar o transporte do acetato em células de CCR, investigando o envolvimento dos transportadores de monocarboxilatos (MCTs) bem como a regulação da sua expressão em resposta ao acetato. Além disso, quisemos avaliar os efeitos do acetato no metabolismo glicolítico e explorar o uso combinado do acetato com o inibidor da glicólise 3-bromopiruvato (3BP) como uma nova estratégia para potenciar o efeito do acetato em células de CCR. Procurou-se também avaliar o efeito do acetato na principal via de sinalização ativada no CCR: RAS-RAF-MEK-ERK.

Foram utilizadas como modelo três linhas celulares derivadas de CCR humano: RKO^{BRAFV600E}, HCT-15^{KRASG13D} e HCT116$^{KRASG13D;\ PI3KCA}$. Mostramos que o tratamento com acetato diminuiu a proliferação celular e induziu a apoptose em células de CCR. O processo de apoptose foi caracterizado pela fragmentação de DNA, activação de caspase-3, exposição de fosfatidilserina para o folheto externo da membrana plasmática e o surgimento de uma população sub-G1. Além disso, verificou-se que o acetato induziu permeabilização parcial da membrana lisossomal com libertação de catepsina D (CatD) para o citosol, e que esta protease, mas não a CatB e a CatL, tem um papel anti-apoptótico na apoptose induzida pelo acetato em células de CCR. Em seguida, mostramos que o tratamento com acetato induziu

disfunções mitocondriais, assim como a acumulação de espécies reativas de oxigênio (nível de ROS, total e mitocondrial) e um aumento da massa mitocondrial acompanhado por uma despolarização da membrana. Foi também possível demonstrar que, tal como o ácido acético em levedura, a apoptose induzida pelo acetato não está associada com a indução de autofagia em células de CCR. Além disso, a inibição da CatD (com siRNA ou pepstatina A) aumentou a apoptose associada a uma disfunção mitocondrial mais proeminente, bem como a massa mitocondrial. Este efeito, parece ser específico da CatD, uma vez que a inibição da CatB e CatL não teve nenhum efeito, não havendo libertação destas proteases para o citosol durante a apoptose induzida pelo acetato. Resumindo, estes resultados identificaram uma nova função da CatD na degradação das mitocôndrias danificadas quando a autofagia é perturbada, protegendo assim as células de CCR da apoptose induzida pelo acetato.

Além disso, mostramos que o acetato entra nas células de CCR por um transportador de monocarboxilato tipo 1 dependente de sódio (SMCT-1) e por difusão passiva mediada por aquaporinas. O MCT-1 está também envolvido no transporte do acetato havendo, na presença de acetato, um aumento na expressão de MCT-1 e MCT-4, bem como da forma glycosilada da CD147, a chaperona do MCT1/4. Adicionalmente, verificou-se que o acetato promove a re-localização do MCT-1 para a membrana plasmática desencadeando alterações no metabolismo da glicose (diminuindo o consumo de glucose e produção de lactato). Além disso, exploramos o tratamento combinado do acetato com o 3BP e demonstramos que o 3BP potencia a apoptose induzida pelo acetato em células de CCR.

Neste trabalho demonstramos um papel protetor da CatD na apoptose induzida pelo acetato que pode afetar a eficácia do acetato e de outros compostos terapêuticos. Propomos assim uma nova estratégia para prevenir e/ou tratar o CCR que inclui a utilização de inibidores da CatD em combinação com outras abordagens para aumentar as concentrações de acetato no cólon, como o uso de nutracêuticos. Os nossos resultados também sugerem uma nova abordagem para a terapia do CCR baseada na eliminação de células de CCR através da sua sensibilização ao acetato pelo 3BP ou por um outro agente quimioterapêutico cujo transporte seja mediado pelo MCT-1.

THESIS OUTLINE

The present thesis is organized in five different chapters as follows:

Chapter 1 is a general introduction and comprises parts of two review articles presenting an overview of the literature related with the scope of the thesis, divided in three main parts. The first part focuses on the current knowledge on colorectal cancer (CRC) (general aspects, incidence, types, the main genetic alterations involved in CRC carcinogenesis, risk factors, available treatment options for pacients with CRC and the use of nutraceuticals as a strategy to prevent/treat CRC). The second part explores the difference between the microbiota from normal colon epithelium to CRC, focusing on short-chain fatty acids (SCFAs, especially butyrate, propionate and acetate), with respect to their production and metabolism at the intestinal level, transporters/receptors and their physiological functions in intestinal epithelial cells. The effect of SCFAs in CRC cells is also described, focusing on how these compounds can be useful in the prevention or treatment of CRC. The last part comprises an overall review about the lysosome as an important player in cancer cell death regulation exploring the role of cathepsins (lysosome proteases), namely cathepsin D (CatD) in this context.

Chapters 2, 3 and 4 describe the experimental work and are part of three articles:

Chapter 2 describes the matherial and methodos used to develop all the experimental work, subdivided according to the type of technique.

In **Chapter 3** the results of the thesis are presented, subdivided in subchapters as follows:

3.1: *Acetate-induced apoptosis in colorectal carcinoma cells involves lysosomal membrane permeabilization and cathepsin D release*.

3.2: *Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy-independent degradation of damaged mitochondria*.

3.3: *The role of acetate on monocarboxylate transporter (MCT) expression and glucose metabolism in colorectal cancer cells: therapeutic implications*.

3.4: Acetate induces downregulation of ERK1/2 pathway in colorectal cancer cells.

Chapter 4 comprises a general discussion of the work.

Chapter 5 presents the overall conclusions of the thesis as well as suggestions for future work.

CHAPTER 1:

General introduction

This chapter comprises parts from the following publications:

Pereira H, **Oliveira CSF**, Castro L, Preto A, Chaves SR, Côrte-Real M (2015). Yeast as a tool to explore cathepsin D function. *Microbial Cell*, 2(7):225-234.
(In this publication I have participated in the writing of the section regarding cathepsins, its role in physiology and pathology and the role of cathepsin D in cancer).

Oliveira CSF, Barreto J, Pereira H, Chaves SR, Baltazar, F, Côrte-Real M, Preto A. The role of diet related short-chain fatty acids in colorectal cancer: prevention or therapeutic strategy? Paper submitted to *Current Pharmaceutical Design*.
(In this publication I am the first author responsible for the design and writing of the manuscript)

General introduction

1. Colorectal cancer

Colorectal cancer (CRC) is a malignant neoplasia localized in the large intestine (that comprise the colon), vermiform appendix or rectum (Figure 1). CRC is also referred as colon cancer or rectal cancer, depending on where the cancer arises and usually have many features in common, which explain the reason why these cancers are generally discussed together.

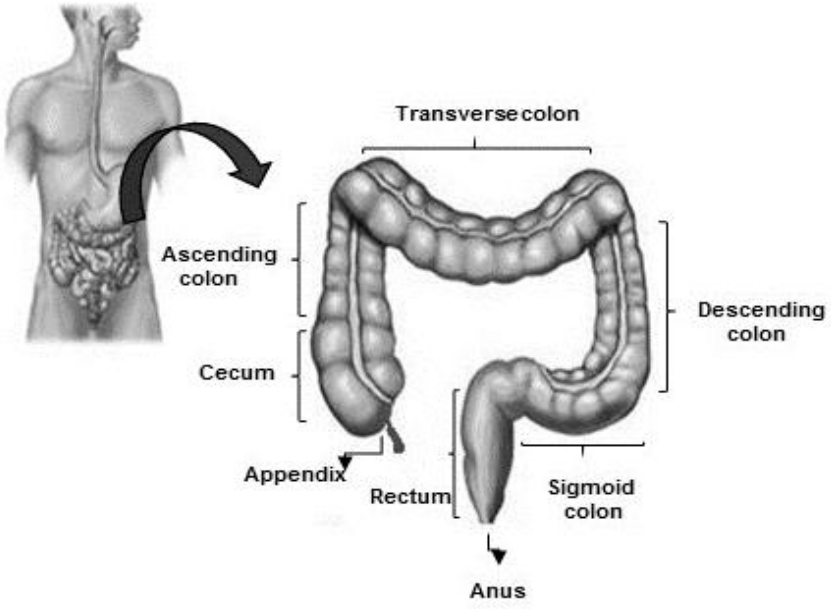

Figure 1: Ilustration of the large intestine. Front of abdomen showing the respective regions of the colon, appendix, rectum and anus.

CRC is among the most common cancers, accounting for over 1 million cases (1.23 million cases, 9.7% of overall cancer) and about half a million deaths annually representing an important health issue worldwide (Zhu, Michelle Luo et al. 2011; Cappellani, Zanghi et al. 2013; Stigliano, Sanchez-Mete et al. 2014). CRC is currently the third most frequent cancer in men, after lung and prostate cancer, and the second most frequent cancer in women, after breast cancer (Samuel Constant 2013; Mudassar, Khan et al. 2014; Tarraga Lopez, Albero et al. 2014).

Epidemiological studies have shown that rates of CRC incidence and mortality vary substantially across regions of the world (Table 1) (Cotter 2013; Bishehsari,

Mahdavinia et al. 2014; Raskov, Pommergaard et al. 2014; Stigliano, Sanchez-Mete et al. 2014), with a higher incidence in North America, Europe, Australia and New Zeland (Raskov, Pommergaard et al. 2014). Almost 60% of cases occur in developed regions, particularly in the United States (where CRC is the third most common cancer) and in Europe (Stigliano, Sanchez-Mete et al. 2014), where CRC is the second cause of death from all cancer types in both genders, with ~446.000 new cases arising each year (Altobelli, Lattanzi et al. 2014; Tarraga Lopez, Albero et al. 2014). In the particular case of Portugal, CRC is the third most common cancer, accounting for 3800 deaths in Portugal in 2013 (Ferreira and Riphaus 2014). In Portugal, like in most European countries, CRC incidence is steadily increasing, and the number of deaths from CRC increased by 3% per year between 2000 and 2005 (Cotter 2013; Ferreira and Riphaus 2014).

Table 1: Estimated rate of incidence and mortality for colorectal cancer, in 2008 in a population of 100 000 persons, for both sexes, in different regions of the world.

Region of the world	***Incidence***	***Mortality***
Norther America	30.1	9.1
Europe	28.1	12.8
Eastern Asia	18.0	8.0
Western Asia	17.9	7.9
Latin America	11.4	6.6
South East Asia	6.9	4.8
Africa	5.9	4.8

Adapted from Cotter J, 2013.

As mentioned above, both incidence and mortality rates are not equally distributed among the world, varying according to diferent primary and secondary CRC prevention strategies, which can be correlated with the differences in health insurance systems and budgets (Cotter 2013). In addition, higher CRC rates have been reported especially among populations that adopt Western-style diets, strongly linked to changes in lifestyle and exposure to carcinogens (Zhu, Michelle Luo et al.

2011; Kim, Coelho et al. 2013; Sobhani, Amiot et al. 2013; Bishehsari, Mahdavinia et al. 2014).

CRC is generally classified into three types: hereditary, familial and sporadic. Hereditary/familial CRC derive, in part, from germline mutations comprising approximately 10-30% of all CRC cases (Jasperson, Tuohy et al. 2010). Hereditary/familial CRC present a well-characterized cancer predisposition syndromes including **Lynch syndrome**, **Familial adenomatous polyposis** (FAP), **MUTYH-associated polyposis** (MAP), **hamartomatous polyposis syndromes** and **hyperplastic polyposis** (Jasperson, Tuohy et al. 2010; Tarraga Lopez, Albero et al. 2014). Most CRC cases are sporadic (about 70%) and derive from somatic mutations, not associated with family history, but linked mainly to environmental causes (Kim, Coelho et al. 2013; Sobhani, Amiot et al. 2013; Bishehsari, Mahdavinia et al. 2014; Tarraga Lopez, Albero et al. 2014).

Usually, CRC develops slowly over a period of 10 to 15 years. It is generally due to an abnormal growth of cells (polyps) from the intestinal mucosa (Figure 2), with approximately 90% of CRCs classified as adenocarcinomas (colon polyps) (Tannapfel, Neid et al. 2010; Fleming, Ravula et al. 2012). These polyps are very common and most are benign, generally located in the rectum or in the sigmoid colon (66% to 77% of cases) (Tannapfel, Neid et al. 2010). If the polyp/adenoma is not removed, cells continue to proliferate and may become cancerous and/or progress into CRC with the ability to invade or metastasize to other parts of the body (Figure 2) (Tannapfel, Neid et al. 2010; Cappellani, Zanghi et al. 2013). Only a small percentage of adenomas progress to CRC (Cappellani, Zanghi et al. 2013) through the involvement of several genetic alterations that require the accumulation of mutations in particular genes that induce genetic and epigenetic changes responsible for cell proliferation, apoptosis, angiogenesis and invasion (Tannapfel, Neid et al. 2010; Gonsalves, Mahoney et al. 2014).

1.1 Genetic alterations associated with colorectal cancer

A genetic model has been proposed to explain the stepwise formation of CRC from normal colonic tissue, a well-known **adenoma-carcinoma sequence** (Mudassar, Khan et al. 2014), which postulates that:

- CRC is the result of mutations of genes with important functions in regulating cell proliferation, repair of DNA damage and apoptosis.
- Mutations in several genes are required over the years.
- The sequence of mutations is very important in determining the eventual formation of CRC.

Genes involved in the genetic paradigm of CRC can be divided into two main classes: tumour suppressor genes (TSGs) and oncogenes (Mudassar, Khan et al. 2014). TSGs are often inactivated and encode proteins that either inhibit cell proliferation or promote apoptosis. In contrast, oncogenes are activated versions of protooncogenes, which are often involved in promoting cell proliferation and cancer development. Once activated, oncogenes can lead to accelerated cell growth and contribute to tumour formation. The main molecular changes involved in the CRC carcinogenesis are illustrated in Figure 2.

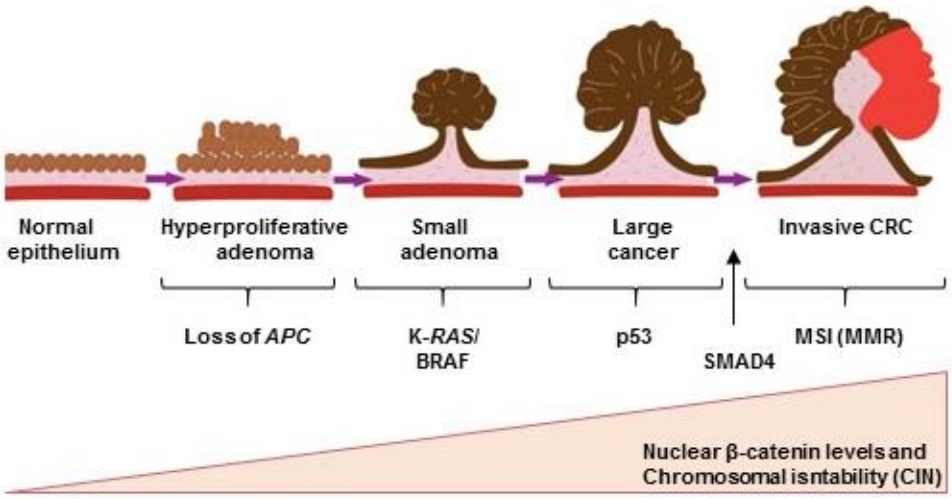

Figure 2: Colorectal carcinogenesis from normal epithelium to colon cancer. The main mutations in CRC development are shown: Adenomatous polyposis coli (*APC),* K-RAS, BRAF, p53, SMAD4, Microsatellite instability (MSI), Mismatch repair (MMR), Chromosomal instability (CIN).

The primary genetic alteration in CRC carcinogenesis involves the adenomatous polyposis coli (*APC*) gene mutation. Secondary genetic alterations occur with activation of the KRAS or BRAF oncogenes, followed by inactivation of the TSG *p*53, as well as microsatellite and chromosomal instability (MSI) (Tannapfel, Neid et al. 2010; Mudassar, Khan et al. 2014).

APC is a tumour supressor gene mutated both in inherited and sporadic CRC forms (Smith, Carey et al. 2002; Waldner and Neurath 2010). Germline mutations in this gene lead to development of a high number of premalignant polyps in the colon of individuals affected with familial adenomatous polyposis (FAP) (Aoki and Taketo 2007; Waldner and Neurath 2010; Raskov, Pommergaard et al. 2014). In cases of sporadic CRC, *APC* mutations are present in microscopic adenomas (~50-60%) (Smith, Carey et al. 2002; Aoki and Taketo 2007). The *APC* gene product is a protein (~312 kDa) that functions as a "gatekeeper" that regulates the entry of intestinal epithelial cells into the adenoma-cancer progression (Smith, Carey et al. 2002). At the molecular level, APC is a negative regulator of the Wnt-signalling pathway, which is critical during cellular proliferation and differentiation (Raskov, Pommergaard et al. 2014; Russo, Catania et al. 2014). APC is a multi-domain protein that contains binding sites for numerous other proteins, including microtubules components, Wnt pathways regulators – axin, β-catenin and the cytoskeletal regulators EB1 (Smith, Carey et al. 2002; Aoki and Taketo 2007). Most *APC* mutations occur in a region referred to as the mutation cluster region (MCR), resulting in C-terminal truncations that cause loss of the protein domain required for binding to β-catenin and microtubules (Aoki and Taketo 2007). This leads to accumulation of nuclear β-catenin and activation of targets of the canonical Wnt signalling pathway (Aoki and Taketo 2007; Raskov, Pommergaard et al. 2014).

Other mutations implicated in CRC development are activation of KRAS and BRAF oncogenes frequently found in sporadic CRC - in approximately 35-40% and 5-10% of the CRC cases, respectively (Figure 3) (Velho, Moutinho et al. 2008; Yokota 2012; Gonsalves, Mahoney et al. 2014; Raskov, Pommergaard et al. 2014; Sridharan, Hubbard et al. 2014). KRAS (Kirsten rat sarcoma) belongs to the RAS protein family. It is a proto-oncogene on chromosome 12 and encodes a GTP-binding protein (21 kDa) capable of receiving signals from tyrosine kinase receptors,

integrating mitogenic messages from the outside of the cell; when KRAS is stimulated, it anchors GTP (releasing GDP), which turns on its active state that triggers a signalling cascade known as **RAS-RAF-MEK-ERK** (Smith, Carey et al. 2002; Yokota 2012). KRAS hotspot mutations are found most commonly at codons 12 or 13, dominated by G→A transitions at the second base of the respective codon resulting in G12D or G13D mutations(Figure 3) (Prior, Lewis et al. 2012). These mutations can usually lead to abnormal cell proliferation and malignant cell transformation (Smith, Carey et al. 2002). It is known that mutated KRAS is constitutively activated, permanently stimulating growth and differentiation in CRC cells (Bos 1989). Altered KRAS is found both in CRC and in large colorectal adenomas, but less frequently in small adenomas, which suggests that the mutation appears before the acquisition of the malignant state and that its presence confers a growth advantage (Leslie et al., 2002). In recent years, it has been described that KRAS mutations limit the effects of anti-EGFR therapy (Tannapfel, Neid et al. 2010; Waldner and Neurath 2010; Yokota 2012), and the use of agents such as Cetuximab have to be limited to patients with *KRAS* wild-type CRC (Waldner and Neurath 2010; Yokota 2012; Raskov, Pommergaard et al. 2014). Our group has also studied the role of KRAS in CRC and recently demonstrated that KRAS hotspot mutations play a role in autophagy regulation, which might have important therapeutic implications (Alves, Castro et al. 2015).

Like in the case of KRAS, mutated BRAF is also involved in the alteration of RAS-RAF-MEK-ERK pathway in CRC cases (Velho, Moutinho et al. 2008). BRAF encodes a protein (94 kDa) of the RAF family of kinases and operates by binding to RAS (Tannapfel, Neid et al. 2010; Yokota 2012). The most common mutation found in CRC carcinogenesis is the classic GTG→GAC substitution at position 1799 of exon 15, which results in the V600E amino acid change and the subsequent constitutive activation of the EGFR signalling pathway (Yokota 2012). In addition, BRAF mutations have been described as a strong negative prognostic marker in patients with CRC, since patients have demonstrated very poor response to conventional chemotherapy, especially to anti-EGFR therapy (Yokota 2012). Preto *et al* have demonstrated that BRAF might be an important therapeutic target in CRC harbouring a $BRAF^{V600E}$ mutation (Preto, Figueiredo et al. 2008).

Furthermore, experimental evidence has shown that KRAS and BRAF mutations do not overlap in colorectal cancer (Rajagopalan et al., 2002), but both lead to the constitutive activation of the pathway (MEK-ERK), which suggests that this is an important hit in the development of this type of cancer.

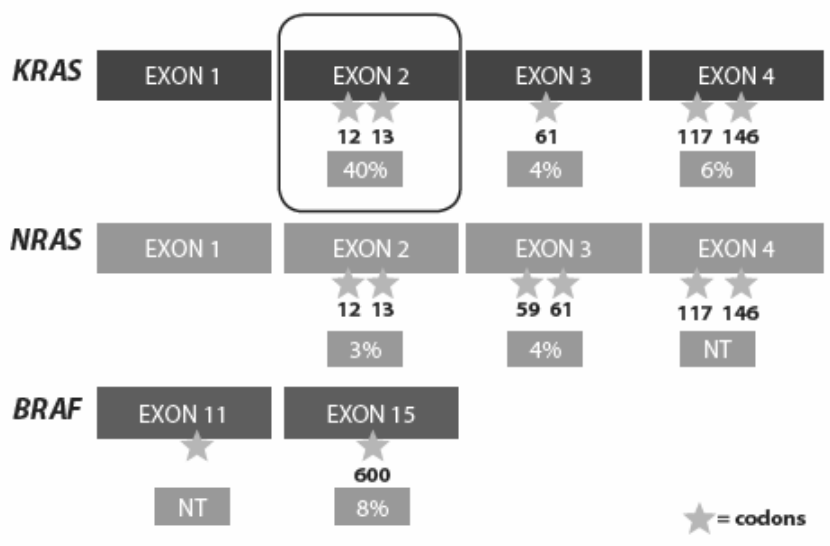

Figure 3: Frequence of KRAS, NRAS and BRAF mutations in CRC cases. The image also shows the main codons mutaded in these genes. (Adapted from Sridharan et al. 2014).

Another important step in colon carcinogenesis is the loss of p53 function which has been reported to occur relatively late in the development of CRC tumors (Smith, Carey et al. 2002). The *TP53* gene, located on chromosome 17p, is mutated in 70% of all CRC tumors (Smith, Carey et al. 2002). The gene encodes the p53 protein, a well-known "guardian of the genome" (Raskov, Pommergaard et al. 2014), responsible for inhibiting cell proliferation whenever DNA damage exists. If the repair mechanisms fail, p53 initiates an apoptotic programme, preventing cell to proliferate (Hollstein, Sidransky et al. 1991). However, if p53 is mutated and DNA damage is not repaired, damage is spread to the daughter cells. In this case, it has been demonstrated that mutation in the *TP53* gene is crucial to progression from non-invasive to invasive disease, found in the following frequences: adenomas (5%), malignant polyps (50%) and invasive CRC (75%) with a increasing frequency correlated to the extent of malignancy (Raskov, Pommergaard et al. 2014).

SMAD4 is another tumor suppressor gene often mutated in colorectal cancer. The SMAD4 protein is part of the transforming growth factor β (TGF-β) signalling

pathway, which is responsible for controlling the expression of cell cycle regulators, differentiation factors and cell adhesion factors (Massaous and Hata 1997). SMAD4 forms heterodimers with other SMAD proteins, acting as transcription factors (Hahn, Schutte et al. 1996; Imamura, Takase et al. 1997). However, mutation of SMAD4 impairs its regulatory activity, blocking the transcription of important genes necessary for cell cycle control and apoptosis (Woodford-Richens, Rowan et al. 2001). As cancer progresses, the mutation rate in this gene increases; it is found mutated in about 31% of the metastatic colorectal cancer (mCRC) cases (Miyaki, Iijima et al. 1999; Maitra, Molberg et al. 2000), suggesting that loss of this gene is important for the development of the metastatic state.

Following these key genetic alterations, a number of acquired genetic mutations in other genes, including *PIK3CA, FBXW7, SMAD2, TCF7L2, NRAS, FAM123B CTNNB1*, have also been described to contribute to colorectal carcinogenesis (Keku, Dulal et al. 2015).

Moreover, genetic instability termed as microsatellite instability (MSI) appears in 12-15% of the CRC cases (Raskov, Pommergaard et al. 2014). Microsatellites are repeated mono to tetra-nucleotide sequences distributed throughout the genome arising from a mismatch repair (MMR) deficiency. During DNA amplification, these sequences can be repeated many times (~10-60) and replication errors in those sequences may occur naturally (Tannapfel, Neid et al. 2010). There are various proteins which recognize incorrect base pairs within DNA and “repair” DNA damage. However, if one of these proteins is altered, incorrect pairs can accumulate in the form of mutations in genes involved in tumor progression (Tannapfel, Neid et al. 2010).

Data have also shown the presence of chromosomal instability (CIN) as a result of structural or numerical chromosomal changes in about 65-70% of CRCs (Waldner and Neurath 2010). These changes can lead to uncontrolled growth and differentiation associated with CRC development (Tannapfel, Neid et al. 2010; Mudassar, Khan et al. 2014).

In addition to these somatic mutations, CRC progression is also due to epigenetic modifications in the expression of genes that regulate proliferation, apoptosis and DNA repair (Cappellani, Zanghi et al. 2013). These epigenetic alterations are

characterized mainly by hypermethylation of the cytosine residues in CpG-rich sequences (CpG-islands) located within the promoter regions of expressed genes. It is important to note that this does not alter DNA integrity, but methylation of these sequences can induce progressive silencing of DNA-repair genes and tumor suppressor genes promoting abnormal cell proliferation (Cappellani, Zanghi et al. 2013).

1.1.2 Colorectal cancer risk factors

CRC has been characterized as a multifactorial disease process which may include hereditary/familial factors, age, environmental lifestyle-related risk factors (diet, physic inactivity, obesity, smoking, alcohol consumption) as well as inflammatory conditions of the digestive tract and, more recently, changes in intestinal microbiota composition (Figure 4) (Bishehsari, Mahdavinia et al. 2014; Mudassar, Khan et al. 2014; Kumar, Sastry et al. 2015). According to the Centre for Disease Control and Prevention, patients with family history of CRC have a higher risk of developing CRC themselves (Mudassar, Khan et al. 2014).

Regarding age, CRC is commonly found in patients aged 50 years and over (Mudassar, Khan et al. 2014). Interestingly, the most common risk factor includes dietary regimen rich in sugar and proteins, especially with high intake of red meat, processed meat, animal fats and usually poor in whole grain, vitamin D, fruits, vegetables, cereals and fibers (Cappellani, Zanghi et al. 2013; Kim, Coelho et al. 2013; Bishehsari, Mahdavinia et al. 2014). The association between high red and processed meat intake and CRC risk includes the content of the meat and compounds generated by the cooking process (e.g. N-nitroso compounds) (Kim, Coelho et al. 2013). These factors can affect the large intestine mucosa with genotoxicity and metabolic disturbances contributing to colon carcinogenesis (Kim, Coelho et al. 2013; Raskov, Pommergaard et al. 2014).

Obesity is another risk factor associated with CRC development (Cappellani, Zanghi et al. 2013). One of the mechanisms involved in the obesity-CRC relationship seems to be an asymptomatic chronic inflammation of the colonic mucosa associated with

an alteration of the dominant phyla of bacteria in the gut and of body weight both in humans and in animal models (Zhu, Michelle Luo et al. 2011).

Another emerging risk factor involved in CRC is altered intestine microbiota composition (Zhu, Michelle Luo et al. 2011; Kumar, Sastry et al. 2015). Since the microbiota impacts numerours physiological functions in the gut, some studies have shown that significant changes in the microbiota composition, especially of some bacterial groups that are able to produce metabolites which cause inflammation, correlated with tumor initiation/progression (Cappellani, Zanghi et al. 2013; Kumar, Sastry et al. 2015).

Tobacco smoking acts on the mucosa though the production of carcinogenic agents (such as acetaldehyde, benzopyrenes, aromatic amines and N-nitrosamines) and also increases the risk of microsatellite instability (Bishehsari, Mahdavinia et al. 2014; Raskov, Pommergaard et al. 2014). Furthermore, excessive alcohol consumption can also accelerate CRC initiation and progression by triggering inflammation and epigenetic changes (Bishehsari, Mahdavinia et al. 2014).

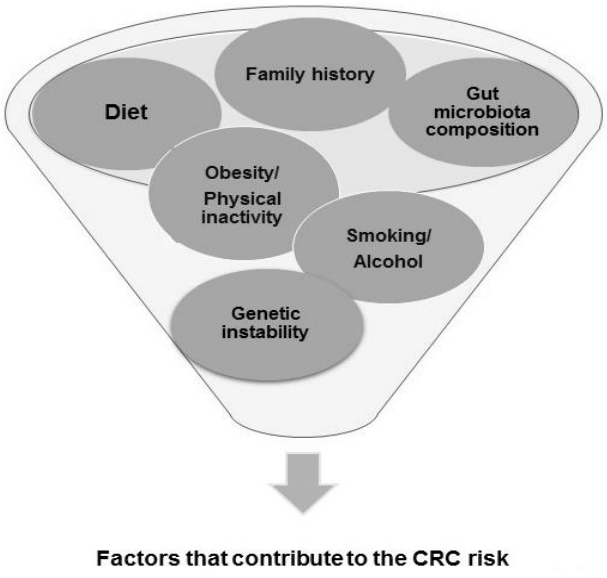

Figure 4: Main factors contributing to the risk of CRC development (Adapted from Kumar et al. 2015).

It has been demonstrated that CRC may be highly amenable to prevention through a dietary fiber consumption (Zeng, Lazarova et al. 2014). Many of these benefits can be attributed to the fermentation of dietary fiber into short chain fatty acids

(SCFAs) (Zeng, Lazarova et al. 2014), described as anti-tumorigenic agents in the colon (Tang, Chen et al. 2011; Adom and Nie 2013; Marques, Oliveira et al. 2013; Zeng, Lazarova et al. 2014). Likewise, primary and secondary prevention, in attention to a healthy lifestyle, physical activity and screening should be enhanced in the general population to decrease the risk of CRC as well as its incidence and mortality.

1.1.2 Colorectal cancer therapy

The main symptoms of CRC are changes in bowel habits, rectal bleeding, abdominal pain, anemia, weight loss and feeling tired all the time. Due to the slow progression of CRC, early detection of polyps and smaller lesions in the colon through several available screening methods, including rectal exam, fecal blood test, colonoscopy and sigmoidoscopy, is possible (Delavari, Mardan et al. 2014).

The majority of CRC cases would benefit from surgical treatment if diagnosed early or at the stage of the premalignant polyps (Pawa, Arulampalam et al. 2011). However, at diagnostic, about 20% of patients are already in a metastatic state; when the disease is considered incurable, palliative care (“best supportive care”) is selected as the best strategy to treat the patient (Lucas, O'Neil et al. 2011). At previous stages of the disease, many therapeutic strategies may be adopted, generally starting with tumor resection, either endoscopically or surgically. In some cases, the tumor is removed completely and no further treatment is needed except follow-up visits. After characterization of the CRC stage and in the case of advanced cancer with lymph nodes or any other tissue metastization, additional treatment is required.

The chemotherapeutic options for this type of cancer may be divided in two main groups. The first group, *cytotoxic chemotherapy*, englobes classical chemotherapeutic agents (5-fluorouracil, 5-FU), Capecitabine, Irinotecan and Oxaliplatin) (Sridharan, Hubbard et al. 2014). The other group is known as *targeted therapy,* and is divided into three main classes of biological agents: **(1)** monoclonal antibodies against the epidermal growth factor receptor (EGFR) on the surface of tumor cells (Cetuximab and Panitumumab); **(2)** inhibitors of the vascular endotelial

growth factor (VEGF) proangiogenic system (Bevacizumab and Aflibercept) and **(3)** small-molecule inhibitor of intracellular kinases involved in various signalling cascades (Regorafenib) (Sridharan, Hubbard et al. 2014). The use of glycolysis inhibitors as potential anticancer agents has also been proposed, since most cancer cells, including CRC cells, exhibit increased glycolysis and use this metabolic pathway for ATP generation as the main source of their energy supply, a phenomenon known as Warburg effect (Pelicano, Martin et al. 2006; Pedersen 2012; Amorim, Pinheiro et al. 2015).

These chemotherapeutic modalities may use one drug at a time (as single-agent chemotherapy) or several drugs at once (combination chemotherapy) (Sridharan, Hubbard et al. 2014). An overview regarding the different treatment options for CRC available today is presented in Table 2. Their main advantages, modes of action and side effects are described in the next sections.

1.1.3.1 Classical chemotherapy relies on cytotoxic agents

Traditional chemotherapeutic agents are cytotoxic i.e, they act by targeting cells that divide rapidly, one of the main properties of most cancer cells. However, cytotoxic agents also harm cells that divide rapidly under normal circumstances: cells in the bone marrow, digestive tract, and hair follicles, resulting in the most common side effects of chemotherapy: myelosuppression (decreased production of blood cells, hence also immunosuppression), mucositis (inflammation of the lining of the digestive tract), and alopecia (hair loss). Despite these severe side effects, these agents have been extensively used in CRC chemotherapy.

The approved conventional cytotoxic agents are classified into three main classes: fluoropyrimidines (5-fluorouracil and capecitabine), topoisomerase I inhibitors (irinotecan) and platinum-containing compounds (oxaliplatin) (Sridharan, Hubbard et al. 2014).

5-fluorouracil (5-FU) is the main anticancer agent used in CRC therapy, and one of the most effective (Gill, Thomas et al. 2003; Lucas, O'Neil et al. 2011). 5-FU abrogates DNA synthesis by inhibiting thymidylate synthase (TS), an enzyme that is essential for *de novo* synthesis of thymidine, the precursor of thymine nucleotide

(Papamichael 1999; Gill, Thomas et al. 2003). This agent is usually administered in combination with other chemical compounds, particularly with folinic acid (leucovorin, LV), which stabilizes the interaction between 5-FU and TS (Lucas, O'Neil et al. 2011) improving the anti-metabolite effect of 5-FU, resulting in a faster response to this chemotherapy (Gill, Thomas et al. 2003). However, by combining 5-FU and LV, the toxicity of the first is also enhanced, resulting in the appearance of several and more hazardous side effects, though the overall survival time is extended in about one year (Lucas, O'Neil et al. 2011). 5-FU treatment raises several side effects, particularly myelosuppression (a decrease in production of blood cells by bone marrow), diarrhea, stomatitis, nausea, vomiting, alopecia and cardiotoxicity, among several others (Board and Valle 2007).

Capecitabine (N -pentyloxycarbonyl-5-deoxy-5-fluorocytidine) (available as Xeloda®) is an oral fluoropyrimidine produced as a pro-drug of fluorouracil (FU) (Koukourakis, Kouloulias et al. 2008). This drug has provided compelling efficacy data for CRC treatment (stage III or IV), both as monotherapy and in combination regimens (Koukourakis, Kouloulias et al. 2008). The preferential conversion of capecitabine to 5-fluorouracil in neoplastic tissues renders this fluoropyrimidine particularly appealing for clinical use (Koukourakis, Zacharias et al. 2010). The enzyme thymidine phosphorylase, necessary to convert this pro-drug, is an active form expressed in higher concentration in neoplastic compared to healthy tissues, which makes capecitabine more tumor-specific than other chemotherapeutic agents (Koukourakis, Zacharias et al. 2010). The main side effects of capecitabine are neutropenia, stomatitis and diarrhea, which often occur due to nonselective cytotoxicity (Koukourakis, Kouloulias et al. 2008).

Irinotecan (also known as CPT-11 and available as Camptosar®) is a semi-synthetic derivative of the natural alkaloid camptothecin which targets the activity of topoisomerase I and exerts its cytotoxic effect by causing DNA damage (Gill, Thomas et al. 2003). Irinotecan has been approved for CRC patients whose tumors did not respond to 5-FU treatment (Oguro, Seki et al. 1990). Normally, this type of treatment is combined with other chemotherapeutical agents, including 5-FU/LV, increasing the response rate, progression-free survival (about seven months) and overall survival (about seventeen months) (Lucas, O'Neil et al. 2011). Irinotecan is

also responsible for several secondary effects, namely diarrhea and myelosuppression (Xu and Villalona-Calero 2002; Gill, Thomas et al. 2003).

Oxaliplatin (available as Eloxatin®) was the first platinum-based therapy approved for CRC treatment and, like other platinum-based therapies, this compound intercalates in DNA, impairing DNA replication and transcription, abrogating cell proliferation (Graham, Mushin et al. 2004). Oxaliplatin alone does not have important anti-cancer properties, but when combined with other anticancer agents, particularly fluoropyrimidines (Lucas et al., 2011) or bevacizumab (Okines and Cunningham 2009), its anti-cancer properties are enhanced. By adding this platinum-based compound to 5-FU, the progression free survival is increased, but the overall survival time remains unaltered (Lucas, O'Neil et al. 2011). The main secondary effects that arise due to the treatment with oxaliplatin are nausea, vomiting and sensory neuropathy, and the most severe cases may present dysesthesia in the hands, feet and in perioral area (Di Francesco, Ruggiero et al. 2002) (Di Francesco et al., 2002).

There are other platinum-based compounds widely used in CRC treatment. In fact, oxaliplatin appeared in response to the emerging necessity of overcoming the secondary effects induced by **cisplatin**, another platinum-based therapy widely used in cancer treatment (Graham, Mushin et al. 2004), despite the fact that it is not the preferred treatment regarding CRC. Cisplatin binds DNA, creating cross-links between purine bases, both in the same DNA strand or between different DNA strands, inducing DNA damage responses (Oliver, Mercer et al. 2010). Likewise, cisplatin treatment also leads to severe side effects, including neurotoxicity, nephrotoxicity, myelotoxicity, nausea, vomiting and auditory impairment (Di Francesco, Ruggiero et al. 2002).

As cytotoxic chemotherapeutic agents target cells in rapid proliferation, normal cells within the organism may also be affected, resulting in the appearance of adverse effects that affect the quality of life (Gerber 2008). To overcome these effects caused by cytotoxic chemotherapy, specific targeted therapies started to be explored.

1.1.3.2 Targeted therapies for colorectal cancer treatment

Targeted therapies comprise chemical agents that interfere specifically with molecules necessary for the appropriate tumor development and growth. Those agents usually have antibody specificity and normally inhibit signalling pathways that are important for cancer development and maintenance, resulting in diminished toxicity for normal tissues (Cohen, Cohen et al. 2005). Furthermore, in normal cells, the targeted molecules are expressed at lower levels than those found in cancer cells, or are not expressed at all. The progress in this field opened the possibility of creating more personalized CRC treatments, since patients can be administered with specific drugs depending on the mutations found (Gerber 2008).

Two types of chemical agents used as targeted therapies have been distinguished: *monoclonal antibodies* and *small molecule inhibitors.* The first are large, water-soluble molecules, with a molecular weight of about 150.000 Da and act by binding to the extracellular part of the cells that contain their receptors (Gerber 2008; Tol and Punt 2010). The latter are small molecule inhibitors which block signals by interfering with intracellular molecules that intervene in signalling transduction pathways in response to extracellular stimuli; these inhibitors have a lower molecular weight (about 500 Da) and must enter the cells to perform their function (Gerber 2008).

The role of targeted therapies on CRC therapy will be explored, focusing particularly on monoclonal antibodies, since they are used widely in CRC treatment. The targeted biologic therapies available for CRC fall into three groups: monoclonal antibodies against the epidermal growth factor receptor (EGFR), inhibitors of the vascular endothelial growth factor (VEGF) proangiogenic system and an oral small-molecule inhibitor of intracellular kinases involved in various signalling cascades.

1.1.3.2.1 Monoclonal antibodies in colorectal cancer treatment

The main targets of the antibodies used in CRC therapy are molecules important in cell proliferation, invasion, migration and angiogenesis. An example is the Epidermal Growth Factor Receptor (EGFR, also known as ErB1 or HER1), a member of the ERbB transmembrane receptor family with tyrosine kinase activity responsible for

stimulating several signalling pathways. Therefore its activation results in a wide range of cellular responses, including mitogenic stimulation, changes in cell adhesion that eventually allow motility or, in some cases, cell death (Mendelsohn and Baselga 2006; Sridharan, Hubbard et al. 2014). The RAS-RAF-MEK-ERK pathway is triggered by activation of EGFR, resulting in cell proliferation and survival, and hence it is not surprising that EGFR plays important roles in cancer development (Alroy and Yarden 1997). EGFR is normally present in epithelial cells, found, for example in skin and mucosa (Gerber 2008), and is overexpressed in 25 to 77% of CRC cases (Hoy and Wagstaff 2006), particularly the most aggressive. Overexpression of EGFR therefore carries a negative prognostic value (Spano, Lagorce et al. 2005). Monoclonal antibodies targeting EGFR have already been developed; **cetuximab** and **panitumumab** are two examples of FDA-approved agents for CRC treatment (Tol and Punt 2010).

Cetuximab (commercially available as Erbitux®) inhibits tumor development by binding to the extracellular domain of EGFR, impairing activation of the receptor (Blick and Scott 2007). This human-mouse chimeric monoclonal antibody exhibits anti-tumor properties in mCRC cells expressing EGFR that do not harbor mutations in KRAS or BRAF by inducing cell-cycle arrest and apoptosis, inhibiting angiogenesis and metastasis (Blick and Scott 2007; Tol and Punt 2010). As KRAS and BRAF are downstream of EGFR in the signalling pathway, mutations in those molecules can trigger activation of the signalling pathway even without ligand-dependent stimulation, circumventing the action of cetuximab (Khambata-Ford, Garrett et al. 2007). Cetuximab can be used both combined with classical chemotherapeutical agents (such as irinotecan) or in monotherapy. Evidence has shown that 22.5% of patients that no longer respond to treatments based on irinotecan start responding to treatments with cetuximab and irinotecan combined, and 10% positively respond to treatments based only on cetuximab (Mendelsohn and Baselga 2006).

EGFR is also present in normal tissues, and inhibition of its downstream signalling pathways may lead to local undesired side effects. In fact, several secondary effects due to cetuximab treatment are reported, particularly associated with dermatological and gastrointestinal toxicities. The most severe secondary effects include

cutaneous rash, diarrhea, nausea and vomiting (Gerber 2008). Normally, the appearance of a cutaneous rash suggests that the treatment is working (Agero, Dusza et al. 2006).

Panitumumab (commercially available as Vectibix®) is another monoclonal antibody highly selective for EGFR, also responsible for blocking the activation of this receptor. Unlike cetuximab, panitumumab is a fully human monoclonal antibody and is used to impair cell proliferation and survival, as well as to diminish pro-inflammatory cytokine production due to EGFR activation (Hoy and Wagstaff 2006). As with cetuximab, this type of treatment is only effective in tumors with non-mutated KRAS (Amado, Wolf et al. 2008). Panitumumab is used as a therapeutic agent both in monotherapy (when the patient fails to respond to other types of chemotherapy, such as fluoropyrimidines and irinotecan) or combined with other chemotherapeutical agents (Kohne, Hofheinz et al. 2012). Several secondary effects are associated with panitumumab treatment, including skin rash, fatigue, abdominal pain, nausea, vomiting, diarrhea, constipation and hypomagnesaemia (Hoy and Wagstaff 2006).

There are many other players in colon carcinogenesis, and thus EGFR is not the only possible target for antibody-based treatments. It is known that angiogenesis is fundamental for tumor development, and some evidence shows that tumors are unable to grow more than 3 millimeters away from the surrounding vasculature (Gerber 2008).

Vascular endothelial growth factor (VEGF) comprises a family of key molecules that regulate angiogenesis and vascular permeability. Their expression is negligible in adult tissues, except in the process of wound healing and in female sexual cycle related processes, such as endometrial development (Rosen 2002). VEGF is overexpressed in about 50% of CRC cases, and it is minimally expressed or absent in normal colon mucosa and in adenomas (Bendardaf, Buhmeida et al. 2008). VEGF overexpression is correlated both with disease progression and with negative prognostic, because tumors that highly express this molecule tend to be more lethal than those that do not (Bendardaf, Buhmeida et al. 2008).

Expression of VEGF is triggered, among other circumstances, under hypoxic conditions and through the activation of EGFR-related pathways (Niu, Wright et al. 2002). The major importance of VEGF overexpression in mCRC lays on the fact that tumor cells need to reach the bloodstream in order to metastasize (Sridharan, Hubbard et al. 2014).

The rationale for targeting VEGF lies on the fact that, by inhibiting its action, tumor growth is circumscribed due to the limited access to blood vessels and nutrients (Gerber 2008). However, it is believed that the mode of action of anti-VEGF agents is far more complex and may involve several other mechanisms (Ellis and Hicklin, 2008). Some side effects may arise due to this type of treatment, namely bleeding, thrombosis and hypertension (Gerber 2008).

In CRC treatment, **bevacizumab** (available as Avastin®) was the first anti-angiogenic agent approved by US Food and Drug Administration (FDA), and is the most common anti-VEGF therapy used today (Sridharan, Hubbard et al. 2014). Bevacizumab is used in mCRC treatment in combination with classical chemotherapeutic agents, including 5-FU/LV, irinotecan and oxaliplatin (Okines and Cunningham 2009). This type of treatment appears to improve the progression free survival time, but has no significant impact in the overall survival time (Okines and Cunningham 2009).

Bevacizumab appears to induce apoptosis, blocking VEGF-mediated stimulation of endothelial progenitor cells and normalizing tumor vasculature, which has significant impact in chemotherapeutic delivery improvement (Okines and Cunningham 2009). Moreover, evidence has shown that by targeting VEGF the immune response against cancer cells is improved by enhancing differentiation of dendritic cells; VEGF appears to inhibit their differentiation, which impairs the ability to present antigens and affects the immune response against cancer cells in VEGF-positive cancers (Ellis and Hicklin 2008). Despite all these advantageous features, targeted treatments against VEGF raise several secondary effects that may compromise the treatment success including hypertension, thrombosis, intestinal perforation and wound healing impairment (Okines and Cunningham 2009).

Due to the success of bevacizumab, several compounds targeting VEGF-signalling such as **sunitinib**, **cediranib** and **sorafenib** have been developed and are currently being tested in clinical trials on human cancers, including CRC (Ferrarotto and Hoff 2013). These compounds include specific anti-VEGF agents, multitargeted agents that selectively inhibit VEGF receptors, and multikinase inhibitors that target VEGF receptors and other kinases that contribute to tumor development (Ferrarotto and Hoff 2013).

1.1.3.2.2 New anti-angiogenesis therapies

Like bevacizumab, another active agent against angiogenesis was recently appoved by the FDA as a second-line therapy for the treatment of mCRC (Wang and Lockhart 2012). **Aflibercept** (available as Zaltrap®) is a recombinant protein consisting of the key domains of VEGF receptors 1 and 2. This agent captures and blocks the isoforms of VEGF-A and VEGF-B, and therefore inhibits angiogenesis (Wang and Lockhart 2012). It has been used particularly in combination with cytotoxic chemotherapy, such as FOLFOX (Leucovirin/5-FLU/Oxaliplatin) and ILV5FU2 (5-FLU/Leucovirin/Irinotecan) (Wang and Lockhart 2012). The most common adverse symptoms are hypertension, proteinuria, fatigue, and diarrhea (Wang and Lockhart 2012).

Regorafenib (available as Stivarga®) is an oral multikinase inhibitor that has shown antitumor activity against a range of solid tumors, including CRC (De Wit, Boers-Doets et al. 2014). Studies have demonstrated that this compound targets cell signalling pathways such as RAF/MEK/ERK signalling and is also involved in the inhibition of protein kinases associated with angiogenesis (e.g., VEGFR 1–3) (Yan and Grothey 2015). In particular, regorafenib blocks several protein kinases that either prompt tumor cells to grow or help form new blood vessels to feed the tumor. This agent is generally used in patients with advanced CRC that cannot be surgically removed and no longer respond to other appropriate treatments (De Wit, Boers-Doets et al. 2014; Yan and Grothey 2015). Common side effects include fatigue, decreased appetite, hand-foot syndrome (redness and irritation of the hands and feet), diarrhea, sores in the mouth and throat, weight loss, infections, and high blood pressure (De Wit, Boers-Doets et al. 2014). Some serious side effects that can occur

include liver damage, severe bleeding and perforations in the stomach or intestines (De Wit, Boers-Doets et al. 2014).

So far, chemotherapeutic agents have been used as the first choice for CRC treatment and also to prolong the survival of pacients. However, as previously mentioned, these chemical anticancer drugs have important adverse effects. To overcome these problems, nutraceuticals have been extensively investigated for the prevention and/or therapy of CRC. These nutritional compounds have the additional benefit of improving the overall health of the patient.

Table 2: FDA-Approved Agents and indications in Metastatic Colorectal Cancer (Dezember 2013).

Class of Agent (s)		***Trade name***	***Target***	***Approved as single agent***	***Approved in combination with other agents***
Cytotoxic chemoterapy	Fluoracil	Efudex Adrucil Flouroplex	DNA synthesis	+	+
	Capecitabine	Xeloda	DNA synthesis	+	
	Irinotecan	Camptosar	DNA synthesis	+	+
	Oxaliplatin	Eloxatin	DNA synthesis		+
EGFR antibody	Cetuximab	Ertibix	Blocks EGFR pathway	+	+
	Panitumumab	Vectibix	Blocks EGFR pathway	+	
VEGF inhibitor	Bevacizumab	Avastin	Blocks VEGF		+
	Aflibercep	Zaltrap	Blocks VEGF		
Multikinase inhibitor	Regorafenib	Stivarga	Inhibits VEGFR (1-3)	+	

Adapted from Sridhran et al. 2014.

1.2 The role of nutraceuticals in colorectal cancer

The term nutraceutical is known as a food (or part of the food) that provides medical or health benefits for the consumer (Arora and Baldi 2015). It is also defined as functional food, ranging from natural diets (medicinal plants, marine organisms, vegetables, fruits) and herbal products to genetically engineered foods and processed products such as cereals, soups and beverages providing the body with the required amount of vitamins, fiber, proteins, carbohydrates, microrganisms, *etc*., needed for body health (Figure 4) (Singh and Sinha 2012). At present, these compounds are available in foods, dietary supplements and as pharmaceutical formulations (capsules, tablets and powders) by the medicinal and commercial industries (Arora and Baldi 2015).

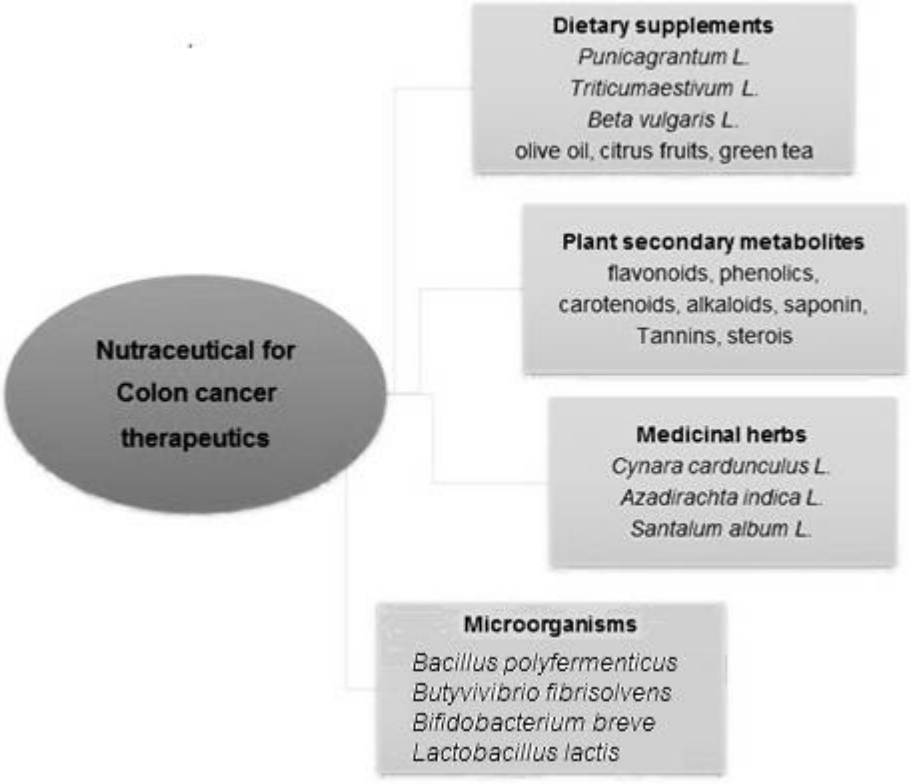

Figure 4: Different classes of nutraceuticals and their uses. Adapted from Kuppusamy, 2014.

Dairy products that contain nutraceuticals have represented a new research area in the last decade due to their presumed safety and potential nutritional properties, especially in the gastrointestinal tract, namely in the prevention of diarrhea or obstipation, stimulation of mineral adsorption, modulation of the metabolism of the

intestinal flora, effects on lipid metabolism, immunomodulatory properties and cancer prevention (Kuppusamy, Mashitah et al. 2014).

It has been suggested that consumption of nutraceuticals (especially fibers and some mircrorganisms) may decrease the risk of colon cancer development (Fotiadis, Stoidis et al. 2008). In this case, the beneficial effects of these components in retarding carcinogenesis is attributed to their anti-carcinogenic activity, which provides a strong support for the acceptance of these nutraceuticals as chemopreventive agents (Cencic and Chingwaru 2010; Kuppusamy, Mashitah et al. 2014). At present, they are classified into three main groups: probiotics, prebiotics and synbiotics (Fotiadis, Stoidis et al. 2008).

Probiotics are characterized by the Food and Agricultural Organization/World Health Organization (FAO/WHO) as "*live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host*" (Arora and Baldi 2015). Additionaly, they are also known as live microorganisms (especially bacterial) belonging to natural microbiota with low or no pathogenicity, but with functions of prime importance to the health and well being of the host. Numerous microorganisms from different genera like *Lactobacillus*, *Bifidobacterium*, *Propionibacterium, Pedicoccus, Leuconostoc*, *Enterococus* and the yeast *Saccharomyces boulardii* are recognised as probiotics (Fotiadis, Stoidis et al. 2008). These bioactive components can be used in probiotic foods, particularly in fermented milk products, and others are being investigated with regard to their medicinal use (Cencic and Chingwaru 2010; Arora and Baldi 2015). Usually, probiotics have high antimicrobial and immunomodulatory capabilities. It has also been demonstrated that their members produce short-chain fatty acids (SCFAs) as fermentation products, which possess several anti-neoplastic properties, especially in the human colon (Cencic and Chingwaru 2010; Zeng, Lazarova et al. 2014).

Prebiotic is "*a selectively fermented ingredient, or a fiber that allows specific changes, particularly in activity of the gastrointestinal microbiota, conferring benefits on the well being and health of host*" (Cencic and Chingwaru 2010). A number of prebiotics include certain fibers and resistant starches, but the most widely described prebiotics are non-digestible oligosaccharides which upon fermentation of beneficial microorganisms produce different amounts of SCFAs in the colon

(Fotiadis, Stoidis et al. 2008). Furthermore, a mixture of probiotics and prebiotics can result in additive or synergistic effects on gastrointestinal function. The term used, in this case, is **synbiotic -** "*a combination of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and activating the metabolism of one or a limited number of health promoting bacteria, and thus improving host welfare*"(Fotiadis, Stoidis et al. 2008).

In summary, it has been established that gut microbiota interact with the host at multiple levels to maintain intestinal homeostasis and disruption in this symbiotic interaction can result in many physiological alterations associated with the onset and progression of chronic intestinal diseases including CRC (Fotiadis, Stoidis et al. 2008). Moreover, scientific evidence (*in vitro* and *in vivo* studies) has shown that modulation of the host gut microbial environment by the use of nutraceuticals (pro, pre and/or synbiotics) is a protective approach for proper maintenance of healthy gut microbiota and reduce the development of colon cancer risk (Fotiadis, Stoidis et al. 2008; Zeng, Lazarova et al. 2014). It is thus important to understand the "normal" gut microbiota and its changes during CRC development to understand how the use of nutraceuticals, notably by increasing the production of short-chain fatty acids, could function as a new strategy to prevent/treat CRC.

1.3 Normal colon microbiota and short-chain fatty acids

The human intestine harbours as many as 10^{12} microorganisms from 500 - 1000 different bacterial species, described by their variation in 16S ribosomal RNA genes (Sobhani, Amiot et al. 2013; Zeng, Lazarova et al. 2014; Cipe, Idiz et al. 2015). Several studies have proposed that the normal human microbiota is formed mainly by strict anaerobes, such as *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Fusobacterium*, *Peptostreptococcus*, and *Atopobium* (Neish 2009; Tiihonen, Ouwehand et al. 2010; Liu, Cao et al. 2013), representative of 2 main divisions: *Bacterodeites* and *Firmicutes* (Neish 2009; Tiihonen, Ouwehand et al. 2010; Holmes, Li et al. 2011; Liu, Cao et al. 2013; Zeng, Lazarova et al. 2014). *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* were also found, but in minor proportions (Liu, Cao et al. 2013; Zeng, Lazarova et al. 2014). It is

known that the intestinal microbiota is a very complex system with numerous beneficial roles to human health, including protection against pathogens, immune system maturation, degradation of toxic substances, digestion of complex carbohydrates and production of short-chain fatty acids (SCFAs) (Holmes, Li et al. 2011; Russell, Hoyles et al. 2013).

In this case, although the main SCFAs (namely acetate, propionate and butyrate) exert multiple beneficial effects in the normal colon physiology, their role as cancer-preventing/treating agents has received special attention by several researchers (Scheppach, Bartram et al. 1995; Kim, Park et al. 2014; Kasubuchi, Hasegawa et al. 2015). These microbial compounds are produced by anaerobic microorganisms able to ferment polysaccharides, oligosaccharides, proteins, peptides and glycoproteins in the colon, including *Firmicutes*, *Clostridium*, *Bifidobacterium, Propionibacterium, Bacteroides* and *Lactobacillus* (Neish 2009; Adom and Nie 2013; Layden, Angueira et al. 2013; Kim, Park et al. 2014). The ability to produce butyrate requires a specific enzymatic process via butyryl-CoA: acetate CoA-transferase (mainly by *Eubacterium*, *Roseburia, Anaerostipes* and *Faecalibacterium prausnitzii* species) or via butyrate kinase (in certain *Clostridium* and *Coprococcus* species) (Russell, Hoyles et al. 2013; Kim, Park et al. 2014; Zeng, Lazarova et al. 2014). *Firmicutes* species are able to produce butyrate by both pathways (Russell, Hoyles et al. 2013; Kim, Park et al. 2014; Zeng, Lazarova et al. 2014). Propionate is generally produced by *Bacteroides, Firmicutes* and *Propionibacterium* species which involve the succinate, acrylate and propanediol pathways (Hosseini, Grootaert et al. 2011; Russell, Hoyles et al. 2013; Kim, Park et al. 2014), while acetate is produced by acetogenic bacteria such as *Acetobacterium* species, *Clostridium aceticum* and *Propionibacterium,* able to perform reductive acetogenesis from formate (Russell, Hoyles et al. 2013; Kim, Park et al. 2014). Normally, these bacteria exist in a mutually beneficial symbiotic relationship in the human colon, providing the proper amount of these SCFAs to help maintain colon homeostasis (Cipe, Idiz et al. 2015).

Indeed, it has recently been shown, that some changes in SCFAs production is directly associated with alterations in the intestinal microbiota modulated by numerous extrinsic factors such as diet, age, medication, treatment (drugs,

radiation, surgery), stress and diseases (Zhu, Michelle Luo et al. 2011; Cipe, Idiz et al. 2015). These changes in the normal symbiotic state (normobiosis) can be associated with many intestinal disorders, such as obesity, inflammatory bowel disease and colorectal cancer (Chen, Liu et al. 2012; Di Mauro, Neu et al. 2013; Sobhani, Amiot et al. 2013; Zeng, Lazarova et al. 2014). In this regard, the main challenge is to characterize the differences between a "healthy" intestinal microbiota and a CRC microbiota and its consequences on SCFAs production. This study would facilitate future studies towards the development of novel strategies for the diagnosis, treatment, and prevention of colorectal cancer (Cipe, Idiz et al. 2015).

1.3.1 Intestinal microbiota and short-chain fatty acids in colorectal cancer patients

Several factors (genetic and environmental) are involved in the transformation of normal colonic epithelium to adenoma and CRC (Keku, Dulal et al. 2015). It has been demonstrated that specific changes in human intestinal microbiota (dysbiosis) can also be associated with CRC development, especially in sporadic cases (Sobhani, Tap et al. 2011; Sobhani, Amiot et al. 2013; Gao, Guo et al. 2015). These changes support the hypothesis that "colon cancer may be a bacteria-related disease" (Sobhani, Amiot et al. 2013; Gao, Guo et al. 2015), and that specific bacterial species can be involved in CRC pathogenesis (Ohigashi, Sudo et al. 2013; Geng, Song et al. 2014; Cipe, Idiz et al. 2015; Gao, Guo et al. 2015) (Figure 5).

The majority of studies have shown a significant difference between the bacteria genera associated with cancerous and non-cancerous intestinal tissue or fecal samples (Sobhani, Amiot et al. 2013; Gao, Guo et al. 2015; Keku, Dulal et al. 2015). The intestine microbial composition of CRC patients is usually increased in the following genera: *Lactococcus*, *Fusobacterium, Prevotella, Streptococcus, Bacteroides* (Sobhani, Tap et al. 2011; Gao, Guo et al. 2015; Keku, Dulal et al. 2015). It has been demonstrated that some bacteria have a complex arsenal of virulence factors which allow them to colonize and persist in the intestine, inducing chronic inflammation, accumulated mutations by DNA damage through superoxide radicals, genotoxin formation, increased T-cell proliferation and biosynthesis of procarcinogenic compounds that interfere with cell cycle regulation contributing to

colorectal carcinogenesis (Yang and Jobin 2014; Cipe, Idiz et al. 2015; Gao, Guo et al. 2015; Leung, Tsoi et al. 2015; Nistal, Fernandez-Fernandez et al. 2015).

Escherichia coli (*E. coli*, a member of Enterobacteriaceae family) and *Fusobacterium nucleatum (F. nucleatum*) are the main bacteria associated with CRC cases and commonly over-represented in many tumor tissues from CRC patients (Sobhani, Amiot et al. 2013; Yang and Jobin 2014; Gao, Guo et al. 2015; Keku, Dulal et al. 2015; Leung, Tsoi et al. 2015). Interestingly, in healthy individuals, Enterobacteriaceae constitute only a small fraction (less than 1%) of the gut microbiota, but *E. coli* in particular becomes dominant in the gut microbiota of individuals with inflammatory bowel disease (IBD), in several animal models of gut inflammation and in CRC cases (Yang and Jobin 2014). An interesting fact is that, *E. coli* strains isolated from individuals with IBD and CRC are often adherent and invasive, displaying several pathogenic properties which modulate host cells and mediate carcinogenesis, and are in this way classified as inflammation drivers of the CRC formation (Leung, Tsoi et al. 2015). Beyond that, *F. nucleatum* is an opportunistic bacterium able to induce chronic inflammation and interact directly or indirectly with colonocytes leading to uncontrolled cell proliferation, dysbiosis and intestinal tumorigenesis (Kostic, Chun et al. 2013; Leung, Tsoi et al. 2015). Recently, *F. nucleatum* has been considered as an indicator of disease progression and tumor severity (Leung, Tsoi et al. 2015). Likewise, it has also been shown that *Streptococcus gallolyticus*/*bovis* (involved with intestinal inflammatory disorders) is present in 20-50% of CRC and in less than 5% of normal colon (Gao, Guo et al. 2015).

In contrast, patterns that preserve microbial intestinal homeostasis, such as *Bifidobacterium*, *Lactobacillus*, *Clostridium* and *Ruminococcus* are under-represented in CRC patients compared to healthy individuals (Sobhani, Tap et al. 2011; Chen, Liu et al. 2012; Sobhani, Amiot et al. 2013; Nistal, Fernandez-Fernandez et al. 2015).

Changes in the intestinal microbial community of CRC patients affect the amount and types of metabolites produced by colonic microbes. In this regard, some studies have reported that changes in colon microbiota are associated with a decrease in SCFA concentrations accompanied by an elevated pH in patients with CRC

compared to healthy individuals (Sobhani, Tap et al. 2011; Ohigashi, Sudo et al. 2013; Sobhani, Amiot et al. 2013) (Figure 5). However, it is not yet understood if the changes in colon microbiota are a cause or a consequence of CRC development. In this context, it is important to characterize the possible over- and under-represented bacteria, exploring the relationship between changes in the colon microbiota composition with the production of bacterial metabolites as well as the tumorigenesis process of CRC. The relationship between diet, intestinal microbiota, SCFA metabolism and colorectal cancer is very complex and remains an important area of research. In the following sections, the role of SCFAs in normal conditions is discussed (their concentrations, metabolism, beneficial effects, transporters and receptors), as well as how these microbial metabolites, namely butyrate, propionate and acetate, can be useful in the prevention and/or treatment of CRC.

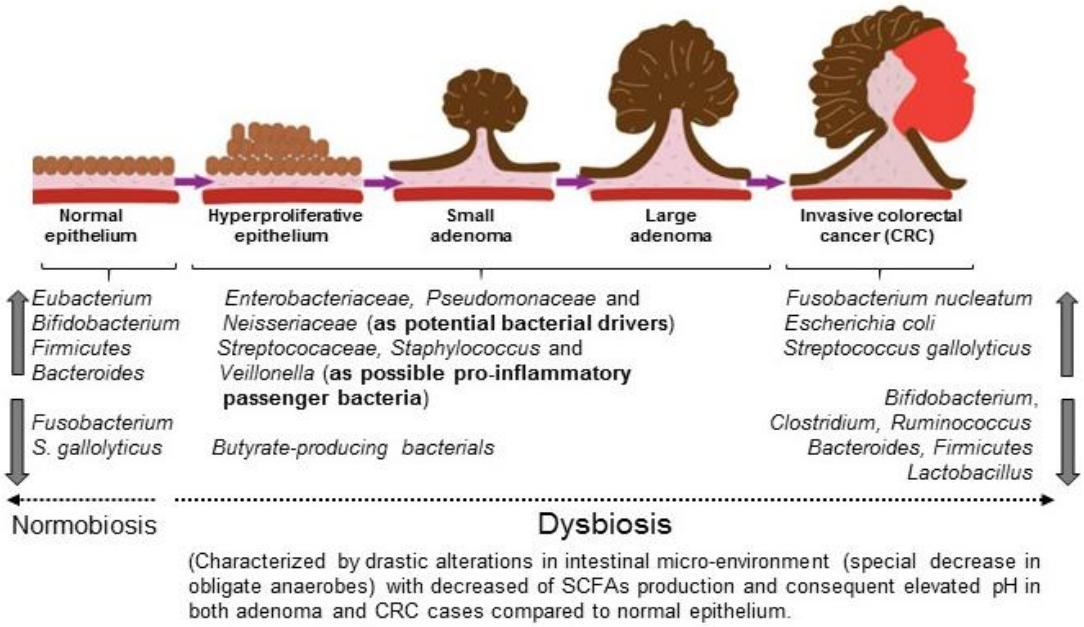

Figure 5: Schematic representation of the most important changes in the microbiota in the transition from normal epithelium to colorectal cancer (CRC).

1.4 Short-chain fatty acid concentrations in colon

Short chain fatty acids (SCFAs) are the major products of bacterial fermentation of undigested dietary fiber and of starches resistant to digestion in the human colon (Adom and Nie 2013; Zeng, Lazarova et al. 2014). Indeed, a diet rich in fiber,

resistant starches and complex carbohydrates leads to increases the levels of colonic SCFAs (Adom and Nie 2013; Layden, Angueira et al. 2013).

SCFAs are carboxylic acids with aliphatic tails ranging from formic (C1), acetic (C_2), propionic (C_3), butyric (C_4), valeric (C_5) and caproic (C_6) acid (Layden, Angueira et al. 2013). SCFAs constitute approximately two-thirds of the colonic anions (~150 mM). At physiological pH, they predominate in a dissociated form, specifically acetate, propionate, butyrate, valerate and caproate (Nedjadi, Moran et al. 2014). The rate and amount of SCFAs produced depend on diet composition (which determines the type of the substrate fermented), on the microbiota present in the colon and on gut transit time (Mortensen and Clausen 1996; Macfarlane and Macfarlane 2012; Zeng, Lazarova et al. 2014). SCFAs are usually generated by carbohydrate fermentation according to the following equation (Zeng, Lazarova et al. 2014) :

$$59\ C_6H_{12}O_6 + 38\ H_2O = 60\ CH_3COO^-\ (\text{Acetate}) + 22\ CH_5COO^-\ (\text{Propionate}) + 18\ CH_7COO^-\ (\text{Butyrate}) + 96\ CO_2 + 256\ H^+ + \text{heat} + \text{gases}$$

Acetate, propionate and butyrate are the three major colonic SCFAs. They are found in the colon at considerably high concentrations, ranging from 40–80 mM to 10–25 mM, respectively (Scheppach, Bartram et al. 1995; Alles, Hartemink et al. 1999; Jenkins, Kendall et al. 1999; Topping and Clifton 2001). Although not constant, the ratio of SCFA concentrations in the colonic lumen is approximately 60% acetate, 25% propionate and 15% butyrate (Scheppach, Bartram et al. 1995; Canani, Costanzo et al. 2011). In addition, the concentration of acetate, propionate and butyrate vary along the human intestine as follows: approximately 130 ± 9 mmol/L in the cecum (where the amounts of fermentable substrates are higher), ~ 80 ± 11 mmol/L in the descending colon and ~ 13 ± 6 mmol/L in the rectum (Cummings, Pomare et al. 1987; Du, Shi et al. 2014). This variation in acid concentration is linked with pH differences along the human colon, namely: ~ 5.5-6.8 in the cecum and ~ 6.6-7 in the descending colon (Figure 6). The higher acidity is associated with more active carbohydrate fermentation in the cecum than in the sigmoid/rectum, where the pH is more alkaline (Cummings, Pomare et al. 1987).

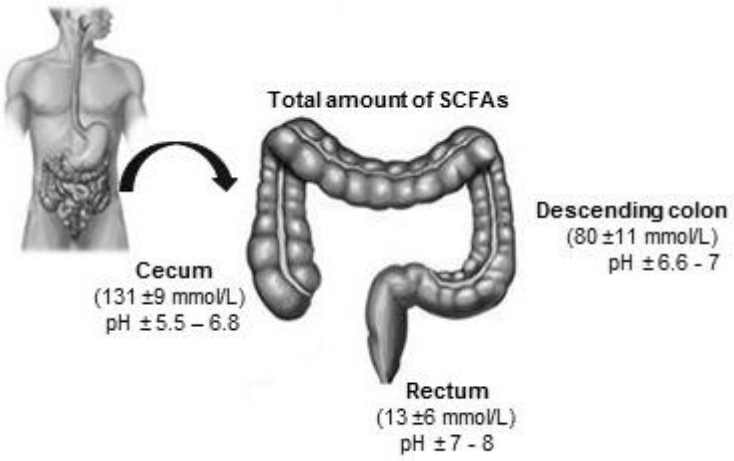

Figure 6: Regions of the colon and rectum with respective total amount of SCFA concentrations and pH.

1.4.1 Metabolism of short-chain fatty acids in the colon

It has been demonstrated that SCFAs derived from microbial metabolism in the gut play a central role in colon homeostasis (den Besten, Lange et al. 2013; den Besten, van Eunen et al. 2013). SCFAs are substrates for energy metabolism by the epithelial cells, converted by colonocytes into glucose, ketone bodies, and amino acids (Hadjiagapiou, Schmidt et al. 2000; den Besten, Lange et al. 2013; Kasubuchi, Hasegawa et al. 2015). Previous studies showed a preferential utilization of SCFAs in the order of butyrate > propionate > acetate by colonocytes (Adom and Nie 2013). Butyrate is the major energy source for colonocytes, but also for other cells from liver and muscle tissues (Ahmad, Krishnan et al. 2000; Adom and Nie 2013; Russell, Hoyles et al. 2013; Zeng, Lazarova et al. 2014). Butyrate is usually metabolized to acetyl-CoA by colonocytes (Donohoe, Collins et al. 2012) while propionate and acetate can modulate lipogenesis and gluconeogenesis (Comalada, Bailon et al. 2006). It has been demonstrated that propionate is largely metabolized in the liver (Russell, Hoyles et al. 2013) and also acts as substrate for gluconeogenesis inhibiting cholesterol synthesis in hepatic tissue (Adom and Nie 2013; Scott, Gratz et al. 2013). Acetate can be oxidized in the tricarboxylic acid (TCA) cycle, used as a substrate for the synthesis of cholesterol, long-chain fatty acids or as a co-substrate for glutamine and glutamate synthesis by colonocytes (Adom and Nie 2013; den Besten, Lange et al. 2013; Zeng, Lazarova et al. 2014). This compound also enters systemic circulation and can be used by many tissues, including heart,

adipose, kidney, liver and muscle (den Besten, Lange et al. 2013; Scott, Gratz et al. 2013).

In contrast to normal colonocytes, CRC cells primarily undergo aerobic glycolysis instead of oxidative metabolism (Donohoe, Collins et al. 2012). In this case, it has been proposed that the preference of CRC cells for glucose metabolism instead SCFAs as an energy source explains the opposite effects on the growth of normal *versus* cancerous colonocytes (Donohoe, Collins et al. 2012). However, this paradox is still poorly understood and more studies are needed to understand how the metabolic differences between normal and cancerous colonocytes can be used to inhibit tumorigenesis in CRC.

1.4.2 Short-chain fatty acid transporters and receptors

Production of total colonic SCFAs is difficult to determine because more than 95% of these SCFAs are water soluble and rapidly absorbed and metabolized by colonocytes (Roy, Kien et al. 2006; Kim, Park et al. 2014; Zeng, Lazarova et al. 2014). Only a small amount of these SCFAs leave the colon intact and can be transported via the blood circulation to other organs (Roy, Kien et al. 2006; Ganapathy, Thangaraju et al. 2013; Kim, Park et al. 2014).

Though distinct mechanisms for SCFAs absorption across the plasma membrane of colonocytes in various species have been postulated (Hijova and Chmelarova 2007; Nedjadi, Moran et al. 2014), most studies on SCFAs transporters both show in intestinal epithelial cells and CRC cells used butyrate transport as a model (Hadjiagapiou, Schmidt et al. 2000; Ganapathy, Thangaraju et al. 2013; Goncalves and Martel 2013) and information concerning other SCFAs like propionate or acetate transport is still rare.

SCFAs enter intestinal epithelial cells through passive diffusion of protonated SCFAs or mediated transport by monocarboxylate transporters (MCTs) (Goncalves and Martel 2013). The MCT family comprises 14 members, however, only the first four (MCT-1 - 4) are known to mediate the proton-coupled transport of monocarboxylic acids across the plasma membrane (Pinheiro, Longatto-Filho et al. 2012; Halestrap 2013). The main function of MCT has been associated with the

uptake or efflux of monocarboxilic acids through the plasma membrane, according to the cell metabolic needs, characterized by a high affinity transport for L-lactate, pyruvate, acetate, propionate, D,L-β-hydroxybutyrate and acetoacetate (Halestrap and Meredith 2004).

SMCT-1 and MCT-1 have been identified as the main monocarboxylate transporters, responsible for the uptake of SCFAs across the membrane of intestinal cells (den Besten, Lange et al. 2013; Goncalves and Martel 2013; Kim, Park et al. 2014). SMCT-1 is a Na^+-coupled transporter for a variety of SCFAs, especially butyrate (Canani, Costanzo et al. 2011; Ganapathy, Thangaraju et al. 2013), while MCT-1 is a H^+-coupled transporter for SCFAs and related organic acids, which transports these molecules across the plasma membrane depending on the H^+ eletrochemical gradient (Kim, Park et al. 2014). MCT-1 is expressed widely in many different cell types and has been characterized as the primary butyrate transporter in colon epithelial cells (Canani, Costanzo et al. 2011; Fung, Cosgrove et al. 2012).

MCT-1 also exports lactate to the extracellular milieu, which is a potentially cytotoxic metabolic by-product of glycolysis, indicating that it plays a dual role to enhance CRC cell survival (Fung, Cosgrove et al. 2012). In contrast, it has been shown that CRC cells silence SMCT-1 by DNA methylation (Hadjiagapiou, Schmidt et al. 2000; Thangaraju, Cresci et al. 2008; Babu, Ramachandran et al. 2011; Ganapathy, Thangaraju et al. 2013; Goncalves and Martel 2013) which confers a selective advantage to escape butyrate-induced cell death through limitation of butyrate uptake (Li, Myeroff et al. 2003; Ganapathy, Thangaraju et al. 2013).

In addition to SCFA transporters, their receptors constitute a new and rapidly growing field of research, as more functions of SCFA receptors have been discovered. SCFAs are able to activate cells through several cell-surface G-protein-coupled receptors (GPRs), especially GPR41 and GPR43 (Kim, Kang et al. 2013; Kuwahara 2014), involved in immune response regulation (Kim, Kang et al. 2013). These two GPRs are expressed not only by intestinal epithelial cells where SCFAs are produced, but also at multiple other sites considered to be metabolically important such as adipose tissue and pancreatic islets (Layden, Angueira et al. 2013). Recent studies have identified the cell-surface receptors GPR41, GPR43 and GPR109A as essential for the biologic effects of SCFAs in the colon (Ganapathy,

Thangaraju et al. 2013; Kim, Kang et al. 2013). GPR41 (also called free fatty acid receptor 3; FFA3) expressed in colon cells is activated by SCFAs, primarily by butyrate and propionate (Layden, Angueira et al. 2013; Kuwahara 2014), while GPR43 (free fatty acid receptor 2; FFA2) has a more potent affinity for propionate and acetate (Ganapathy, Thangaraju et al. 2013; Layden, Angueira et al. 2013). In addition, GPR109A (hydroxycarboxylic acid receptor 2), a receptor for niacin and vitamin B3, is also a receptor for butyrate in colon cells (Kim, Park et al. 2014; Kuwahara 2014). Nevertheless, the regulation of SCFA transporters and receptors in CRC cells is still poorly understood, and more studies are needed to grasp how these metabolites can influence the regulation of their transporters and receptors in CRC cells.

1.4.3 Physiological effects of short-chain fatty acids in normal colon

It is known that SCFAs play a significant role in maintaining the normal physiological functions of the colonic mucosa, serving as regulators of intracellular pH, cell volume and other functions associated with ion absorption and gut motility of intestinal epithelial cells (Kim, Park et al. 2014; Ohtani 2015). SCFAs are the major energy source for colonocytes and can also be used for *de novo* synthesis of lipids as referred before (Scharlau, Borowicki et al. 2009; Kim, Kang et al. 2013; Kasubuchi, Hasegawa et al. 2015).

In addition, it has been demonstrated that SCFAs have a suppressive role in inflammation and cancer of the intestine (Kim, Park et al. 2014; Keku, Dulal et al. 2015). Though the anti-inflammatory mechanism of SCFAs is still not adequately clarified, a recent study showed that, once absorbed by intestinal epithelial cells and/or immune cells, SCFAs (especially butyrate and propionate) inhibit the activity of histone deacetylases (HDACs), promoting the hyperacetylation of histones and trans-activating gene expression, which results in downregulation of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and IL-12 (Ohtani 2015).

Interestingly, it has been demonstrated that total SCFAs and/or local differences in SCFA concentrations along the intestinal tract might be implicated in diseases of the colon, especially gastrointestinal disorders and colorectal cancer (Wong, de

Souza et al. 2006). For example, a decreased level of butyrate in the environment of colonocytes has been suggested to contribute to the genesis of ulcerative colitis (UC) and to a significantly increased risk of colon cancer (Huda-Faujan, Abdulamir et al. 2010). Therefore, increased production and greater distal delivery of SCFAs may result in a protective effect against these diseases (Wong, de Souza et al. 2006).

SCFAs, especially butyrate, are involved not only in the regulation of expression of several genes, cell proliferation, differentiation and apoptosis to promote colonic wellbeing in epithelial cells, but in the prevention of colon cancer by promoting growth inhibition, cell-cycle arrest and apoptosis in transformed colonocytes (Sakata 1987; Comalada, Bailon et al. 2006; Fung, Cosgrove et al. 2012; Ganapathy, Thangaraju et al. 2013; Kim, Park et al. 2014). Furthermore, as described in more detail below, it has been shown that SCFAs exhibit anti-cancer activities acting through different mechanisms to induce cell death in CRC cells (Comalada, Bailon et al. 2006; Lan, Lagadic-Gossmann et al. 2007; Zhang, Zhou et al. 2010; Adom and Nie 2013; Marques, Oliveira et al. 2013; Oliveira, Pereira et al. 2015).

1.4.4 Mechanisms of action of short-chain fatty acids in colorectal cancer cells: prevention and therapeutic implications

A variety of biological effects of SCFA have been reported, and a vast number of experimental studies keep showing new aspects of these molecules. Most notably, it has been shown that the SCFAs butyrate, propionate and acetate induce apoptosis in CRC cells but not in normal cells (Sakata 1987; Comalada, Bailon et al. 2006; Sauer, Richter et al. 2007; Zhang, Zhou et al. 2010; Tang, Chen et al. 2011; Imbernon, Whyte et al. 2014). The anti-cancer effect of SCFAs is also supported by epidemiological studies suggesting an inverse relation between the level of dietary fibers and the incidence of CRC (Scheppach, Bartram et al. 1995; Sengupta, Muir et al. 2006; Zeng, Lazarova et al. 2014). These dietary components influence the risk of human colon cancer through diverse mechanisms, which include activation of cell death through different processes depending on the concentration, pH and cell type. The mechanisms by which these SCFA are

metabolized and regulate cell proliferation, differentiation, cell death and their role in inflammation in CRC cells will be discussed below.

1.4.4.1 Butyrate

Butyrate is considered the most potent of the SCFAs regarding prevention and inhibition of colon carcinogenesis, and its antitumor effects have been the most studied (Scheppach, Bartram et al. 1995; Comalada, Bailon et al. 2006; Scharlau, Borowicki et al. 2009; Zhang, Zhou et al. 2010; Matthews, Howarth et al. 2012; Goncalves and Martel 2013). Butyrate's protective effects against human colon cancer cells involve inhibition of cell differentiation, promotion of cell-cycle arrest, apoptosis and of inhibition of histone deacetylases (HDACs) (Table 3) (Hinnebusch, Meng et al. 2002; Comalada, Bailon et al. 2006; Zhang, Zhou et al. 2010; Donohoe, Collins et al. 2012; Fung, Cosgrove et al. 2012; Matthews, Howarth et al. 2012).

Previous studies in CRC cell lines showed that induction of apoptosis and cell cycle arrest in G0-G1 or G2-M by butyrate could occur *via* p53-dependent or p53-independent pathways (Archer, Meng et al. 1998; Mariadason, Velcich et al. 2001; Matthews, Howarth et al. 2012). Zhang and co-workers analyzed the cytotoxicity mechanism of butyrate (0-40 mM) in human colon cancer (RKO cells) and showed that it exhibited a strong growth inhibitory effect against RKO cells, inducing the intrinsic apoptosis pathway characterized by DNA fragmentation and activation of caspase-9 and caspase-3 (Zhang, Zhou et al. 2010). The expression of anti-apoptotic protein Bcl-2 decreased, whereas the apoptotic protein Bax increased in a dose-dependent manner during butyrate-induced apoptosis (Zhang, Zhou et al. 2010).

Table 3: Effects of SCFA butyrate in colorectal cancer cells.

Butyrate			
Human colorectal cancer cell lines	**Concentration (mM)**	**Effects**	***Reference***
HCT116 *HT-29*	0 - 20	p21 is required for butyrate-mediated growth arrest	*Archer et al, 1998*
Caco-2 *SW620*	2 - 10	Cell cycle arrest and apoptosis more effective in undifferentiated cells, whereas differentiated cells were essentially resistant to butyrate effects	*Mariadason et al, 2001*
HT-29		Growth inhibition by the downregulation of ERK1/2	*Davido et al, 2001*
HCT116 *HT-29*	1 5	Cell growth arrest, differentiation, apoptosis, induction of histone H4 hyperacetylation	*Hinnebusch et al, 2002*
HT-29	1 - 8	Inhibition of cell proliferation, induction of differentiation and apoptosis	*Comalada, 2006*
HT-29	5 - 40	Modulation of histone acetylation	*Kiefer et al, 2006*
HT-29	5	Downregulation of the GLUT-1 expression mediates apoptosis	*He et al, 2007*
LT92 *HT-29*	0 - 50	Induction of GSTs as a possible mechanism of chemoprevention	*Scharlau et al, 2009*
RKO	0 - 40	Inhibition of cell proliferation involving inactivation of ERK MAPK, induction of apoptosis via activation of caspase-9 and caspase-3	*Zhang et al, 2010*
Caco-2	5	Induction of apoptosis, G2-M arrest, alterations in the oxidative pentose pathway	*Matthews et al, 2012*
*RAW264.7**	0 – 1.200 µmol/L	Decrease of proinflammatory factors with an increase in the antiinflammatory cytokine	*Liu et al, 2012*

*Murine macrophage cell line

Critical tumorigenesis signalling pathways that regulate cell proliferation, cell migration and apoptosis, including extracellular signal-regulated protein kinase 1/2 (ERK1/2), C-Jun N-terminal kinase (JNK) and p38 MAPK (p38) were also studied

(Zhang, Zhou et al. 2010). An important point is that a high levels of ERK1/2 activation/expression are associated with cell proliferation and survival in various cancer cells, including CRC (Davido, Richter et al. 2001; Zhang, Zhou et al. 2010). However, it was demonstrated that butyrate-induced growth inhibition occurs with inactivation or downregulation of ERKs in RKO and HT-29 cells, respectively (Davido, Richter et al. 2001; Zhang, Zhou et al. 2010). Moreover, activation of the JNK MAPK pathway played an important role in butyrate-induced apoptosis in RKO cells (Zhang, Zhou et al. 2010).

Matthews *et al* 2012, showed that butyrate (5 mM) induced apoptosis and G2-M arrest mediated by alterations in the oxidative pentose pathway, reduction in glutathione availability and glucose consumption, and increased levels of reactive oxygen species (ROS) (Matthews, Howarth et al. 2012). He *et al* (2007) observed that downregulation of GLUT-1 expression was associated with apoptosis-induced by butyrate (5 mM) in HT-29 cells, also correlated with an increase in the expression and activity of MCT-1 as a mechanism to maximize intracellular availability of butyrate (He, Li et al. 2007). Some authors state that butyrate inhibits CRC cell proliferation and induces cell death due to its inefficient metabolism associated with the Warburg effect and nuclear accumulation in transformed colonocytes, where it acts as a HDAC inhibitor (Shao, Gao et al. 2004; Wong, de Souza et al. 2006; Donohoe, Collins et al. 2012). Robert Li and co-workers (2006) confirmed accumulation of acetylated histone 3 (H3) as a reslt of butyrate treatment in CRC cells. Butyrate further contributes to hyperacetylation through conversion to acetyl-CoA and stimulation of histone acetyltransferase (HAT) activity (Barshishat, Polak-Charcon et al. 2000; Li and Li 2006). However, the metabolic state of the cell influences the amount of intranuclear butyrate and acetyl-CoA levels determining whether butyrate functions as a HDAC inhibitor or stimulate HATs, thus epigenetically regulating the expression of different target genes (Mariadason, Velcich et al. 2001; Donohoe, Collins et al. 2012). Deregulation of the expression or activity of HATs and HDACs may lead to alterations in gene expression profiles, associated with reactivation or silencing of important genes for cancer progression, differentiation and apoptosis (Sambucetti, Fischer et al. 1999; Iacomino, Tecce et al. 2001; Marchion and Munster 2007). In this context, Archer *et al* 1998, showed that butyrate (0-20 mM) increased p21 expression through a process involving

histone hyperacetylation, and that p21 was required for butyrate-mediated growth arrest in colon cancer cells (HT-29 and HCT116).

Furthermore, compared to propionate and acetate, butyrate has strong anti-inflammatory properties, and this effect is mediated mainly by inhibition of TNF-α production, nuclear factor-kappa B (NF-κB) activity and IL-8, 10 -12 expression in immune and colonic epithelial cells (Tedelind, Westberg et al. 2007; Kim, Park et al. 2014; Zeng, Lazarova et al. 2014). Yet, little is known about the role of butyrate on inflammation in CRC cells.

The main conclusion is that the exposure of the human colon to butyrate might protect against CRC by reducing survival and inducing cell death in CRC cells by several mechanisms discussed above. It is worth noting that cells that metabolize butyrate at a higher rate, are usually less susceptible to its apoptosis-inducing effects. This may explain why normal colonocytes are unaffected by high levels of this SCFA in the colon, since they preferentially use butyrate as an energy source, in contrast with CRC cells which seem to prefer glucose.

1.4.4.2 Propionate

Though propionate exerts an anti-neoplastic effect in colorectal cancer cells and has a mechanisms of action similar to butyrate, there are less published studies with this SCFA than with butyrate (Kiefer, Beyer-Sehlmeyer et al. 2006; Hosseini, Grootaert et al. 2011) (Table 4).

Jan and co-workers showed that propionate (10 - 40 mM) induced typical signs of apoptosis in human CRC cell lines, with loss of mitochondrial membrane potential ($\Delta\Psi m$), generation of ROS, cytochrome c release, caspase-3-processing and nuclear chromatin condensation (Jan, Belzacq et al. 2002). Matthews and co-workers demonstrated that at lower doses (5 mM) propionate also induced apoptosis, characterized by an elevated ROS production and decreased glucose oxidation after 48 h of treatment in the Caco-2 colon cancer cell line (Matthews, Howarth et al. 2012). Tang *et al*. showed that propionate treatment (3 -10 mM) induces ROS generation and loss of mitochondrial membrane potential ($\Delta\Psi m$) with autophagy induction to degrade damaged mitochondria in HCT116 cells, showing

in this case that propionate-triggered autophagy serves as an adaptive strategy to delay mitochondria-mediated cell death in CRC cells (Tang, Chen et al. 2011).

Table 4: Effects of SCFA propionate in colorectal cancer cells.

		Propionate	
Human colorectal cancer cell lines	**Concentration (mM)**	**Effects**	***Reference***
HT-29		Growth inhibition by the downregulation of ERK1/2	*Davido et al. 2002*
HT-29 *Caco-2*	0 - 40	Decrease of viability/apoptosis with mitochondrial alterations	*Jan et al. 2002*
HT-29	20 - 40	Modulation of histone acetylation	*Kiefer et al, 2006*
HT-29	2 - 16	Antiproliferative effect	*Comalada, 2006*
HT-29	30	Apoptosis at pH 7.5 Necrosis at pH 5.5	*Lan et al, 2007*
Colo320DM	0,3 - 30	Inhibition of the NF-$_k$B pathway	*Tedelind et al, 2007*
HCT116	0 - 10	Mitochondrial defects and autophagy	*Tang et al, 2011b*
HCT116 *SW480*	0 - 3	Two types of programmed cell death (autophagy and apoptosis)	*Tang et al, 2011a*
Caco-2	5	Induction of apoptosis, G2-M arrest, alterations in the oxidative pentose pathway.	*Matthews et al, 2012*
*RAW264.7**	0 – 1.200 µmol/L	Decrease of proinflammatory factors with an increase in the antiinflammatory cytokine	*Liu et al, 2012*

*Murine macrophage cell line

It has been demonstrated that propionate, like butyrate, also acts as an inducer of histone acetylation in CRC cells, which is possibly associated with a modulated growth response of tumorigenic lesions in the gut (Kiefer, Beyer-Sehlmeyer et al. 2006; Hosseini, Grootaert et al. 2011; Kim, Park et al. 2014). In this regard, Kiefer *et al* showed that propionate alone (2.5 – 40 mM) or in combination with butyrate or acetate induced significant histone acetylation in HT-29 cells. In addition, another

study demonstrated that propionate-induced growth inhibition, like butyrate, involves downregulation of ERK1/2 in HT-29 cells (Davido, Richter et al. 2001).

Furthermore, it has been shown that propionate has anti-inflammatory properties in the gut, comparable to those already described for butyrate (Tedelind, Westberg et al. 2007; Liu, Li et al. 2012; Kim, Park et al. 2014). Tedelind *et al* 2007, observed that the anti-inflammatory activity of propionate (0.3 - 30 mM) involves the inhibition of the NF-κB pathway and suppression of IL-6 release in Colo320DM cells.

Taken together, these findings suggest that propionate could be effective in the prevention and treatment of some colon alterations, including CRC. Nevertheless, further studies both *in vitro* and *in vivo* are required to understand its real role alone and in combination with other SCFAs, as discussed below.

1.4.4.3 Acetate

Acetate has been the least studied of the three most relevant SCFAs (table 5). Initially, Jan and co-workers showed that acetate (0-40 mM) decreased the viability and induced typical signs of apoptosis in the colon adenocarcinoma cell line HT-29, including loss of mitochondrial membrane potential, generation of ROS, caspase-3 processing and nuclear chromatin condensation (Jan, Belzacq et al. 2002).

As already referred for butyrate and propionate, the mechanism through which acetate mediates cell death and its anti-inflammatory effects are currently poorly understood. However, it has been shown that acetate also reduces the production of pro-inflammatory factors, including TNF-α, IL-1β, IL-6, while enhancing the production of the anti-inflammatory cytokine IL-10 (Tedelind, Westberg et al. 2007; Liu, Li et al. 2012; Kim, Park et al. 2014) and induces inhibition of the NF-κB pathway in CRC cells (Tedelind, Westberg et al. 2007). Although it has been demonstrated that acetate can affect the immune response in the colon, more studies are necessary in order to understand the precise role of this SCFA and its interaction with intestinal microbiota, host immune cells, colonocytes and CRC cells.

Table 5: Effects of SCFA acetate in colorectal cancer cells.

Acetate			
Human colorectal cancer cell lines	**Concentration (mM)**	**Effects**	***Reference***
HT-29 *Caco-2*	0 - 40	Decrease of viability/Apoptosis with mitochondrial alterations	*Jan et al, 2002*
HT-29	4 - 32	Ineffective in these concentrations	*Comalada, 2006*
HT-29	80	Does not induce histone acetylation	*Kiefer et al, 2006*
Colo320DM	2,4	Inhibition of the NF-$_k$B pathway	*Tedelind et al, 2007*
HT-29	15	Apoptosis at pH 7.5 Necrosis at pH 5.5	*Lan et al, 2007*
*RAW264.7**	0 – 1.200 µmol/L	Decrease of proinflammatory factors with an increase in the antiinflammatory cytokine	*Liu et al, 2012*

*Murine macrophage cell line

Studies in the yeast species *Saccharomyces cerevisiae* first demonstrated that acetic acid induced a mitochondria-mediated apoptotic process (Pereira, Silva et al. 2008; Guaragnella, Zdralevic et al. 2012) with several features similar to apoptosis mediated by SCFAs in CRC cells. Indeed, alterations in mitochondria were identified in yeast, including production of ROS, mitochondrial swelling, decrease of $\Delta\Psi m$ (Ludovico, Rodrigues et al. 2002), mitochondrial fragmentation/degradation (Fannjiang, Cheng et al. 2004), mitochondrial outer membrane permeabilization (MOMP) with consequent release of pro-apoptotic factors like cytochrome *c*, yeast apoptosis inducing factor 1 (Aif1p) and Nuc1p (yeast ortholog of EndoG) (Ludovico, Rodrigues et al. 2002; Wissing, Ludovico et al. 2004; Kroemer, Galluzzi et al. 2007). The yeast orthologs of the mammalian VDAC (voltage-dependent anion channel) and ANT (adenine nucleotide transporter) were shown to play a role in MOMP and cytochrome *c* release during acetic acid-induced apoptosis in yeast (Pereira, Camougrand et al. 2007) Later, vacuolar membrane permeabilization (VMP) and release of Pep4p, yeast cathepsin D (CatD), from the lysosome-like vacuole to the cytosol, were observed in yeast cells exhibiting apoptotic cell death induced by acetic acid (Pereira, Chaves et al. 2010). In that study, it was also shown that, once

in the cytosol, Pep4p played an important role in mitochondrial degradation through an autophagic-independent process, which protected yeast cells from acetic acid-induced apoptosis. Recently, it was demonstrated that both the protective function of Pep4p and its role in mitochondrial degradation during acetic acid-induced apoptosis in yeast depend on Pep4p proteolytic activity, and is complemented by heterologous expression of human CatD (Pereira, Azevedo et al. 2013; Oliveira, Pereira et al. 2015).

Taking into account the aforementioned results, we hypothesized that similar cellular events could occur in response to acetate in CRC cells. As mentioned before, acetate, like butyrate and propionate, may be useful as a chemopreventive agent, but more studies are necessary to understand the mechanisms underlying acetate-induced apoptosis in CRC cells.

1.4.4.4 Combined effects of short-chain fatty acids

Neither butyrate, propionate or acetate are available as the only metabolite present in the human colon, and therefore their combined effect should be taken into consideration. Although there are few studies on the combined effect of SCFAs performed in CRC cells, Tang *et al* 2011a, using a combination of butyrate and propionate (1-3 mM), showed for the first time that SCFAs orchestrate two types of programmed cell death in colon cancer cells (HCT116 and SW480). Butyrate and propionate treatment induced autophagy to dampen apoptosis, whereas inhibition of autophagy potentiated SCFA-induced apoptosis (Tang, Chen et al. 2011). Matthews and co-workers demonstrated that the combination (butyrate and propionate: 5 mM) induced cell cycle arrest in G2-M associated with a rapid and extensive apoptosis with changes in redox state and D-glucose metabolism in Caco-2 cells, compared to single treatments (Matthews, Howarth et al. 2012). In addition, Kiefer *et al*. showed that mixtures of acetate, butyrate and propionate (at molar ratios 75: 11 : 14, 69 : 16 : 15 and 43 : 24 : 33, respectively) which mimic the relative concentrations occurring in the gut, modulated histone acetylation. However, they concluded that the histone acetylation was mainly due to additive effects of butyrate and propionate, but not due to acetate (Kiefer, Beyer-Sehlmeyer et al. 2006). Lan and co-workers showed that acetate and propionate (15 mM and 30 mM,

respectively) produced by *Propionibacterium freudereichii* decreased proliferation and induced cell cycle arrest in G2/M, followed by a sequence of cellular events characteristic of apoptosis at pH 7.7, but induced necrosis at pH 5.5 in colon cancer cells (HT-29) (Lan, Lagadic-Gossmann et al. 2007). This study demonstrated for the first time the impact of the extracellular pH prevailing within the colon (from 5.5 to 7.5) on the mode of cell death triggered by propionibacteria-produced SCFA in CRC cells (Lan, Lagadic-Gossmann et al. 2007). In this regard, our group has recently characterized the potential of *P. freudenreichii* subsp. *freudenreichii* DSM 20271 as a producer of SCFAs (propionate and acetate) using several culture media to simulate the digestive stress process and the composition of digested products present in the colon (Marta *et al* unpublished results). Pure SCFAs, as well as the bacterial fermented broth, inhibited CRC cell proliferation and increased cell death. The results showed that co-culture of *P. freudenreichii* and CRC cells is possible and favourable for the bacteria and that *P. freudenreichii* could potentially be used as a probiotic in CRC prevention and/or treatment through its ability to produce SCFAs (Marta *et al* unpublished results).

Although studies on the combined effects of SCFAs are still few, it seems that they have a greater effect in colorectal cancer cells compared with treatment with each SCFA separately. In summary, there is increasing evidence of the anti-neoplastic effects of SCFAs in CRC cells, which may be modulated by several aspects, namely the cell type, SCFA concentration/availability, extracellular pH, time of exposure to SCFAs, their absorption and metabolism.

1.5 Lysosomes, lysosomal membrane permeabilization and its role in cell death regulation

The role of lysosomes as regulators of cell death in cancer cells has been a subject of increased interest in the last years. Lysosomes (greek: digestive body) are cytoplasmic membrane-enclosed organelles that contain hydrolytic enzymes (Cesen, Pegan et al. 2012). The primary function of lysosomes is to degrade macromolecules; for this purpose, lysosomes contain more than 50 acid hydrolases, including phosphatases, nucleases, glycosidases, proteases, peptidases, sulphatases and lipases (Johansson, Appelqvist et al. 2010). For this reason,

lysosomes have an acidic environment (pH 4.6–5.0), which allows a maximal enzymatic activity (Boya and Kroemer 2008; Cesen, Pegan et al. 2012). In addition, over the last years, the lysosome has emerged as a significant player of the cellular death machinery (Johansson, Appelqvist et al. 2010; Repnik, Hafner Cesen et al. 2014). The major hallmark of the lysosomal cell death pathway is characterized by the partial/selective loss of lysosomal membrane integrity, which allows the release of lysosomal hydrolases into the cytosol,a process known as lysosomal membrane permeabilization (LMP) (Groth-Pedersen and Jaattela 2013; Repnik, Hafner Cesen et al. 2014).

Depending of the initial stimuli, dose and cell type, LMP can initiate different cell death pathways (Figure 7), characterized by the release of lysosomal proteases, especially cathepsins into the cytosol (Repnik, Stoka et al. 2012; Repnik, Cesen et al. 2013). Lysosomal cathepsins translocate from the lysosomal lumen into the cytosol in response to a variety of signals such as TNF receptor ligation (the best-studied inducer of LMP) (Groth-Pedersen and Jaattela 2013), sphingosine (Cesen, Pegan et al. 2012; Repnik, Hafner Cesen et al. 2014), staurosporine (Johansson, Steen et al. 2003), reactive oxygen species (Johansson, Appelqvist et al. 2010) p53 (Johansson, Appelqvist et al. 2010), lysosomotropic agents (ciprofloxacin, norfloxacin and hydroxychlotoquine) and some cancer drugs (resveratrol and etoposide) (Trincheri, Nicotra et al. 2007; Hsu, Wu et al. 2009).

Accumulated data have shown that a partial or moderate LMP with subsequent release of cathepsins into the cytosol can activate either the intrinsic caspase-dependent apoptosis pathway or the caspase-independent alternative cell death pathway (Jaattela, Cande et al. 2004; Boya and Kroemer 2008; Groth-Pedersen and Jaattela 2013). In the case of necrosis, LMP is an early event associated with a massive release of contents to the cytosol (Boya and Kroemer 2008; Groth-Pedersen and Jaattela 2013; Repnik, Cesen et al. 2013). In the necroptosis process, LMP is a late process, which coincides with the disintegration phase through proteolysis (Groth-Pedersen and Jaattela 2013; Repnik, Cesen et al. 2013). Moreover, some studies have reported the involvement of cathepsins in autophagy to enhance cell survival under some death stimuli (Hsu, Wu et al. 2009; Hah, Noh et al. 2012; Tan, Peng et al. 2013).

LMP is still incompletely understood. The hallmark of LMP is considered to be the reversible or irreversible loss of membrane integrity with the release of luminal contents into the cytosol (Repnik, Stoka et al. 2012; Repnik, Cesen et al. 2013). The release of lysosomal components through a mediated process involving pores or translocators has not been precluded yet. It has been demonstrated that LMP can be a result of osmotic lysis or direct membranolytic activity of the compounds that accumulate in the lumen of lysosomes (Cesen, Pegan et al. 2012; Repnik, Hafner Cesen et al. 2014).

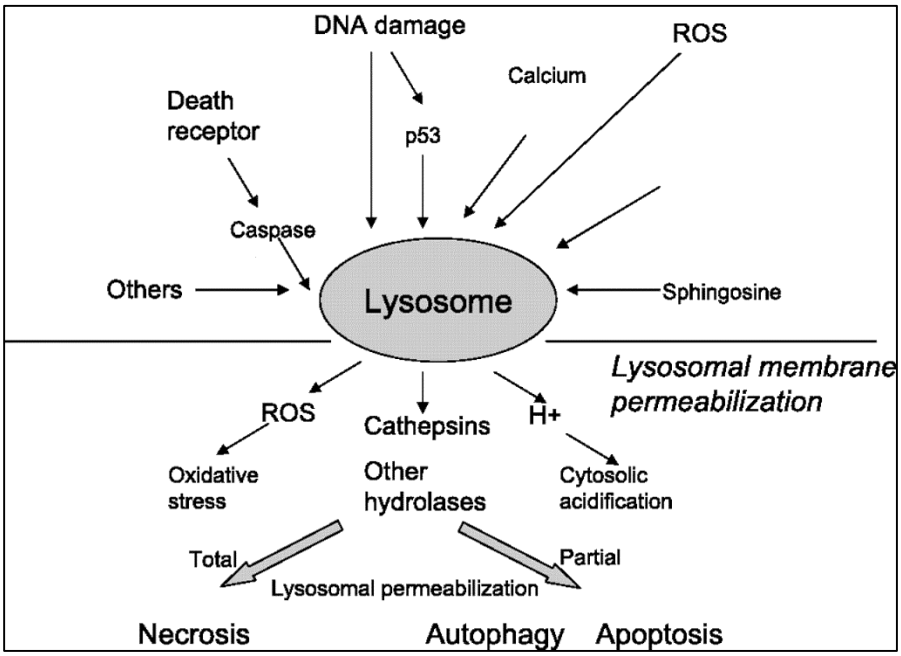

Figure 7: Regulation of lysosome membrane permeabilization (LMP) and the role of lysosomal proteins in cell death. LMP can be induced by death receptor-mediated pathway, DNA damages, intracellular calcium, ROS and other cellular stress factors. Cathepsins and other hydrolases released to the cytosol are involved in different types of cell death, depending of the LMP intensity. (Adapted from Tang et al. 2008).

Likewise, the lysosomal membrane composition, including sphingolipid and cholesterol levels, plays a key role in the maintenance of lysosomal integrity (Repnik, Hafner Cesen et al. 2014). In this case, a high content of sphingolipid and cholesterol makes the lysosomal membrane more ordered, rigid and thicker (Repnik, Stoka et al. 2012; Repnik, Hafner Cesen et al. 2014). It is known that damage in lysosomal membrane components or changes in the membrane structure and fluidity could result in lysosomal destabilization (Johansson, Appelqvist et al. 2010; Repnik, Hafner Cesen et al. 2014). An example is the LMP

induced by oxidative stress characterized by an increase of hydrogen peroxide diffusion into the lysosome. In the lysosome lumen, the acidic milieu and the presence of low-molecular-weight iron, derived from degraded iron- containing protein, promote reduction of iron and generation of hydroxyl radicals. The hydroxyl radicals induce peroxidation of membrane lipids and thereby cause leakage of lysosomal constituents into the cytosol (Johansson, Appelqvist et al. 2010).

In this regard, it has been demonstrated that cancer cell lysosomes are more prone to LMP than normal cells (Boya and Kroemer 2008; Groth-Pedersen and Jaattela 2013), but the reasons are still poorly understood. Some authors indicate that the susceptibility of cancer cells to LMP is due to the abnormal size of the lysosomes, which could make them more fragile than normal-sized lysosomes (Boya and Kroemer 2008). Another possibility is that higher metabolic rates and an increased turnover of iron-containing proteins in cancer cells lead to an accumulation of iron in the lysosomal compartment, with consequent sensitization to ROS-induced LMP (Boya and Kroemer 2008). Remarkably, induction of LMP has emerged as an effective way to kill resistant cancer cells, which could have multiple implications for the future treatment of apoptosis-defective and multidrug resistant cancer cells, including CRC cells.

1.6 Cathepsins

Cathepsins are members of a large protease family, which can be subdivided according to their structure and active-site amino acid into cysteine (cathepsins B, C, F, H, K, L, O, S, V, W, and X), serine (cathepsins A and G), and aspartic cathepsins (cathepsins D and E). While cathepsins B, L, H, C and D are ubiquitously expressed in human tissues, expression of cathepsins A, G, K, S, V, X and W is tissue and cell type specific (Wex, Levy et al. 1998; Bromme, Li et al. 1999; Turk, Turk et al. 2000; Lecaille, Kaleta et al. 2002). In general, cathepsins are found in acidic cellular organelles, lysosomes and endosomes. Initially, their function was though to be limited to bulk degradation of proteins delivered to the lysosome by endocytosis or autophagocytosis. However, it was later demonstrated that cathepsins possess highly specific and directed proteolytic activity, and that they can be found in other cellular compartments (Brix, Dunkhorst et al. 2008; Duncan,

Muratore-Schroeder et al. 2008; Pratt, Sekedat et al. 2009; Hook, Funkelstein et al. 2012; Turk, Stoka et al. 2012). Numerous physiological functions of cathepsins have been uncovered, including a role in hormone and antigen processing, bone and tissue remodeling, growth factor and proenzyme activation and, more recently, in the immune response (Brix, Dunkhorst et al. 2008; Conus and Simon 2010; Reiser, Adair et al. 2010; Turk, Stoka et al. 2012; Jacobson, Lima et al. 2013). Cathepsins also participate in apoptosis through LMP in response to a variety of apoptotic signals (Boya and Kroemer 2008; Cesen, Pegan et al. 2012; Repnik, Hafner Cesen et al. 2014). These lysosomal proteases can also be secreted from the cell and degrade extracellular matrix proteins such as collagen, fibronectin, proteoglycans and laminin (Fonovic and Turk 2014).

In addition to their physiological function, cathepsins also have been associated with several pathologies such as cardiovascular diseases, osteoporosis, rheumatoid arthritis, artherosclerosis and cancer (Benes, Vetvicka et al. 2008; Reiser, Adair et al. 2010; Turk, Stoka et al. 2012; Fonovic and Turk 2014). Elucidating the mechanisms underlying the involvement of cathepsins in the pathogenesis of these diseases, and how they can be modulated to develop new prevention and therapeutic strategies, has therefore taken center stage. Among cathepsins, cathepsin D (CatD) has attracted increased attention in recent years due to its importance in the mediation of lysosomal cell death pathways and in cancer. In the next part, it will be given special attention to the physiological and pathological functions of CatD, focusing in their role in cancer such as in apoptosis process.

1.6.1 Role of cathepsin D in cellular physiology and pathology

CatD is a soluble aspartic endopeptidase found in the lysosomes of most mammalian cells. Like other cathepsins, CatD is activated by proteolytic cleavage of the synthetized inactive zymogen (preproCatD), which is composed of an N-terminal signal peptide, a propeptide, and a catalytic domain (Gieselmann, Pohlmann et al. 1983; Erickson 1989). The signal peptide directs the nascent chain to the endoplasmic reticulum, where it is cleaved in the lumen. ProCatD is then N-glycosylated and transported to the Golgi, where the N-glycan structures acquire mannose-6phosphate (Man-6P) residues that can bind to Man-6P receptor(s) (Man-

6PR), and the complex is directed to the lysosomal compartment (Baranski, Koelsch et al. 1991). In the acidic milieu, proCatD (52 kDa) undergoes further proteolytic processing by cleavage of the proregion, resulting in the 48 kDa single chain intermediate active form. Finally, this chain is processed into mature active CatD, composed of heavy (34 kDa) and light (14 kDa) chains linked by non-covalent interactions (Erickson, Conner et al. 1981; Gieselmann, Hasilik et al. 1985; Conner and Richo 1992). It has been shown that CatD processing involves cysteine cathepsins (Gieselmann, Hasilik et al. 1985) and, more recently, that it is independent of its catalytic function and auto-activation but requires CatL and CatB (Laurent-Matha, Derocq et al. 2006). Although proCatD and CatD are mostly intracellular, they can also localize in the extracellular matrix and synovial fluid of cartilage (Poole, Hembry et al. 1974; Bjelle and Osterlin 1976; Vittorio, Crissman et al. 1986). ProCatD/CatD are also found in human, bovine and rat milk (Vetvicka, Vagner et al. 1993; Larsen and Petersen 1995; Benes, Koelsch et al. 2002), serum, sweat and urine (Zuhlsdorf, Imort et al. 1983; Baechle, Flad et al. 2006), and extracellularly in macrophage-rich regions of atherosclerotic lesions (Hakala, Oksjoki et al. 2003). ProCatD secretion by human keranocytes (Vashishta, Saraswat Ohri et al. 2007), mammalian epithelial cells (Lkhider, Castino et al. 2004) and different types of cancer cells (Benes, Vetvicka et al. 2008; Masson, Bach et al. 2010) was also demonstrated.

It is widely accepted that the major function of CatD is its involvement in general protein degradation and turnover within the lysosomal compartment. However, CatD has also emerged as an important regulator and signalling molecule with numerous physiological functions. These include activation of enzymatic precursors, prohormones and growth factors, processing of brain-specific antigens, tissue homeostasis, and participation in apoptosis (Minarowska, Minarowski et al. 2007; Benes, Vetvicka et al. 2008). CatD has also been associated with different pathological scenarios such as Alzheimer’s disease, artherosclerosis some inflammatory disorders, cancer progression and metastasis (Conus and Simon 2010; Masson, Bach et al. 2010; Reiser, Adair et al. 2010; Tan, Peng et al. 2013), being considered now as a specific biomarker for several pathologies. Although the involvement of CatD in both physiological and pathological processes in multiple studies, a detailed description of the role of CatD in cancer is given below.

1.6.2 The role of cathepsin D in cancer

Numerous reports have demonstrated that CatD is overexpressed in several cancer types (Gyrd-Hansen, Nylandsted et al. 2004; Leto, Tumminello et al. 2004; Benes, Vetvicka et al. 2008; Palermo and Joyce 2008; Masson, Bach et al. 2010; Tan, Peng et al. 2013), often correlating with poor prognosis. In particular, CatD is considered an independent prognostic marker in breast cancer associated with metastatic risk (Bossard, Descotes et al. 2003; Liaudet-Coopman, Beaujouin et al. 2006) and in colorectal cancer (CRC) (Kirana, Shi et al. 2012; Shin, Sung et al. 2014). Mechanistically, the majority of reports attribute its role in cancer to overexpression of proCatD. As an example, transfection of rat tumor cells with human proCatD cDNA leads to increased proliferation, invasion and metastasis *in vitro* and *in vivo* (Berchem, Glondu et al. 2002). Accordingly, anti-proCatD antibodies can inhibit tumor growth both *in vitro* and *in vivo* (Fusek and Vetvicka 1994; Bazzett, Watkins et al. 1999; Vetvicka, Vetvickova et al. 2000). Overexpressed proCatD escapes normal targeting routes and is hypersecreted to the extracellular milieu, where it can act in multiple fashions (Figure 8) (Masson, Bach et al. 2010). On one hand, it can exert an autocrine effect, inducing cancer cell growth by interacting with cell surface receptors (Mathieu, Rochefort et al. 1990; Fusek and Vetvicka 1994; Vetvicka, Benes et al. 2002; Vetvicka, Vetvickova et al. 2004). This autocrine role has so far been observed in breast, prostate, ovarian and lung cancer cells (Fusek and Vetvicka 1994; Bazzett, Watkins et al. 1999; Vetvicka, Vetvickova et al. 2000). In addition, proCatD can plays a crucial paracrine role in the tumor microenvironment by stimulating fibroblast outgrowth and tumor angiogenesis (Berchem, Glondu et al. 2002; Hu, Roth et al. 2008), as well as inhibiting anti-tumor responses (Wolf, Clark-Lewis et al. 2003). When in the tumor microenvironment, proCatD may also affect stromal cell behavior and/or degrade components from the extracellular matrix (Vetvicka, Vashishta et al. 2010; Khalkhali-Ellis and Hendrix 2014), including the release of growth factors (Briozzo, Badet et al. 1991). Although it has been suggested that proCatD can be processed in the acidic extracellular space to catalytically active CatD (Achour, Bridiau et al. 2013), the enzymatic activity of CatD is reportedly not required for its mitogenic role. Indeed, a proteolytically inactive mutant of CatD (D231N) is still mitogenic for fibroblasts (Laurent-Matha, Maruani-Herrmann et al. 2005), as well as for cancer cells both *in vitro*, in three-dimensional

matrices, and in athymic nude mice (Glondu, Coopman et al. 2001; Berchem, Glondu et al. 2002). Similarly, proCatD stimulates angiogenesis in tumor xenografts of athymic nude mice independently on its catalytic activity (Laurent-Matha, Maruani-Herrmann et al. 2005), also suggesting that CatD can signal through protein-protein interactions.

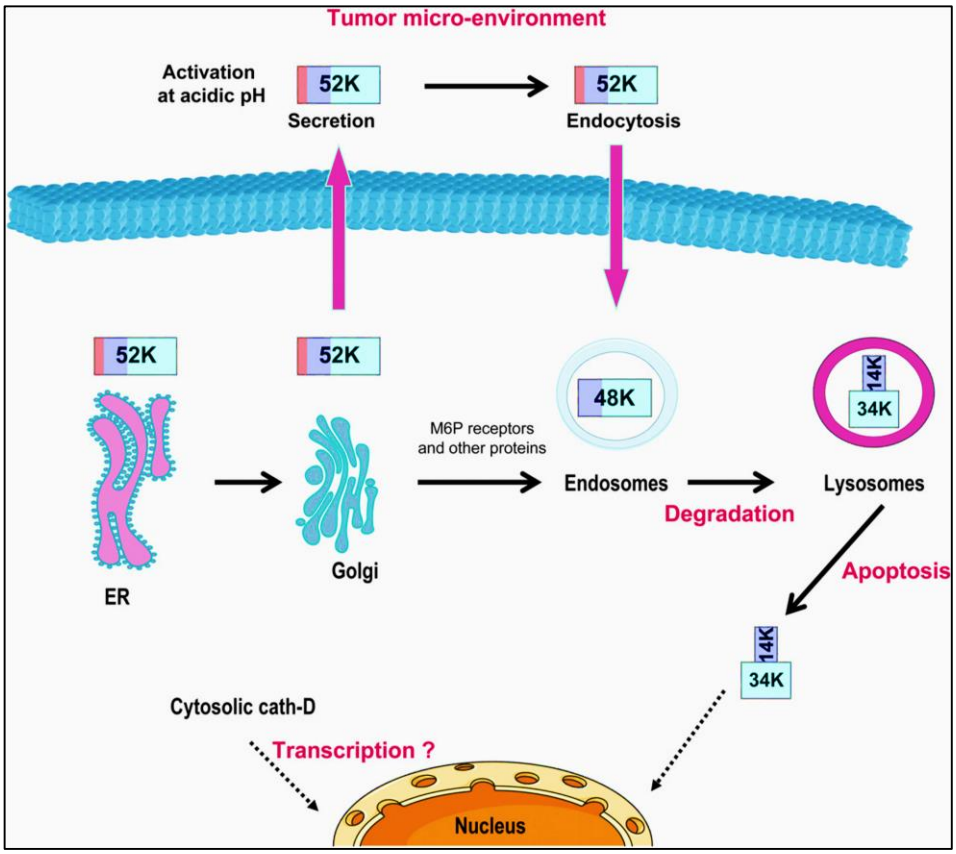

Figure 8: Localization of CatD in cancer cells. In cancer cells, overexpressed CatD accumulates intracellularly, which may affect the cell degradative capacities. The proCatD (52 kDa) is also hyper-secreted into the tumor micro-environment thus affecting it through the modulation of stromal cell behavior and/or of components of the extracellular matrix. CatD hyper-secreted by cancer cells may be captured back by both cancer and stromal cells. After lysosomal membrane permeabilization (LMP) during apoptosis, lysosomal mature CatD (34 kDa) is released into the cytosol and there may interact with and/or degrade pro-apoptotic or anti-apoptotic proteins. A cytoplasmic form of CatD may also be involved in the regulation of transcription in cancer cells by interacting with nuclear proteins and modulating their activity. Adapted from Masson et al. 2010.

Though less extensive, there are also examples of CatD roles in cancer cells that are not attributed to proCatD. For instance, intracellular CatD can stimulate cancer cell growth by inactivating secreted growth inhibitors (Liaudet, Derocq et al. 1995; Nirde, Derocq et al. 2010). Moreover, mature CatD released into the cytosol as a consequence of the reportedly higher susceptibility of cancer cells to LMP (Fehrenbacher, Gyrd-Hansen et al. 2004; Boya and Kroemer 2008) may interact

with and/or degrade pro- and anti-apoptotic proteins, modulating cell death (Minarowska, Minarowski et al. 2007).

Targeting CatD is a promising strategy in the clinic, but requires further detailed elucidation of its mechanisms of action. In the following section, we focus on the role of CatD in the apoptotic process, of particular relevance for cancer research. These studies may however also offer clues into the function of CatD in other physiological and pathological scenarios.

1.6.3 Opposing functions of cathepsin D in apoptosis

In recent years, multiple studies have shown that CatD is a central player in the apoptotic response, both under physiological and pathological conditions. In fact, depending on the cell type and context, CatD can induce or inhibit apoptosis, acting through different mechanisms (Minarowska, Minarowski et al. 2007). In this regard, CatD can directly induce apoptosis triggered by several stimuli such as staurosporine (Johansson, Steen et al. 2003), etoposide, 5-fluorouracil and cisplatin (Emert-Sedlak, Shangary et al. 2005), as well as resveratrol (Trincheri, Nicotra et al. 2007) and others, mediated by intrinsic or extrinsic pathways (Minarowska, Minarowski et al. 2007). In the intrinsic pathway, the role of CatD is linked to the release of mature CatD (34 kDa) into the cytosol and cleavage of Bid to form tBid, triggering insertion of the pro-apoptotic protein Bax into the mitochondrial membrane (Boya and Kroemer 2008). Subsequent mitochondrial outer membrane permeabilization leads to the release of pro-apoptotic molecules such as cytochrome *c* and apoptosis inducing factor (AIF) to the cytosol (Boya and Kroemer 2008). For instance, it has been shown that CatD mediates cytochrome *c* release and caspase activation in human fibroblasts undergoing staurosporine-induced apoptosis (Johansson, Steen et al. 2003), and cleaves Bid and promotes apoptosis via oxidative stress-induced LMP in human neutrophils (Blomgran, Zheng et al. 2007). In addition, Pepstatin A and/or knockdown of CatD expression by RNA interference prevent resveratrol toxicity, impeding Bax oligomerization, mitochondrial membrane permeabilization, cytochrome *c* release and caspase 3 activation in DLD1 and HT29 CRC cell lines (Trincheri, Nicotra et al. 2007). One study also reports that CatD mediates selective release of AIF in T lymphocytes

entering the apoptosis early commitment phase through activation of Bax in a Bid-independent manner (Bidere, Lorenzo et al. 2003). This shows that CatD can be involved in caspase-independent apoptosis by activating Bax independently of Bid cleavage. Other studies strongly suggest that cytosolic CatD may have an additional role involving protein-protein interactions (Masson, Bach et al. 2010). As examples, it has been shown that overexpression of either catalytically active or inactive CatD by cancer cells enhances apoptosis-dependent chemo-sensitivity (Beaujouin, Baghdiguian et al. 2006), and that stress-induced apoptosis is not affected in fibroblasts synthesizing a catalytically inactive CatD (Tardy, Tyynela et al. 2003). Additionally, microinjection of inactive proCatD into the cytosol of both human fibroblasts and HeLa cells induces apoptosis (Schestkowa, Geisel et al. 2007). Interestingly, one report also indicates that cytosolic mature CatD may reach the nucleus during cell death (Zhao, Aviles et al. 2010).

In contrast with the multiple studies showing CatD is pro-apoptotic, other studies describe an anti-apoptotic function of CatD. Most of these suggest it plays an anti-apoptotic role in cancer cells. For example, CatD downregulation sensitizes human neuroblastoma cells to doxorubicin-induced apoptosis, while CatD overexpression has the opposite effect (Sagulenko, Muth et al. 2008). Accordingly, inhibition of CatD with pepstatin A induces caspase-dependent apoptosis in neuroblastoma cell lines (Castino, Bellio et al. 2007). Moreover, overexpression of intracellular CatD in mouse xenografs using rat-derived cell lines inhibits apoptosis (Berchem, Glondu et al. 2002) and expression of wild type or a catalytic mutant of CatD promotes survival and invasive growth of CatD-deficient fibroblasts (Laurent-Matha, Maruani-Herrmann et al. 2005). Another study in glioblastoma cells proposes that CatD stimulates autophagy induction, inhibiting apoptotic cell death under genotoxic conditions (Hah, Noh et al. 2012). An anti-apoptotic role of CatD has also been described under physiological conditions using CatD-deficient mice (Saftig, Hetman et al. 1995; Koike, Nakanishi et al. 2000; Koike, Shibata et al. 2003). Indeed, mutant mice developed apoptosis in the thymus, thalamus and retina.

In summary, it is documented that CatD plays an important role in apoptosis regulation, both with and without involvement of its proteolytic activity. However, the

exact role of CatD in apoptosis in CRC cells, particularly what determines whether this protease plays an anti-or pro-apoptotic function remains poorly understood.

RESEARCH PROJECT:

Rationale and aims

Rationale

Colorectal cancer (CRC) is one of the most common causes of cancer-related mortality, and thus finding new prevention and therapeutic approaches is of prime importance. Propionibacteria, found in fiber-rich food and dairy products, produce short-chain fatty acids (SCFAs), namely butyrate, propionate and acetate. It has been demonstrated that SCFAs induce apoptosis in human CRC cells (Jan, Belzacq et al. 2002; Lan, Bruneau et al. 2008) to a higher extent than in normal intestinal cells (Lan, Lagadic-Gossmann et al. 2007). In the present thesis, we proposed to further characterize the effect of acetate in CRC cells.

In cancer cells, lysosomes have emerged as key players in apoptosis through selective lysosomal membrane permeabilization (LMP) with subsequent release of cathepsins into cytosol (Kirkegaard and Jaattela 2009; Groth-Pedersen and Jaattela 2013). However, the role of this pathway in cancer is controversial. In a previous study, it was demonstrated in *Sacharomyces cerevisiae* that acetic acid-induced apoptosis involves partial vacuole permeabilization with release of Pep4p, the yeast ortholog of lysosomal human cathepsin D (CatD), into the cytosol, which has a protective role in this process (Pereira, Chaves et al. 2010). Indeed, this protease was required for cell survival in a manner depedent on its catalytic activity and for efficient mitochondrial degradation indendently of autophagy (Pereira, Azevedo et al. 2013). These results raised the possibility that partial LMP and consequent CatD release could also be involved in acetate-induced apoptosis in CRC cells. Furthermore, understanding the mechanisms underlying acetate-induced cell death may provide the possibility to use acetate with specific cathepsin inhibitors as a novel prevention/therapeutic strategy in CRC.

In addition, it is known that monocarboxylate transporters (MCTs), namely MCT-1 to 4, are transmembrane proteins involved in intracellular pH regulation by the transmembrane transport of lactate, pyruvate and some SCFAs, especially butyrate in normal colon cells and CRC cells (Pinheiro, Longatto-Filho et al. 2008). There is also evidence that MCTs are upregulated in colorectal cancer (Pinheiro, Longatto-Filho et al. 2008; Sonveaux, Vegran et al. 2008) to maintain the glycolytic metabolism. Thus, we thought it was important to study the role of MCTs in acetate

transport into CRC cells, as well as MCT regulation and cellular localization in presence of acetate in CRC cells. Finally, since the majority of cancer cells, including CRC, have a glycolytic phenotype, it was important to assess the effects of acetate on glucose metabolism and explore the combined use of acetate with a glycolysis inhibitor like 3-bromopyruvate (3BP) as a potentiator of acetate effects in CRC cells.

Aims

The general aim of the present study was to elucidate the mechanisms by which acetate mediates cell death, focusing on LMP induction, the main cathepsins involved, and in the crosstalk between the lysosome and mitochondria such as the involvement of autophagy in this process. We also aimed to study acetate transport in CRC cells, characterizing the involvement of monocarboxylate transporters (MCTs) as well as MCT regulation in response to acetate treatment. Moreover, we aimed to verify the effects of acetate in the glycolytic metabolism and to explore the combined use of acetate with the glycolysis inhibitor 3BP in CRC cells as a new strategy to potentiate the effect of acetate in CRC cells. Furthermore, we aimed to understand the effect of acetate in the main signalling pathway activated in CRC: RAS-RAF-MEK-ERK.

The specific aims of the present thesis are as follows:

- ✓ To ascertain whether lysosomal membrane permeabilization (LMP) and CatD are involved in acetate-induced apoptosis in CRC cells.

- ✓ To assess the role of CatD in acetate-induced mitochondrial alterations and the involvelment of autophagy in acetate-induced apoptosis in CRC cells**.**

- ✓ To investigate the mechanism of acetate transport as well as the role of acetate on MCT expression/regulation and glucose metabolism. To explore the use of 3BP and acetate as a possible therapeutic approach for CRC.

- ✓ To determine the role of acetate in the regulation of the RAS-RAF-MEK-ERK signalling pathway in acetate-induced apoptosis in CRC cells.

CHAPTER 2:

Materials and methods

2. Materials and methods

2.1 Cell lines and culture conditions

We used three cell lines derived from human CRC, namely RKO^{BRAFV600E}, HCT-15^{KRASG13D} and HCT116$^{KRASG13D;\ PI3KCA}$. Cells were maintained at 37 ºC under a humidified atmosphere containing 5% CO_2. HCT-15 cells were grown in Roswell Park Memorial Institute (RPMI, Biowest) medium with L-glutamine and HEPES. RKO cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Biowest) with high-glucose and supplemented with 1 mM sodium pyruvate and 1.5 g/l sodium bicarbonate. HCT116 cells were grown in McCoy's 5A medium (Biowest). All culture media were supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. For autophagy studies, we used HBSS (Gibco) medium supplemented with 7.5% sodium bicarbonate. Cells were seeded and adhered onto appropriate sterile plates for 24 h prior to the onset of any treatments.

2.1.1 Cell treatments with acetate

Cells were seeded and adhered onto appropriate sterile plates for 24 hours before treatments in all experiments. We previously determined the half-maximal inhibitory concentration (IC_{50}), IC intermediate (IC inter) and 2×IC_{50} of acetate after 48 hours of treatment: 70 mM, 100 mM and 140 mM for HCT-15; 110 mM, 140 mM and 220 mM for RKO cell lines, respectively (Marques, Oliveira et al. 2013). In addition, in some experiences we used values for the IC_{30}, 45 mM and 75 mM, respectively for HCT-15 and RKO cells. The IC_{50}, IC inter and 2×IC_{50} values for HCT116 (100 mM, 150 mM and 200 mM, respectively) were calculated from the mean values of sulforhodamine B (SRB) reduction (Supplementary Figure S3) (Oliveira, Pereira et al. 2015). The HCT116 cells were used only in the autophagic studies.

2.1.2 Cell treatments to inhibit the activity of Cathepsins (B, L and D)

Before the treatments, cells were pre-incubated with 10 µM (2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E-64d; Sigma-Aldrich) (a cathepsin B and cathepsin L inhibitor) for 1 hour or with 100 µM Pepstatin A (PstA;

Sigma-Aldrich) (a CatD inhibitor) for 16 hours, respectively, and then co-incubated without or with or different acetate concentrations or etoposide for 12, 24 or 48 hours to perform the assays.

2.1.3 Cell treatments with acetate and 3-bromopyruvate (3BP)

Cells were treated during 48 hours, using the concentration (IC) of acetate previously determined (Marques, Oliveira et al. 2013): 45 mM, 70 mM, for HCT-15 and 75 mM, 110 mM for RKO cell lines (values for the IC_{30} and IC_{50}, respectively). The concentrations of 3-bromopyruvate (3BP) used (17,5 µM, 35 µM and 75 µM, 150 µM; IC_{25} and IC_{50}, respectively for HCT-15 and RKO cells) in the assay were calculated from the mean values obtained in the SRB assays. For the assays with acetate and 3BP, the 3BP was co-incubated 16 hours before completing 48 hours of acetate treatment.

2.2 Cell viability assay

2.2.1 MTT reduction assay

Cells were incubated with different concentrations of sodium acetate, henceforth referred to as acetate, for 48 hours. Freshly prepared 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 0.5 mg/ml in PBS pH 7.4 and plates incubated in the dark for 2 hours at 37ºC. To dissolve the formazan crystals, 500 µl of acidic isopropanol (0.04 M HCl in absolute isopropanol) was added to each well, followed by 30 min of orbital shaking in the dark, to solubilise the formazan crystals. Absorbance was read at 570 nm in a microplate reader (Spectra Max 340PC, Molecular Devices). Results were expressed as a percentage of sample absorbance in relation to the negative control (untreated cells).

2.3 Cell proliferation assays

2.3.1 Sulforhodamine B (SRB) assay

After the treatments, the cells were fixed in ice-cold methanol containing 1% acetic acid, and incubated with 0.5% (w/v) sulforhodamine B dissolved in 1% acetic acid for 1.5h at 37°C. After washing with 1% acetic acid, SRB was solubilised with 10 mM Tris, pH 10. Absorbance was read at 540 nm in a microplate reader (Spectromax 340 PC, Molecular Devices). Results were expressed relatively to the negative control (untreated cells), which was considered as 100% of cell proliferation.

2.3.2 BrdU incorporation

Cells were seeded in plates containing glass coverslips, exposed to acetate for 48 h and incubated with 10 mM BrdU for 1 h. Cells were fixed with 4% paraformaldehyde and nuclear incorporation of BrdU was detected by immunofluorescence. Coverslips were mounted on Vectashield Mounting Medium with DAPI (4',6-diamidino-2-phenylindole) and the percentage of positive nuclei (BrdU index) was determined from > 500 cells/datum point.

2.3.3 Colony formation assay (CFA)

Cells were seeded in 6-well plates at 600 cells/mL, 300 cells/mL (respectively for HCT-15 and RKO cell lines) and incubated 24 h at 37º C with 5% CO_2 before the treatments. Cells were treated as described for the SRB assay (the same conditions). After 48 hours of treatment, the medium was replaced by fresh medium twice per week during 14 days. To evaluate the colony numbers formed for each condition, the cells were washed with 1x PBS and then fixed with 6% glutaraldehyde/0.5% crystal violet solution for 30 min, at room temperature (RT). The cells were then washed with water and air-dried.

2.4 Cell death assays

2.4.1 TUNEL assay

Cells were exposed to DMSO control, etoposide or different concentrations of acetate for 48 hours. When cathepsin inhibitors were used, cells were pre-incubated with pepstatinA (Pst A) or (2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d) for 16 hours and 1 hour, respectively, and then co-incubated with acetate or etoposide for 48 hours. Cytospins of both floating and attached cells, from each sample, were fixed with 4% paraformaldehyde for 15 min at room temperature (RT). Cells were washed in PBS and permeabilized with ice-cold 0.1% Triton X-100 in 0.1% sodium citrate. TdT-mediated dUTP Nick End Labelling (TUNEL) was performed following the manufacturer's instructions ("*In situ* cell death detection kit, fluorescein", Roche, Mannheim, Germany). Slides were mounted on Vectashield Mounting Medium with DAPI and maintained at -20ºC until visualization in a fluorescence microscope (Leica DM 5000B, Leica Microsystems, Wetzlar, Germany).

2.4.2 Caspase 3 activity assay

Cells were exposed to etoposide or acetate for 48h. Both floating cells and attached cells were collected from each sample, washed twice with PBS, and lysed in Lysis Buffer (10 mM Tris, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% Triton X-100) through 3 freeze/thaw cycles. In all, 50 µl of total extracts (1 mg/ml) were incubated with 50 µl 200 µM z-DEVD-AFC (Biomol, Plymouth Meeting, PA, USA) in 2× reaction buffer (20 mM PIPES, pH 7.4, 4 mM EDTA, 10 mM DTT) and fluorescence of cleaved AFC was measured using a microplate reader (Flouroskan Ascent FL, Thermo Scientific Inc., Waltham, MA, USA).

2.4.3 Annexin V/ Propidium iodide by flow cytometry

After the treatments, both floating and attached cells were collected from each sample and washed in PBS. 1×10^6 cells were resuspended in 200 µl 1× binding buffer and incubated with 8 µl Annexin V-FITC (BD Biosciences, San Jose, CA,

USA) and 15 µl 50 µg/ml propidium iodide (PI) for 15 min in the dark. Samples were analysed in a flow cytometer (Epics XL, Beckman Coulter, Miami, FL, USA) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Monoparametric detection of red fluorescence was performed using FL-3 (488/620 nm) and detection of green fluorescence was performed using FL-1 (488/525 nm). Cell viability or cell death were categorized by the level of Annexin-V FITC and/or PI fluorescence: viable cells (AV-/PI-), early apoptotic cells (AV+/PI-), late apoptotic cells (AV+/PI+) or necrotic cells (AV-/PI+). In all, 20 000 cells were analysed *per* sample and data analysed using FlowJo software (version 7.6) or Flowing software (version 2.5.1).

2.4.4 Sub-G1 cell population analysis by flow cytometry

Cells were treated with DMSO, etoposide or acetate for 48 hours. When PstA was used, cells were pre-incubated as described above. Both floating and attached cells were collected from each sample, washed with PBS, and incubated with 70% cold ethanol for 15 minutes. Cells were then washed twice with PBS, incubated with RNase A (200 µg/ml) for 15 minutes in the dark at 37ºC and with propidium iodide (0.5 mg/ml) for 30 minutes at RT before analysis on a flow cytometer, as described above. FlowJo software was used to generate DNA content frequency histograms and quantify the amount of cells in the individual cell cycle phases, including the sub-G1 population assumed as corresponding to apoptotic cells.

2.5 Lysosome membrane permeabilization (LMP) assessment

2.5.1 Acridine Orange staining analysis by fluorescence microscopy

Cells were seeded in 6-well plates containing glass coverslips, and exposed to etoposide or acetate for 48 hours. Cells were then incubated with 1 µM Acridine Orange (AO) for 15 minutes at 37ºC and washed with PBS. Coverslips were mounted over glass slides with PBS and immediately observed and photographed using a fluorescence microscope (Leica DM 5000B).

2.5.2 Acridine Orange staining analysis by flow cytometry

Cells were exposed to etoposide or acetate for 48 hours. Both floating and attached cells were collected from each sample, washed with PBS, and ressuspended in PBS to a final concentration of 1×10^6 cells in 1.5 ml. Cells were then incubated with 1 µM AO (or without AO to measure autofluorescence) for 15 minutes at 37ºC.

2.6 Preparation of extracts to immunoblot detections

2.6.1 Total protein extracts

After the treatments, both floating and attached cells were collected, washed with PBS and centrifuged at 650 x g for 5 min at 4 ºC. The supernatant was discarded and cells lysed in 80 ml Ripa Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40) supplemented with 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF and 40 µl/ml Protease inhibitor cocktail. Supernatants were cleared by centrifugation at 4500 x g for 10 min. Total protein extracts were stored at - 80 ºC.

2.6.2 Cytosolic protein extracts

Cells were exposed to etoposide or acetate for 48 h. Both floating and attached cells were collected, washed with PBS and centrifuged at 650 x g for 10 min at 4 ºC. All subsequent steps were performed at 4 ºC. Cells were resuspended in 1 ml PBS, centrifuged at 1500 x g for 5 min and washed twice in 800 ml Isotonic Buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES) by centrifugation at 3000 x g for 5 min. The cell pellet was then resuspended in 300 ml Isotonic Buffer with 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF and 40 ml/ml Protease inhibitor cocktail and homogenized by passing through a 26-G needle 70 times. Nuclei and unbroken cells were removed by centrifugation at 1000 x g for 5 min, followed by two consecutive centrifugations, first at 10 000 x g for 15 min to remove lysosomes and mitochondria and then at 100 000 x g for 1 h in an ultracentrifuge. Cytosolic protein extracts were stored at - 80 ºC.

2.7 Western blot analysis

Protein samples (25 µg) were separated by sodium dodecyl sulfate 12,5% polyacrylamide gel electrophoresis and electroblotted onto PVDF (polyvinylidene difluoride) membranes. All the primary antibodies were incubated overnight at 4º C. The primary antibodies used are represented in Table 1.

Secondary antibodies used were peroxidase conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) and horseradish peroxidase-labeled goat anti-mouse immunoglobulin IgG (Jackson ImmunoResearch). Subsequent chemiluminescence detection was performed using the ECL detection system (Amersham, Biosciences, Buckinghamshire, UK) and a molecular imager by Chemi-Doc XRS system (Bio-Rad, Laboratories Inc., Hercules, CA, USA). When performed, densitometry analysis of protein bands was performed using the Quantity One software (version 4.6.9) and levels of actin used as a normalization control for protein loading.

Table 1 – List of primary antibodies used in the present study.

Protein	***Primary antibody (Company, dilution)***
Cathepsin D	Calbiochem, 1:100
Cathepsin B	Abcam, 1:300
Cathepsin L	Abcam, 1:1000
Beclin-1	Cell Signalling, 1:3000
Atg-5	Sigma-Aldrich, 1:5000
LC3I/II	Sigma-Aldrich, 1:3000
AIF	Chemicon International, 1:500
VDAC-1	MitoSciences, 1:2000
TOM22	Santa Cruz Biotechnology, 1:1000
MCT-1	Santa Cruz Biotechnology, 1:500
MCT-2	Santa Cruz Biotechnology, 1:200
MCT-4	Santa Cruz Biotechnology,1:500
SMCT-1	Santa Cruz Biotechnology, 1:250
CD147	Santa Cruz Biotechnology, 1:500

2.8 RNA interference-mediated inhibition of cathepsin D

RKO cells were plated in 6 well plates at a density of 3×10^4 cells per well. After 24 hours, about 70% confluence was confirmed before transfection with small interfering RNA (siRNA) oligonucleotides. Cells were transfected with 100 nM on-target plus SMART pool siRNA against CatD (A-003649-16; Thermo Fisher Scientific, Lafayette, CO, USA). Transfection performance was monitored using a validated Silencer Select Negative Control (scrambled siRNA control, nº 4390843; Life Technologies, CARLSBAD, CA, USA). Transfection was performed with 6 µl Lipofectamine 2000 (Invitrogen Corp., Paisley, UK). After 14 hours, the transfection mixture was removed and cells were left untreated (blank) or treated with acetate (110 mM) or etoposide (50 µM) and incubated for a further 48 hours in fresh medium. MitoSOx, Dihydroethidium (DHE) and double staining with MitoTracker Green and Mitotracker Red CMXRos were performed as described below. The experiments were carried out in three replicates, and CatD levels were monitored by Western blotting.

2.9 Determination of Intracellular Reactive Oxygen Species (ROS)

2.9.1 Dichlorofluorescein (DCF) assay

Cells were seeded in a 12-well plate at a density of 1.4×10^5 cells/well and 1.8×10^5 cells/well (RKO and HCT-15 cells respectively). When indicated, cells were pre-incubated with PstA for 16 hours and then co-incubated with PstA without and with different acetate concentrations for 12, 24 and 48 hours. After treatment, cells were washed twice with 1x PBS and 2*'*,7*'*-dihydrodichlorofluorescein diacetate (H_2DCF-DA; Sigma-Aldrich) was added to the culture plates at a final concentration of 100 µM. Plates were incubated at 37 ºC for 30 min in the dark. Then, cells were lysed with 500 µL of 90% DMSO/10 % PBS (lysis solution) for 10 min at room temperature in the dark, with agitation. H_2O_2 (500 µM or 1 mM for HCT-15 or RKO cell lines, respectively) was used as positive control. H_2DCF-DA fluorescence intensity was detected with emission wavelength at 538 nm and excitation wavelength at 485 nm using a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc., Waltham, MA, USA). Values are expressed as the mean fluorescence

intensity normalized to the cell number in each condition at the end of the experiment. Three independent experiments were performed.

2.9.2 Dihydroethidium (DHE) assay

Non-treated cells (negative control) or cells exposed to different acetate concentrations for 12, 24 and 48 hours were analyzed. When used, cells were pre-incubated with PstA for 16 hours and then co-incubated with PstA without and with different acetate concentrations for 24 hours. Inhibition of cathepsin D in RKO cells was performed as described above. Approximately 1×10^6 floating and attached cells were collected, washed with 1x PBS, centrifuged at 1500 x g for 5 minutes and incubated with 150 nM dihydroethidium (DHE) (Molecular Probes, Eugene, USA) (30 minutes, 37ºC, in the dark) to detect superoxide anion (O_2^-). Fluorescence emission of oxidized DHE was analyzed by flow cytometry. H_2O_2 (500 µM or 1 mM for HCT-15 or RKO cell lines, respectively) was used as positive control. Values are expressed as the mean of fluorescence intensity normalized to T0 (control for ROS level before the treatment). Three independent experiments were carried out and data were analyzed using Flowing software (version 2.5.1).

2.9.3 MitoSOX assay

MitoSOX Red (Molecular Probes) was used to detect mitochondrial superoxide. Approximately 1×10^6 floating and attached cells were collected, washed with 1x PBS, centrifuged at 1500 x g for 5 minutes and incubated with 2.5 µM MitoSOX Red for 30 min at room temperature. Fluorescence emission of oxidized MitoSOX Red was analyzed by flow cytometry. H_2O_2 (1 mM) was used as a positive control. Values are expressed as the percentage of cells with positive staining normalized to T0 (control for ROS level before the treatment).

2.10 Analysis of mitochondrial alterations

2.10.1 Mitochondrial mass and mitochondrial membrane potential (ΔΨm) analysis

MitoTracker Green FM (Molecular Probes) was used to analyze the relative mitochondrial mass. MitoTracker Green is a green-fluorescent dye that localizes to the mitochondrial matrix regardless of the ΔΨ*m* and covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues. MitoTracker Red CMXRos (Molecular Probes) was used simultaneously, to monitor the changes in ΔΨ*m*. MitoTracker Red CMXRos is a red-fluorescent dye that stains mitochondria in live cells through accumulation in mitochondria in a membrane potential-dependent manner. Untreated cells (negative control), exposed to etoposide (50 µM), an inducer of LMP, or different acetate concentrations were analyzed at different time points. When used, cells were pre-incubated and co-incubated with E-64d and PstA without and with different acetate concentrations as described before. Both floating cells and attached cells were collected, washed with 1x PBS, centrifuged at 1500 x g for 5 minutes and incubated with 400 nM MitoTracker Green FM and 200 nM MitoTracker Red CMXRos (30 minutes, 37ºC, in the dark). The uncoupling agent CCCP (50 µM, 30 min) was used as positive control for mitochondrial membrane depolarization. Fluorescence emission was analyzed by flow cytometry. The values of mitochondrial mass for each time point were expressed as the ratio between the mean green fluorescence intensity and the one correspondent to time zero (T0). The values of the ΔΨ*m* for each time point were expressed as the ratio between the mean red fluorescence intensity and the mean green fluorescence intensity normalized to the correspondent one at T0. Three independent experiments were carried out.

2.10.2 Fluorescence microscopy studies

Cells were seeded in 6-well plates containing glass coverslips and exposed to acetate for 48 hours. At the end of the experiment, cells were washed with 1x PBS and incubated with 20 nM $DiOC_6(3)$ (Molecular Probes, Eugene, OR) or with 400 nM MitoTracker Green and 200 nM MitoTracker Red CMXRos for 30 minutes, at 37ºC, in the dark. CCCP (50 µM, 30 min) was also used as a positive control for

mitochondrial membrane potential loss. Coverslips were mounted in 1x PBS and immediately observed under the fluorescence microscope (Leica DM 5000B, Leica Microsystems, Wetzlar, Germany). Three coverslips were prepared for each experimental condition and representative images are shown.

2.11 Flow cytometry analysis

Samples were analyzed in a flow cytometer (Epics XL, Beckman Coulter, Miami, FL, USA) equipped with an argon-ion laser emitting a 488-nm beam at 15mW. Detection of red fluorescence was performed using FL-4 (488/620 nm) and detection of green fluorescence was performed using FL-1 (488/525 nm). 30 000 or 20. 000 cells were acquired *per* sample and data were analyzed using FlowJo software (version 7.6) or Flowing software (version 2.5.1).

2.12 Assessing autophagy in CRC cell lines

Cell lines untreated (Blank) or treated with IC_{50}, IC intermediate (IC inter) or $2\times IC_{50}$ acetate concentrations were incubated for 42 hours in complete medium. Afterwards, cells were incubated in HBSS medium for 6 hours in the presence or absence of 20 nM Bafilomycin A1 (BafA1, Acros Organics, Geel, Belgium) and in the presence or absence of acetate. After the treatments, were performed total protein extracts and Western blotting to detect some proteins involved with autophagy, such as Beclin-1, LC3I/II and Atg-5.

2.13 Measurement of acetate uptake

The protocol for [^{14}C] acetate uptake was adapted from Kobayashi et al. (Kobayashi, Itagaki et al. 2004) as described in Azevedo-Silva et al. (Azevedo-Silva, Queiros et al. 2015). Briefly, cells were seeded in 24-well plates at $2–3\times10^5$ cells/well, incubated overnight at 37◦C with 5% CO_2, washed twice and incubated with HEPES buffer (pH 7.4) for 10min at 37ºC. HEPES buffer was removed and cells were incubated in MES buffer (pH 6.0) with the radiolabelled [^{14}C] acetate (4000 d.p.m./nmol) for the appropriate period of time at 37◦C. Afterwards, the [^{14}C] acetate solution was removed, the plate was incubated on ice and the cultures washed with

ice-cold HEPES buffer, pH 7.4. Cells were then solubilized with 1% SDS/0.2 M NaOH and mixed with scintillation liquid for measuring radioactivity in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer with d.p.m. correction. For normalization, protein of all samples was quantified using a BCA Protein Assay Kit (Pierce). As [^{14}C] acetate uptake was linear up to 5 min, 3 min of incubation of the cells with the radiolabelled substrate was adopted in the present study. The effect of distinct inhibitors AR-C155858, CHC, DIDS, CCCP, monensin, valinomycin and $HgCl_2$ was evaluated in cells incubated for 3 min with each compound in MES buffer, pH 6.0, prior to incubation with 1.0 mM [^{14}C] acetate for 3 min.

2.14 Immunofluorescence microscopy analysis of MCT-1, MCT-4 and CD147

Cells were seeded in 12-well plates containing glass coverslips and exposed to acetate for 48 hours (IC_{30} and IC_{50} acetate doses for both cell lines). As negative control, cells with fresh medium were used. At the end of the experiment, cells were washed with 1x PBS and fixed with 4% paraformaldehyde for 15 min at RT. Then, coverslips were washed in 1x PBS and permeabilised with 0,1% Triton X-100 in 1x PBS for 2 min. After this, coverslips were washed in 1x PBS and blocked with 5% BSA for 30 min. Coverslips were incubated with anti-MCT-1 (1:250, Santa Cruz Biotechnology) or anti-MCT-4 (1:500, Santa Cruz Biotechnology) or anti-CD147 (1:250, Santa Cruz Biotechnology) diluted in 5% BSA, overnight. At the end, coverslips were washed twice with 1x PBS and incubated with fluorescence anti-rabbit (for MCT-4, 1:500, Alexa Fluor® 488, Invitrogen A-11008) and fluorescence anti-mouse (MCT-1 and CD147, 1:250, Alexa Fluor® 594, Invitrogen A-11032) for 1 h. So, the coverslips were washed twice with 1x PBS and finally mounted on Vectashield Mounting Medium with DAPI and immediately observed under the fluorescence microscope (Olympus BX61 fluorescence microscope, Tokyo, Japan). Three coverslips were prepared for each experimental condition and representative images are shown.

2.15 Glucose consumption and lactate production assays

Cells cultured in 48-well plates were pre-incubated in glucose-free media for 2 h. Then, cells were washed with 1x PBS and incubated with the doses of acetate - IC_{30}

and IC_{50}, as mentioned before). Non-treated cells (negative control) or exposed to acetate concentrations were analyzed. After incubation, conditioned medium was collected at 3, 6, 12, 24 and 48 hours. Glucose consumption and extracellular lactate were measured by the enzymatic colorimetric kits: Glucose Assay Kit (Roche, Mannheim, Germany) and Lactate Assay kit (SpinReact, Sant Esteve de Bas, Girona, Spain), following the manufacturer's instructions. Glucose and lactate fluorescence intensity were detected with absorbance emission at 490 nm using a fluorescence microplate reader. Values are expressed as the mean of fluorescence intensity normalized to T0 (control for glucose and lactate levels before the treatment with acetate) and the cell biomass for each condition (by Sulforhodamine (SRB) assay). The SRB protocol was performed as previously described (Marques, Oliveira et al. 2013).

2.16 Statistical analysis

Statistical significance analysis was determined by two-way ANOVA or by one-way ANOVA followed by Dunnett or Bonferroni's test for multiple comparisons using Prism software version 6 (GraphPad La Jolla, CA, USA). Kinetic parameters were determined using GraphPad for the non-linear regression of the plots of the initial uptake rates of acetate as a function of the acid concentration. All results are presented as mean ± standard deviation (SD) of three independent experiments. Differences were considered significant for *P* values lower than 0.05.

CHAPTER 3:

Results

This chapter includes part of the results presented in the following publications:

Marques C*, **Oliveira CSF***, Alves S, Chaves SR, Coutinho OP, Côrte-Real M, Preto A (2013). Acetate-induced apoptosis in colorectal carcinoma cells involves lysosomal membrane permeabilization and cathepsin D release. *Cell death & disease*, 4: e507. *These authors contributed equally to this article.

Oliveira CSF, Pereira H, Alves S, Castro L, Baltazar F, Chaves SR, Preto A, Côrte-Real M (2015). Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy-independent degradation of damaged mitochondria. *Cell death & disease*, 6: e1788.

Oliveira CSF, Azevedo-Silva J, Casal M, Côrte-Real M, Baltazar F, Preto A. The role of acetate on monocarboxylate transporter (MCT) expression and glucose metabolism in colorectal cancer cells: therapeutic implications. Submitted to *Cancer Letters.*

In all these publications I actively participated in the design, laboratory work, such as in the writing of the manuscript and in the process of journal submission.

3.1: Acetate-induced apoptosis in colorectal carcinoma cells involves lysosomal membrane permeabilization and cathepsin D release

One of the goals of this thesis project was to further characterize the previously described apoptotic death pathway in colorectal cancer (CRC) cells induced by acetate (Jan, Belzacq et al. 2002; Lan, Lagadic-Gossmann et al. 2007). Furthermore, as we previously reported that acetic acid-induced apoptosis in *Saccharomyces cerevisiae* cells involves partial vacuole permeabilization and release of Pep4p, the yeast cathepsin D (CatD), showing a protective role in this process, we also aimed to investigate whether lysosomal membrane permeabilization (LMP) and CatD could be involved in acetate-induced apoptosis in CRC cells.

Our results showed that acetate per se inhibits proliferation and induces apoptosis in HCT-15 and RKO (colon cancer cell lines selected in the present sudy). More importantly, we uncovered that acetate triggers LMP and CatD release to the cytosol. Pepstatin A (a CatD inhibitor) but not E64d (a cathepsin B and L inhibitor) increased acetate-induced apoptosis of CRC cells, suggesting that CatD has a protective role in this process. Our data indicate that acetate induces LMP and subsequent release of CatD in CRC cells undergoing apoptosis, and suggest exploiting novel strategies using acetate as a prevention/therapeutic agent in CRC, through simultaneous treatment with CatD inhibitors.

3.1.1 Acetate induces apoptosis and inhibits cell proliferation in CRC cell lines

CRC-derived cell lines HCT-15 and RKO were treated with different concentrations of acetate for 24 and 48 h and cell viability assessed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction test. After 24 h, there was no statistically significant decrease in viability of acetate-treated cells in either cell line, in comparison with untreated cells (not shown). The half-maximal inhibitory concentration (IC50) of acetate was therefore calculated from the mean values of MTT reduction after 48 h of treatment: 70mM and 110mM for HCT-15 and RKO cells, respectively (Figure 1a). IC50, 2xIC50 and an intermediate concentration of acetate were used in subsequent studies.

The decrease in cell viability determined by MTT assay might be due to decreased cell proliferation, increased cell death or both. We therefore assessed cell proliferation after exposure to acetate using sulforhodamine B (SRB) and bromodeoxyuridine (BrdU) assays and apoptosis using Annexin V/propidium iodide (AV/PI), caspase activity and terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) assays, as well as sub-G1 population analysis by flow cytometry. The IC50 of acetate reduced cell proliferation by approximately 30% and 65% in HCT-15 and RKO cells, respectively, as determined by SRB assay (Figure 1b). In HCT-15 cells, IC50 and 2xIC50 of acetate reduced proliferation by approximately 17% and 75%, respectively, as determined by BrdU assay (Figures 1c and d).

We next analysed cell death through AV/PI staining and found that acetate induced exposure of phosphatidylserine to the outer leaflet of the plasma membrane in HCT-15 cells in a dose-dependent manner (Figure 2a). The number of cells stained with AV (AV+PI- plus AV+PI+) increased from 3.9% in the untreated control to 8.0, 18.2 and 47.9% after exposure to 70, 100 and 140 mM acetate, respectively, and to 49.1% when cells were exposed to etoposide for 48 h (Figure 2b). Levels of necrotic cells (AV- PI+) after exposure to 70 or 100mM acetate were very low. .A higher dose of acetate (140 mM) increased the number of necrotic cells (4% AV-/PI+), similarly to etoposide, although the majority of the population (~48%) was in early and late apoptosis (AV+PI- plus AV+PI+) (Figure 2b). These results led us to conclude that acetate induces apoptosis rather than necrosis.

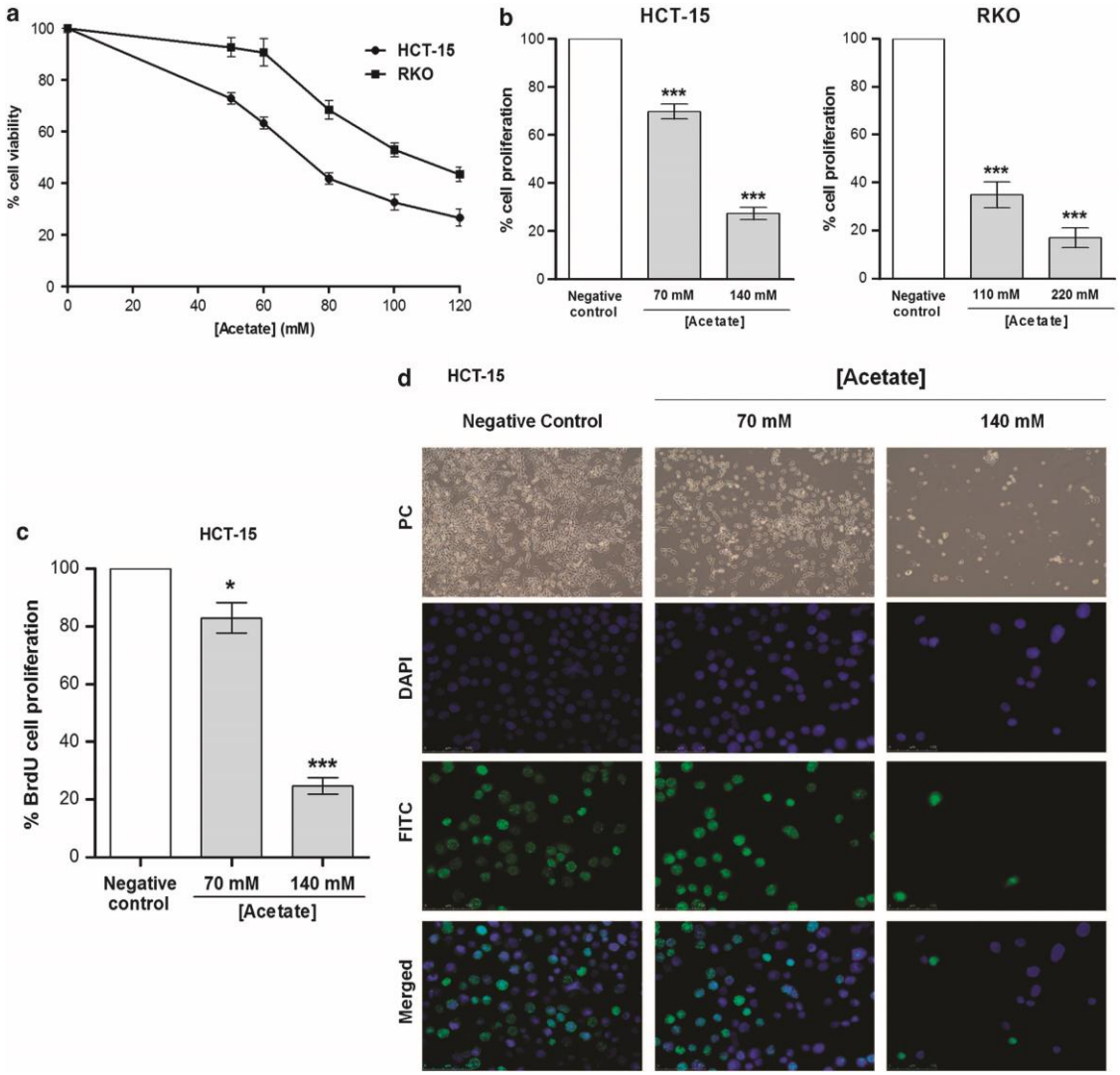

Figure 1: Determination of acetate IC50 values and proliferation analysis in CRC cell lines treated with acetate. (**a**) HCT-15 and RKO cells were incubated with different concentrations of acetate for 48 h or with fresh complete medium as a negative control, and IC50 values determined by MTT reduction assay. (**b**) Cell proliferation analysis by SRB assay in CRC cells treated with acetate. Cells were incubated with IC50 and 2xIC50 concentrations of acetate (respectively, 70 mM and 140 mM for HCT-15 and 110 mM and 220 mM for RKO) for 48 h. Values represent mean ± S.E.M. of at least three independent experiments. ***P ≤ 0.001, compared with negative control cells. (**c**) Cell proliferation analysis by BrdU incorporation assay in HCT-15 cells treated with acetate (70 mM and 140 mM) for 48 h; values represent mean ± S.E.M. of at least three independent experiments *P ≤ 0.05; ***P ≤ 0.001, compared with negative control cells. (**d**) Representative photographs of BrdU incorporation assay in HCT-15 cells treated with acetate (70 mM and 140 mM) for 48 h. phase contrast (PC; x 100); DAPI (4',6-diamidino-2-phenylindole), FITC (fluorescein isothiocyanate) and merged (x 200) were obtained by fluorescence microscopy. (Some of these results were obtained in collaboration with C. Marques).

Accordingly, caspase 3 was also activated in HCT-15 cells, as cleavage of the fluorogenic caspase 3 substrate z-DEVD-AMC, expressed in arbitrary fluorescence units/min/mg protein, increased from 3.6 in HCT-15 control cells (untreated) to 24.4 after exposure to 140 mM acetate (Figure 2c). Similar results were obtained when

activation of total caspases was assessed by labelling with the fluorescent pan-caspase inhibitor FICT-VAD-fmk (not shown).

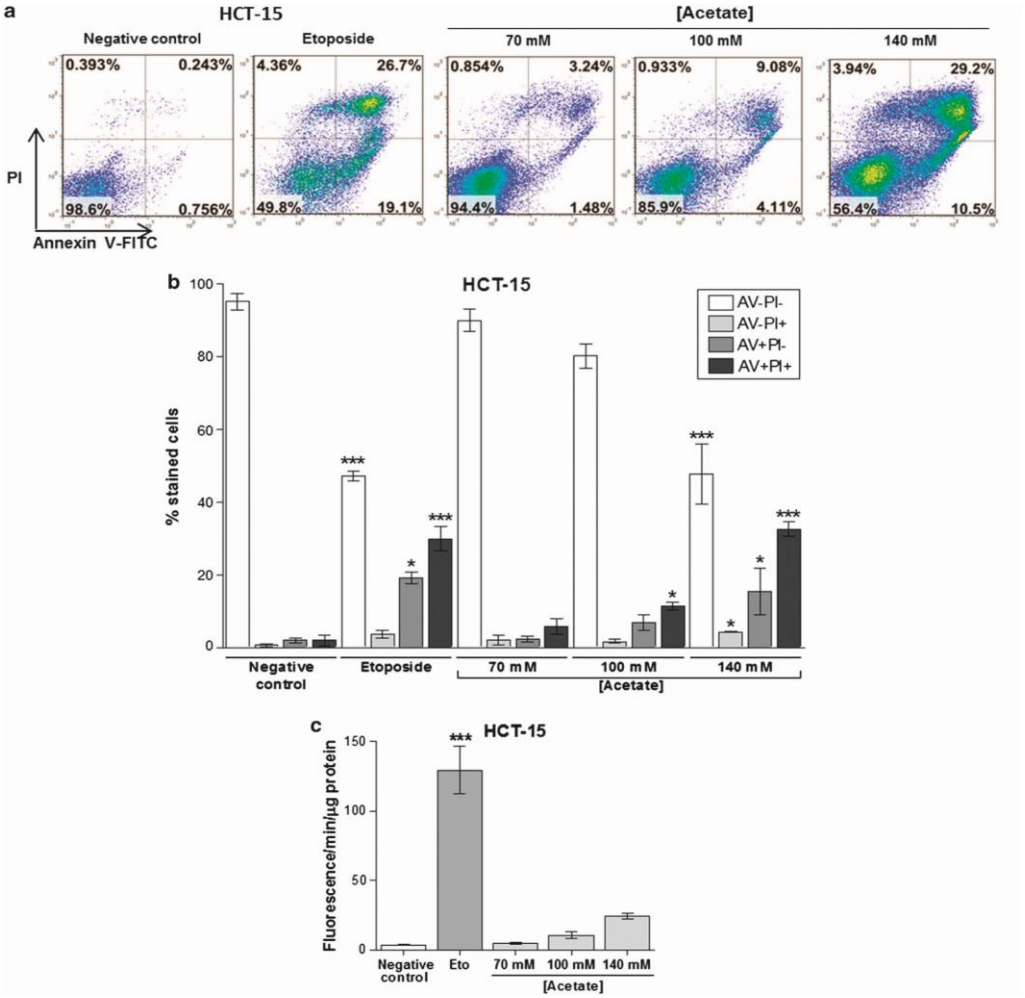

Figure 2: Acetate induces apoptosis and not necrosis in CRC cells. Apoptosis determined by Annexin V fluorescein isothiocyanate (AV-FITC) and propidium iodide (PI) assay in HCT-15 cells after incubation with 70 mM, 100 mM and 140 mM of acetate for 48 h. Cells were incubated with fresh complete medium or etoposide (50 µM) as a negative and positive control, respectively. (**a**) Representative histograms of HCT-15 cells double-labeled with AV and PI. Percentages of apoptotic cells (positive for AV) are the sum of the lower and upper right panels. (**b**) Quantitative analysis of AV/PI staining in HCT-15 cells. Values represent mean ± S.E.M. of at least three independent experiments. *P ≤ 0.05; ***P ≤ 0.001, comparing each subset of cells (AV- PI -, AV - PI +, AV + PI -, AV + PI +) to the respective negative control cells.(**c**) Quantitative analysis of caspase 3 activity in HCT-15 cells after incubation with 70 mM, 100 mM and 140 mM of acetate for 48 h. Values represent mean ± S.E.M. of three independent experiments. *P ≤ 0 .05; **P ≤ 0.01; ***P ≤ 0.001, compared with negative control cells.

We also assessed the levels of DNA strand breaks in both the cell lines by TUNEL assay. In comparison to the negative control, there were no significant differences in the number of HCT-15 apoptotic cells after 48 h of treatment with 70 mM acetate

(IC50) (0.5% versus 1.6%; Figure 3a), though phenotypic alterations typical of apoptosis (such as apoptotic bodies) were observed (Figure 3b).

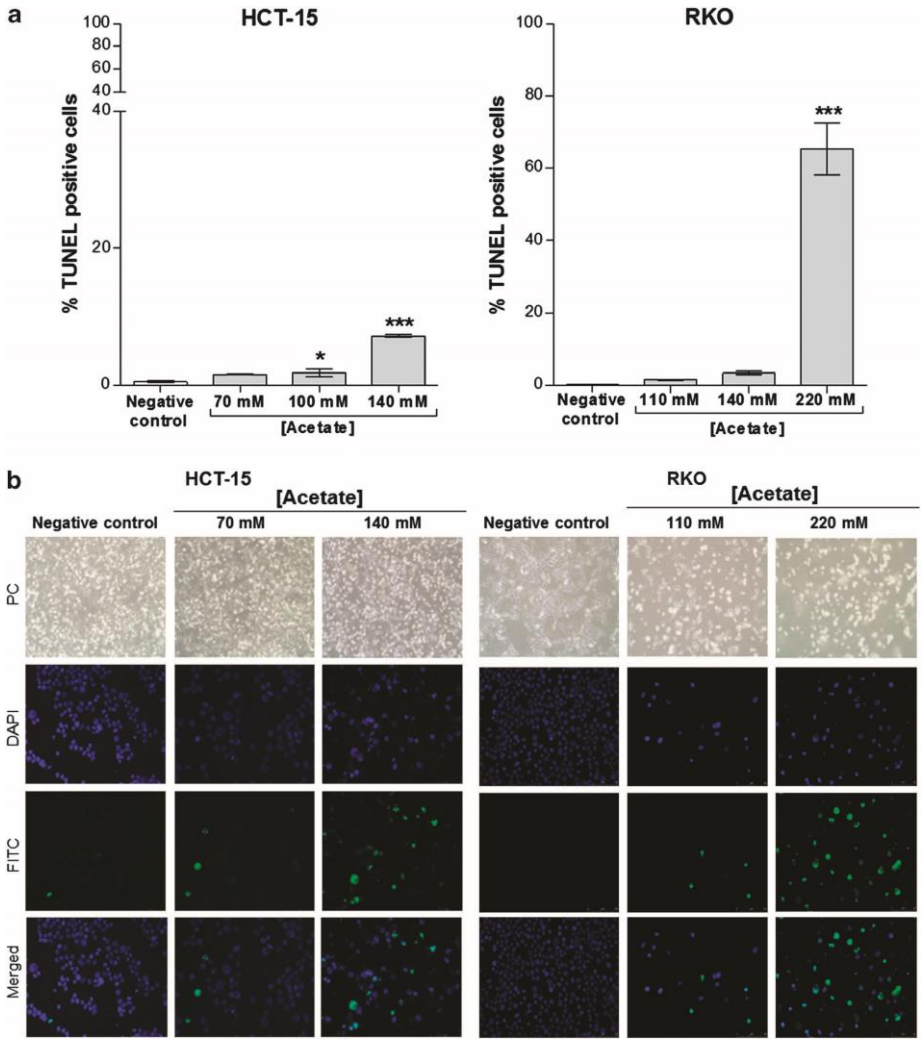

Figure 3: Acetate increases levels of TUNEL-positive cells. Apoptosis analysis in HCT-15 and RKO cells by TUNEL assay, after incubation with IC50 and 2xIC50 acetate concentrations (70 mM and 140 mM for HCT-15; 110 mM and 220 mM for RKO) for 48 h. (**a**) Analysis of TUNEL assay in HCT15 and RKO cells. Values represent mean ± S.E.M. of at least three independent experiments. *P ≤ 0.05; ***P ≤ 0.001 compared with negative control cells. (**b**) Representative images (x 200) of DAPI (4',6- diamidino-2-phenylindole), FITC (fluorescein isothiocyanate) and merged were obtained by fluorescence microscopy. PC, phase contrast. (These results were obtained in collaboration with C. Marques).

However, the number of apoptotic cells increased significantly (7.2%) after 48 h of treatment with 140 mM acetate (2xIC50) (Figure 3a). Exposure to 110 mM acetate (IC50) induced a minor increase in the number of apoptotic RKO cells, compared with low basal apoptotic levels (1.6% versus 0.3%; Figure 3a), but again with evident phenotypic alterations (Figure 3b). When treated with 220 mM acetate (2xIC50), the number of apoptotic RKO cells increased significantly (65.5%; Figure 3a). Acetate

also led to a dose-dependent increase in the sub-G1 peak of HCT-15 cells, indicative of an apoptotic sub-population, and similar to that of cells treated with etoposide (Figure 5b). Two peaks corresponding to the G1 and G2/M phases of the cell cycle were evident in DNA content histograms of HCT-15 control (untreated) cells, with very few cells in the hypodiploid sub-G1 cell-cycle phase (2% of the total events acquired). Exposure to etoposide or 140 mM acetate greatly increased the percentage of hypodiploid cells to approximately 70% and 35%, respectively (Figure 5b). It has been reported that the pH influences the cell death pathway induced by SCFA produced by propionibacteria, that is, SCFA trigger apoptosis at pH 7.5 in HT-29 cells but necrosis at pH 5.5.1 In our study, the pH of the culture medium after 48 h of incubation with acetate was closer to 7.5 than to 5.5 in both HCT-15 and RKO cell lines and was similar to that of the control culture (Table 1). In RKO cells, the pH of the control culture medium (pH = 8.00) was higher than that of HCT-15 cells (pH = 7.45), probably due to the different composition of the growth medium (DMEM, Dulbecco's Modified Eagle's Medium) for RKO cells and RPMI for HCT-15 cells) and to the different growth rates of these cell lines. Taken together, our results show that, under our experimental conditions, acetate induced apoptosis.

Table 1: Measurements of pH in acetate-treated cells.

Condition	pH
HCT-15	
RPMI	7.45
Untreated cells	7.07
Acetate	
70 mM	7.18
100 mM	6.93
140 mM	7.06
RKO	
DMEM	8.00
Untreated cells	8.01
Acetate	
110 mM	8.01
140 mM	8.20
220 mM	8.16

Determination of pH values in the culture medium of CRC cells, after incubation with acetate for 48 h. Untreated cells were incubated with fresh complete medium. Data represent means of at least three independent experiments. (These results were obtained in collaboration with C. Marques).

3.1.2 Acetate induces lysosomal permeabilization and cathepsin D release to the cytosol

We next investigated whether the lysosomal pathway was involved in acetate-induced apoptosis in CRC cells. We measured LMP both by staining with the lysosomotropic agent Acridine Orange (AO) and by immunoblot detection of CatD in cytosolic fractions. AO is a weak base that moves freely across membranes when uncharged and accumulates in acidic compartments like lysosomes in its protonated form, where it forms aggregates that fluoresce bright red.

LMP is associated with proton release, which renders lysosomes more alkaline and hence with decreased red fluorescence. LMP was monitored qualitatively by fluorescence microscopy and quantitatively by flow cytometry, by measuring the percentage of cells with loss of lysosomal AO red fluorescence. Etoposide was used as a positive control, as it destabilizes lysosomes and induces LMP through CatD release to the cytosol (Emert-Sedlak, Shangary et al. 2005; Groth-Pedersen, Ostenfeld et al. 2007; Fehrenbacher, Bastholm et al. 2008). Fluorescence microscopy analysis of both the cell lines stained with AO showed that a high percentage of control cells exhibited intact acidic lysosomes (i. e., high levels of red fluorescence corresponding to accumulation of AO in acidic lysosomes) and a reduced percentage of cells with diffused green fluorescence (corresponding to non-lysosomal AO) (Figure 4a). By contrast, exposure to acetate led to a decrease in the percentage of cells with red fluorescence, associated with an increase in the percentage of cells with green fluorescence, indicative of LMP (Figure 4a). Similar results were obtained in cells treated with etoposide. These differences were more pronounced in cells treated with 2xIC50 acetate than in cells treated with IC50 concentrations. Quantification of AO-stained cells was performed by flow cytometry; cells with red fluorescence levels lower than the threshold of AO-positive staining of untreated cells were considered to exhibit LMP. Cells with LMP increased from approximately 3.6% in untreated cells to 11.7, 25.8 and 49.1% in cells exposed to 70 mM (IC50), 100 mM and 140 mM (2xIC50) acetate, respectively (Figure 4c). These results demonstrate that acetate induces LMP in a dose-dependent manner. As lysosomal proteases such as CatD are released from lysosomes to the cytosol after LMP, their detection in the cytosolic fraction is indicative of LMP. We therefore

treated HCT-15 and RKO CRC cells with two concentrations of acetate (IC50 and 2xIC50 for each cell line), or with etoposide, and detected CatD in whole-cell extracts and in cytosolic fractions by western blot (Figure 4d). We found that CatD is expressed in whole-cell lysates and that untreated cells exhibited very low levels of CatD in the cytosol. Notably, we found that exposure to etoposide and to IC50 and 2xIC50 concentrations of acetate led to CatD release to the cytosol, indicating that acetate induces LMP in a concentration-dependent manner and further suggesting that acetate induces a lysosomal pathway of apoptosis in both the cell lines. We also observed that exposure to acetate increased Pro-CatD levels in the cytosol of both the cell lines (Figure 4d). The apparent decrease in cytosolic Pro-CatD levels in RKO cells treated with 2xIC50 compared with IC50 concentrations of acetate seems to be due to increased processing to mature CatD.

3.1.3 Cathepsin D release protects cells from acetate-induced apoptosis

To test whether the apoptotic phenotype of HCT-15 and RKO cells exposed to acetate depends on CatD, we inhibited lysosomal CatD with pepstatin A (PstA) and cathepsins B (CatB) and L (CatL) with (2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d). PstA, but not E64d, increased apoptotic levels induced by acetate (Figure 5a). Indeed, incubation of HCT-15 cells and RKO with PstA before incubation with the 2xIC50 concentration of acetate increased the number of TUNEL-positive cells, though only reaching statistical significance in HCT-15. E64d did not significantly alter apoptotic levels of either cell line under the same conditions (Figure 5a). PstA-treated HCT-15 cells exposed to acetate also exhibited a higher percentage of cells with sub-G1 DNA content than cells treated with acetate alone, indicative of higher apoptotic levels (Figures 5b and c). By contrast, inhibition of CatD in cells exposed to etoposide decreased the sub-G1 population (Figures 5b and c), in agreement with previous reports showing that CatD has a pro-apoptotic role in etoposide-induced cell death (Emert-Sedlak, Shangary et al. 2005). These results therefore indicate that CatD has a protective role in acetate-induced apoptosis in CRC cells, as has been observed in S. cerevisiae cells for Pep4p, the human CatD ortholog (Pereira, Chaves et al. 2010).

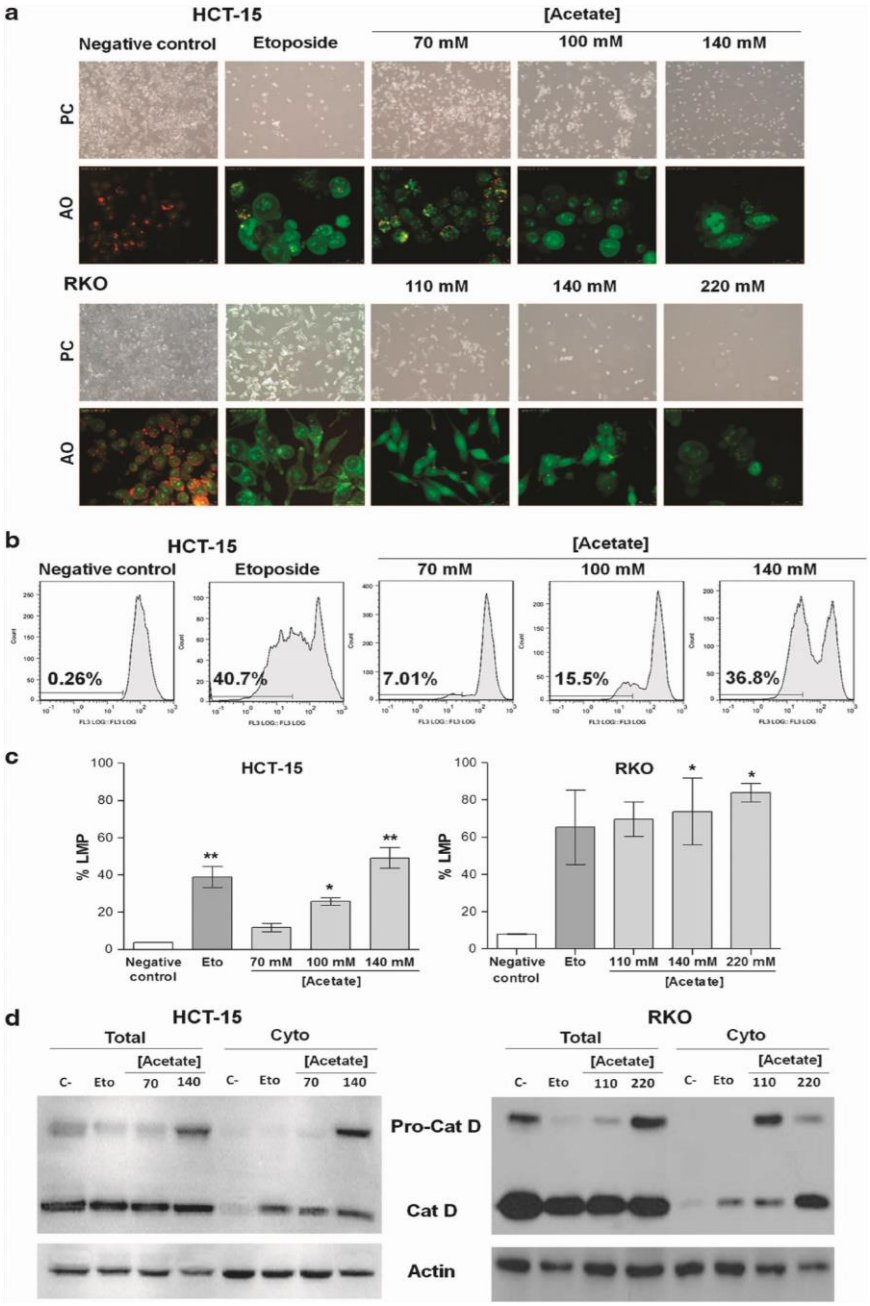

Figure 4: Acetate induces LMP, analyzed by lysosomal pH alterations and CatD release to the cytosol. (**a**) HCT-15 and RKO cells were incubated with acetate (70 mM, 100 mM and 140 mM, for HCT-15 cells; 110 mM, 140 mM and 220 mM, for RKO cells) for 48 h or with fresh complete medium or etoposide (50 µM) as a negative and positive control, respectively. LMP was detected by AO staining and visualization by fluorescence microscopy. Representative images (x400) are shown. (**b**) Representative images of monoparametric histograms of green fluorescence (FL3 area (log)) in HCT-15 cells treated as in (**a**). (**c**) Percentage of HCT-15 and RKO cells displaying LMP quantified by flow cytometry analysis of AO staining after exposure to acetate as described in (**a**). Values represent mean ± S.E.M. of three independent experiments. *P ≤ 0.05; **P ≤ 0.01 compared with negative control cells. (d) Effect of acetate on the expression and release of cathepsin D to the cytosol in HCT-15 and RKO cells, comparing whole-cell lysates (total) and cytosolic fractions (cyto). Cells were treated with IC50 and 2xIC50 acetate concentrations (respectively, 70 mM and 140 mM for HCT-15 cells and 110 mM and 220 mM for RKO cells) for 48 h or with fresh complete medium or etoposide (50 µM) as a negative and positive control, respectively. Actin was used as a loading control. (Some of these results were obtained in collaboration with C. Marques).

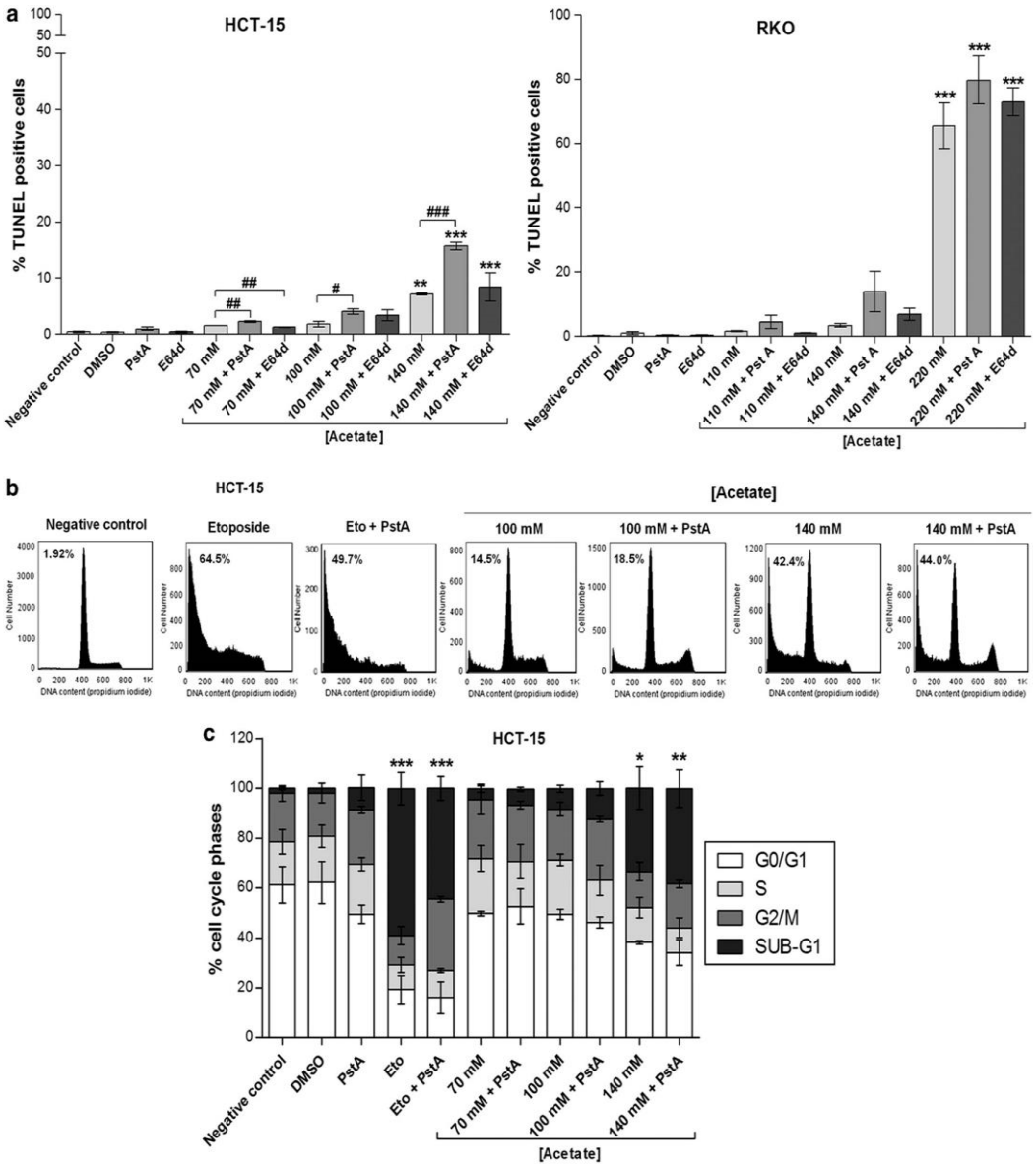

Figure 5: CatD has a protective role in acetate-induced apoptosis. Effect of the specific CatD inhibitor (PstA) and CatB and CatL inhibitor (E64d) on apoptosis in HCT-15 and RKO cells treated with acetate. PstA (100 µM) was pre-incubated for 16 h and then co-incubated with acetate for 48 h. E64d (10 µM) was pre-incubated for 1 h and then co-incubated with acetate for 48 h. (**a**) Apoptosis analysis by TUNEL assay. Values represent mean ± S.E.M. of at least three independent experiments. **P ≤ 0.01; ***P ≤ 0.001 compared with negative control cells. # P ≤ 0.05; ## P ≤ 0.01; ### P ≤ 0.001 comparing acetate treatment with or without PstA or E64d. (**b**) Analysis of the effect PstA on the sub-G1 subpopulation of acetate-treated HCT-15 cells by flow cytometry. Representative histograms corresponding to HCT-15 cells treated with 100 mM and 140 mM acetate with or without PstA. Percentage of sub-G1 cells are shown. Cells were incubated with fresh complete medium or etoposide (50 µM) as a negative and positive control, respectively. (**c**) Analysis of the distribution of cell-cycle phases in HCT-15 cells after acetate treatment in absence/presence of PstA. Values represent mean ± S.E.M. of at least three independent experiments. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 comparing the percentage of sub-G1 populations of treated cells with that of untreated cells (negative control/dimethyl sulfoxide (DMSO)). (Some of these results were obtained in collaboration with C. Marques).

3.2: Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy-independent degradation of damaged mitochondria

As we previously established that acetate induces lysosome membrane permeabilization (LMP) in CRC cells, associated with release of the lysosomal protease Cathepsin D (CatD), which has a well-established role in the mitochondrial apoptotic cascade. Unexpectedly, we showed that CatD plays an anti-apoptotic role in this process, since pepstatin A (a CatD inhibitor) increased acetate-induced apoptosis. These results mimicked our previous data in the yeast system showing that acetic acid activates a mitochondria-dependent apoptosis process associated with vacuolar membrane permeabilization and release of the vacuolar protease Pep4p, ortholog of mammalian CatD. Indeed, this protease was required for cell survival in a manner dependent on its catalytic activity and for efficient mitochondrial degradation independently of autophagy. In this study, we therefore assessed the role of CatD in acetate-induced mitochondrial alterations. We found that, like acetic acid in yeast, acetate-induced apoptosis is not associated with autophagy induction in CRC cells. Moreover, inhibition of CatD with siRNA or pepstatin A enhanced apoptosis associated with higher mitochondrial dysfunction and increased mitochondrial mass. This effect seems to be specific, since inhibition of Cathepsins B and L with E-64d had no effect, nor were these proteases significantly released to the cytosol during acetate-induced apoptosis. Using yeast cells, we further show that the role of Pep4p in mitochondrial degradation depends on its protease activity and is complemented by CatD, indicating this mechanism is conserved.

In summary, the clues provided by the yeast model unveiled a novel CatD function in the degradation of damaged mitochondria when autophagy is impaired, which protects CRC cells from acetate-induced apoptosis. CatD inhibitors could therefore enhance acetate-mediated cancer cell death, presenting a novel strategy for prevention or therapy of CRC.

3.2.1 Acetate leads to oxidative stress, changes in ΔΨ*m* and increased mitochondrial mass in CRC cells

Acetate-induced apoptosis has been associated with mitochondrial dysfunction, including increased ROS and ΔΨ*m* alterations, though only in HT-29 cells (Jan, Belzacq et al. 2002; Lan, Lagadic-Gossmann et al. 2007). We also previously showed that CRC-derived cells HCT-15 and RKO exposed to physiological concentrations of acetate exhibit characteristic apoptotic markers (Marques, Oliveira et al. 2013). However, an effect on mitochondrial mass, as suggested by our studies in yeast (Pereira, Chaves et al. 2010) has never been described. We therefore sought to comprehensively determine how acetate affects mitochondrial function in HCT-15 and RKO cells, in particular ROS accumulation and changes in ΔΨ*m* and mitochondrial mass. For this purpose, cells exposed to different concentrations of acetate were stained with the reactive oxygen species (ROS) probes 2,7-dihydrodichlorofluorescein diacetate (H_2DCF-DA) or dihydroethidium (DHE), to detect hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-), respectively. ROS levels in HCT-15 and RKO cells consistently increased in response to acetate in a dose-dependent manner after 12 and 24 hours of exposure, and then slightly decreased after 48h (H_2O_2 in HCT-15 (Figure 1a) and RKO cells (not shown), and O_2^- in RKO cells (Figure 1b)). To determine if the acetate-induced ROS accumulation could be due to inhibition of cellular antioxidant defenses, we used a qualitative assay to measure catalase activity (Iwase, Tajima et al. 2013). We found that exposure to IC_{50} concentrations of acetate slightly decreased catalase activity in HCT-15 and RKO cells, though more evidently in the latter (Supplementary Figure S1).

In addition to resulting from a decrease in antioxidant defenses, ROS accumulation can be an early event of the apoptotic process, associated with other mitochondrial dysfunctions.(Handy and Loscalzo 2012) We therefore assessed if acetate induces further mitochondrial alterations in CRC cells. We found that acetate, like CCCP, decreased the mitochondrial potential - ΔΨ*m*, as it lowered mitochondrial accumulation of $DiOC_6(3)$ and of MitoTracker® Red CMXRos in RKO cells and of $DiOC_6(3)$ in HCT-15 cells (Supplementary Figure S2), while increasing cytosolic fluorescence of the probes. Since changes in ΔΨ*m* can result from changes in

mitochondrial mass, we quantitatively assessed these two parameters simultaneously in RKO cells stained with both MitoTracker® Red CMXRos and MitoTracker® Green FM, by flow cytometry. In addition to a decrease in the mean red fluorescence, indicative of decreased ΔΨ*m* (Figure 1c), acetate increased the mean green fluorescence, indicative of an increase in mitochondrial mass (Figures 1c and d). After normalizing acetate-induced changes in ΔΨ*m* to mitochondrial mass, and in relation to time zero (Figure 1e), it became evident that a short exposure to acetate induces a small increase in ΔΨ*m* (Figure 1e). This hyperpolarization was apparent after 12 hours of exposure to 110 and 140 mM but not to 220 mM acetate. For longer exposure times, this ratio significantly decreased for all concentrations, indicative of ΔΨ*m* dissipation (as verified for the control CCCP treatment). We also investigated the effect of acetate on the levels of mitochondrial proteins, namely the apoptosis inducing factor (AIF), the voltage dependent anion channel (VDAC1), and a subunit of the outer mitochondrial membrane translocator (TOM22). We found all doses of acetate, as well as etoposide, increased the levels of AIF and VDAC1 in RKO and, to a higher extent, in HCT-15 cells (Supplementary Figure S3). In addition, acetate, but not etoposide, led to increased levels of TOM22.

Our results indicate that acetate causes mitochondrial dysfunction, increasing ROS accumulation, perturbing ΔΨ*m* and increasing mitochondrial mass in a time- and dose-dependent manner, suggesting that acetate impairs the turnover of harmful mitochondria.

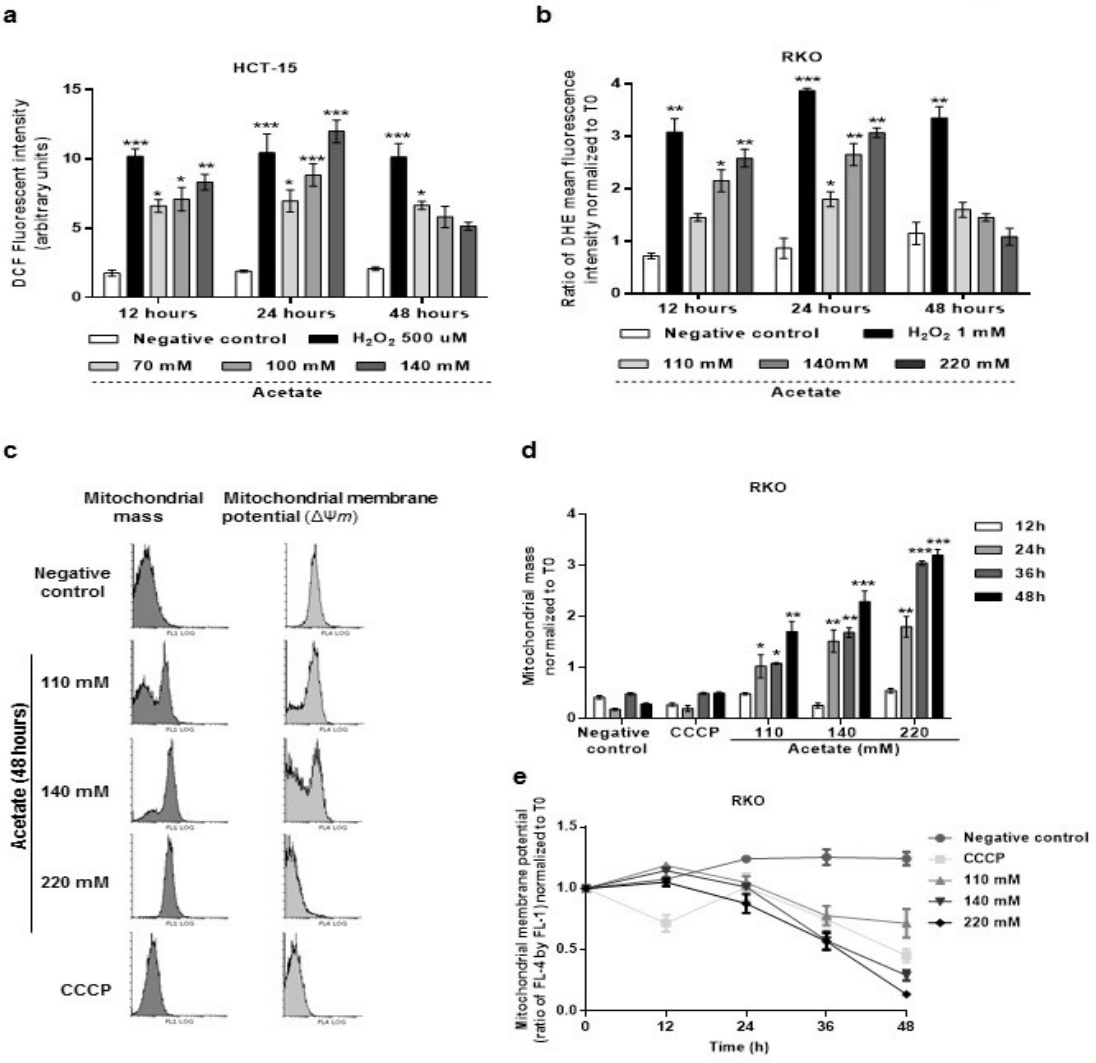

Figure 1: Acetate induces oxidative stress and mitochondrial dysfunction in CRC cells. (a) and **(b**) HCT-15 and RKO cells were incubated with acetate (70, 100 or 140 mM for HCT-15 cells; 110, 140 or 220 mM for RKO cells) for 12, 24 and 48 hours. HCT-15 and RKO negative control cells were incubated with fresh complete medium for the same times, while positive control cells were incubated with 500 µM and 1 mM H_2O_2, respectively. **(a)** Accumulation of hydrogen peroxide (H_2O_2) in HCT-15 cells was detected with 2',7'-dihydrodichlorofluorescein diacetate (H_2DCF-DA) using a fluorescence microplate reader. **(b)** Accumulation of superoxide anion (O_2^-) in RKO cells was detected with dihydroethidium (DHE) and quantified by flow cytometry. **(c)**, **(d)** and **(e)** Detection of mitochondrial dysfunction in RKO cells using MitoTracker® Green FM and MitoTracker® Red CMXROs double staining. Cells were incubated with 110, 140 or 220 mM of acetate for 48 hours and then stained with 400 nM MitoTracker® Green and 200 nM MitoTracker® Red for 30 min at 37º in the dark. **(c)** Representative histograms of mitochondrial dysfunction in cell exposed to 110, 140 or 220 mM of acetate for 48 hours. There was a significant increase in the green mean fluorescence intensity (MitoTracker® Green), indicative of an increase in mitochondrial mass, and a decrease in the mean red fluorescence intensity (Mito Tracker® Red CMXRos), indicative of changes in the mitochondrial membrane potential. **(d)** Acetate treatment induces an increase in the mean green fluorescence intensity normalized to the green mean fluorescence intensity at time 0 (T0), indicating an increase in mitochondrial mass. **(e)** Acetate treatment induces an alteration in the mitochondrial membrane potential normalized to mitochondrial mass, assessed by the ratio between the mean red fluorescence intensity (FL-4) and the mean green fluorescence intensity (Fl-1) normalized to T0. For each time point, the values of at least three independent experiments are represented. Bonferroni's test *P ≤ 0.05; **P ≤ 0.01 and ***P ≤ 0.001 compared to negative control cells. In **(c)**, **(d)** and **(e)**, cells incubated with fresh complete medium or with 50 µM CCCP were used as a negative and positive control, respectively.

3.2.2 Acetate inhibits basal autophagy and impairs autophagy induction

To determine if the increased mitochondrial mass resulting from acetate exposure could be due to autophagy inhibition, we monitored how it affects the levels of Beclin-1, a well-known autophagic player that is part of the class III PI3K complex (PI3KC3). Acetate lowered Beclin-1 levels in both RKO and HCT-15 cells, consistent with inhibition of autophagy and possibly explaining the increased levels of mitochondrial proteins in acetate-treated cells (Figure 2a). We next determined whether acetate affects autophagy induction. We monitored starvation-induced autophagy (in HBSS medium) by assessing an autophagy hallmark: conversion of LC3-I to LC3-II (in the presence and absence of bafilomycin A1 (Baf. A1) (Mathew et al., 2007), a specific inhibitor of vacuolar type H+ -ATPase used to inhibit the fusion of autophagosomes with lysosomes (Yamamoto, Tagawa et al. 1998).

HCT-15 and RKO cell lines had high basal autophagy levels and did not induce autophagy in response to starvation, evidenced by the increased accumulation of LC3-II in the presence of Baf. A1 already in control cells, which was not altered in response to starvation (Figure 2b, c and d). Therefore, we could not determine whether acetate affects autophagy induction in these cells. For that purpose, we instead used the HCT116 cell line, which displays increased autophagic flux in response to starvation (Figure 2b, c and d). We found that a 48h exposure of HCT116 cells to half maximal inhibitory concentration (IC_{50}), intermediate concentration and 2x IC_{50} of acetate (Supplementary Figure S4) decreased conversion of LC3-I to LC3-II in the presence of Baf. A1, which was even more pronounced under starvation conditions (Figure 2e and 2f). Increasing doses of acetate also progressively decreased the levels of Beclin1 and Atg5 proteins, essential for autophagosome completion (Xie and Klionsky 2007; Mehrpour, Esclatine et al. 2010) in both complete and starvation media (Figure 2g). Taken together, our results indicate not only that exposure to acetate does not induce autophagy under our experimental conditions, but also that it can inhibit both basal autophagy and autophagy induction.

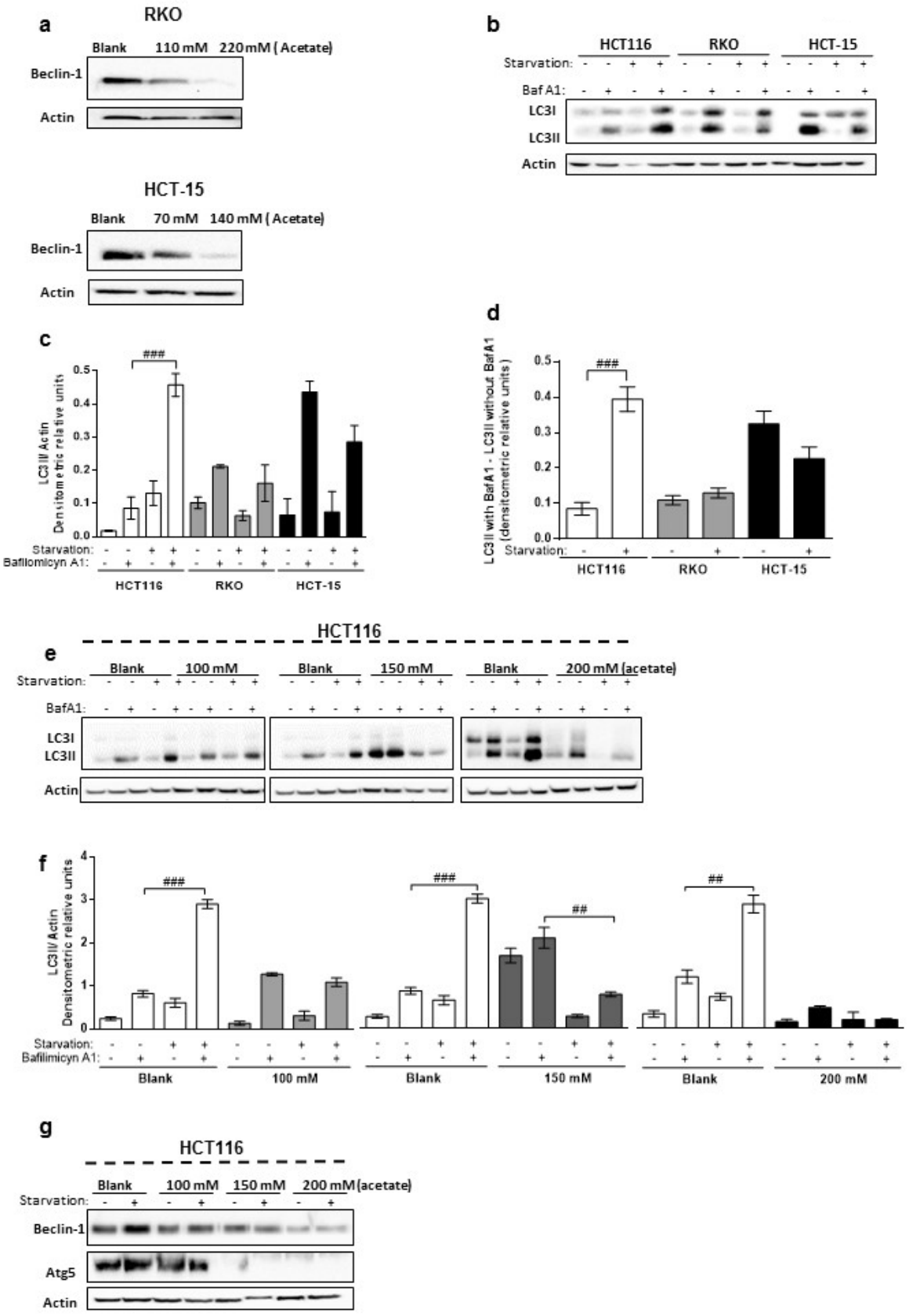

Figure 2: Acetate impairs autophagy in CRC cells. (a) Levels of Beclin-1 in cells exposed to acetate for 48h (110 and 220 mM; 70 and 140 mM for RKO and HCT-15 cells respectively)**. (b)** Immunoblot analysis of LC3-I/II in HCT116, RKO and HCT-15 cell lines. Cells were maintained in complete medium or incubated in HBSS medium (starvation) for 6h in the presence and absence of

20 nM Bafilomycin A1 (Baf. A1). The HCT116 cell line exhibited the strongest autophagic response to nutrient limitation (starvation) and thus was chosen to address the effect of acetate on starvation-induced autophagy. **(c)** LC3-II/Actin ratios of HCT116, RKO and HCT-15 cells were determined using Quantity One software. **(d)** The autophagic flux was determined by subtracting the normalized LC3-II levels in the presence of Baf. A1 from the corresponding levels obtained in the absence of Baf. A1. **(e)** Levels of LC3-I/II and (**g)** of Beclin-1 and Atg5 in HCT116 cells exposed to acetate. Cells were left untreated (Blank) or treated with 100, 150 or 200 mM acetate for 42 hours. Afterwards, cells were maintained in complete medium or HBSS medium (starvation) with acetate for another 6 hours in presence or absence of 20 nM Baf. A1. These data demonstrate that acetate decreases the autophagic flux in CRC cells in a dose-dependent manner, as shown by the decrease in LC3-II delivery to the lysosome, and in Beclin-1 and Atg5 protein levels. **(f)** The LC3-II/Actin ratios of HCT116 cells were determined using Quantity One software. Actin was used as a loading control. Values are mean ± SEM of three independent experiments. Representative immunoblots are shown in **(a)**, **(b)**, **(e)** and **(g)**. One-way ANOVA followed by Bonferroni's test, ## $P \leq 0.01$ and ### $P \leq 0.001$ compared to control cells treated with HBSS (starvation medium).

3.2.3 Active Cathepsin D mediates mitochondrial degradation and protects CRC cells from acetate-induced mitochondrial dysfunction

The cellular event or signal that determines whether mitochondrial degradation in cancer cells undergoing apoptosis occurs through selective or non-selective autophagy or by an autophagy-independent process, and which are the consequences to cell fate, is still unclear. In a previous study in *Saccharomyces cerevisiae*, we showed that the yeast CatD (Pep4p) plays an anti-apoptotic role in acetic acid-induced cell death that depends on its proteolytic activity, and is required for mitochondrial degradation independently of autophagy (Pereira, Azevedo et al. 2013). We therefore hypothesized that CatD, like Pep4p, is required for efficient degradation of damaged mitochondria. To test this hypothesis, we assessed how CatD affects mitochondrial dysfunctions induced by acetate in CRC cells.

We first analyzed whether Pepstatin A (PstA), a known specific pharmacological inhibitor of CatD, or transfection with specific RNA interference (siRNA) targeting CatD interfered with acetate-induced accumulation of ROS in RKO cells. We found that PstA increased total O_2^- and H_2O_2 accumulation in cells exposed to the three acetate concentrations for 24h (Figure 3a and 3b). Down-regulation of CatD was even more effective, as it significantly increased total and mitochondrial O_2^- accumulation in cells treated with 110 mM acetate (detected with DHE or MitoSOX, respectively) (Figure 3c and 3d). In contrast, inhibition of CatD did not significantly affect etoposide-induced ROS accumulation (Figure 3c and 3d).

We next tested the effect of depleting CatD on mitochondrial mass. Transfection with CatD siRNA clearly increased the levels of the mitochondrial protein AIF in both acetate- and etoposide-treated RKO cells, though not in untreated controls (Figure 4a). Using the double staining protocol described above, we also found that depletion of CatD in acetate-treated RKO cells significantly increased mitochondrial mass and decreased $\Delta\Psi m$. In contrast, it had no significant effect in cells treated with etoposide (Figure 4b and 4c). Altogether, these data indicate that CatD protects RKO cells from mitochondrial dysfunction induced by acetate through its involvement in the degradation of damaged mitochondria.

To further analyze whether the role of CatD in mitochondrial degradation depends on its proteolytic activity, we assessed how this parameter was affected by inhibition with PstA. PstA significantly increased the mitochondrial mass of RKO cells exposed to 110 and 140 mM acetate for 48 hours, but decreased the $\Delta\Psi m$ (Figure 4d and 4e). In contrast, inhibition of CatD in RKO cells exposed to etoposide did not affect mitochondrial mass or the $\Delta\Psi m$. Moreover, E-64d, an inhibitor of CatB and CatL (also over-expressed in CRC cells) (Troy, Sheahan et al. 2004) had no effect in the mitochondrial mass or $\Delta\Psi m$ of either acetate- or etoposide-treated cells (Figure 4d and 4e). We confirmed E-64d was active under our experimental conditions because it decreased caspase-3 activity in cells exposed to etoposide (not shown), confirming the previously described pro-apoptotic function of CatB and CatL in this process (Oberle, Huai et al. 2010). These results indicate CatD, but not CatL or CatB, is involved in mitochondrial degradation in response to acetate. Accordingly, we found that only the active form of CatD, but not CatL and only trace amounts of CatB, is released to the cytosol after acetate treatment (Figure 5).

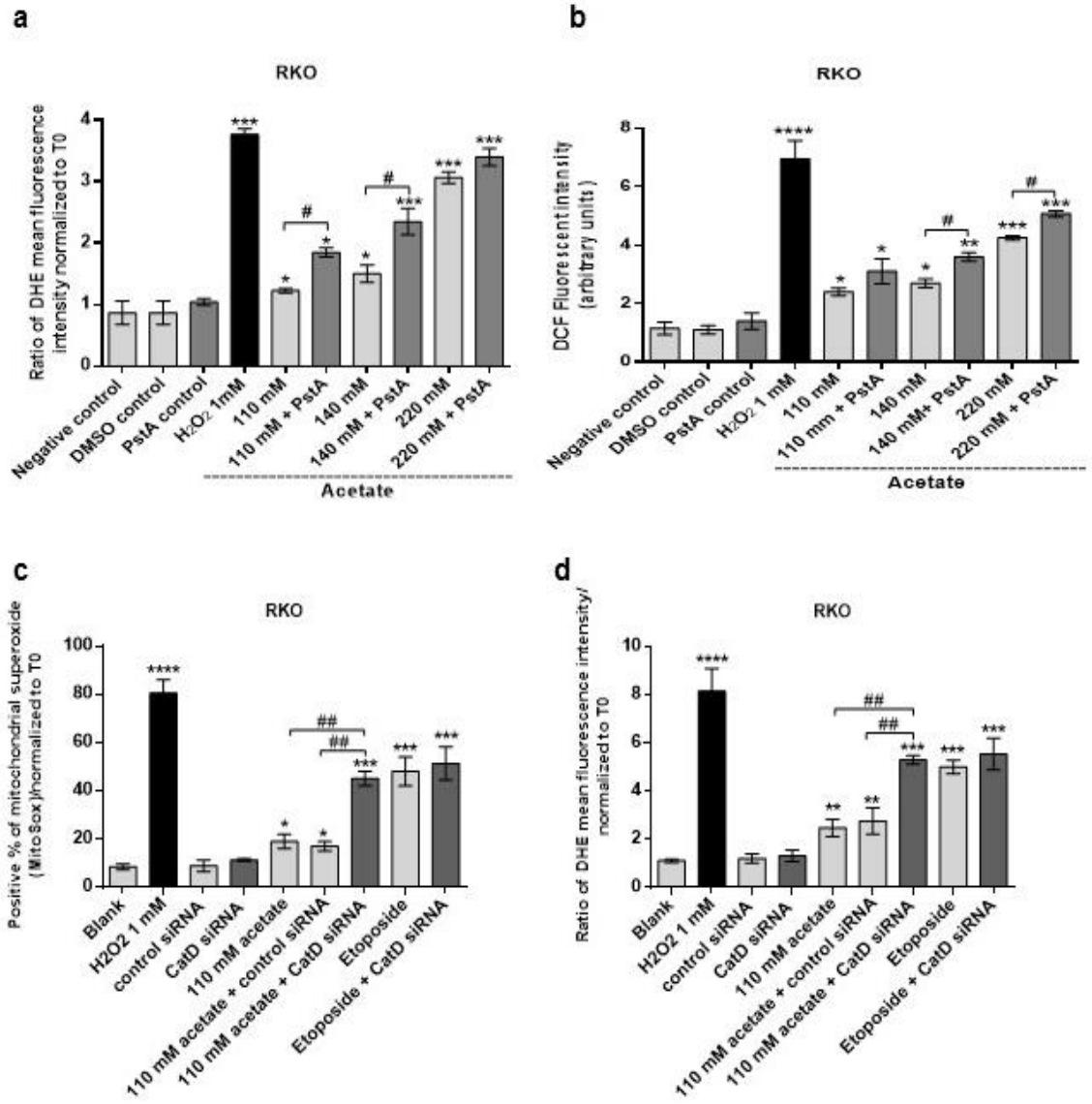

Figure 3: CatD protects CRC cells from oxidative stress. (a) and (b) Effect of Pepstatin A (PstA), a specific inhibitor of CatD catalytic activity, on ROS levels of RKO cells treated with acetate. PstA (100 µM) was pre-incubated for 16 hours and then co-incubated with acetate for 24 hours. **(a)** Accumulation of superoxide anion (O_2^-) in RKO cells was detected with dihydroethidium (DHE) and quantified by flow cytometry. **(b)** Accumulation of H_2O_2 in RKO cells was detected with H_2DCF-DA using a fluorescence microplate reader. **(c)** and **(d)** Effect of CatD silencing on mitochondrial superoxide and total superoxide production in RKO cells treated with acetate (110 mM) or etoposide (50 µM) for 48 hours. As controls, RKO cells were not transfected (blank), transfected with scrambled control siRNA or transfected with CatD siRNA. 1 mM H_2O_2 was used as a positive control for ROS production. **(c)** Accumulation of mitochondrial superoxide in RKO cells was detected with MitoSOX™ and quantified by flow cytometry. **(c)** Downregulation of CatD induces a significant increase in mitochondrial superoxide production normalized to time 0 (T0) in RKO cells treated with 110 mM acetate and **(d)** a significant accumulation of total superoxide, normalized to T0 cells. Values represent mean ± S.E.M. of at least three independent experiments. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 compared with control cells. # P ≤ 0.05 and ## P ≤ 0.001 comparing acetate treatment with acetate/PstA or acetate treatment with acetate/ CatD siRNA.

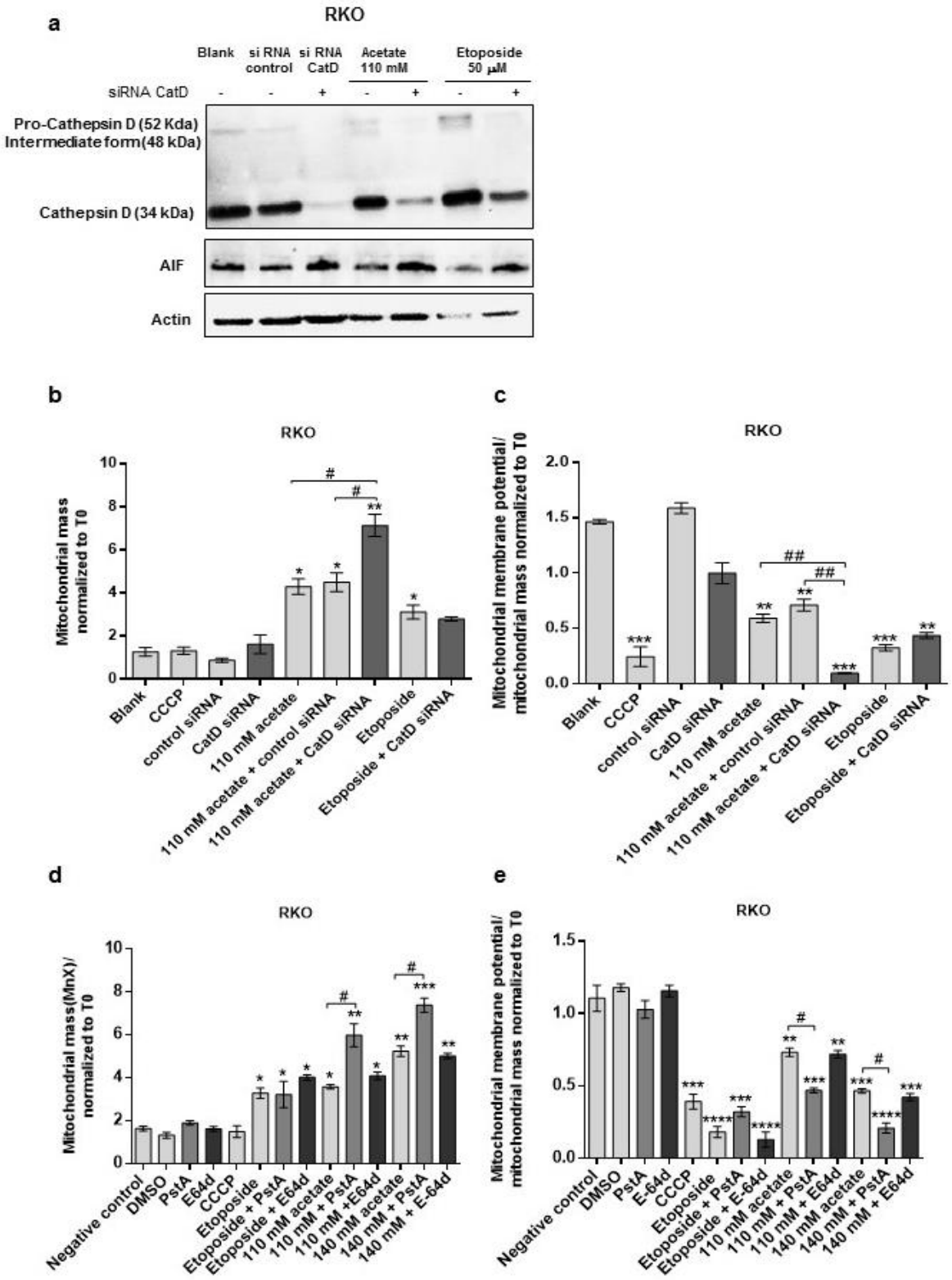

Figure 4: CatD is involved in autophagy-independent mitochondrial degradation in CRC cells. **(a)** Western blot of CatD isoforms (Pro-Cathepsin D, Intermediate form and mature CatD) and AIF with or without CatD silencing in the absence or presence of acetate (110 mM) or etoposide (50 µM). As controls, RKO cells were not transfected (blank), transfected with scrambled control siRNA or transfected with CatD siRNA. Actin was used as a loading control. **(b)** and **(c)** effect of CatD silencing on mitochondrial dysfunction in RKO cells treated with acetate (110 mM) or etoposide (50 µM) for 48 hours. **(b)** Downregulation of CatD induces a significant increase in mitochondrial mass normalized

to time 0 (T0) in RKO cells treated with 110 mM acetate and **(c)** a significant mitochondrial membrane depolarization normalized to mitochondrial mass, in relation to T0. For each bar, the mean value for at least three independent experiments is represented. Bonferroni's test *P ≤ 0.05; **P ≤ 0.01 and ***P ≤ 0.001 compared to negative control cells. # P ≤ 0.05, ## P≤ 0.01 comparing acetate treatment with acetate/CatD siRNA. **(d)** and **(e)** effect of the specific CatD inhibitor (PstA) and the CatB and CatL inhibitor (E-64d) on mitochondrial dysfunction in RKO cells treated with acetate (110 and 140 mM) or etoposide (50 µM) for 48 hours. E-64d (10 uM) and PstA (100 µM) were pre-incubated for 1 or 16 hours, respectively, and then co-incubated with acetate or etoposide for 48 hours. Cells were incubated with fresh complete medium or with 50 µM CCCP as a negative and positive control, respectively. Cells were then double stained with 400 nM MitoTracker® Green and 200 nM MitoTracker® Red CMXRos for 30 min at 37º in the dark and analyzed by flow cytometry. Inhibition of Cathepsin D activity with PstA induces an increase in mitochondrial mass **(d)** and membrane depolarization normalized to mitochondrial mass in relation to time 0 (T0) in RKO cells **(e)**. For each bar, the mean value for at least three independent experiments is represented. Bonferroni's test *P ≤ 0.05; **P ≤ 0.01 and ***P ≤ 0.001 compared to negative control cells. # P ≤ 0.05 comparing acetate treatment with acetate/PstA.

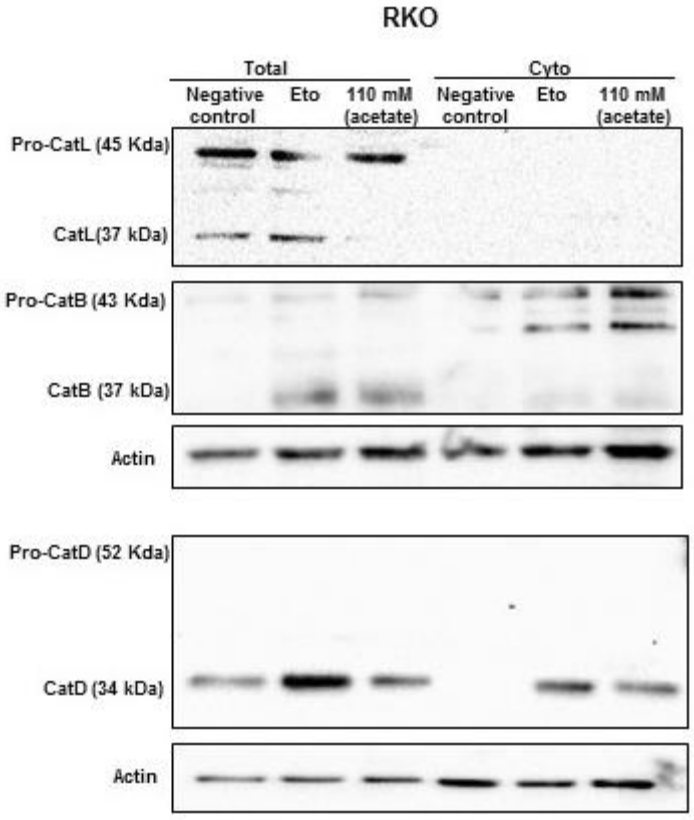

Figure 5: Acetate induces specific CatD release to the cytosol in CRC cells. Effect of acetate on the expression and release of CatL, CatB and CatD to the cytosol in RKO cells. Cells were treated with 110 mM acetate or 50 uM etoposide for 48 hours, or with fresh medium as negative control. Total and cytosolic fractions (Cyto) are showed. Actin was used as a loading control.

Supplementary figures

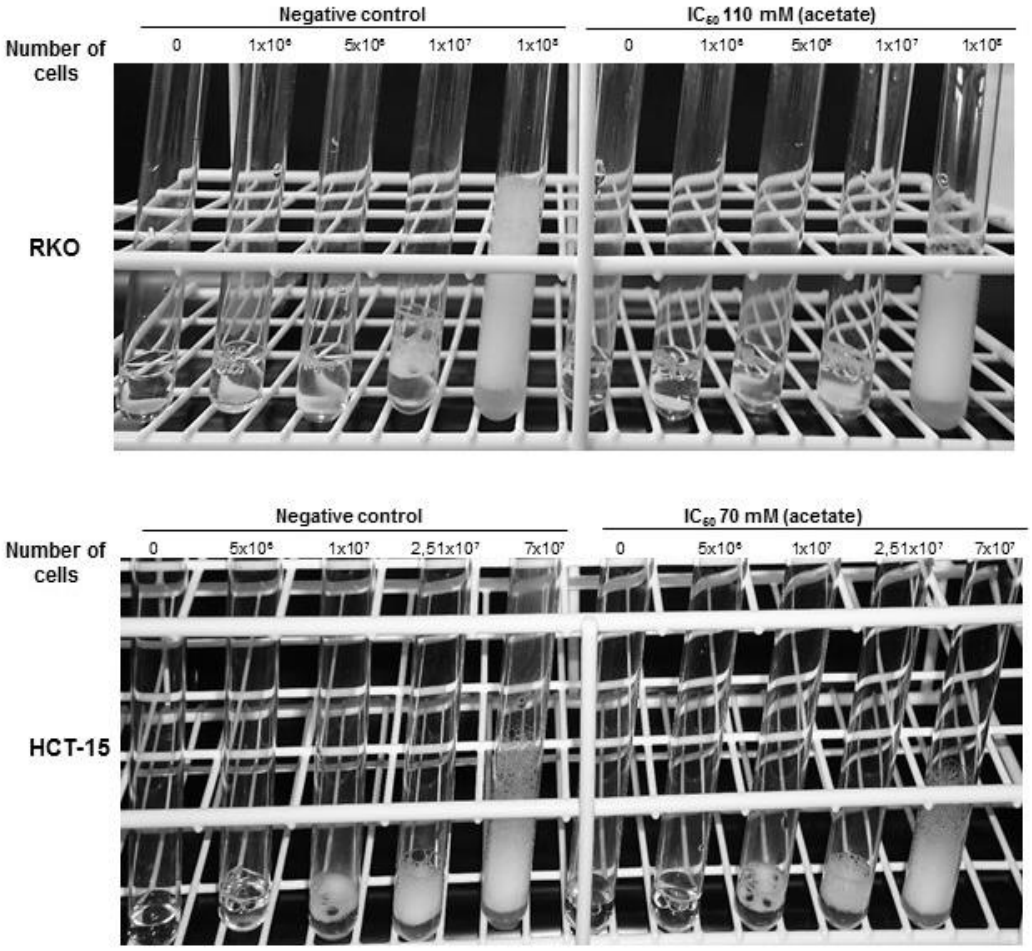

Figure S1: Acetate decreases catalase activity in CRC cells. RKO and HCT-15 cells were treated with 110 or 70 mM of acetate, respectively, or with medium fresh as negative control. After 48 hours, cells were washed with PBS and 4 different cell concentrations per condition in a final volume of 500 ul were added to glass tube. Subsequently, 100 ul of Triton X-100 and 100 ul of undiluted hydrogen peroxide (30%) were added, mixed and tubes were incubated at room temperature for 15 min.

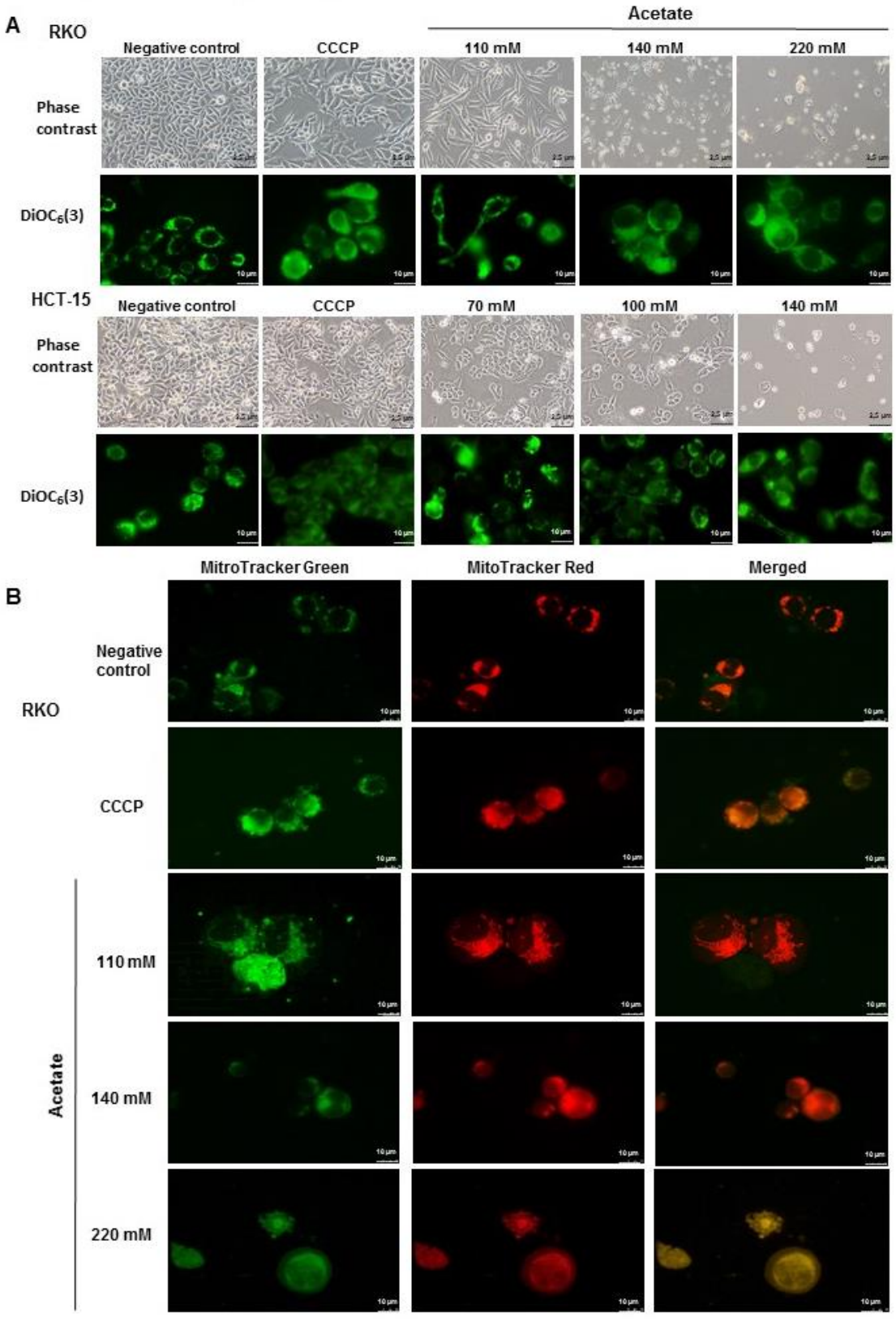

Figure S2: Representative fluorescence microscopy images of changes in DY*m* of RKO and HCT-15 cells. Cells were plated on coverslips and incubated with different acetate concentrations for 48 hours, or with fresh medium or CCCP as negative and positive controls, respectively. Cells were then stained with **(a)** 20 nM $DiOC_6(3)$ or **(b)** 200 nM MitoTracker® Red and 400 nM MitoTracker®Green (30 min, 37º in the dark).

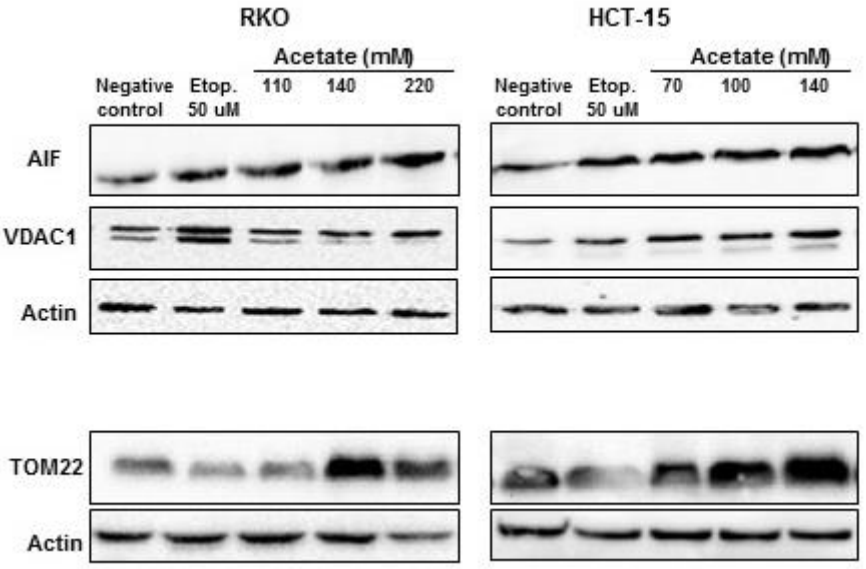

Figure S3: Acetate causes accumulation of mitochondrial proteins in RKO and HCT-15 cells. RKO and HCT-15 cells were treated with 110, 140 and 220 mM or 70, 100 and 140 mM of acetate, respectively, for 48 hours, or with fresh medium as a negative control, or etoposide (50 µM) as a positive control. Levels of AIF, VDAC1 and TOM22 were assessed by Western Blot. Actin was used as a loading control.

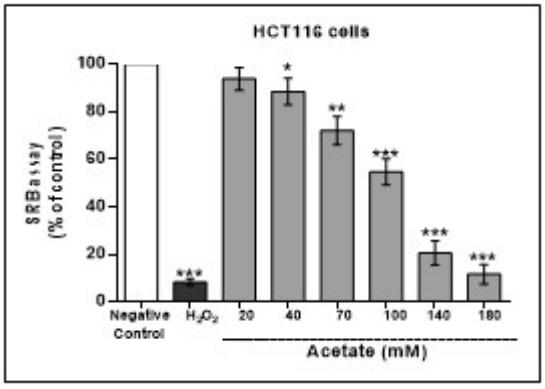

Figure S4: Acetate inhibits proliferation of HCT116 cells. HCT116 cells were seeded ($7x10^4$ cells/well) and incubated for 48 hours with acetate, or with fresh medium or H_2O_2 used as positive and negative controls, respectively. The half-maximal inhibitory concentration (IC_{50}), IC inter and $2×IC_{50}$ values were determined using the sulforhodamine B assay.

3.3: The role of acetate on monocarboxylate transporter (MCT) expression and glucose metabolism in colorectal cancer cells: therapeutic implications

Acetate is a short-chain fatty acid relevant in the regulation of colorectal cancer (CRC) cells survival. Monocarboxylate transporters (MCTs) have gained increased interest in cancer contributing to their metabolic phenotype and to the uptake of drugs such as 3-bromopyruvate (3BP). The mechanism by which acetate enters CRC cells is not well understood. Here, we aimed to investigate the mechanism of acetate transport as well as the role of acetate on MCTs expression and glucose metabolism. We further wanted to explore the use of 3BP and acetate as a possible therapeutic approach for CRC.

Our results show that the transport of acetate occurs through the sodium dependent monocarboxylate transporter (SMCT-1) and by passive diffusion via aquaporins. MCT-1 and MCT-2 are also involved in acetate uptake, indeed acetate increased the expression of MCT-1, MCT-4 and of the highly glycosylated CD147 chaperone, promoting membrane re-localization of MCT-1 and changes in glucose metabolism. We further verified that 3BP potentiates acetate-induced apoptosis in CRC cells. Altogether we provide new evidences for the role of acetate in the regulation of MCTs expression and glycolytic profile, which influence acetate transport and increases the sensitivity of CRC cells to 3BP therapy.

3.3.1 Kinetics and energetics of acetate uptake across the plasma membrane of CRC cells

The initial uptake rates of [^{14}C] acetate were evaluated at pH 6.0 in HCT-15 and RKO cell lines (Figure 1a and b). The non-linear regression analysis shows that HCT-15 cells transport acetate follows a second order kinetics with an affinity constant (K_m) of 1.97 ± 0.57 mM and a transport capacity (V_{max}) of 62 ± 9 nmol/mg of protein/min. In RKO cells, acetate follows a first order kinetics with a diffusion constant of 5.19 ± 0.16 µL/mg of protein/min.

To evaluate the energetics of [^{14}C] acetate transport we tested the influence of agents known to disrupt different ion membrane electrochemical potentials (Figure 1c), such as CCCP (a protonophore which disrupts both the pH and the electric potentials), monensin (an ionophore that specifically disrupts a Na^+ gradient) and valinomycin (an ionophore which preferentially affects a K^+ gradient and, to a lesser extent, a Na^+ gradient across biological membranes). We also tested the inhibition of acetate transport using monocarboxylate transporters (MCTs) inhibitors such as CHC (inhibitor of MCT-1 and SMCT-1), DIDS (inhibitor of MCTs and anion exchangers) and AR-C155858 (specific inhibitor of MCT-1 and MCT-2). We also used $HgCl_2$, which is described as an inhibitor of aquaporin activity (Yukutake, Tsuji et al. 2008; Hirano, Okimoto et al. 2010). Our results showed that CCCP inhibited significantly acetate transport in both HCT-15 (71%) and RKO (52%) cells; monensin also inhibited acetate uptake in HCT-15 (78%) and RKO (36%) and valinomycin presented only a small inhibitory effect in both cell lines (22% inhibition in HCT-15 and 21% in RKO cells). Regarding MCT inhibitors, CHC inhibited both HCT-15 (30%) and RKO cells (35%) but DIDS and AR-C155858 did not prevent acetate uptake. Interestingly, $HgCl_2$ (aquaporin inhibitor) inhibited acetate uptake in HCT-15 (51%) and RKO (56%) cells indicating the contribution of aquaporins in acetate diffusion into CRC cells.

We further investigated if this inhibition was independent of the inhibition detected for CCCP or monensin. HCT-15 and RKO cells were used to evaluate the inhibition of acetate uptake using double combinations of CCCP (1 µM), $HgCl_2$ (5 µM) and monensin (20 µM) (Figure 2a and b). However, in these assays lower concentrations than described above were used. We verified that in HCT-15 cells CCCP inhibited

34%, $HgCl_2$ 37% and monensin 69% of acetate transport. Combination of CCCP and $HgCl_2$ resulted in a 56% inhibition of acetate uptake, $HgCl_2$ and monensin in 76%, and CCCP with monensin inhibited 75%. In RKO cells acetate uptake was inhibited in 37% with CCCP, 28% with $HgCl_2$ and 68% with monensin. Combination of CCCP with $HgCl_2$ and monensin with $HgCl_2$ resulted in 46% inhibition of acetate transport while CCCP with monensin inhibited 81%.

In conclusion, these results point for the involvement of at least two mechanisms of transport in CRC cells: a secondary active transport dependent on electrochemical Na^+ gradient and diffusion mediated by aquaporins.

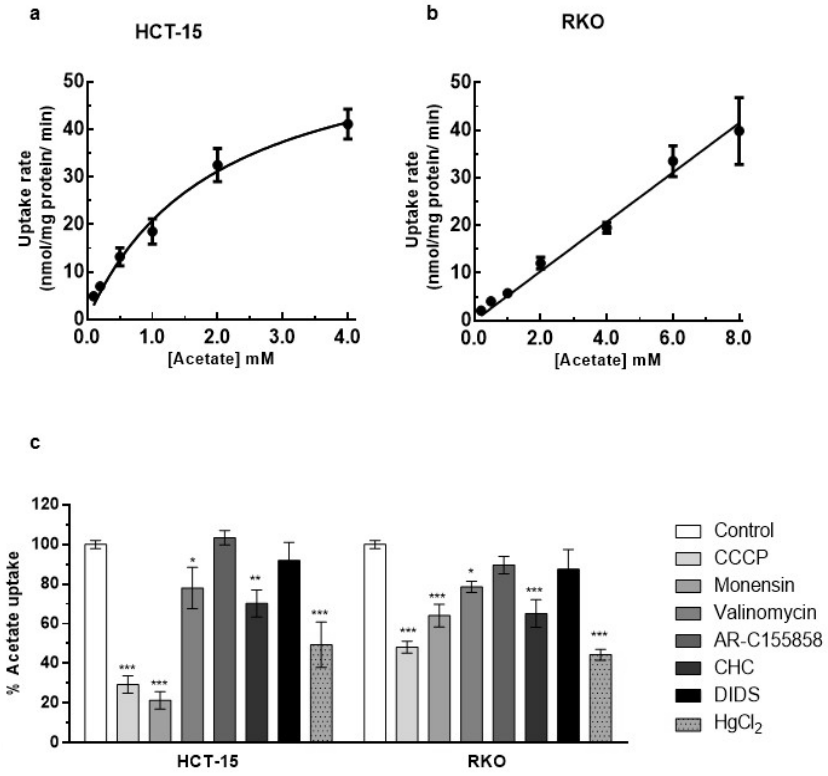

Figure 1: Characterization of acetate uptake in CRC cells. **(a)** and **(b)** Plots of the initial uptake rates of labeled acetate, as a function of the acid concentration at pH 6.0 in HCT-15 and RKO, respectively. Kinetic parameters, as affinity constant (K_m) and transport capacity (V_{max}) or Diffusion constant (K_d) for the uptake of acetate were based on the non-linear regression for the Michaelis-Menten equation f [V]=(Vmax x [Acetate])/(Km + [Acetate]) and Passive diffusion equation f[V] = Kd.[Acetate]. In HCT-15 a transporter system exists with a K_m of 1.97 ± 0.57 mM and a V_{max} of 62 ± 9 nmol/ mg of protein/ min. In RKO acetate enters cells by passive diffusion with a K_d of 5.19 ± 0.16. **(c)** Effect of CCCP (100 µM), Monensin (100 µM), Valinomycin (100 µM), AR-C155858 (1 µM), CHC (10 mM), DIDS (1 mM) and $HgCl_2$ (100 µM) in the uptake of 1 mM of acetate. Statistical analysis was performed by two-way ANOVA: ***, ** and * indicate significant differences with a respective P-values of <0.001, <0.01 and <0.05 (n=3). (These results were obtained in collaboration with J. Azevedo-Silva).

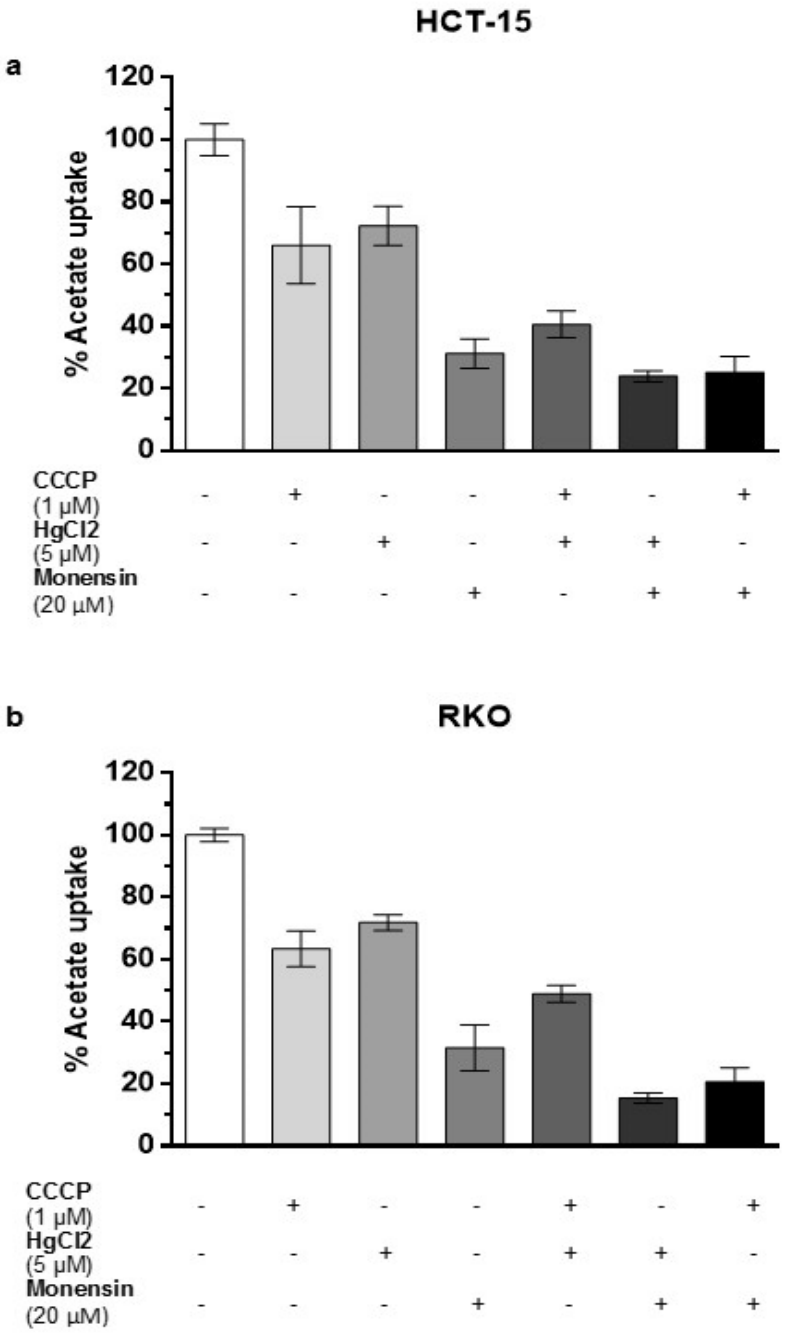

Figure 2: Inhibition of 1 mM of acetate uptake by CCCP (1 µM), $HgCl_2$ (5 µM) and Monensin (20 µM) alone or in combination in HCT-15 (a) and RKO (b) cells. Statistical analysis was performed by two-way ANOVA: ***, ** and * indicate significant differences with a respective P-values of <0.001, <0.01 and <0.05 (n=3). (These results were obtained in collaboration with J. Azevedo-Silva).

3.3.2 Acetate pre-treatment alters the kinetics and the energetics of acetate uptake by CRC cells

To evaluate if acetate treatment could influence the kinetics and energetics of acetate, we tested the influence of CCCP, monensin, AR-C155858 and $HgCl_2$ in the uptake of 1 mM of acetate in cells treated with IC_{30} of acetate for 48h (Figure 3). Untreated cells of HCT-15 and RKO cell lines behaved as expected with acetate transport being inhibited with CCCP (HCT-15: 54%; RKO: 62%), monensin (HCT-15: 60%; RKO: 59%) and $HgCl_2$ (HCT-15: 51%; RKO: 56%) but not with AR-C155858. Cell treated with the IC_{30} of acetate for 48 hours also presented inhibition for acetate uptake using CCCP (HCT-15: 52%; RKO: 53%), monensin (HCT-15: 60%; RKO: 38%) and HgCl2 (HCT-15: 60%; RKO: 55%). Acetate transport in HCT-15 (29%) and RKO (23%) cells treated with acetate was significantly inhibited by AR-C155858. These results led us to conclude that in cells treated with acetate, MCT-1 and MCT-2, besides other transport mechanisms described above, are also involved in acetate transport.

3.3.3 Acetate upregulates MCT-1, MCT-4 and CD147 expression in CRC cells

We wanted to study if acetate influences the expression of MCTs as it was already reported by us for butyrate in breast cancer cells (Queiros, Preto et al. 2012; Azevedo-Silva, Queiros et al. 2015). We assessed the expression profile for the SMCT-1, MCT-1, MCT-2, MCT-4 and the chaperone CD147 (an important protein for MCT activity) in response to acetate by western blot analysis of cell extracts (Figure 4). We analyzed CRC cells treated with both IC_{30} and IC_{50} acetate doses (45 mM and 70 mM; 75 mM and 110 mM, respectively for HCT-15 and RKO) after 24 and 48 hours of incubation. We found that both CRC cell lines express SMCT-1 and MCT-2 but no difference in expression after acetate treatment was observed in both cell lines. We found an elevated expression of MCT-1 and MCT-4 after 48 hours but not after 24 hours acetate treatment in both cell lines compared to the negative control (Figure 4). The increase of MCT-1 and MCT-4 expression was accompanied by an increased expression of the chaperone cluster of differentiation (CD)147, important for MCT-1 and MCT-4 activity. The chaperone CD147, was present in both fully (FG) and core-glycosylated forms in both CRC cell lines after 48 hours of

acetate treatment compared to the negative control. A higher level of CD147 expression was observed in RKO compared to HCT-15 cells (Figure 4). We also evaluated the expression of GLUT-1 after acetate treatment. GLUT-1 is responsible for basal glucose transport across the plasma membrane into the cytosol in many cancer cells (Zhang, Zhao et al. 2015). We observed that both cells expressed GLUT-1 transporter, but no difference in the expression of this transporter was observed after acetate treatment. Our results suggest that acetate regulates MCT-1, MCT-4 and CD147 expression in CRC cells.

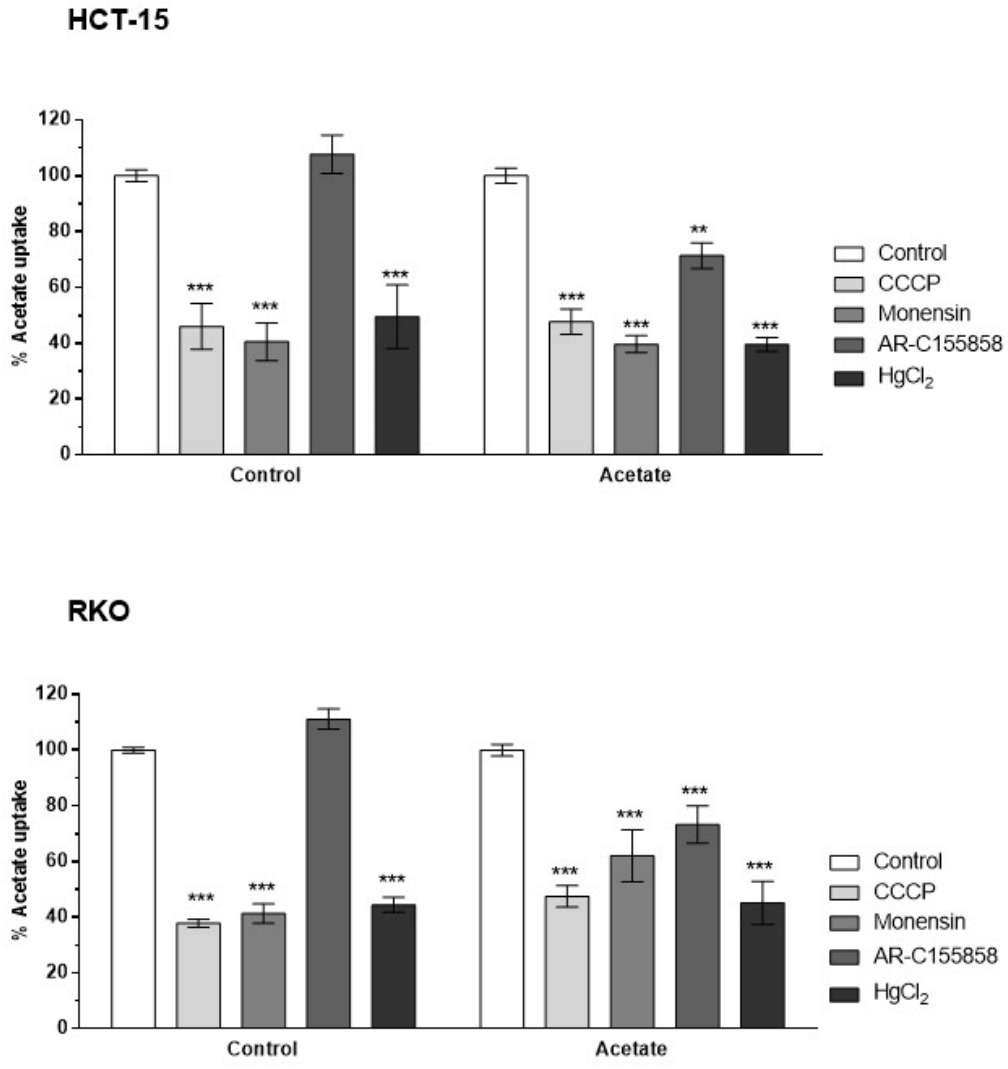

Figure 3: Inhibition of acetate (1 mM) uptake in HCT-15 and RKO cell lines treated with IC_{30} of acetate for 48h with CCCP (100 µM), Monensin (100 µM), AR-C155858 (1 µM) and $HgCl_2$ (100 µM). Statistical analysis was performed by two-way ANOVA: ***, ** and * indicate significant differences with a respective P -values of <0.001, <0.01 and <0.05 (n=3). (These results were obtained in collaboration with J. Azevedo-Silva).

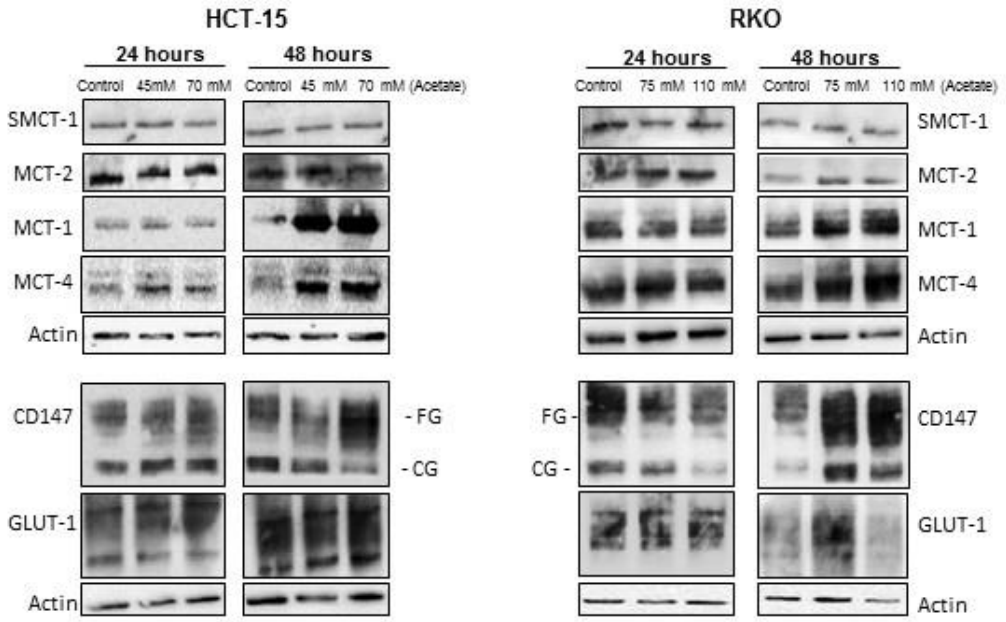

Figure 4: Characterization of MCTs expression and other glycolytic markers in CRC cells after acetate treatment. HCT-15 and RKO cells were incubated with acetate (45 mM and 70 mM for HCT-15 cells; 75 mM and 110 mM for RKO cells, respective doses for the IC_{30} and IC_{50}) or with fresh medium (as negative control) for 24 and 48 hours. Western blotting images of the SMCT-1, MCT-1, MCT-2, MCT-4, CD147 and GLUT-1 expression. β-actin was used as loading control. A representative experiment of at least three independent experiments is shown.

3.3.4 Acetate induces MCT-1 plasma membrane localization in CRC cells

We analyzed by immunofluorescence the cellular localization changes induced by acetate in MCT-1, MCT-4 and CD147. CRC cells were treated with acetate IC_{30} and IC_{50} doses during 48 hours, as described above for immunodetection. In agreement with the western blot analysis we could observe that acetate treatment leads to an elevated expression of these transporters and an increase in MCT-1 localization at the plasma membrane in comparison to the cytoplasm in both cell lines (Figure 5), being more evident in HCT-15 than in RKO cells. Moreover, we found no differences between MCT-4 and CD147 co-localization after acetate treatment. Our results suggest that acetate treatment (in physiological concentrations) induces MCT-1 plasma membrane localization, which might explain that the plasma membrane transport of acetate also occurs by MCT-1 in CRC cells.

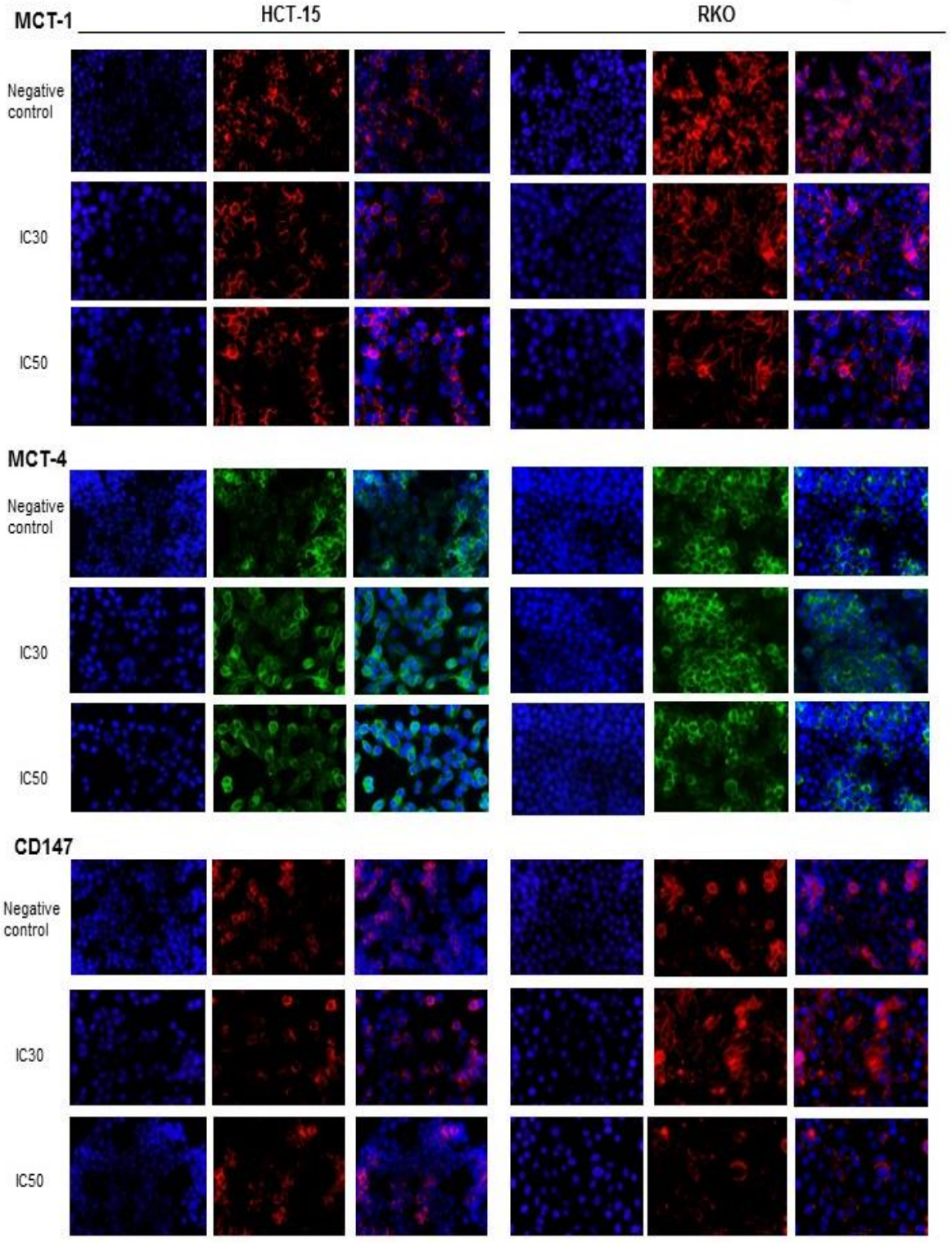

Figure 5: Localization of MCT-1, MCT-4 and CD147 in CRC cells after acetate treatment. HCT-15 and RKO cells were incubated with acetate (45 mM and 70 mM for HCT-15 cells; 75 mM and 110 mM for RKO cells, respective doses for the IC_{30} and IC_{50}) or with fresh medium (as negative control) for 48 hours. Representative images of immunofluorescence are shown (400× magnification).

3.3.5 Acetate treatment induces changes in the glycolytic metabolic profile in CRC cells

MCTs are known to be overexpressed in cancer cells to maintain high glycolytic rates by performing lactate efflux from glycolysis as well as pH regulation by the co-transport of protons (Pinheiro, Longatto-Filho et al. 2012). Herein, we showed that acetate increases MCT-1 localization at the plasma membrane upon acetate treatment being accompanied by an increase in MCT-1, MCT-4 and CD147 expression. We therefore wonder if acetate might induce metabolic changes like glucose consumption and lactate production. To test this hypothesis, CRC cell lines were exposed to IC_{30} and IC_{50} doses of acetate and the relative rate of glucose consumption and extracellular lactate production were measured at 3, 6, 12, 24 and 48 hours in triplicate and normalized to T0 (amount of glucose and lactate at time 0) and to cell biomass (by SRB assay). We found that CRC cells in response to acetate, exhibited a higher consumption of glucose ($p < 0.05$) and lactate production ($p < 0.05$; $p < 0.01$) up to 24 hours in a dose-dependent manner and then significantly decreased at 48 hours (Figure 6). RKO cells appear to be more glycolytic than HCT-15 cells since they exhibited a higher consumption of glucose ($p < 0.01$) and lactate production up to 12 hours after acetate treatment.

Glycolytic metabolism is a well-known characteristic of cancer cells which contributes to their aggressive phenotype, such as increased proliferation, suppression of anti-cancer immune response, invasion, angiogenesis and metastasis (Pelicano, Martin et al. 2006; Amorim, Pinheiro et al. 2015). Our results indicate that there is an increase in the glycolytic metabolism in response to short incubation periods of acetate, although after 48 hours of treatment, acetate induces a significant decrease in the glycolytic metabolism of CRC cells.

3.3.6 3BP potentiates acetate-induced apoptosis

It has been demonstrated that cancer cells exhibit the Warburg effect switching their metabolism to anaerobic glycolysis, characterized by glucose conversion into lactate even in the presence of oxygen (Jones and Bianchi 2015). Regarding our results on the metabolic profile and the elevated expression of MCT-1 and MCT-4

in CRC cells after acetate treatment we decided to study the effect of 3-bromopyruvate (3BP) in combination with acetate. 3BP is a small molecule analogue of lactate and pyruvate that acts as a glycolysis inhibitor and which is transported by MCTs in cancer cells (Ko, Smith et al. 2004; Pedersen 2012; Azevedo-Silva, Queiros et al. 2015). It was previously described by us the capability of butyrate to sensitize breast cancer cells to 3BP by increasing the expression and activity of MCTs (Queiros, Preto et al. 2012; Azevedo-Silva, Queiros et al. 2015). Our present results indicate that acetate have a similar effect in CRC cells regarding the expression of MCTs and thus we hypothesize that acetate could also sensitize these cells to 3BP.

To test this hypothesis, we used the colony formation assay (CFA) to analyze CRC cells treated with IC_{25} and IC_{50} values for the 3BP (17,5 µM, 35 µM and 75 µM, 150 µM, respectively for HCT-15 and RKO cells) alone or in combination with acetate treatment (only with the IC_{50} value for acetate: 70 mM, for HCT-15 and 110 mM for RKO cell lines) after 48 hours of incubation. We found that the combined treatment (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate) decreases cell proliferation (number of colonies formed) in both CRC cell lines in a 3BP dose-dependent manner in comparison to the negative control and to the same dose of 3BP or acetate alone (Figure 7a). The effect of the combined treatment was more evident in RKO cells compared to HCT15 cells.

Analysis of cell proliferation by SRB assay (Figure 7b) showed that IC_{25} and IC_{50} of 3BP reduced proliferation of HCT-15 and RKO cell being only significant with IC_{50} of 3BP. In addition, we found that combination of IC_{25} of 3BP/ IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate also reduced cell proliferation in both cell lines with a significant difference compared to the negative control. We also observed a significant difference between acetate treatment alone compared with the 3BP+acetate combination in both CRC cell lines ($p < 0.01$ and $p < 0.0001$), being more evident in RKO cells as reported previously.

We next analyzed cell death through AV/PI staining (Figure 7c) and found that the number of apoptotic (early and late) cells stained with AV (AV + PI - plus AV + PI +) were 11%, 13% and 7% for IC_{25} of 3BP, IC_{50} of 3BP and IC_{50} of acetate, respectively for HCT-15 cells in comparison to 3% in the negative control. In RKO cells the levels

of apoptotic cells stained with AV were 4%, 13% and 18% for IC_{25} of 3BP, IC_{50} of 3BP and IC_{50} of acetate, respectively in comparison to 2% in the negative control. In addition, the treatment with 3BP alone induces a significant increase ($p < 0.05$ and $p < 0.01$) in cells stained with AV – PI + in a dose-dependent manner in both CRC cells. We could observe that the combined treatment of 3BP and acetate (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate) potentiates apoptosis, as the number of cells stained with AV + PI - and AV + PI + showed a significant increase comparing with acetate alone ($p < 0.01$ and $p < 0.001$) in both CRC cells, being more evident in RKO cells. In summary, our results show that the combination treatments (acetate in physiologic concentration + 3BP) potentiate apoptosis in CRC cells.

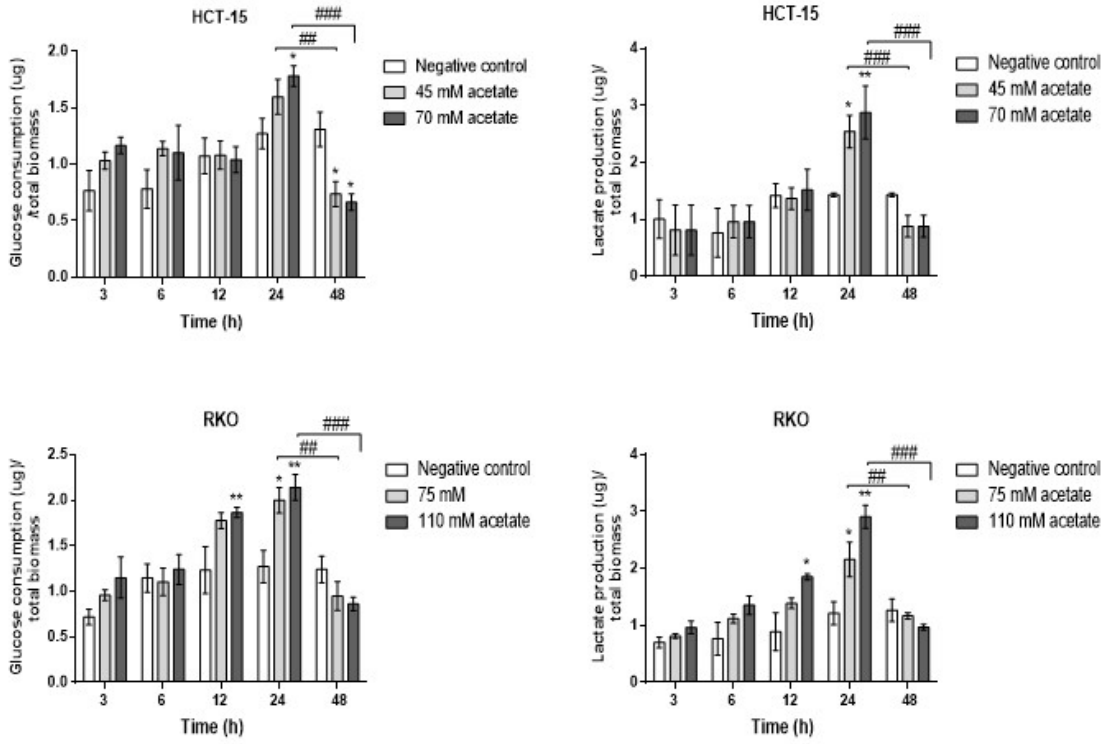

Figure 6: Acetate induces metabolic changes in CRC cells. HCT-15 and RKO cells were incubated with acetate (45 mM and 70 mM for HCT-15 cells; 75 mM and 110 mM for RKO cells, respective doses for the IC_{30} and IC_{50}) or with fresh medium (as negative control). Extracellular amounts of glucose consumption and lactate production overtimes 3, 6, 12, 24 and 48 hours are shown. Values are expressed as mean ± SD of at least three independent experiments. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 compared with control cells and # P ≤ 0.05, ## P ≤ 0.01 and ### P ≤ 0.001 comparing acetate treatment at 24 h with acetate treatment at 48 h.

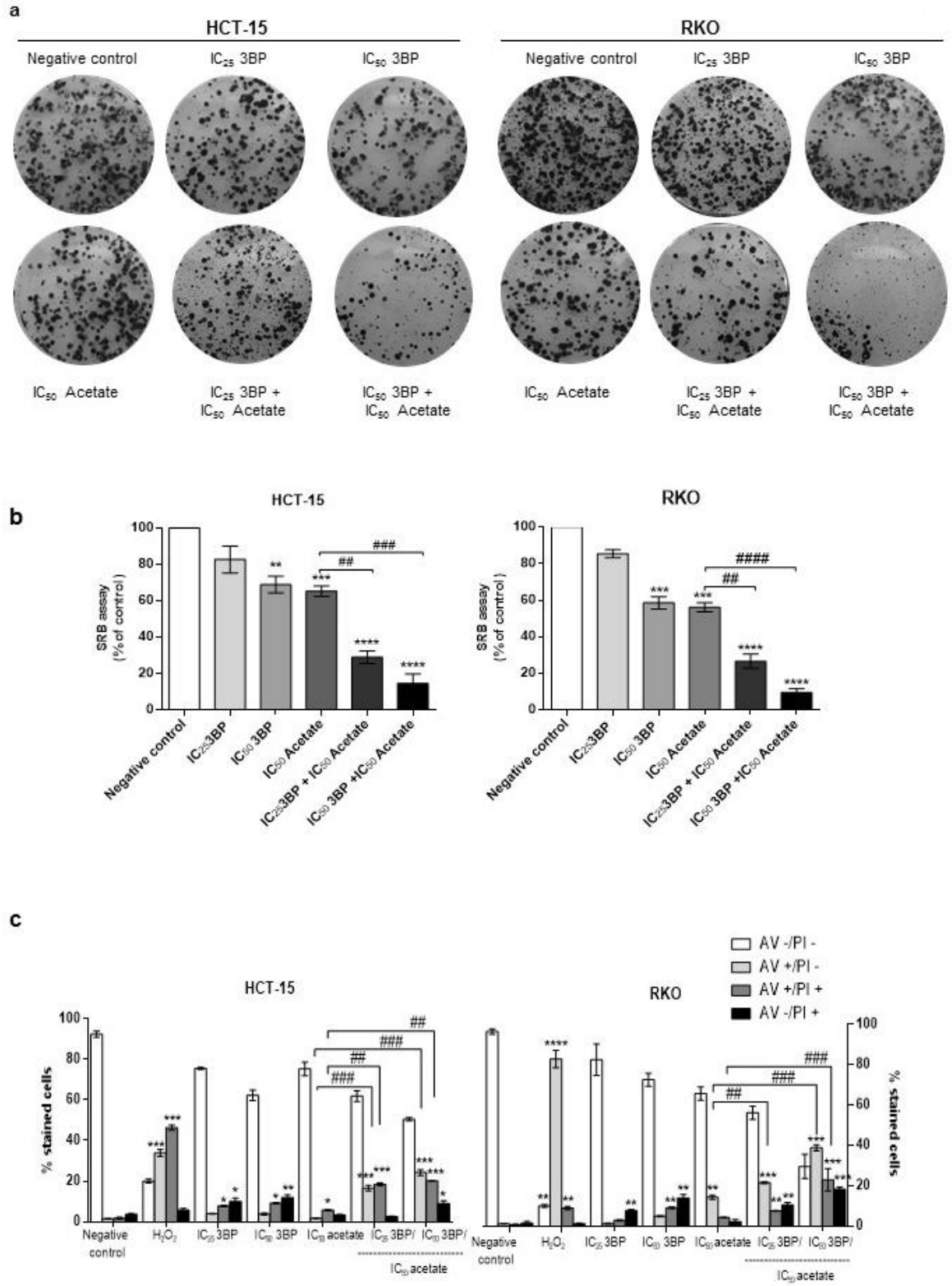

Figure 7: Effect of acetate with 3-bromopyruvate (3BP; a glycolysis inhibitor) in CRC cells. (a, b and c) CRC cells were treated with IC_{25} and IC_{50} values for the 3BP (17,5 uM, 35 uM and 75 uM, 150 uM, respectively for HCT-15 and RKO cells). The treatments were performed with 3BP alone or with acetate treatment combination (only the IC_{50} value for the acetate: 70 mM for HCT-15 and 110 mM for RKO cell line) after 48 h of incubation. 3BP was added 16 hours before to complete 48 hours of the treatment. As negative control, cells were treated with fresh medium. H_2O_2 (500 uM or 1 mM for HCT-15 or RKO cell lines, respectively) was used as positive control. **(a)** Colony formation assay shows that the combined treatment (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate) decreases cell proliferation (number of colony formed at the end of the assay) in both CRC cell lines

in a dose-dependent manner compared to the negative control and with the same dose of the 3BP or acetate alone. **(b)** Sulforhodamine B (SRB) assay analyzes the cell proliferation of the same conditions. Values represent mean ± SD of at least three independent experiments. **P ≤ 0.01, ***P ≤ 0.001 and ***P ≤ 0.0001 compared with negative control cells and ## P ≤ 0.01 and #### P ≤ 0.0001 comparing acetate alone with combined treatment (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate). **(c)** Quantitative analysis of AV/PI staining in HCT-15 and RKO cells. Values represent mean ± SD (AV – PI -, AV + PI -, AV + PI + and AV – PI +) of each condition (n=3). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ***P ≤ 0.0001 compared with negative control cells and ## P ≤ 0.01 and #### P ≤ 0.0001 comparing acetate alone with combined treatment (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate).

3.4: Acetate induces downregulation of ERK1/2 in colorectal cancer cells

CRC arises from a multistep process involving several genes and in particular alterations in the KRAS-BRAF-ERK signalling pathway. High levels of extracellular signal-regulated kinases 1/2 (ERK1/2) activation/expression is associated with cell proliferation and cell survival in various cancer cells, including CRC. SCFAs, namely butyrate and propionate inhibit cell proliferation through the inactivation or downregulation of ERK1/2 in colon cancer cells. However, little is know about how acetate regulates this pathway. Here, we aimed to study the effects of acetate on the levels of ERK expression in CRC cells. Our preliminary results show that acetate downregulates the levels of active ERKs in these cells, which is in accordance with previous published results using butyrate and propionate.

Preliminary results and discussion

3.4.1 Acetate treatment decreases the levels of ERKs in CRC cells

Activating mutations of KRAS and BRAF are frequently found in sporadic CRC - in approximately 35-40% and 5-10% of CRC cases, respectively (Velho, Moutinho et al. 2008; Yokota 2012). These mutation proteins are constitutively activated and trigger activation of the signalling cascade **RAS-RAF-MEK-ERK**, which permanently stimulates growth and differentiation of CRC cells (Yokota 2012). Furthermore, it has been described that ERK1 and ERK2 are key signalling molecules in the regulation of cell proliferation, transformation and cancer cell metastasis (Lee, Lee et al. 2015). It has been demonstrated that butyrate and propionate inhibit cell proliferation through the inactivation or downregulation of ERK1/2 in colon cancer cells HT-29 and RKO (Davido, Richter et al. 2001; Zhang, Zhou et al. 2010). Little is known about how acetate regulates ERK levels in CRC cell lines harbouring different genetic backgrounds. For this purpose, we used two CRC-derived cell lines, with a $KRAS^{G13D}$ mutation (HCT15) or a $BRAF^{V600E}$ mutation (RKO), and analysed the levels of phosphorylated ERKs in response to acetate treatment.

We found that acetate decreased the levels of phosphorytated ERKs in a dose-dependent manner in both CRC cell lines after 48 hours of acetate treatment (Figure 1). This result can be correlated with the decreased proliferation induced by acetate in CRC cells reported previously by us and others (Jan, Belzacq et al. 2002; Lan, Lagadic-Gossmann et al. 2007; Marques, Oliveira et al. 2013) and prompt for a possible role of acetate in the regulation of the ERK signalling pathway. Our results are in accordance with the results already obtained with butyrate and propionate in colon cancer cells (Davido, Richter et al. 2001; Zhang, Zhou et al. 2010). It would be interesting to assess the levels of MAPK/ERK kinase MEK (1/2) dual-specificity protein kinases which activate ERK1/2, to confirm our results that acetate is involved in the regulation of the **RAS-RAF-MEK-ERK** signalling pathway in CRC cells.

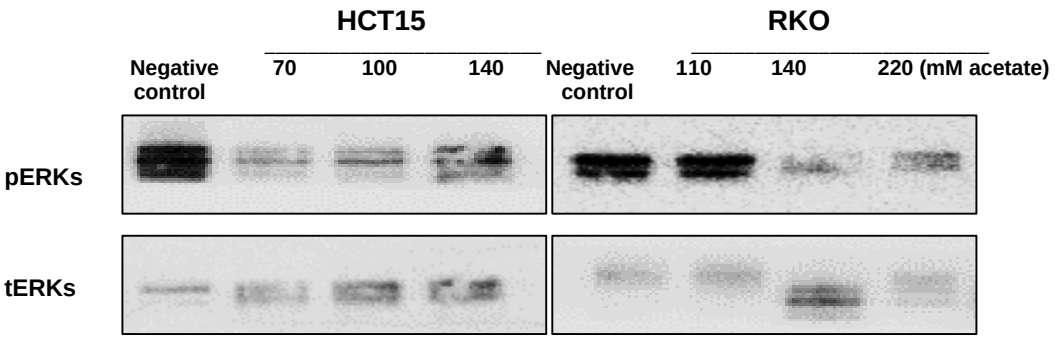

Figure 1: Acetate decreases the levels of ERKs in CRC cells. HCT-15 and RKO cells were treated with 70, 100 and 140 mM or 110, 140 and 220 mM of acetate, respectively, for 48 hours, or with fresh medium as a negative control. Representative immunoblot shows the levels of phosphorylated ERKs (pERKs) and total ERKs (tERKs). Total ERKs were used as a loading control.

CHAPTER 4:

General discussion

General discussion

Colorectal cancer is one of the most common solid tumors worldwide (Goncalves and Martel 2013; Altobelli, Lattanzi et al. 2014). Worldwide variations in colorectal cancer incidence suggest that dietary and lifestyle factors contribute to its etiology and are important risk factors for CRC. CRC is most susceptible to dietary influence, and a higher intake of dietary fibers can reduce the risk of CRC (Bingham, Day et al. 2003). Some of the significant health benefits of dietary fiber can be attributed to its microbial fermentation, namely by Propionibacteria in the colon into short-chain fatty acids (SCFAs: acetate, propionate and butyrate) (Comalada, Bailon et al. 2006; Macfarlane, Steed et al. 2008; Tang, Chen et al. 2011; Tang, Chen et al. 2011; Zhu, Michelle Luo et al. 2011). Both pure SCFA and propionibacteria culture supernatants from the dairy species *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici* induce apoptosis in CRC cells in vitro (Jan, Belzacq et al. 2002; Ruemmele, Schwartz et al. 2003; Lan, Lagadic-Gossmann et al. 2007). Moreover, administration of *P. freudenreichii* in vivo significantly increased the number of apoptotic epithelial cells damaged by 1,2-dimethylhydrazine, a carcinogenic agent, without affecting the survival of healthy normal colonic mucosa (Lan, Bruneau et al. 2007; Lan, Bruneau et al. 2008). Indeed, many studies suggest these SCFAs protect against carcinogenesis, since they reduce human colon cancer cell growth and differentiation and stimulate apoptosis in CRC cells (Comalada, Bailon et al. 2006; Lan, Lagadic-Gossmann et al. 2007; Janku, McConkey et al. 2011; Zeng, Lazarova et al. 2014). These characteristics indicate that propionibacteria might have a protective role against colon cancer, acting as a probiotic, and point to a useful role of propionibacteria and their metabolites propionate, butyrate and acetate as powerful agents for CRC prevention or therapy.

Acetate is one of the most important SCFAs, but has been less investigated than propionate and butyrate. For this purpose, we showed that acetate per se induces apoptosis in CRC-derived cell lines HCT-15 and RKO, by inducing DNA fragmentation, caspase activation, phosphatidylserine exposure to the outer leaflet of the plasma membrane and the appearance of a sub-G1 population. The concentrations used in our study were in the physiological range; total SCFA concentrations from 50–200 mM have been reported in the intestinal lumen of a

wide range of vertebrates and in most cases remain at constant high levels throughout the bowel length (Titus and Ahearn 1988; Scharrer, Amstutz et al. 1999). Our results are thus in agreement with previous reports showing that propionibacteria supernatants, as well as pure acetate and/or propionate, induce apoptosis in HT-29 and CACO-2 CRC cell lines (Jan, Belzacq et al. 2002). In these cells, a mixture of acetate and propionate induced caspase 3 activation, nuclei shrinkage, chromatin condensation and nuclei fragmentation into apoptotic bodies.

Furthermore, the cell microenvironment may be a determinant for the type of cell death induced by toxic stimuli. The pH of the human colon lumen ranges from 5.5–7.5. SCFA produced by *P. freudenreichii* trigger apoptosis in HT-29 cells at pH 7.5 but necrosis at pH 5.5. At pH 7.5, propionibacterial SCFA were shown to induce cell-cycle arrest in the G2/M phase and morphological characteristics of apoptotic cell death, like membrane blebbing, chromatin condensation and fragmentation, phosphatidylserine exposure and formation of apoptotic bodies (Lan, Lagadic-Gossmann et al. 2007). In accordance with these reports, we showed that acetate induces apoptosis in HCT-15 and RKO cells without altering the extracellular pH (complete medium). We also demonstrated that acetate alone inhibits proliferation in both the cell lines, in agreement with previous reports (Lan, Lagadic-Gossmann et al. 2007).

We have previously demonstrated in the yeast S. cerevisiae that acetic acid induces a mitochondria-mediated apoptotic process (Pereira, Chaves et al. 2010) with several features similar to apoptosis induced by SCFA in CRC cells. Notably, the mitochondrial inner membrane AAC carrier, the yeast ortholog of mammalian ANT, was required for mitochondrial outer membrane permeabilization and cytochrome c release in yeast cells committed to apoptosis induced by acetic acid. We also observed that Pep4p, the yeast CatD, was released from the lysosome-like vacuole to the cytosol in response to acetic acid (Pereira, Chaves et al. 2010). As acetic acid and acetate trigger apoptosis through an analogous pathway in both yeast and CRC cells, respectively, we hypothesized that partial LMP with CatD release might also be involved in acetate-induced apoptosis in CRC cells. The involvement of lysosomes in apoptotic cell death, mainly through partial LMP, has gained increased attention (Guicciardi, Leist et al. 2004). It is now established that they are important

contributors to cancer cell death, increasing interest in exploiting lysosomal cell death pathways as a potential target in cancer therapy (Kirkegaard and Jaattela 2009). LMP followed by release of lysosomal contents to the cytosol, especially cathepsins, seems to be the critical step of the lysosomal death pathway. The most relevant human cathepsins are the proteases CatB and CatL and the sole lysosomal aspartic protease CatD. They are most abundant in the lysosome (Turk, Stoka et al. 2002) and remain active at a neutral pH (Boya and Kroemer 2008). Overexpression of cathepsins often occurs in human cancers, and high levels of their expression can be associated with increased risk of relapse and poor prognosis (Palermo and Joyce 2008). However, besides the tumor-promoting effects of these proteases, there is evidence that cathepsins may also function as tumor suppressors (Lopez-Otin and Matrisian 2007).

Little is known about the role of the lysosomal pathway in cell death regulation in CRC. It has been shown that resveratrol, a naturally occurring polyphenol, triggers a caspase-dependent intrinsic pathway of apoptosis involving lysosomal CatD in CRC cells. The authors provide evidence that CatD, though not CatB or CatL, mediates resveratrol cytotoxicity in DLD1 and HT29 cell lines, inducing lysosome leakage with increased cytosolic CatD (Trincheri, Nicotra et al. 2007). In this study, we aimed to investigate the mechanisms underlying acetate induced apoptosis in CRC cells, focusing on the role of the lysosomal pathway through LMP and CatD release. We showed, for the first time, by different approaches that exposure of CRC cells to acetate leads to LMP, release of CatD to the cytosol and accumulation of Pro-CatD and mature CatD. We also showed that inhibiting CatD with PstA, a widely used specific inhibitor of CatD enzymatic activity (Emert-Sedlak, Shangary et al. 2005; Trincheri, Nicotra et al. 2007) increased acetate-induced apoptosis in CRC cells. Interestingly, in yeast, deletion of Pep4p confers higher susceptibility to acetic acid, while cells overexpressing Pep4p display higher resistance (Pereira, Chaves et al. 2010). Recently, it has also been demonstrated that Pep4p has a dual cytoprotective function, anti-apoptotic and anti-necrotic, during S. cerevisiae chronological aging via the polyamine pathway (Carmona-Gutierrez, Bauer et al. 2011). Our data in CRC cells are thus in agreement with the results obtained in yeast. Taken together, our results point to a function of CatD in cell protection rather than in the execution of acetate-induced cell death, in both CRC and yeast.

The role of CatD expression in colon carcinogenesis is controversial and poorly understood. Likewise, CatD has emerged as an important molecular target in cancer therapy, since it is overexpressed and secreted by cells of various tumor types, including CRC (Talieri, Papadopoulou et al. 2004; Kirana, Shi et al. 2012). CatD plays a vital role in extracellular matrix degradation and cancer cell survival, and actively participates in the invasion of carcinoma cells during both local invasion and metastasis formation (Szajda, Snarska et al. 2008; Carmona-Gutierrez, Bauer et al. 2011). However, its role in apoptosis depends on cellular types, specific contexts, stimulus and catalytic activity (Carmona-Gutierrez, Bauer et al. 2011; Hah, Noh et al. 2012). As SCFAs, and in particular acetate, have garnered increased interest as potential prevention/therapeutic agents in CRC, we sought to further understand the mechanism underlying the anti-apoptotic role of this lysosomal protease under this specific cellular and stimulus context. Based on data from our group and others, as well as on the present study, we propose that the lysosome and mitochondria communicate during acetate-induced apoptosis in CRC cells through permeabilization of both organelles and selective leakage of proteins. Indeed, we show that acetate triggers release of CatD to the cytosol, but not of CatL and only a small amount of CatB, which does not seem to have major consequences. Besides lysosomal alterations, acetate triggered mitochondrial dysfunction in a dose- and time-dependent-manner, characterized by increased mitochondrial ROS, changes in $\Delta\Psi m$ and an increase in mitochondrial mass, which were enhanced when CatD was inhibited. This mitochondrial dysfunction is in agreement with that reported during apoptosis induction by acetate and a mixture of acetate and propionate produced by *Propionibacterium freudenreichii* in other CRC cells (HT-29), including increased ROS and $\Delta\Psi m$ dissipation, as well as swelling in isolated mitochondria (Lan, Lagadic-Gossmann et al. 2007). However, the role of CatD or the lysosome in that context was not evaluated.

The increase in mitochondrial mass observed after exposure of cells to acetate led us to investigate whether the presumed decrease in mitochondrial turnover was associated with modulation of autophagy by this SCFA. Previous studies demonstrated that apoptosis triggered by low doses (1-10 mM) of propionate and butyrate can be delayed because autophagy is also induced, which can potentially impair the therapeutic efficacy of SCFAs in colon cancer (Tang, Chen et al. 2011;

Tang, Chen et al. 2011; Adom and Nie 2013). Indeed, autophagy has been implicated in cancer progression, used by cells for autophagic degradation of damaged organelles, long-lived proteins and pathogens, and in this way maintain homeostasis (Klionsky 2005; Mathew, Karantza-Wadsworth et al. 2007; Janku, McConkey et al. 2011). Further assays are required to ascertain whether low concentrations of acetate can cause a similar adaptive response in CRC cells. However, we previously showed that autophagy is not induced during acetic acid-induced apoptosis in yeast (Pereira, Chaves et al. 2010). In the present study, we demonstrate that acetate impairs the autophagic flux of HCT116 cells and decreases Beclin-1 levels in all three cell lines. Therefore, we show that concentrations of acetate normally present in the human intestine have a previously uncharacterized effect in CRC cells: inhibition of autophagy, most likely through impairment of autophagosome and lysosome fusion during acetate induced-apoptosis.

It is known that malfunctioning mitochondria are selectively targeted for autophagic degradation (Tang, Chen et al. 2011). However, it is conceivable that cells use alternative pathways to clear damaged mitochondria when autophagy is inactive. We therefore investigated if CatD is involved in mitochondrial degradation, like Pep4p in yeast, possibly playing a pro-survival role through elimination of dysfunctional mitochondria. We show that CatD protects RKO cells from acetate-induced oxidative stress and mitochondrial depolarization, since its inhibition with PstA or down-regulation with siRNA cause a significant increase in the levels of H_2O_2 and O_2^- and a decrease in $\Delta\Psi m$. Hah *et al.* 2012 have previously suggested that CatD has a protective role in H_2O_2-induced cell death in M059J glioblastoma cells through its involvement in autophagy, which enhances cell survival under oxidative stress. Accordingly, we had shown that Pep4p deletion increased the sensitivity of yeast cells to acetic acid associated with increased ROS accumulation (Pereira, Azevedo et al. 2013), though autophagy was not induced (Pereira, Chaves et al. 2010). These data, together with our results, support the notion that reduced CatD activity leads to increased ROS accumulation and mitochondrial depolarization. We therefore proceeded to determine whether CatD is involved in the degradation of damaged mitochondria, as we presumed to be the case during acetate-induced apoptosis in CRC cells. We found that down-regulation of CatD in

acetate-treated RKO cells increased mitochondrial mass, as well as the cellular pool of AIF. These findings corroborate our hypothesis that CatD is involved in the degradation of damaged mitochondria during acetate induced-apoptosis through an autophagy-independent process. Though not sufficiently efficient to maintain mitochondrial mass homeostasis, as an acetate-induced increase in mitochondrial mass is still observed, this alternative degradation process allows the cell to dispose of dysfunctional mitochondria and delay cell death. It will be interesting in the future to determine if CatD is also released and is involved in mitochondrial degradation in response to other SCFAs, either directly or through downstream substrates. Inhibition of CatD with PstA mimicked the results obtained with CatD siRNA, indicating the catalytic activity of CatD is required for this function, as we previously found for the anti-apoptotic role of both yeast and human CatD (Marques, Oliveira et al. 2013; Pereira, Azevedo et al. 2013). We now also show that the role of yeast CatD in acetic acid-induced mitochondrial degradation depends on its proteolytic activity and can be complemented by human CatD, indicating this mechanism is conserved through evolution.

As discussed above, partial LMP followed by a release of lysosomal hydrolases into the cytosol can activate intrinsic caspase-dependent apoptosis or a caspase-independent alternative cell death pathway (Repnik, Stoka et al. 2012; Groth-Pedersen and Jaattela 2013). Thus, disabling lysosome function is under investigation as an adjuvant therapeutic approach to sensitize cells to apoptosis-inducing agents (Repnik, Stoka et al. 2012). Destabilization of the lysosomal membrane has been proposed as a promising strategy, since it would promote apoptosis through initiation of the lysosomal pathway in cancer cells (Groth-Pedersen and Jaattela 2013). However, our results show that the release of lysosomal proteases, in particular of CatD (Marques, Oliveira et al. 2013), may enable a degradation process alternative to autophagy with a similar protective role in cell survival, thus counteracting activation of the lysosomal death pathway. This would negatively impact the efficacy of acetate, and probably of other therapeutic compounds that trigger LMP and impair autophagy, and would require inhibiting CatD in combination treatments.

In summary, these first findings based on the clues provided by the yeast system unveiled a novel pro-survival function of CatD in autophagy-independent mitochondrial degradation, which can enhance cell survival in CRC cells undergoing acetate-induced apoptosis. These results offer new perspectives in the intricate regulation of life and death, and suggest using CatD inhibitors as adjunct therapy with SCFAs for prevention/therapy of colorectal cancer.

In addition to these findings, acetate is a monocarboxylic acid, which figures among the most abundant SCFA in the human organism and is absorbed at the intestinal level being able to reach distant organs (Moschen, Broer et al. 2012; Kasubuchi, Hasegawa et al. 2015). Acetate transport across biological membranes in the colon model is not well understood, thus here we deepen the study of the kinetics and energetics of acetate transport across CRC cells. Our results indicate the participation of sodium dependent transporters such as SMCT-1 or anion-exchangers (AE-1 for example) in the uptake of acetate in CRC cells. These results are in accordance with several reports showing that SMCT-1 transport monocarboxylic acids such as butyrate, 3BP and dichloroacetate in cancer cells (Thangaraju, Cresci et al. 2008; Thangaraju, Karunakaran et al. 2009; Babu, Ramachandran et al. 2011) and it has also been described that anion-exchangers might be involved in acetate transport in HT29-18-C1, a CRC cell line (Rowe, Lesho et al. 1994).

Our results also show that at pH 6.0 (similar to the gut environment) acetate transport across CRC cells membrane occurs preferentially via a sodium-dependent mechanism possibly by SMCT-1. Indeed this is in agreement to the fact that monocarboxylate transport family members (MCT 1-4) are proton symporters, involved in the uptake and/or efflux of pyruvate, lactate, ketone bodies and SCFA through the plasma membrane (Pinheiro, Longatto-Filho et al. 2012; Kim, Park et al. 2014). The literature on acetate transport is scarce and little is known about SCFA transport mediated by MCTs in CRC cells, being that the majority of the studies use butyrate as model to study SCFA transport, both in normal intestinal epithelial and CRC cells (Hadjiagapiou, Schmidt et al. 2000; Ganapathy, Thangaraju et al. 2013; Goncalves and Martel 2013). In this regard, some studies demonstrated that butyrate can be transported into colon cancer cells mainly by MCT-1

(Hadjiagapiou, Schmidt et al. 2000; He, Li et al. 2007; Goncalves and Martel 2013). It has also been reported that MCT-1 is responsible for acetate transport in mouse cancer cells with an affinity constant of 3.7 mM (Carpenter and Halestrap 1994). Herein we showed for the first time that MCT-1 participates actively in the transport of acetate into CRC cells.

Interestingly our data suggest that other transport mechanisms such as passive diffusion through aquaporins might also contribute to acetate uptake. Aquaporins are important membrane proteins for cellular homeostasis, being known to be upregulated in different types of cancer including CRC (Papadopoulos and Saadoun 2014), suggesting their capacity to transport not only water but other compounds. To the best of our knowledge, this is also the first report suggesting that aquaporins might play a novel role in acetate transport in colorectal cancer.

Acetate is an important messenger in the symbiotic relationship between intestinal microbiota and colonocytes and is thus predictable that may play an important role in the regulation of transporters expression and metabolism of colon human cells (Moschen, Broer et al. 2012; Kasubuchi, Hasegawa et al. 2015). These issues are poorly understood and needed further understanding namely in CRC cells. Our data suggest that acetate might interfere with MCT-1 expression and with its cellular localization influencing the total acetate uptake/transport. Accordingly, it has been reported that butyrate and other SCFA, may modulate cell behavior by activation of several specific proteins depending of concentration and cell models used (Hadjiagapiou, Schmidt et al. 2000; Canani, Costanzo et al. 2011; Kasubuchi, Hasegawa et al. 2015).

Despite the controversy in the literature concerning MCT expression in CRC, it has been shown that MCTs (especially MCT-1, MCT-2 and MCT-4) are usually upregulated in CRC tumors compared to normal epithelium samples (Pinheiro, Longatto-Filho et al. 2008; Pinheiro, Reis et al. 2010; Pinheiro, Longatto-Filho et al. 2012). We have previously shown that butyrate led to an increase in MCT-1, MCT-4 and CD147 expression with an increased localization of MCT-1 expression at the plasma membrane in breast cancer cells (Queiros, Preto et al. 2012; Azevedo-Silva, Queiros et al. 2015). Our results on CRC cells demonstrated that acetate is also capable of inducing the expression of MCT-1, MCT-4 and CD147 with an increase

in plasma membrane localization of MCT-1. Our observations suggest that MCT-1 might be involved in acetate transport across the membrane by regulating MCT-1 expression and activation at the plasma membrane.

The mechanisms involved in the regulation of MCTs in CRC cells in response to SCFA (especially acetate), are not well clarified, being the majority of the reports focused in the role of butyrate in CRC cells (Hadjiagapiou, Schmidt et al. 2000; He, Li et al. 2007; Ganapathy, Thangaraju et al. 2013; Goncalves and Martel 2013). Some studies have reported that MCT-1, MCT-4, MCT-5 and MCT-6 isoforms are also expressed in CRC cells (Hadjiagapiou, Schmidt et al. 2000). In this regard, it has been shown that upon butyrate treatment, MCT-1 is the most abundant MCT isoform expressed in Caco-2, a CRC derived cell line (Hadjiagapiou, Schmidt et al. 2000). Furthermore, some investigators have shown that SMCT-1 was silenced by DNA methylation in CRC cells (Thangaraju, Cresci et al. 2008; Babu, Ramachandran et al. 2011; Ganapathy, Thangaraju et al. 2013; Goncalves and Martel 2013), which conferred a selective advantage to escape butyrate-induced cell death (Li, Myeroff et al. 2003; Ganapathy, Thangaraju et al. 2013). Taking into account our results, we cannot exclude the involvement of SMCT-1 as the Na^+-linked symporter for acetate in CRC cells, since SMCT-1 was expressed after acetate treatment in CRC cells. To the best of our knowledge, this is the first report showing that the SCFA acetate leads to an increase in MCTs expression and to a cellular re-localization of MCT-1 in CRC cells. Our results put forward a possible regulatory role of acetate at both MCT-1 expression and localization in CRC cells, similarly to what was described by us with butyrate in breast cancer cells (Azevedo-Silva, Queiros et al. 2015).

Our data also show that MCT-1 re-localization to the plasma membrane is associated with increased expression of MCT-1 and CD147 in response to acetate treatment. Indeed it has been described that MCT-1 and MCT-4 require the presence of CD147, for proper localization and function (Halestrap and Meredith 2004).

MCT-4 expression has been associated with highly glycolytic tissues (including CRC), where it is essentially responsible for lactate efflux (Pinheiro, Longatto-Filho et al. 2012; Baltazar, Pinheiro et al. 2014). We observed an elevated MCT-4

expression after 48 hours of acetate treatment that can be related with the increased glucose consumption and lactate production upon 24h of acetate treatment in CRC cells. Our findings are in agreement with Matthews and co-workers, which described that butyrate and propionate, alone or in combination, significantly increased glucose consumption at 24 hours with a considerable decrease 48 hours after treatment (Matthews, Howarth et al. 2012). The authors explained the increased rate in glucose consumption as a way to provide energy efficiently and then the decreased rate in glucose consumption associated with the use of butyrate as second source of energy in CRC cells (Matthews, Howarth et al. 2012).

It is known that CRC cells use 30 to 40 times more glucose for energy metabolism than normal colon cells (He, Li et al. 2007). As glucose becomes the primary source in CRC cells, we also evaluated the expression of GLUT-1, the main glucose transporter detected in colorectal cancer tissues (Lambert, Wood et al. 2002; He, Li et al. 2007; Amorim, Pinheiro et al. 2015). Interestingly, we found that both CRC cells express GLUT-1 but the levels remain unchanged after acetate treatment over the time. Therefore the decrease in glucose consumption is not due to a decrease uptake and further studies are required to assess whether it relates to inhibition of the glycolytic flux as already reported in yeast in response to acetic acid (Pampulha and Loureiro-Dias 2000).

It is known that cancer cells usually express high levels of MCTs to maintain a highly glycolytic profile and an appropriate pH environment essential for tumor growth. MCTs became attractive targets in cancer therapy, especially in cancer with a hyper-glycolytic phenotype (Halestrap 2013; Baltazar, Pinheiro et al. 2014). Furthermore, MCTs can also be seen as Trojan horses and recent reports have demonstrated that MCTs are also involved in the transport of drugs such as 3BP (Pedersen 2012; Azevedo-Silva, Queiros et al. 2015). In this case, the elevated MCT expression is used to mediate the entry of the chemotherapeutic agent 3BP into the cells and then selectively kill cancer cells (Baltazar, Pinheiro et al. 2014; Azevedo-Silva, Queiros et al. 2015). 3BP is an anti-cancer agent that targets energy metabolism of cancer cells (Azevedo-Silva, Queiros et al. 2015). Our results showed that the combination of acetate and 3BP potentiates the effect of acetate or 3BP alone in cell proliferation inhibition and apoptosis induction in CRC cells. This result is in agreement with our

previous data showing that butyrate also sensitize breast cancer cancer cells to 3BP treatment (Queiros, Preto et al. 2012; Azevedo-Silva, Queiros et al. 2015).

Summing up, here we show that acetate uptake involves at least two mechanisms of transport in CRC cells: mediated by MCTs and diffusion by aquaporins. Indeed, acetate induces changes in glycolytic metabolism, upregulation of MCT-1, MCT-4 and CD147 and plasma membrane localization of MCT-1. These findings may provide a novel strategy for CRC therapy based on the elimination of CRC cells in the presence of acetate through their sensitization by 3BP or other glycolytic metabolism inhibitor whose transport is mediated by MCTs.

CHAPTER 5:

Concluding remarks and future perspectives

5.1 Concluding remarks

The main conclusions of the results addressed in the present study are represented as a squematic model in Figure 1. In summary the results obtained in this thesis allowed us to conclude that:

I. Acetate inhibits cell proliferation and induces apoptosis in CRC cells. Acetate-induced apoptosis is characterized by DNA fragmentation, caspase activation, phosphatidylserine exposure to the plasma membrane leaflet and the appearance of a population with sub-G1 DNA content.

II. Acetate-induced apoptosis also involves the lysosomal pathway, with lysosome membrane permebilization (LMP), lysosome pH variation and specific release of CatD into the cytosol in CRC cells. CatD, but not CatB and CatL, has a protective role in this process.

III. Acetate-induced apoptosis in CRC cells involves a crosstalk between the lysosome and mitochondria, characterized by mitochondrial alterations, such as Reactive Oxygen Species (ROS) (H_2O_2 and O_2^-) accumulation, an increase in mitochondrial mass, mitochondrial membrane depolarization, elevated expression of mitochondrial proteins, namely the apoptosis inducing factor (AIF), the voltage dependent anion channel (VDAC1) and a subunit of the outer mitochondrial membrane translocator (TOM22). Active CatD, but not CatB and CatL, mediates the degradation of damaged mitochondria protecting CRC cells from acetate-induced apoptosis.

IV. Autophagy is impaired during acetate-induced apoptosis in CRC cells.

V. The plasma membrane transport of acetate is mediated by a sodium dependent transporter (SMCT-1) and might also occur through passive diffusion by aquaporins. MCT-1 is also involved in acetate uptake.

VI. Acetate increases the expression of MCT-1, MCT-4 and CD147 with an increase in MCT-1 localization at the plasma membrane of CRC cells, which can be correlated with the regulation of acetate transport.

VII. Acetate induces significant changes in glycolytic metabolism (decreasing glucose consumption and lactate production).

VIII. The combination treatment of acetate in a physiologic concentration and the glycolysis inhibitor 3BP potentiates acetate-induced apoptosis in CRC cells.

IX. Although preliminary, our results also suggest that acetate decreases ERK levels in CRC cells, which is consistent with its role in inhibition of proliferation and a possible role of acetate in the regulation of the ERK signalling pathway.

Taken together, our findings led us to conclude that the use of CatD inhibitors in combination with strategies to increase acetate concentrations in the colon, namely emerging nutraceuticals, could potentiate acetate-mediated cancer cell death, presenting a novel strategy for prevention or therapy of CRC. Furthermore, as acetate increase MCTs membrane expression, the use of chemotherapeutic agents transported by MCTs like 3BP may be as a new way to increase the sensitivity of CRC cells to 3BP therapy or similar drugs.

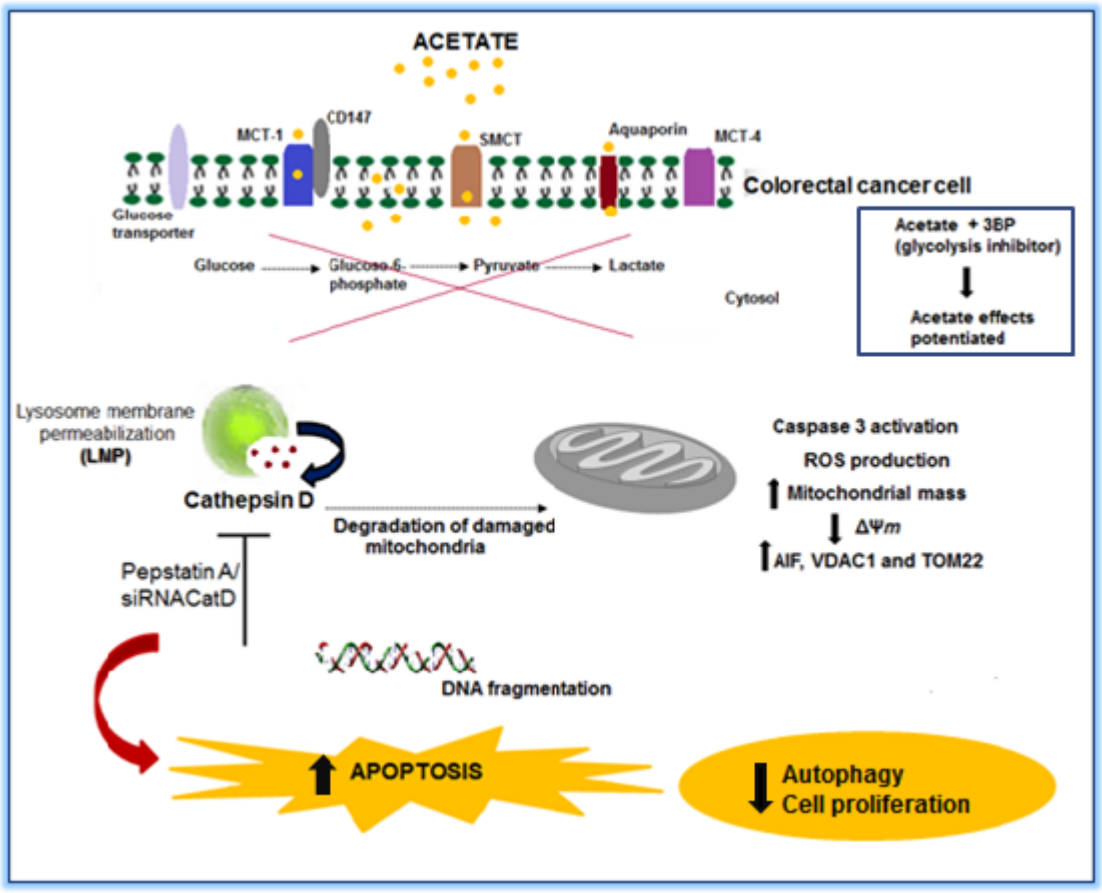

Figure 1: Schematic representation of different cell processes involved in apoptosis triggered by acetate in colorectal cancer cells. Different transporter systems which may mediate acetate uptake by CRC cells are represented. Acetate transport can be mediated by mocarboxylate transporters (MCT), such as SMCT-1 and MCT-1 (only in acetate pre-treated cells) or via passive diffusion by aquaporins. Acetate induces an increase in the expression of MCT-1, MCT-4 as well as of CD147 that after glycosylation leads to plasma membrane re-localization of MCT-1 and glycolysis perturbation. Acetate also induces partial lysosome membrane permeabilization (LMP) with specific release of cathepsin D (CatD) into the cytosol, which protects cells from acetate-induced apoptosis. DNA fragmentation and mitochondrial dysfunctions (accumulation of reactive oxygen species ROS; increase of mitochondrial mass, decrease of mitochondrial membrane potential, $\Delta\Psi m$; caspase 3 activation, and increase in the levels of different mitochondrial proteins such as apoptosis inducing factor, AIF; voltage dependent anion channel, VDAC1 and outer mitochondrial membrane translocator, TOM22) are other cellular events associated with acetate-induced apoptosis. Under these conditions autophagy is not induced and the inhibition of CatD (with pepstatin A or siRNA) enhanced acetate-induced apoptosis associated with a higher mitochondrial dysfunction. CatD plays a role in the degradation of damaged mitochondria when autophagy is impaired in CRC cells. Combined treatment (acetate + 3-bromopyruvate, 3BP, a well-known glycolysis inhibitor) potentiates the acetate effects, decreasing cell proliferation and inducing elevated rate of apoptosis.

5.2 Future perspectives

Our results identified several mechanisms involved in acetate-induced apoptosis in CRC cells, but more studies are still needed to understand how acetate (alone or in combination with other SCFAs) can be used as a nutraceutical approach in CRC. In order to pursue the work here presented, the following studies are suggested:

I. Although the main SCFAs (butyrate, propionate and acetate) are not produced alone at the intestine level, there are still few studies using the combination of these compounds in CRC cells. Thus, it is important to explore their combinatory effects as a strategy to prevent and/or to treat CRC.

II. To determine if the lysosome pathway is involved in butyrate and propionate-induced cell death in CRC cells, since these two SCFAs also induce apoptosis with mitochondrial alterations/dysfunctions (Jan, Belzacq et al. 2002; Comalada, Bailon et al. 2006; Lan, Lagadic-Gossmann et al. 2007; Tang, Chen et al. 2011; Tang, Chen et al. 2011). It will also be important to define the role of cathepsins, especially CatD, CatB and CatL in apoptosis induced by butyrate and propionate in CRC cells. We have already determined the inhibitory concentration (IC_{50}) for butyrate and propionate in CRC cells (see **Supplementary results, Figures 1** and **2**).

III. Our group recently demonstrated that expression of CatD in yeast compensates the loss of Pep4p (the yeast ortholog of human CatD), indicating that these two proteins have a similar role in mitochondrial degradation independently of autophagy (Oliveira, Pereira et al. 2015). This finding provides new perspectives by using yeast expressing CatD to explore the role of CatD in degradation of damaged mitochondria and to uncover possible CatD partners in this process.

IV. It has been demonstrated that hypoxia (oxygen deficiency) is a feature of solid tumors, including CRC, often resistant to conventional cancer therapies and correlated with advanced stages of malignancy (Kim, Lin et al. 2009; Foster, Wong et al. 2014). Thus, regarding our results in normal conditions, it could be important

to study the effects of acetate focusing in the role of CatD under hypoxia conditions in CRC cells.

V. It has been demonstrated that acetylation of histones in cancer cells is usually associated with the reactivation or silencing of genes critical for cancer progression, differentiation and apoptosis (Kiefer, Beyer-Sehlmeyer et al. 2006). In this regard, it has been shown that SCFAs, namely butyrate and propionate, induce hyperacetylation of H3 and H4 core histones by inhibiting histone deacetylases (HDACs) in CRC cells (Hinnebusch, Meng et al. 2002; Kiefer, Beyer-Sehlmeyer et al. 2006), but little is know about the effect of acetate in that process. Thus, we suggest assessing the role of acetate as an inhibitor of histone deacetylases by evaluating H3 and H4 acetylation using immunoblot analysis in CRC cells.

VI. Regarding our results showing downregulation of ERKs upon acetate treatment, it could be interesting to verify the levels of MEKs under the same conditions, to confirm that acetate is also involved in the regulation of the **RAS-RAF-MEK-ERK** signalling pathway in CRC cells. Furthermore, to understand the role of KRAS and BRAF oncogenes signalling pathway in acetate-induced apoptosis by downregulating their expression using RNAi in CRC cells.

VII. According to our results regarding acetate transport, it is necessary to study the rates of acetate uptake by downregulation (RNAi) of the main MCTs involved in that process, namely SMCT-1 and MCT-1 to verify the dependence of acetate in use these transporters. This, coud be useful in future works to explore the use of combined treatment (acetate and chemotherapeutic agents which use the same transporters as acetate) to potentiate its effects in CRC cells.

VIII. It is know that 5-fluorouracil (5-FU) is the main anticancer agent used in CRC treatment, but resistance to this drug in CRC patients still occurs (Zhang, Yin et al. 2008). Taking our results into consideration, we suggest evaluating several combination treatments (such as 5-FU plus acetate, 5-FU plus CatD inhibitors – Pepstatin A or the downregulation of CatD by RNAi and 5-FU plus acetate and

CatD inhibitors) as new possible strategy to improve CRC treatment reducing the chemoresistance described by the use of 5-FU.

Supplementary results

5.2.1 Determination of half-maximal inhibitory concentration (IC_{50}) values for butyrate and propionate in CRC cells.

We determined the values of half-maximal inhibitory concentration (IC_{50}) for butyrate and propionate in HCT-15 and RKO cells, important for future studies. The IC_{50} values of butyrate and propionate were calculated by SRB assay after 48 hours of treatment (Figure S1 and S2). The IC_{50} values for butyrate were: 14 mM and 48.7 mM for HCT-15 and RKO cells, respectively (Figure S1). The IC_{50} values for propionate were 20 mM and 59 mM for HCT-15 and RKO cells, respectively (Figure S2). We could observe that both butyrate and propionate led to a decrease in cell proliferation, like acetate (but in lower concentrations than acetate). Since these SCFAs are also produced and have an important role at the intestine level, it becomes important to study their effect alone and in combination to gain understanding of the role of these compounds in the CRC context.

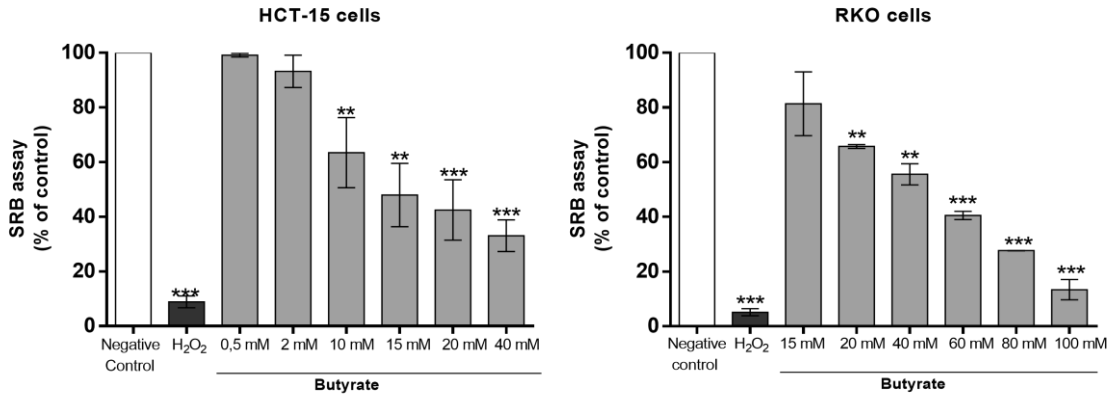

Figure S1: Effect of butyrate on cell proliferation, determined by SRB assay. HCT-15 and RKO cells were seeded at a density of $7x10^4$ and $5x10^4$cells/well, respectively, and incubated with different concentrations of butyrate (48 hours). As positive control were used 500 µM and 1mM of H_2O_2, respectively for HCT-15 and RKO cells. As a negative control cells were incubated with fresh complete medium. For each bar, the mean for at least three indepent experiments is represented. (Bonferroni's test; **P ≤ 0.01 and ***P ≤ 0.001 compared to control cells).

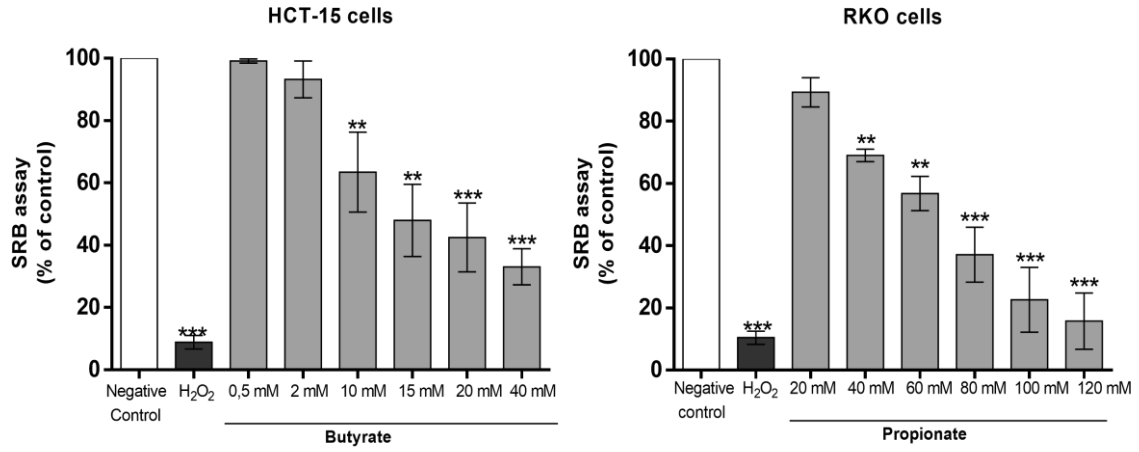

Figure S2: Effect of propionate on cell proliferation, determined by SRB assay. HCT-15 and RKO cells were seeded at a density of $7x10^4$ and $5x10^4$cells/well, respectively, and incubated with different concentrations of propionate (48 hours). As positive control were used 500 µM and 1mM of H_2O_2, respectively for HCT-15 and RKO cells. As a negative control cells were incubated with fresh complete medium. For each bar, the mean for at least three indepent experiments is represented. (Bonferroni's test; **P ≤ 0.01 and ***P ≤ 0.001 compared to control cells).

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ANNEX:

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Acetate-induced apoptosis in colorectal carcinoma cells involves lysosomal membrane permeabilization and cathepsin D release

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Colorectal carcinoma (CRC) is one of the most common causes of cancer-related mortality. Short-chain fatty acids secreted by dietary propionibacteria from the intestine, such as acetate, induce apoptosis in CRC cells and may therefore be relevant in CRC prevention and therapy. We previously reported that acetic acid-induced apoptosis in *Saccharomyces cerevisiae* cells involves partial vacuole permeabilization and release of Pep4p, the yeast cathepsin D (CatD), which has a protective role in this process. In cancer cells, lysosomes have emerged as key players in apoptosis through selective lysosomal membrane permeabilization (LMP) and release of cathepsins. However, the role of CatD in CRC survival is controversial and has not been assessed in response to acetate. We aimed to ascertain whether LMP and CatD are involved in acetate-induced apoptosis in CRC cells. We showed that acetate *per se* inhibits proliferation and induces apoptosis. More importantly, we uncovered that acetate triggers LMP and CatD release to the cytosol. Pepstatin A (a CatD inhibitor) but not E64d (a cathepsin B and L inhibitor) increased acetate-induced apoptosis of CRC cells, suggesting that CatD has a protective role in this process. Our data indicate that acetate induces LMP and subsequent release of CatD in CRC cells undergoing apoptosis, and suggest exploiting novel strategies using acetate as a prevention/therapeutic agent in CRC, through simultaneous treatment with CatD inhibitors.
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Colorectal carcinoma (CRC) is a leading cause of cancer death worldwide (GLOBOCAN 2008), and thus finding new prevention and therapeutic approaches is of prime importance. Propionibacteria, found in fiber-rich food and dairy products, produce short-chain fatty acids (SCFA), mainly propionate and acetate, which induce apoptosis in CRC cells. Their potential in cancer prevention and therapy has thus been proposed by several authors[1–6] and understanding the mechanisms underlying acetate-induced cell death should provide new prevention/therapeutic strategies in CRC.

The lysosomal pathway of apoptosis involves partial lysosomal membrane permeabilization (LMP), with subsequent release of proteases (such as cathepsins) into the cytosol.[7,8] However, the role of this pathway in cancer is controversial. Transformation can lead to several lysosomal changes, such as increased lysosomal volume, secretion of proteases and total protease activity,[9] and changes in the subcellular localization of cathepsins B, D and L (CatB, CatD and CatL, respectively).[10] Such alterations become pro-oncogenic when enhanced secretion of cathepsins initiates proteolytic pathways that increase neoplastic progression.[11] Increased cysteine cathepsin activity is important for tumor angiogenesis, proliferation, growth and invasion.[12] Cathepsins are often overexpressed in human cancers, and high expression levels have been associated with increased risk of relapse and poor prognosis.[13] In contrast to their tumor-promoting effects, there is also evidence that they function as tumor suppressors.[14] This opposing role depends on the context: if cathepsins are released intracellularly they contribute to cancer cell death, but if released extracellularly they break down the extracellular matrix, stimulating angiogenesis and migration.[10]

CatD is a lysosomal aspartyl protease involved in autophagy and apoptosis, thus having a crucial role in cell fate and tissue homeostasis.[15] CatD has emerged as a central player in the apoptotic response, although its role is cell-type and context-dependent. Expression patterns of CatD protein are divergent in CRC, suggesting a complex regulation and function of this protease,[16,17] though its precise role remains poorly understood.

We previously showed that the vacuolar protease Pep4p, the *Saccharomyces cerevisiae* CatD, translocates to the cytosol during acetic acid-induced apoptosis, suggesting that the release of a vacuolar protease during regulated cell death is also conserved in yeast.[18] We additionally showed that Pep4p has a role in cell protection rather than in the execution

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Keywords: Cathepsin-D; lysosomal membrane permeabilization (LMP); apoptosis; acetate; colorectal carcinoma
Abbreviations: AO, Acridine Orange; AV, annexin V; BrdU, bromodeoxyuridine; CatB, cathepsin B; CatD, cathepsin D; CatL, cathepsin L; CRC, colorectal carcinoma; E64d, (2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; LMP, lysosomal membrane permeabilization; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PstA, pepstatin A; SCFA, short-chain fatty acids; SRB, sulforhodamine B; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling
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of acetic acid-induced cell death. These results raised the possibility that partial LMP and consequent CatD release was involved in the response of CRC cells to acetate. Here, we show that CatD is released from lysosomes and might protect CRC cells from acetate-induced apoptosis. Our data therefore establish the lysosome and CatD as novel targets of acetate in CRC cells and indicate that CatD activity has important repercussions in the sensitivity of CRC to acetate produced in the intestine that might have prevention/therapeutic implications.

Results

Acetate induces apoptosis and inhibits cell proliferation in CRC cell lines. CRC-derived cell lines HCT-15 and RKO were treated with different concentrations of acetate for 24 and 48 h and cell viability assessed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction test. After 24 h, there was no statistically significant decrease in viability of acetate-treated cells in either cell line, in comparison with untreated cells (not shown). The half-maximal inhibitory concentration (IC_{50}) of acetate was therefore calculated from the mean values of MTT reduction after 48 h of treatment: 70 mM and 110 mM for HCT-15 and RKO cells, respectively (Figure 1a). IC_{50}, $2 \times IC_{50}$ and an intermediate concentration of acetate were used in subsequent studies.

The decrease in cell viability determined by MTT assay might be due to decreased cell proliferation, increased cell death or both. We therefore assessed cell proliferation after exposure to acetate using sulforhodamine B (SRB) and bromodeoxyuridine (BrdU) assays and apoptosis using Annexin V/propidium iodide (AV/PI), caspase activity and terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) assays, as well as sub-G1 population analysis by flow cytometry. The IC_{50} of acetate reduced cell proliferation by approximately 30% and 65% in HCT-15 and RKO cells, respectively, as determined by SRB assay (Figure 1b). In HCT-15 cells, IC_{50} and $2 \times IC_{50}$ of acetate reduced proliferation by approximately 17% and 75%, respectively, as determined by BrdU assay (Figures 1c and d).

We next analyzed cell death through AV/PI staining and found that acetate induced exposure of phosphatidylserine to the outer leaflet of the plasma membrane in HCT-15 cells in a dose-dependent manner (Figure 2a). The number of cells stained with AV ($AV+PI-$ plus $AV+PI+$) increased from 3.9% in the untreated control to 8.0, 18.2 and 47.9% after exposure to 70, 100 and 140 mM acetate, respectively, and to 49.1% when cells were exposed to etoposide for 48 h (Figure 2b). Levels of necrotic cells ($AV-PI+$) after exposure to 70 or 100 mM acetate were very low. A higher dose of acetate (140 mM) increased the number of necrotic cells ($\sim$4% $AV-/PI+$), similarly to etoposide, although the majority of the population ($\sim$48%) was in early and late apoptosis ($AV+PI-$ plus $AV+PI+$) (Figure 2b). These results led us to conclude that acetate induces apoptosis rather than necrosis. Accordingly, caspase 3 was also activated in HCT-15 cells, as cleavage of the fluorogenic caspase 3 substrate z-DEVD-AMC, expressed in arbitrary fluorescence units/min/μg protein, increased from 3.6 in HCT-15 control cells (untreated) to 24.4 after exposure to 140 mM acetate (Figure 2c). Similar results were obtained when activation of total caspases was assessed by labeling with the fluorescent pan-caspase inhibitor FICT-VAD-fmk (not shown). We also assessed the levels of DNA strand breaks in both the cell lines by TUNEL assay. In comparison to the negative control, there were no significant differences in the number of HCT-15 apoptotic cells after 48 h of treatment with 70 mM acetate (IC_{50}) (0.5% *versus* 1.6%; Figure 3a), though phenotypic alterations typical of apoptosis (such as apoptotic bodies) were observed (Figure 3b). However, the number of apoptotic cells increased significantly (7.2%) after 48 h of treatment with 140 mM acetate ($2 \times IC_{50}$) (Figure 3a). Exposure to 110 mM acetate (IC_{50}) induced a minor increase in the number of apoptotic RKO cells, compared with low basal apoptotic levels (1.6% *versus* 0.3%; Figure 3a), but again with evident phenotypic alterations (Figure 3b). When treated with 220 mM acetate ($2 \times IC_{50}$), the number of apoptotic RKO cells increased significantly (65.5%; Figure 3a). Acetate also led to a dose-dependent increase in the sub-G1 peak of HCT-15 cells, indicative of an apoptotic sub-population, and similar to that of cells treated with etoposide (Figure 5b). Two peaks corresponding to the G1 and G2/M phases of the cell cycle were evident in DNA content histograms of HCT-15 control (untreated) cells, with very few cells in the hypodiploid sub-G1 cell-cycle phase (2% of the total events acquired). Exposure to etoposide or 140 mM acetate greatly increased the percentage of hypodiploid cells to approximately 70% and 35%, respectively (Figure 5b).

It has been reported that the pH influences the cell death pathway induced by SCFA produced by propionibacteria, that is, SCFA trigger apoptosis at pH 7.5 in HT-29 cells but necrosis at pH 5.5.[1] In our study, the pH of the culture medium after 48 h of incubation with acetate was closer to 7.5 than to 5.5 in both HCT-15 and RKO cell lines and was similar to that of the control culture (Table 1). In RKO cells, the pH of the control culture medium ($pH = 8.00$) was higher than that of HCT-15 cells ($pH = 7.45$), probably due to the different composition of the growth medium (DMEM (Dulbecco's Modified Eagle's Medium) for RKO cells and RPMI for HCT-15 cells) and to the different growth rates of these cell lines. Taken together, our results show that, under our experimental conditions, acetate induced apoptosis.

Acetate induces lysosomal permeabilization and cathepsin D release to the cytosol. We next investigated whether the lysosomal pathway was involved in acetate-induced apoptosis in CRC cells. We measured LMP both by staining with the lysosomotropic agent Acridine Orange (AO) and by immunoblot detection of CatD in cytosolic fractions. AO is a weak base that moves freely across membranes when uncharged and accumulates in acidic compartments like lysosomes in its protonated form, where it forms aggregates that fluoresce bright red. LMP is associated with proton release, which renders lysosomes more alkaline and hence with decreased red fluorescence. LMP was monitored qualitatively by fluorescence microscopy and quantitatively by flow cytometry, by measuring the percentage of cells with loss of lysosomal AO red fluorescence. Etoposide was used as a positive control, as it destabilizes lysosomes and induces LMP through CatD release to the cytosol.[19–21]

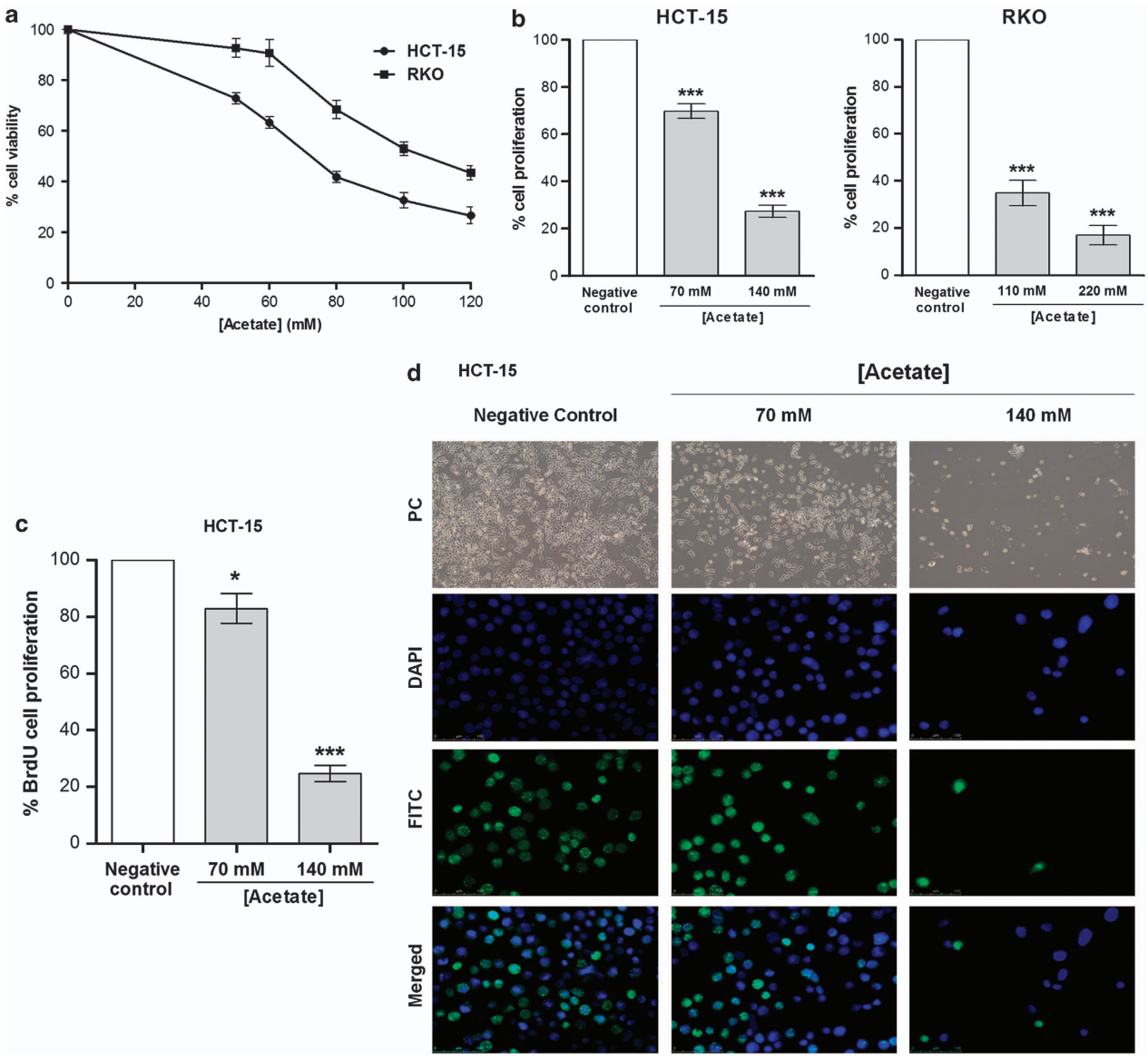

Figure 1 Determination of acetate IC_{50} values and proliferation analysis in CRC cell lines treated with acetate. (**a**) HCT-15 and RKO cells were incubated with different concentrations of acetate for 48 h or with fresh complete medium as a negative control, and IC_{50} values determined by MTT reduction assay. (**b**) Cell proliferation analysis by SRB assay in CRC cells treated with acetate. Cells were incubated with IC_{50} and $2 \times IC_{50}$ concentrations of acetate (respectively, 70 mM and 140 mM for HCT-15 and 110 mM and 220 mM for RKO) for 48 h. Values represent mean ± S.E.M. of at least three independent experiments. $^{***}P \leq 0.001$, compared with negative control cells. (**c**) Cell proliferation analysis by BrdU incorporation assay in HCT-15 cells treated with acetate (70 mM and 140 mM) for 48 h; values represent mean ± S.E.M. of at least three independent experiments $^{*}P \leq 0.05$; $^{***}P \leq 0.001$, compared with negative control cells. (**d**) Representative photographs of BrdU incorporation assay in HCT-15 cells treated with acetate (70 mM and 140 mM) for 48 h. phase contrast (PC; × 100); DAPI (4',6-diamidino-2-phenylindole), FITC (fluorescein isothiocyanate) and merged (× 200) were obtained by fluorescence microscopy

Fluorescence microscopy analysis of both the cell lines stained with AO showed that a high percentage of control cells exhibited intact acidic lysosomes (i. e., high levels of red fluorescence corresponding to accumulation of AO in acidic lysosomes) and a reduced percentage of cells with diffused green fluorescence (corresponding to non-lysosomal AO) (Figure 4a). By contrast, exposure to acetate led to a decrease in the percentage of cells with red fluorescence, associated with an increase in the percentage of cells with green fluorescence, indicative of LMP (Figure 4a). Similar results were obtained in cells treated with etoposide. These differences were more pronounced in cells treated with $2 \times IC_{50}$ acetate than in cells treated with IC_{50} concentrations. Quantification of AO-stained cells was performed by flow cytometry; cells with red fluorescence levels lower than the threshold of AO-positive staining of untreated cells were considered to exhibit LMP. Cells with LMP increased from approximately 3.6% in untreated cells to 11.7, 25.8 and 49.1% in cells exposed to 70 mM (IC_{50}), 100 mM and 140 mM ($2 \times IC_{50}$) acetate, respectively (Figure 4c). These results demonstrate that acetate induces LMP in a dose-dependent manner.

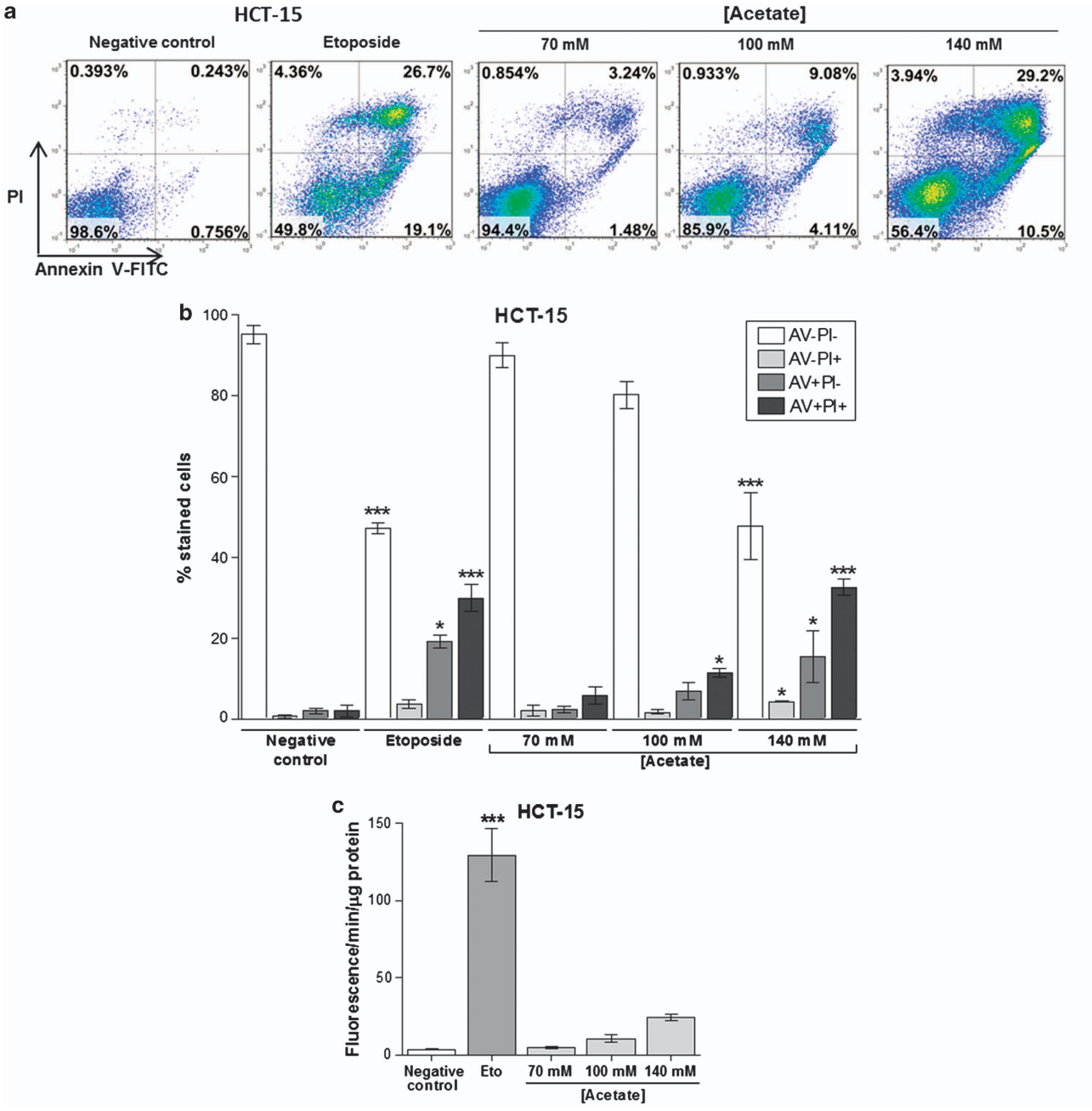

Figure 2 Acetate induces apoptosis and not necrosis in CRC cells. Apoptosis determined by Annexin V fluorescein isothiocyanate (AV-FITC) and propidium iodide (PI) assay in HCT-15 cells after incubation with 70 mM, 100 mM and 140 mM of acetate for 48 h. Cells were incubated with fresh complete medium or etoposide (50 μM) as a negative and positive control, respectively. (**a**) Representative histograms of HCT-15 cells double-labeled with AV and PI. Percentages of apoptotic cells (positive for AV) are the sum of the lower and upper right panels. (**b**) Quantitative analysis of AV/PI staining in HCT-15 cells. Values represent mean ± S.E.M. of at least three independent experiments. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$, comparing each subset of cells ($AV-PI-$, $AV-PI+$, $AV+PI-$, $AV+PI+$) to the respective negative control cells. (**c**) Quantitative analysis of caspase 3 activity in HCT-15 cells after incubation with 70 mM, 100 mM and 140 mM of acetate for 48 h. Values represent mean ± S.E.M. of three independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, compared with negative control cells

As lysosomal proteases such as CatD are released from lysosomes to the cytosol after LMP, their detection in the cytosolic fraction is indicative of LMP. We therefore treated HCT-15 and RKO CRC cells with two concentrations of acetate (IC_{50} and $2 \times IC_{50}$ for each cell line), or with etoposide, and detected CatD in whole-cell extracts and in cytosolic fractions by western blot (Figure 4d). We found that CatD is expressed in whole-cell lysates and that untreated cells exhibited very low levels of CatD in the cytosol. Notably, we found that exposure to etoposide and to IC_{50} and $2 \times IC_{50}$ concentrations of acetate led to CatD release to the cytosol, indicating that acetate induces LMP in a concentration-dependent manner and further suggesting that acetate induces a lysosomal pathway of apoptosis in both the cell lines. We also observed that exposure to acetate

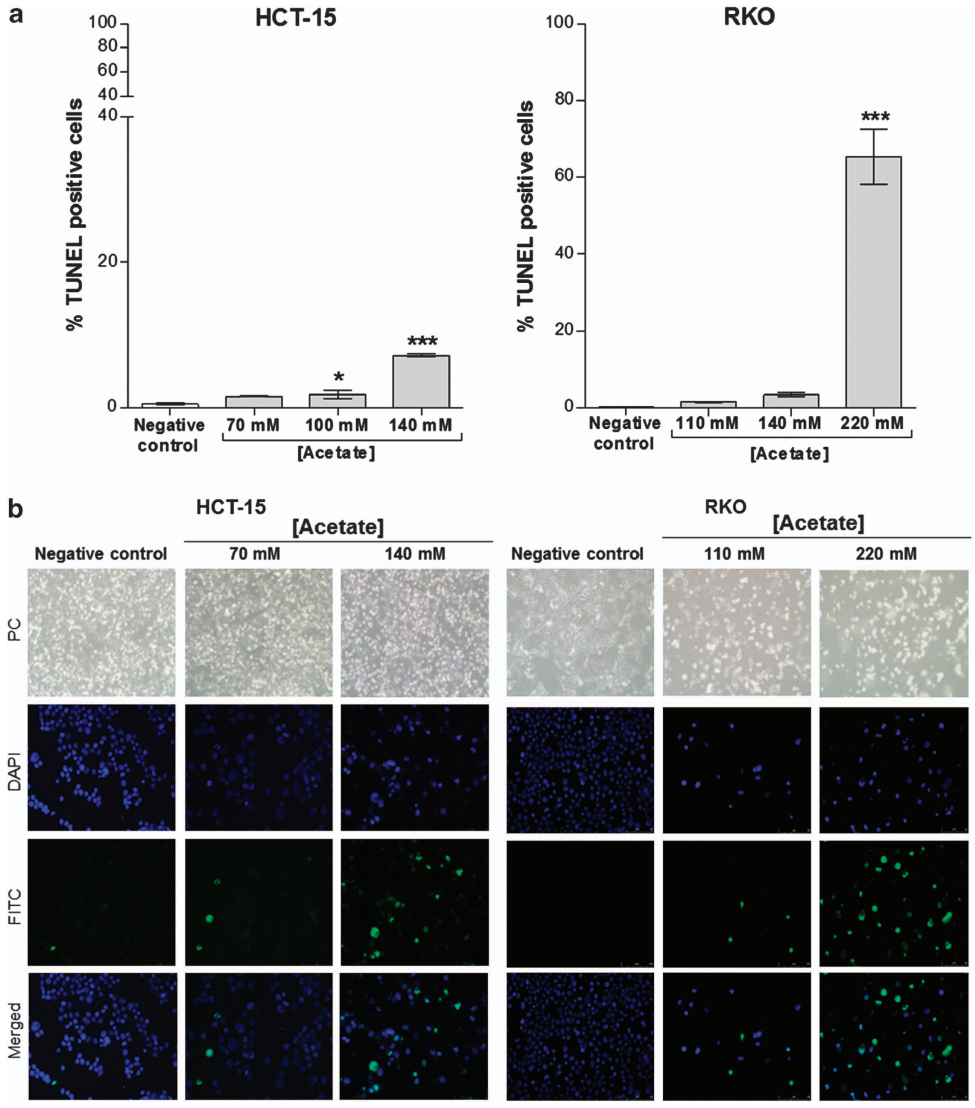

Figure 3 Acetate increases levels of TUNEL-positive cells. Apoptosis analysis in HCT-15 and RKO cells by TUNEL assay, after incubation with IC_{50} and $2 \times IC_{50}$ acetate concentrations (70 mM and 140 mM for HCT-15; 110 mM and 220 mM for RKO) for 48 h. (**a**) Analysis of TUNEL assay in HCT15 and RKO cells. Values represent mean ± S.E.M. of at least three independent experiments. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$ compared with negative control cells. (**b**) Representative images (× 200) of DAPI (4',6-diamidino-2-phenylindole), FITC (fluorescein isothiocyanate) and merged were obtained by fluorescence microscopy. PC, phase contrast

increased Pro-CatD levels in the cytosol of both the cell lines (Figure 4d). The apparent decrease in cytosolic Pro-CatD levels in RKO cells treated with $2 \times IC_{50}$ compared with IC_{50} concentrations of acetate seems to be due to increased processing to mature CatD.

Cathepsin D release protects cells from acetate-induced apoptosis. To test whether the apoptotic phenotype of HCT-15 and RKO cells exposed to acetate depends on CatD, we inhibited lysosomal CatD with pepstatin A (PstA) and cathepsins B (CatB) and L (CatL) with (2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d). PstA, but not E64d, increased apoptotic levels induced by acetate (Figure 5a). Indeed, incubation of HCT-15 cells and RKO with PstA before incubation with the $2 \times IC_{50}$ concentration of acetate increased the number of TUNEL-positive cells, though only reaching statistical significance in HCT-15. E64d did not significantly alter apoptotic levels of either cell line under the same conditions (Figure 5a). PstA-treated HCT-15 cells exposed to acetate also exhibited a higher percentage of cells with sub-G1 DNA content than cells treated with acetate alone, indicative of higher apoptotic levels (Figures 5b and c). By contrast, inhibition of CatD in cells exposed to etoposide decreased the sub-G1 population (Figures 5b and c), in agreement with previous reports showing that CatD has a pro-apoptotic role in etoposide-induced cell death.[21] These results therefore indicate that CatD has a protective role in acetate-induced apoptosis in CRC cells, as has been observed in *S. cerevisiae* cells for Pep4p, the human CatD ortholog.[18]

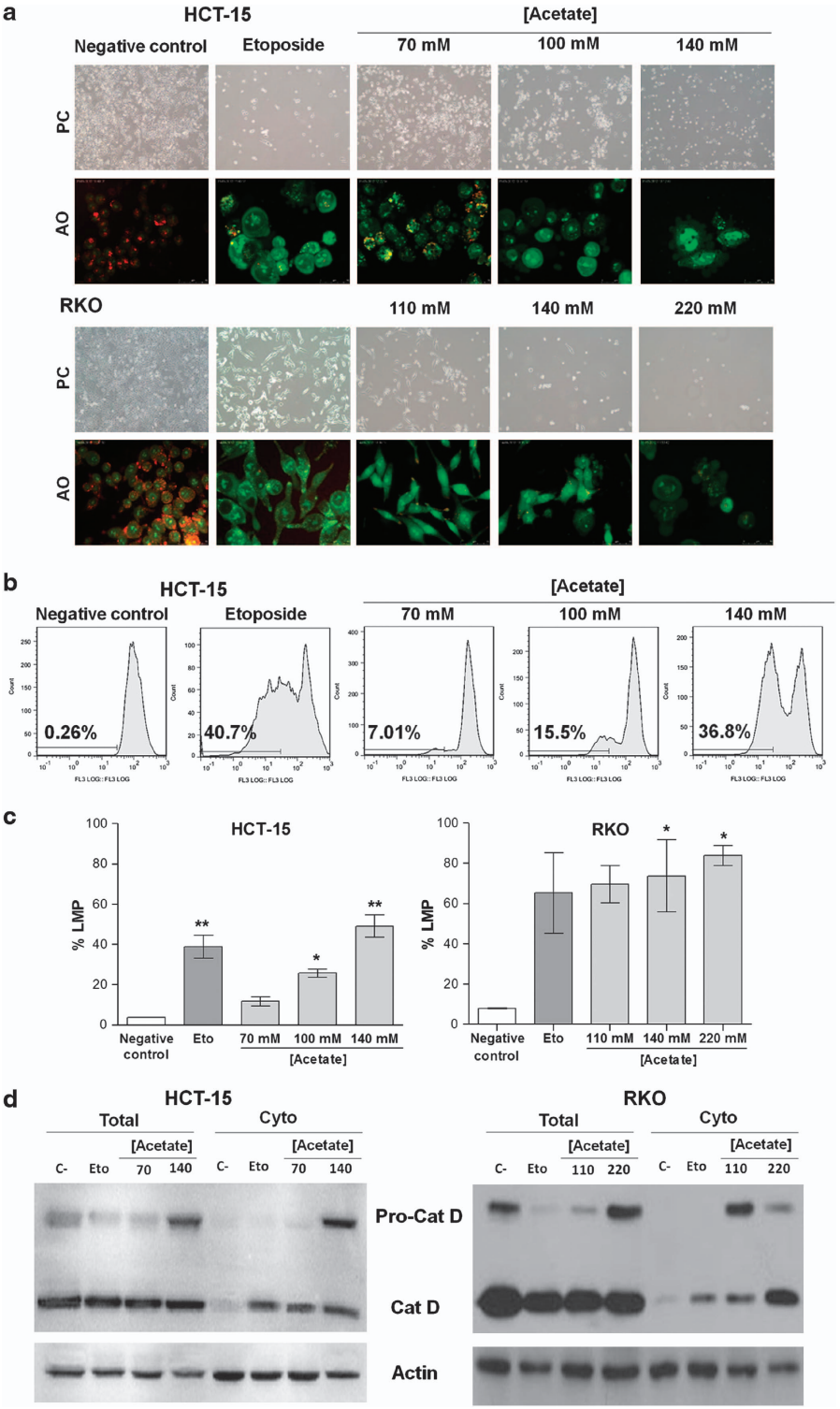

Figure 4 Acetate induces LMP, analyzed by lysosomal pH alterations and CatD release to the cytosol. (**a**) HCT-15 and RKO cells were incubated with acetate (70 mM, 100 mM and 140 mM, for HCT-15 cells; 110 mM, 140 mM and 220 mM, for RKO cells) for 48 h or with fresh complete medium or etoposide (50 μM) as a negative and positive control, respectively. LMP was detected by AO staining and visualization by fluorescence microscopy. Representative images ($\times$ 400) are shown. (**b**) Representative images of monoparametric histograms of green fluorescence (FL3 area (log)) in HCT-15 cells treated as in (**a**). (**c**) Percentage of HCT-15 and RKO cells displaying LMP quantified by flow cytometry analysis of AO staining after exposure to acetate as described in (**a**). Values represent mean $\pm$ S.E.M. of three independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ compared with negative control cells. (**d**) Effect of acetate on the expression and release of cathepsin D to the cytosol in HCT-15 and RKO cells, comparing whole-cell lysates (total) and cytosolic fractions (cyto). Cells were treated with IC_{50} and 2 $\times$ IC_{50} acetate concentrations (respectively, 70 mM and 140 mM for HCT-15 cells and 110 mM and 220 mM for RKO cells) for 48 h or with fresh complete medium or etoposide (50 μM) as a negative and positive control, respectively. Actin was used as a loading control

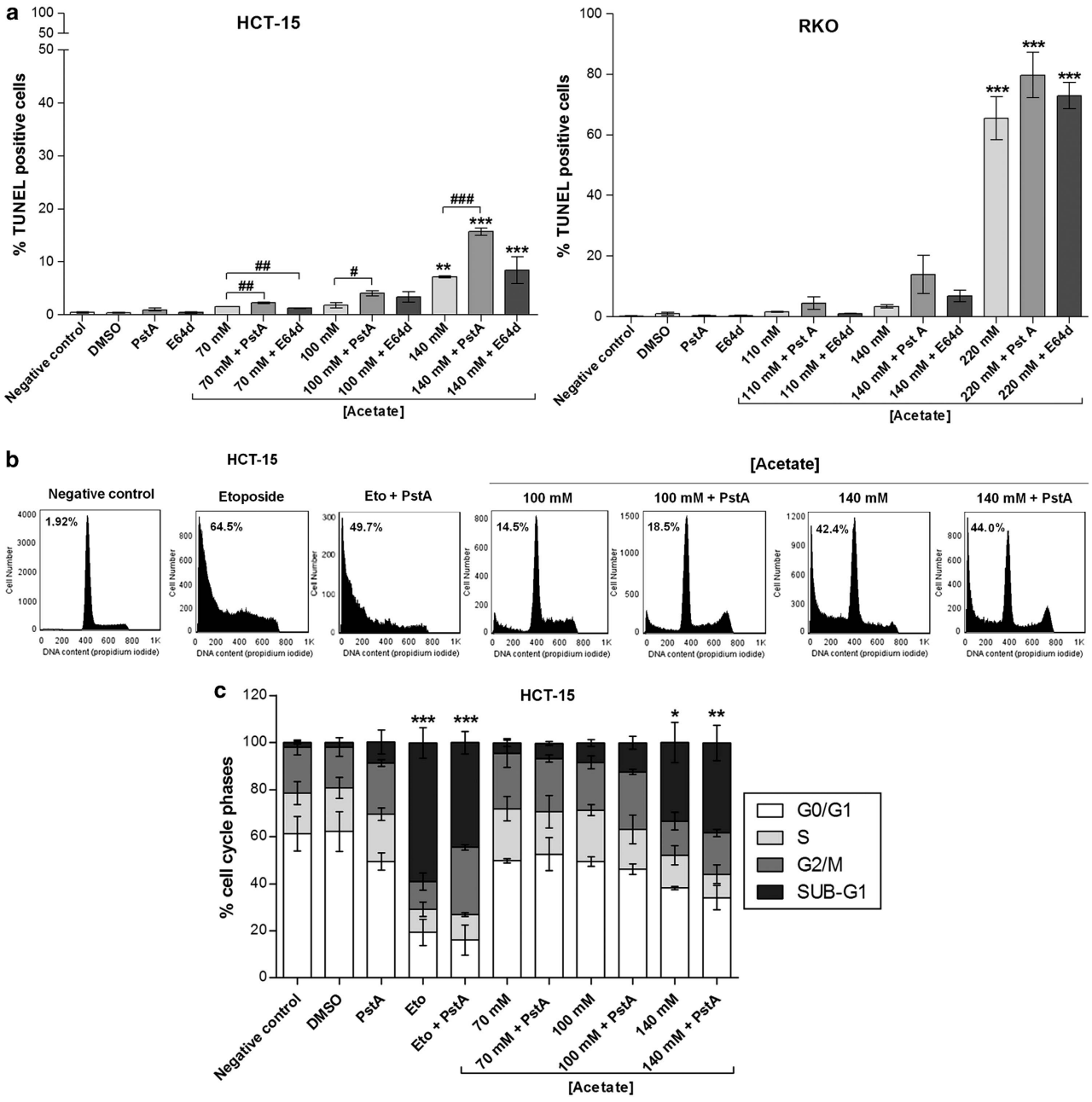

Figure 5 CatD has a protective role in acetate-induced apoptosis. Effect of the specific CatD inhibitor (PstA) and CatB and CatL inhibitor (E64d) on apoptosis in HCT-15 and RKO cells treated with acetate. PstA (100 μM) was pre-incubated for 16 h and then co-incubated with acetate for 48 h. E64d (10 μM) was pre-incubated for 1 h and then co-incubated with acetate for 48 h. (**a**) Apoptosis analysis by TUNEL assay. Values represent mean ± S.E.M. of at least three independent experiments. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ compared with negative control cells. # $P \leq 0.05$; ## $P \leq 0.01$; ### $P \leq 0.001$ comparing acetate treatment with or without PstA or E64d. (**b**) Analysis of the effect PstA on the sub-G1 subpopulation of acetate-treated HCT-15 cells by flow cytometry. Representative histograms corresponding to HCT-15 cells treated with 100 mM and 140 mM acetate with or without PstA. Percentage of sub-G1 cells are shown. Cells were incubated with fresh complete medium or etoposide (50 μM) as a negative and positive control, respectively. (**c**) Analysis of the distribution of cell-cycle phases in HCT-15 cells after acetate treatment in absence/presence of PstA. Values represent mean ± S.E.M. of at least three independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ comparing the percentage of sub-G1 populations of treated cells with that of untreated cells (negative control/dimethyl sulfoxide (DMSO))

Discussion

CRC is a leading cause of mortality in Europe, and its global therapeutics market is worth billions of Euros (GLOBOCAN). Worldwide variations in colorectal cancer incidence suggest that dietary and lifestyle factors contribute to its etiology and are important risk factors for CRC. CRC is most susceptible to dietary influence, and a higher intake of dietary fibers can reduce the risk of CRC.[22] SCFA, particularly propionate and acetate, are the end products of fermentation of physiological bacteria, namely propionibacteria, which are present in dietary fibers and several dairy products frequently consumed.[23] Both

Table 1 Measurement of pH in acetate-treated cells

Condition	pH
HCT-15	
RPMI	7.45
Untreated cells	7.07
Acetate	
70 mM	7.18
100 mM	6.93
140 mM	7.06
RKO	
DMEM	8.00
Untreated cells	8.01
Acetate	
110 mM	8.01
140 mM	8.20
220 mM	8.16

Determination of pH values in the culture medium of CRC cells, after incubation with acetate for 48 h. Untreated cells were incubated with fresh complete medium. Data represent means of at least three independent experiments

pure SCFA and propionibacteria culture supernatants from the dairy species *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici* induce apoptosis in CRC cells *in vitro*.[1–3] Moreover, administration of *P. freudenreichii in vivo* significantly increased the number of apoptotic epithelial cells damaged by 1,2-dimethylhydrazine, a carcinogenic agent, without affecting the survival of healthy normal colonic mucosa.[4,5] These characteristics indicate that propionibacteria might have a protective role against colon cancer, acting as a probiotic, and point to a useful role of propionibacteria and their metabolites propionate, butyrate and acetate as powerful agents for CRC prevention or therapy.

We showed that acetate *per se* induces apoptosis in CRC-derived cell lines HCT-15 and RKO, by inducing DNA fragmentation, caspase activation, phosphatidylserine exposure to the outer leaflet of the plasma membrane and the appearance of a sub-G1 population. The concentrations used in our study were in the physiological range; total SCFA concentrations from 50–200 mM have been reported in the intestinal lumen of a wide range of vertebrates and in most cases remain at constant high levels throughout the bowel length.[24,25] Our results are thus in agreement with previous reports showing that propionibacteria supernatants, as well as pure acetate and/or propionate, induce apoptosis in HT-29 and CACO-2 CRC cell lines.[3] In these cells, a mixture of acetate and propionate induced caspase 3 activation, nuclei shrinkage, chromatin condensation and nuclei fragmentation into apoptotic bodies. It was also demonstrated that mitochondria have a critical role in this process, evidenced by increased mitochondrial outer membrane permeability and enhanced reactive oxygen species accumulation in response to acetate and propionate, alone or in combination.[3] These authors also identified the mitochondrial adenine nucleotide translocator (ANT) as a potential SCFA target, suggesting that mitochondria and ANT are involved in the cell death pathway.[3]

The cell microenvironment may be a determinant for the type of cell death induced by toxic stimuli. The pH of the human colon lumen ranges from 5.5–7.5. SCFA produced by *P. freudenreichii* trigger apoptosis in HT-29 cells at pH 7.5 but necrosis at pH 5.5. At pH 7.5, propionibacterial SCFA were shown to induce cell-cycle arrest in the G2/M phase and morphological characteristics of apoptotic cell death, like membrane blebbing, chromatin condensation and fragmentation, phosphatidylserine exposure and formation of apoptotic bodies.[1] In accordance with these reports, we showed that acetate induces apoptosis in HCT-15 and RKO cells without altering the extracellular pH (complete medium). We also demonstrated that acetate alone inhibits proliferation in both the cell lines, in agreement with previous reports.[1]

We have previously demonstrated in the yeast *S. cerevisiae* that acetic acid induces a mitochondria-mediated apoptotic process[18] with several features similar to apoptosis induced by SCFA in CRC cells. Notably, the mitochondrial inner membrane AAC carrier, the yeast ortholog of mammalian ANT, was required for mitochondrial outer membrane permeabilization and cytochrome *c* release in yeast cells committed to apoptosis induced by acetic acid. We also observed that Pep4p, the yeast CatD, was released from the lysosome-like vacuole to the cytosol in response to acetic acid.[18] As acetic acid and acetate trigger apoptosis through an analogous pathway in both yeast and CRC cells, respectively, we hypothesized that partial LMP with CatD release might also be involved in acetate-induced apoptosis in CRC cells. The involvement of lysosomes in apoptotic cell death, mainly through partial LMP, has gained increased attention.[7] It is now established that they are important contributors to cancer cell death, increasing interest in exploiting lysosomal cell death pathways as a potential target in cancer therapy.[10] LMP followed by release of lysosomal contents to the cytosol, especially cathepsins, seems to be the critical step of the lysosomal death pathway. The most relevant human cathepsins are the proteases CatB and CatL and the sole lysosomal aspartic protease CatD. They are most abundant in the lysosome[26] and remain active at a neutral pH.[27] Overexpression of cathepsins often occurs in human cancers, and high levels of their expression can be associated with increased risk of relapse and poor prognosis.[13] However, besides the tumor-promoting effects of these proteases, there is evidence that cathepsins may also function as tumor suppressors.[14]

Little is known about the role of the lysosomal pathway in cell death regulation in CRC. It has been shown that resveratrol, a naturally occurring polyphenol, triggers a caspase-dependent intrinsic pathway of apoptosis involving lysosomal CatD in CRC cells. The authors provide evidence that CatD, though not CatB or CatL, mediates resveratrol cytotoxicity in DLD1 and HT29 cell lines, inducing lysosome leakage with increased cytosolic CatD.[28] In this study, we aimed to investigate the mechanisms underlying acetate-induced apoptosis in CRC cells, focusing on the role of the lysosomal pathway through LMP and CatD release. We showed, for the first time, by different approaches that exposure of CRC cells to acetate leads to LMP, release of CatD to the cytosol and accumulation of Pro-CatD and mature CatD. We also showed that inhibiting CatD with PstA, a widely used specific inhibitor of CatD enzymatic activity,[21,28–33] increased acetate-induced apoptosis in CRC cells. Interestingly, in yeast, deletion of Pep4p confers higher susceptibility to acetic acid, while cells overexpressing Pep4p display higher resistance.[18] Recently, it has also been demonstrated that Pep4p has a dual cytoprotective function, anti-apoptotic

and anti-necrotic, during *S. cerevisiae* chronological aging via the polyamine pathway.[34] Our data in CRC cells are thus in agreement with the results obtained in yeast. Taken together, our results point to a function of CatD in cell protection rather than in the execution of acetate-induced cell death, in both CRC and yeast.

The role of CatD expression in colon carcinogenesis is controversial and poorly understood. CatD protein levels in CRC clinical cases show divergent expression patterns, suggesting a complex regulation and function of this protease.[17,28,35–41] One study showed that the average CatD protein content was not different in 59 CRC when compared with normal mucosa, but individual tumors demonstrated marked changes in CatD expression levels.[16] Measurement of protein content of tumor *versus* normal pairs on western blots revealed loss of CatD in $>50\%$ of CRC. However, two-fold increases in CatD protein levels were also observed in $\sim 1/3$ of tumors, supporting the concept that CRC develop via divergent molecular pathways and that CatD may function differently in different cancers.[16] Another study analyzed surgical specimens of 100 primary CRC with different grades of differentiation and determined that immunoreactivity for CatD was negative in the cytoplasm of normal colorectal epithelial cells adjacent to carcinoma. CatD expression in carcinoma cells was present in 68% of the cases, and CatD positivity was higher when the grade of differentiation was higher.[17] Yet another study showed that the majority of CRC (93/106) stained positive for CatD, whereas normal colon was almost completely negative.[35] It is therefore apparent that some, but not all, CRC cells overexpress CatD in comparison to normal colon mucosa.

In the present study, we showed that CRC cell lines HCT-15 and RKO express CatD and are sensitive to physiological levels of acetate, suggesting that the levels of CatD observed might differ from those of normal colon cells. However, we could not test this hypothesis '*in vitro*' because the only available 'normal' human colon mucosal epithelium-derived cells NCM460 (INCELL Corporation, San Antonio, TX, USA)[42] also express CatD and behave similarly to RKO cells in response to acetate (data not shown). According to information provided by INCELL, NCM460 cells have acquired a tumorigenic phenotype due to their extended time in culture.

The protective role of CatD demonstrated by our data might partly explain why CatD is overexpressed in some CRC clinical cases in comparison to normal colon mucosa. We therefore hypothesize that increased expression of this protease might be beneficial to cancer cells and thus that CatD might have an 'oncogenic-like effect', allowing CRC cells to survive in the presence of physiological levels of SCFA in the colon.

In summary, our novel findings show that acetate induces partial LMP and consequent release of CatD to the cytosol in CRC cells. Our results also indicate that inhibition of CatD enzymatic activity sensitizes CRC cells to acetate-induced apoptosis, suggesting that CatD, like Pep4p in yeast, might have a protective role in this process. However, the mechanism involved remains elusive and will warrant further investigation. Our observations indicate that CatD protects cells from acetate exposure, prompting further exploiting the differential sensitivity of CRC to acetate. Inhibiting CatD function therefore emerges as a novel prevention/therapeutic strategy in CRCs, particularly in the case of CRCs with decreased levels of CatD. Specifically increasing the sensitivity of CatD-overexpressing CRCs without affecting normal colon mucosa cells will likely require nanodelivery systems of CatD inhibitors such as PstA to specifically target CRC cells, in combination with strategies to increase acetate concentrations in the colon, namely emerging nutraceuticals.

Materials and Methods

Cell lines and culture conditions. HCT-15 and RKO cell lines derived from human CRC were maintained at 37 °C under a humidified atmosphere containing 5% CO_2. HCT-15 cells were grown in RPMI medium with L-glutamine and HEPES supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin/streptomycin. RKO cells were grown in DMEM supplemented with 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, 10% fetal bovine serum, 100 U/ml penicillin/streptomycin. Cells were seeded and adhered onto appropriate sterile plates for 24 h before treatments.

Assessment of cell viability by MTT reduction assay. Cells were incubated with different concentrations of sodium acetate, henceforth referred to as acetate, for 48 h. Freshly prepared MTT was added at a final concentration of 0.5 mg/ml in PBS pH 7.4 and plates incubated in the dark for 2 h at 37 °C. In all, 500 μl of acidic isopropanol (0.04 M HCl in absolute isopropanol) were added to each well, followed by 30 min of orbital shaking in the dark, to solubilize the formazan crystals. Absorbance was read at 570 nm in a microplate reader (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA, USA). Results were expressed as a percentage of sample absorbance in relation to the negative control (untreated cells).

Cell proliferation assays

SRB: Cells were incubated with different acetate concentrations for 48 h, fixed in ice-cold methanol containing 1% acetic acid, and incubated with 0.5% (w/v) SRB dissolved in 1% acetic acid for 1.5 h at 37 °C. After washing with 1% acetic acid, SRB was solubilized with 10 mM Tris, pH 10. Absorbance was read at 540 nm in a microplate reader (SpectraMax 340PC Molecular Devices). Results were expressed relatively to the negative control (untreated cells), which was considered as 100% of cell proliferation.

BrdU incorporation: Cells were seeded in plates containing glass coverslips, exposed to acetate for 48 h and incubated with 10 μM BrdU for 1 h. Cells were fixed with 4% paraformaldehyde and nuclear incorporation of BrdU was detected by immunofluorescence. Coverslips were mounted on Vectashield Mounting Medium with DAPI (4',6-diamidino-2-phenylindole) and the percentage of positive nuclei (BrdU index) was determined from >500 cells/datum point.

Apoptosis assays

TUNEL: Cells were exposed to DMSO control, etoposide or different concentrations of acetate for 48 h. When cathepsin inhibitors were used, cells were pre-incubated with PstA or E64d for 16 h and 1 h, respectively, and then co-incubated with acetate or etoposide for 48 h. Cytospins of both floating and attached cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT). Cells were washed in PBS and permeabilized with ice-cold 0.1% Triton X-100 in 0.1% sodium citrate. TUNEL was performed following the manufacturer's instructions ('*In situ* cell death detection kit, fluorescein', Roche, Mannheim, Germany). Slides were mounted on Vectashield Mounting Medium with DAPI and maintained at -20 °C until visualization in a fluorescence microscope (Leica DM 5000B, Leica Microsystems, Wetzlar, Germany).

Caspase 3 activity: Cells were exposed to etoposide or acetate for 48 h. Both floating cells and attached cells were collected, washed twice with PBS, and lysed in Lysis Buffer (10 mM Tris, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% Triton X-100) through three freeze/thaw cycles. In all, 50 μl of total extracts (1 mg/ml) were incubated with 50 μl 200 μM z-DEVD-AFC (Biomol, Plymouth Meeting, PA, USA)

in 2 × reaction buffer (20 mM PIPES, pH 7.4, 4 mM EDTA, 10 mM DTT) and fluorescence of cleaved AFC was measured using a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc., Waltham, MA, USA).

AV/PI staining: Cells were treated with etoposide or acetate for 48 h. Both floating and attached cells were collected and washed in PBS. In all, 1×10^6 cells were resuspended in 200 μl 1 × binding buffer and incubated with 8 μl AV-fluorescein isothiocyanate (BD Biosciences, San Jose, CA, USA) and 15 μl 50 μg/ml PI for 15 min in the dark. Samples were analyzed in a flow cytometer (Epics XL, Beckman Coulter, Miami, FL, USA) equipped with an argon-ion laser emitting a 488-nm beam at 15 mW. Monoparametric detection of red fluorescence was performed using FL-3 (488/620 nm) and detection of green fluorescence was performed using FL-1 (488/525 nm). In all, 20 000 cells were analyzed *per* sample and data analyzed using FlowJo software (version 7.6, Tree Star Inc., Ashland, OR, USA).

Sub-G1 cell population analysis: Cells were treated with DMSO, etoposide or acetate for 48 h. When PstA was used, cells were pre-incubated as described above. Both floating and attached cells were collected, washed with PBS and incubated with 70% cold ethanol for 15 min. Cells were then washed twice with PBS, incubated with RNase A (200 μg/ml) for 15 min in the dark at 37 °C and with propidium iodide (0.5 mg/ml) for 30 min at RT before analysis on a flow cytometer, as described above. FlowJo software was used to generate DNA content frequency histograms and quantify the amount of cells in the individual cell-cycle phases, including the sub-G1 population assumed as corresponding to apoptotic cells.

LMP assessment

AO staining analysis by fluorescence microscopy: Cells were seeded in six-well plates containing glass coverslips, and exposed to etoposide or acetate for 48 h. Cells were then incubated with 1 μM AO for 15 min at 37 °C and washed with PBS. Coverslips were mounted over glass slides with PBS and immediately observed and photographed using a fluorescence microscope (Leica DM 5000B).

AO staining analysis by flow cytometry: Cells were exposed to etoposide or acetate for 48 h. Both floating and attached cells were collected, washed with PBS and resuspended in PBS to a final concentration of 1×10^6 cells in 1.5 ml. Cells were then incubated with 1 μM AO (or without AO to measure autofluorescence) for 15 min at 37 °C.

Immunoblot detection of cathepsin D

Preparation of cytosolic protein extracts: Cells were exposed to etoposide or acetate for 48 h. Both floating and attached cells were collected, washed with PBS and centrifuged at 650 × *g* for 10 min at 4 °C. All subsequent steps were performed at 4 °C. Cells were resuspended in 1 ml PBS, centrifuged at 1500 × *g* for 5 min and washed twice in 800 μl Isotonic Buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES) by centrifugation at 3000 × *g* for 5 min. The cell pellet was then resuspended in 300 μl Isotonic Buffer with 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF and 40 μl/ml Protease inhibitor cocktail and homogenized by passing through a 26-G needle 70 times. Nuclei and unbroken cells were removed by centrifugation at 1000 × *g* for 5 min, followed by two consecutive centrifugations, first at 10 000 × *g* for 15 min to remove lysosomes and mitochondria and then at 100 000 × *g* for 1 h in an ultracentrifuge. Cytosolic protein extracts were stored at − 80 °C.

Preparation of total protein extracts: Cells were exposed to etoposide or acetate for 48 h. Both floating and attached cells were collected, washed with PBS and centrifuged at 650 × *g* for 5 min at 4 °C. The supernatant was discarded and cells lysed in 80 μl Ripa Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40) supplemented with 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF and 40 μl/ml Protease inhibitor cocktail. Supernatants were cleared by centrifugation at 4500 × *g* for 10 min. Total protein extracts were stored at − 80 °C.

Western blot analysis: Protein samples (25 μg) were separated by sodium dodecyl sulfate 12,5% polyacrylamide gel electrophoresis and electroblotted onto PVDF (polyvinylidene difluoride) membranes. Primary antibodies were anti-cathepsin D (Calbiochem, San Diego, CA, USA) and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) and horseradish peroxidase-labeled goat anti-mouse immunoglobulin IgG (Jackson ImmunoResearch). Subsequent chemiluminescence detection was performed using the ECL detection system (Amersham, Biosciences, Buckinghamshire, UK) and a molecular imager (Chemi-Doc XRS system, Bio-Rad, Laboratories Inc., Hercules, CA, USA).

Statistical analysis: Data are expressed as means ± S.E.M. of at least three independent experiments. Statistical analysis between negative control and treated samples was performed by one-way ANOVA followed by Dunnett's test and the comparison between PstA- or E64d-treated and untreated cells was performed by unpaired two-tailed Student's *t*-test. Differences were considered significant when $P \leq 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy-independent degradation of damaged mitochondria

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Acetate is a short-chain fatty acid secreted by Propionibacteria from the human intestine, known to induce mitochondrial apoptotic death in colorectal cancer (CRC) cells. We previously established that acetate also induces lysosome membrane permeabilization in CRC cells, associated with release of the lysosomal protease cathepsin D (CatD), which has a well-established role in the mitochondrial apoptotic cascade. Unexpectedly, we showed that CatD has an antiapoptotic role in this process, as pepstatin A (a CatD inhibitor) increased acetate-induced apoptosis. These results mimicked our previous data in the yeast system showing that acetic acid activates a mitochondria-dependent apoptosis process associated with vacuolar membrane permeabilization and release of the vacuolar protease Pep4p, ortholog of mammalian CatD. Indeed, this protease was required for cell survival in a manner dependent on its catalytic activity and for efficient mitochondrial degradation independently of autophagy. In this study, we therefore assessed the role of CatD in acetate-induced mitochondrial alterations. We found that, similar to acetic acid in yeast, acetate-induced apoptosis is not associated with autophagy induction in CRC cells. Moreover, inhibition of CatD with small interfering RNA or pepstatin A enhanced apoptosis associated with higher mitochondrial dysfunction and increased mitochondrial mass. This effect seems to be specific, as inhibition of CatB and CatL with E-64d had no effect, nor were these proteases significantly released to the cytosol during acetate-induced apoptosis. Using yeast cells, we further show that the role of Pep4p in mitochondrial degradation depends on its protease activity and is complemented by CatD, indicating that this mechanism is conserved. In summary, the clues provided by the yeast model unveiled a novel CatD function in the degradation of damaged mitochondria when autophagy is impaired, which protects CRC cells from acetate-induced apoptosis. CatD inhibitors could therefore enhance acetate-mediated cancer cell death, presenting a novel strategy for prevention or therapy of CRC.
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Colorectal cancer (CRC) is one of the most common cancers worldwide.[1,2] In Europe, it is the most diagnosed malignancy and the second cause of cancer mortality in both genders,[2] highlighting the need for novel strategies to prevent and treat CRC. Short-chain fatty acids (SCFA), namely butyrate, propionate and acetate, are the major by-products of anaerobic bacterial fermentation of undigested fibers in the human colon. As they were reported as antiproliferative and antineoplastic agents that induce differentiation, growth arrest and apoptosis in CRC cell lines,[3–6] there has been increased interest in exploiting these natural products in CRC prevention and therapy. The antitumor effect of SCFAs stems from their ability to induce cell death involving mitochondria-mediated apoptosis (caspase-dependent/independent) or necrosis in colon cancer cells.[3,4,6] We also previously implicated another organelle in acetate-induced apoptosis, the lysosome. Indeed, lysosomal membrane permeabilization (LMP) and release of cathepsins into the cytosol can initiate the lysosomal apoptotic pathway either in a mitochondria-independent manner or mediated by mitochondrial destabilization with subsequent release of apoptotic factors.[7,8] Among the cathepsins released by LMP, cathepsin D (CatD), originally considered a 'house-keeping enzyme'[9] necessary for autophagy[10] can act as an antiapoptotic or proapoptotic mediator depending on the cell type and context.[10–12] However, the exact mechanisms triggered by CatD following LMP in cancer cells, as well as the signaling to and/or from mitochondria, remain to be clarified.

In a previous study, we demonstrated that CatD is released into the cytosol and protects cells undergoing acetate-induced apoptosis.[5] These results were in agreement with our data showing that Pep4p, the yeast ortholog of human CatD, translocates from the vacuole to the cytosol during mitochondria-mediated acetic acid-induced apoptosis in *Saccharomyces cerevisiae*,[13] and that it has a protective role in this process dependent on its catalytic activity.[14] However,

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Abbreviations: BafA1, bafilomycin A1; CatB, cathepsin B; CatD, cathepsin D; CatL, cathepsin L; CRC, colorectal carcinoma; CCCP, carbonyl cyanide m-chlorophenylhydrazone; E-64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; $\Delta\Psi m$, mitochondrial membrane potential; LMP, lysosomal membrane permeabilization; ROS, reactive oxygen species; PstA, pepstatin A; SCFA, short-chain fatty acids; SRB, sulforhodamine B

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the mechanisms by which CatD protects CRC cells from acetate exposure are still unknown. It had been shown that SCFAs (namely butyrate and propionate) induce autophagy in CRC cells, which serves as an adaptive strategy to delay mitochondria-mediated apoptotic cell death.[15–17] In contrast, we showed that yeast CatD is involved in acetic acid-induced mitochondrial degradation independently of autophagy.[13] We therefore hypothesized the same would occur in the response of CRC cells to acetate. In this work, we assessed how CatD affects the mitochondrial alterations induced by acetate in CRC cell lines, and specifically evaluated its role in mitochondrial degradation. Our results indicate that the antineoplastic mechanism of acetate is characterized by an accumulation of reactive oxygen species (ROS) and changes in mitochondrial mass and mitochondrial membrane potential (ΔΨm). We further show that CatD, but not CatB or CatL, is involved in the degradation of the dysfunctional mitochondria to enhance CRC cell survival in response to acetate, which impairs autophagy. Our data therefore indicate that acetate-induced apoptosis involves cross-talk between the lysosome and mitochondria, and support using probiotics and inhibiting CatD as a prevention or therapeutic strategy in CRC.

Results

Acetate leads to oxidative stress, changes in ΔΨm and increased mitochondrial mass in CRC cells. Acetate-induced apoptosis has been associated with mitochondrial dysfunction, including increased ROS and ΔΨm alterations, although only in HT-29 cells.[3,4] We also previously showed that CRC-derived cells HCT-15 and RKO exposed to physiological concentrations of acetate exhibit characteristic apoptotic markers.[5] However, an effect on mitochondrial mass, as suggested by our studies in yeast,[13] has never been described. We therefore sought to determine comprehensively how acetate affects mitochondrial function in HCT-15 and RKO cells, in particular ROS accumulation and changes in ΔΨm and mitochondrial mass. For this purpose, cells exposed to different concentrations of acetate were stained with the ROS probes 2,7-dihydrodichlorofluorescein diacetate (H_2DCF-DA) or dihydroethidium (DHE), to detect hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-), respectively. ROS levels in HCT-15 and RKO cells consistently increased in response to acetate in a dose-dependent manner after 12 and 24 h of exposure, and then slightly decreased after 48 h (H_2O_2 in HCT-15 (Figure 1a) and RKO cells (not shown), and O_2^- in RKO cells (Figure 1b)). To determine if the acetate-induced ROS accumulation could be due to inhibition of cellular antioxidant defenses, we used a qualitative assay to measure catalase activity.[18] We found that exposure to a half-maximal inhibitory concentration (IC_{50}) of acetate slightly decreased catalase activity in HCT-15 and RKO cells, although more evidently in the latter (Supplementary Figure S1).

In addition to resulting from a decrease in antioxidant defenses, ROS accumulation can be an early event of the apoptotic process, associated with other mitochondrial dysfunctions.[19] We therefore assessed if acetate induces further mitochondrial alterations in CRC cells. We found that acetate, similar to CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), decreased the ΔΨm, as it lowered mitochondrial accumulation of $DiOC_6(3)$ and of MitoTracker Red CMXRos in RKO cells and of $DiOC_6(3)$ in HCT-15 cells (Supplementary Figure S2), while increasing cytosolic fluorescence of the probes. As changes in ΔΨm can result from changes in mitochondrial mass, we quantitatively assessed these two parameters simultaneously in RKO cells stained with both MitoTracker Red CMXRos and MitoTracker Green FM, by flow cytometry. In addition to a decrease in the mean red fluorescence, indicative of decreased ΔΨm (Figure 1c), acetate increased the mean green fluorescence, indicative of an increase in mitochondrial mass (Figures 1c and d). After normalizing acetate-induced changes in ΔΨm to mitochondrial mass, and in relation to time zero (Figure 1e), it became evident that a short exposure to acetate induces a small increase in ΔΨm (Figure 1e). This hyperpolarization was apparent after 12 h of exposure to 110 and 140 mM but not to 220 mM acetate. For longer exposure times, this ratio significantly decreased for all concentrations, indicative of ΔΨm dissipation (as verified for the control CCCP treatment). We also investigated the effect of acetate on the levels of mitochondrial proteins, namely the apoptosis-inducing factor (AIF), the voltage dependent anion channel (VDAC1) and a subunit of the outer mitochondrial membrane translocator (TOM22). We found all doses of acetate, as well as etoposide, increased the levels of AIF and VDAC1 in RKO and, to a higher extent, in HCT-15 cells (Supplementary Figure S3). In addition, acetate, but not etoposide, led to increased levels of TOM22.

Our results indicate that acetate causes mitochondrial dysfunction, increasing ROS accumulation, perturbing ΔΨm and increasing mitochondrial mass in a time- and dose-dependent manner, suggesting that acetate impairs the turnover of harmful mitochondria.

Acetate inhibits basal autophagy and impairs autophagy induction. To determine if the increased mitochondrial mass resulting from acetate exposure could be due to autophagy inhibition, we monitored how it affects the levels of Beclin-1, a well-known autophagic player that is part of the class III PI3K complex. Acetate lowered Beclin-1 levels in both RKO and HCT-15 cells, consistent with inhibition of autophagy and possibly explaining the increased levels of mitochondrial proteins in acetate-treated cells (Figure 2a). We next determined whether acetate affects autophagy induction. We monitored starvation-induced autophagy (in HBSS medium) by assessing an autophagy hallmark: conversion of LC3-I to LC3-II[20] (in the presence and absence of bafilomycin A1 (Baf. A1), a specific inhibitor of vacuolar type H^+-ATPase used to inhibit the fusion of autophagosomes with lysosomes).[21] HCT-15 and RKO cell lines had high basal autophagy levels and did not induce autophagy in response to starvation, evidenced by the increased accumulation of LC3-II in the presence of Baf. A1 already in control cells, which was not altered in response to starvation (Figures 2b–d). Therefore, we could not determine whether acetate affects autophagy induction in these cells. For that purpose, we instead used the HCT116 cell line, which displays increased autophagic flux in response to starvation (Figures 2b–d). We found that a 48 h exposure of HCT116 cells to IC_{50},

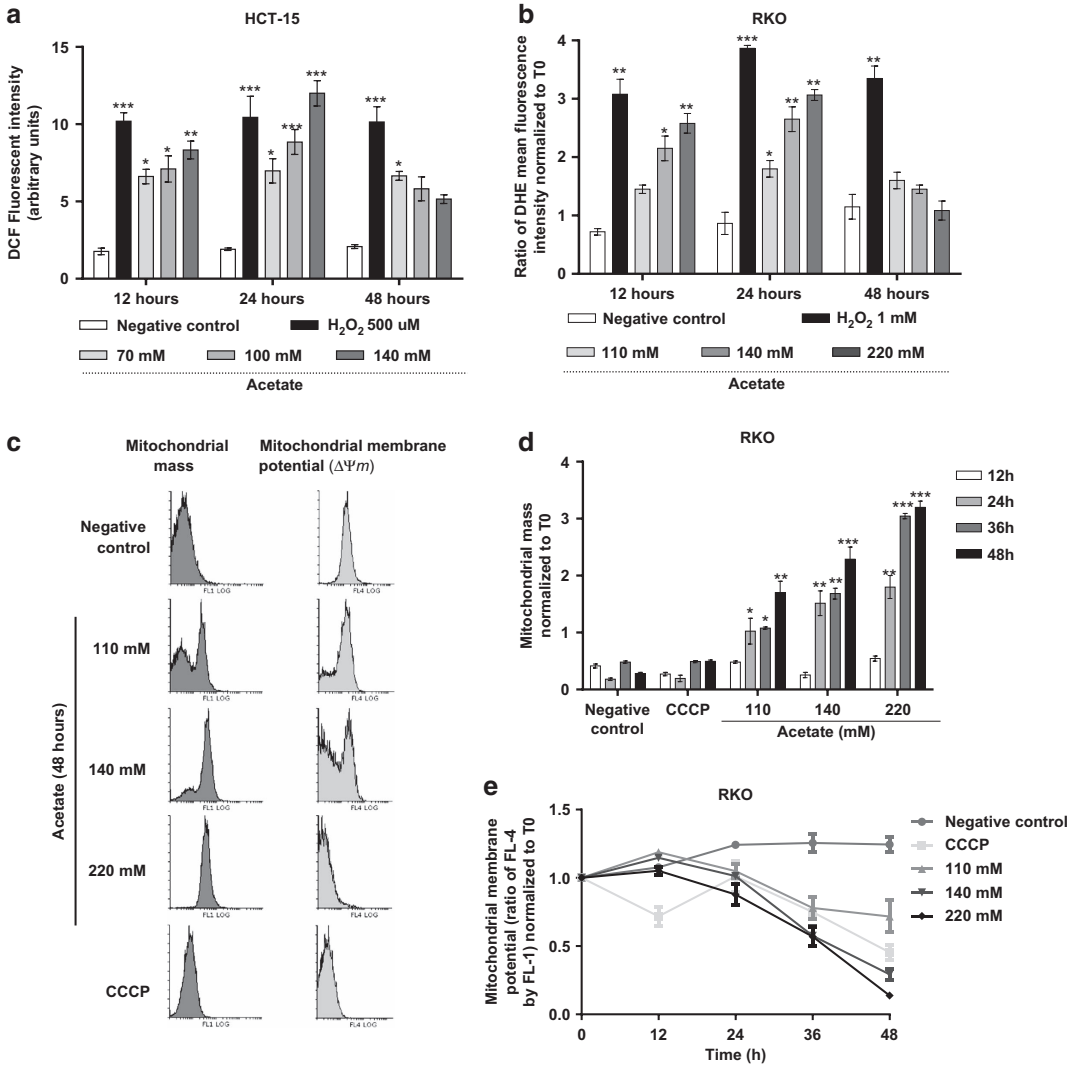
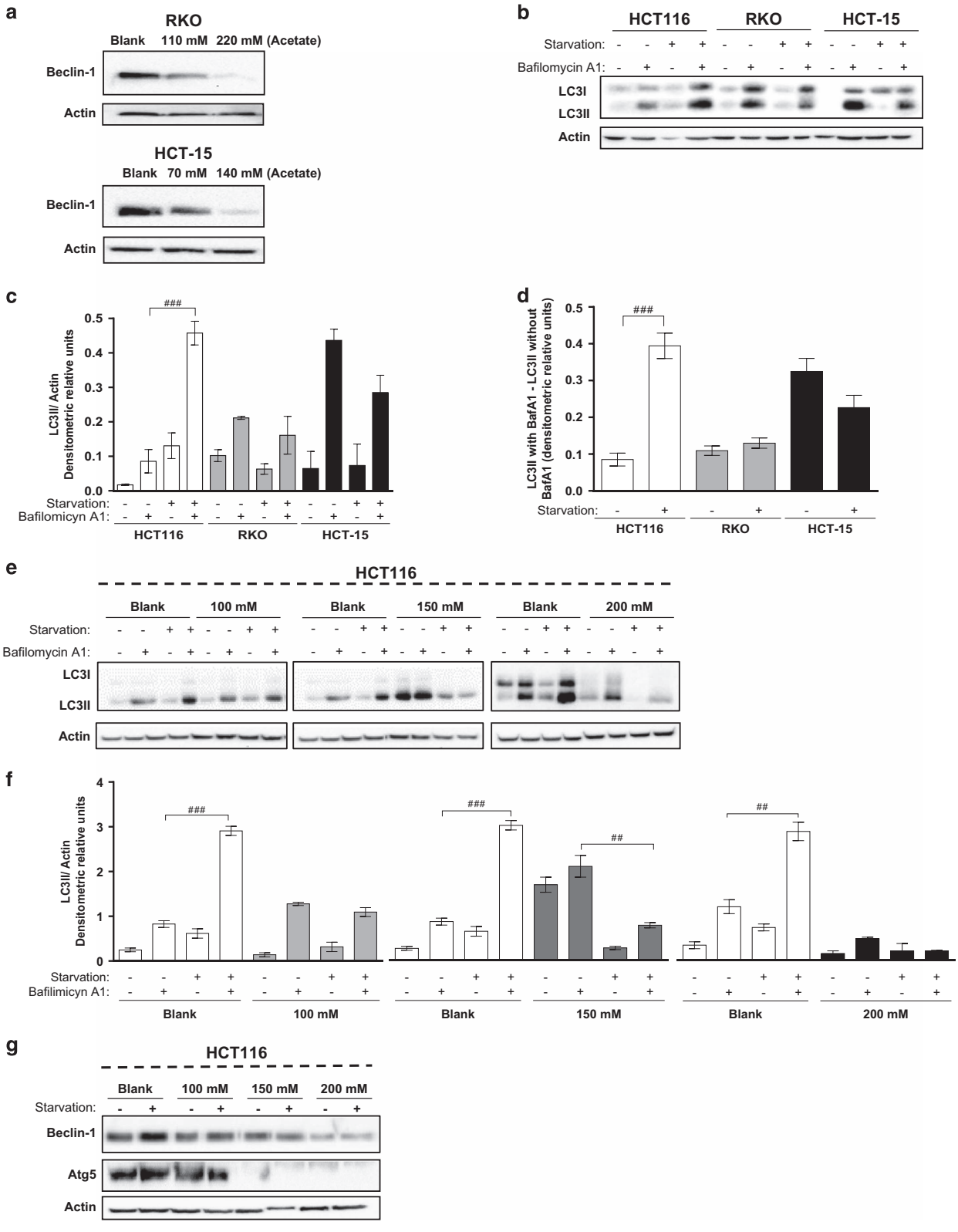


Figure 1 Acetate induces oxidative stress and mitochondrial dysfunction in CRC cells. (**a** and **b**) HCT-15 and RKO cells were incubated with acetate (70, 100 or 140 mM for HCT-15 cells; 110, 140 or 220 mM for RKO cells) for 12, 24 and 48 h. HCT-15- and RKO-negative control cells were incubated with fresh complete medium for the same times, whereas positive control cells were incubated with 500 μM and 1 mM H_2O_2, respectively. (**a**) Accumulation of H_2O_2 in HCT-15 cells was detected with H_2DCF-DA using a fluorescence microplate reader. (**b**) Accumulation of superoxide anion (O_2^-) in RKO cells was detected with DHE and quantified by flow cytometry. (**c**–**e**) Detection of mitochondrial dysfunction in RKO cells using MitoTracker Green FM and MitoTracker Red CMXROs double staining. Cells were incubated with 110, 140 or 220 mM of acetate for up to 48 h and then stained with 400 nM MitoTracker Green and 200 nM MitoTracker Red for 30 min at 37 °C in the dark. (**c**) Representative histograms of mitochondrial dysfunction in cell exposed to 110, 140 or 220 mM of acetate for 48 h. There was a significant increase in the green mean fluorescence intensity (MitoTracker Green), indicative of an increase in mitochondrial mass, and a decrease in the mean red fluorescence intensity (MitoTracker Red CMXRos), indicative of changes in the $\Delta\Psi$m. (**d**) Acetate treatment induces an increase in the mean green fluorescence intensity normalized to the green mean fluorescence intensity at time 0 (*T*0), indicating an increase in mitochondrial mass. (**e**) Acetate treatment induces an alteration in the $\Delta\Psi$m normalized to mitochondrial mass, assessed by the ratio between the mean red fluorescence intensity (FL-4) and the mean green fluorescence intensity (Fl-1) normalized to *T*0. For each time point, the values of at least three independent experiments are represented. Bonferroni's test $^*P \leq 0.05$; $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ compared with negative control cells. In (**c**–**e**), cells were incubated with fresh complete medium or with 50 μM CCCP, which were used as a negative and positive control, respectively

intermediate concentration and 2× IC_{50} of acetate (Supplementary Figure S4) decreased conversion of LC3-I to LC3-II in the presence of Baf. A1, which was even more pronounced under starvation conditions (Figures 2e and f). Increasing doses of acetate also progressively decreased the levels of Beclin1 and Atg5 proteins, essential for autophagosome completion,[22,23] in both complete and starvation media (Figure 2g). Taken together, our results indicate not only that exposure to acetate does not induce autophagy under our experimental conditions but also that it can inhibit both basal autophagy and autophagy induction.

Active CatD mediates mitochondrial degradation and protects CRC cells from acetate-induced mitochondrial dysfunction. The cellular event or signal that determines whether mitochondrial degradation in cancer cells undergoing apoptosis occurs through selective or nonselective autophagy or by an autophagy-independent process, and


a
RKO
Blank 110 mM 220 mM (Acetate)
Beclin-1
Actin
HCT-15
Blank 70 mM 140 mM (Acetate)
Beclin-1
Actin
b
HCT116
RKO
HCT-15
Starvation:
Bafilomycin A1:
LC3I
LC3II
Actin
c
LC3II/ Actin Densitometric relative units
###
Starvation:
Bafilomicyn A1:
HCT116
RKO
HCT-15
d
LC3II with BafA1 - LC3II without BafA1 (densitometric relative units)
###
Starvation:
HCT116
RKO
HCT-15
e
HCT116
Blank
100 mM
Blank
150 mM
Blank
200 mM
Starvation:
Bafilomycin A1:
LC3I
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HCT116
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Starvation:
Beclin-1
Atg5
Actin

which are the consequences to cell fate, is still unclear. In a previous study in *S. cerevisiae*, we showed that the yeast CatD (Pep4p) has an antiapoptotic role in acetic acid-induced cell death that depends on its proteolytic activity, and is required for mitochondrial degradation independently of autophagy.[14] We therefore hypothesized that CatD, similarly to Pep4p, is required for efficient degradation of damaged mitochondria. To test this hypothesis, we assessed how CatD affects mitochondrial dysfunctions induced by acetate in CRC cells.

We first analyzed whether Pepstatin A (PstA), a known specific pharmacological inhibitor of CatD, or transfection with specific RNA interference (siRNA) targeting CatD interfered with acetate-induced accumulation of ROS in RKO cells. We found that PstA increased total O_2^- and H_2O_2 accumulation in cells exposed to the three acetate concentrations for 24 h (Figures 3a and b). Downregulation of CatD was even more effective, as it significantly increased total and mitochondrial O_2^- accumulation in cells treated with 110 mM acetate (detected with DHE or MitoSOX, respectively) (Figures 3c and d). In contrast, inhibition of CatD did not significantly affect etoposide-induced ROS accumulation (Figures 3c and d).

We next tested the effect of depleting CatD on mitochondrial mass. Transfection with CatD siRNA clearly increased the levels of the mitochondrial protein AIF in both acetate- and etoposide-treated RKO cells, although not in untreated controls (Figure 4a). Using the double-staining protocol described above, we also found that depletion of CatD in acetate-treated RKO cells significantly increased mitochondrial mass and decreased $\Delta\Psi m$. In contrast, it had no significant effect in cells treated with etoposide (Figures 4b and c). Altogether, these data indicate that CatD protects RKO cells from mitochondrial dysfunction induced by acetate through its involvement in the degradation of damaged mitochondria.

To further analyze whether the role of CatD in mitochondrial degradation depends on its proteolytic activity, we assessed how this parameter was affected by inhibition with PstA. PstA significantly increased the mitochondrial mass of RKO cells exposed to 110 and 140 mM acetate for 48 h, but decreased the $\Delta\Psi m$ (Figures 4d and e). In contrast, inhibition of CatD in RKO cells exposed to etoposide did not affect mitochondrial mass or the $\Delta\Psi m$. Moreover, E-64d, an inhibitor of CatB and CatL (also overexpressed in CRC cells),[24] had no effect on the mitochondrial mass or $\Delta\Psi m$ of either acetate- or etoposide-treated cells (Figures 4d and e). We confirmed that E-64d was active under our experimental conditions because it decreased caspase-3 activity in cells exposed to etoposide (not shown), confirming the previously described proapoptotic function of CatB and CatL in this process.[25] These results indicate that CatD, but not CatL or CatB, is involved in mitochondrial degradation in response to acetate. Accordingly, we found that only the active form of CatD, but not CatL and only trace amounts of CatB, is released to the cytosol after acetate treatment (Figure 5).

The aforementioned results indicate that the role of CatD in mitochondrial degradation depends on its proteolytic activity. To further support this conclusion and determine whether this mechanism is conserved, we assessed if the same is true for the yeast CatD, and if human and yeast CatD are functionally equivalent. For this purpose, we constructed strains deficient in Pep4p expressing the empty vector control, and equivalent levels of FLAG-tagged wild-type Pep4p, a double-point mutant form deficient in proteolytic activity, and human CatD (Figure 6a). We then compared their sensitivity to acetic acid with that of wild-type W303 cells expressing the empty vector. In accordance with our previous data,[14] expression of wild-type Pep4p, but not of the catalytically inactive mutant, reverted the sensitivity of Pep4p-deficient cells to acetic acid-induced apoptosis. Now, we further show that expression of human CatD also compensates for the loss of Pep4p, indicating that the two proteins have a similar role in this process (Figure 6b). All strains were then transformed with a plasmid expressing mitochondrial GFP, and mitochondrial degradation in response to acetic acid was assessed by estimating the percentage of cells with preserved green fluorescence, as described previously.[14] While expression of wild-type Pep4p and CatD reverted the delay in mitochondrial degradation of Pep4p-deficient cells exposed to acetic acid, expression of double-point mutant Pep4p did not (Figure 6c). Although the precise mechanism underlying the role of Pep4p in cell survival and in mitochondrial degradation is still elusive, we have now determined that both depend on its proteolytic activity and are complemented by CatD. Taken together, our results show that Pep4p/CatD is involved in acetic acid/acetate-induced mitochondrial degradation independently of autophagy but dependently of its catalytic activity in both yeast and CRC cells.

Discussion

CRC is one of the most common solid tumors worldwide.[2,26] A diet rich in dietary fiber is associated with a reduction in CRC incidence,[1,27] indicating CRC may be amenable to prevention through a dietary regimen.[15,16,27] Some of the significant health benefits of dietary fiber can be attributed to its microbial

Figure 2 Acetate impairs autophagy in CRC cells. (**a**) Levels of Beclin-1 in cells exposed to acetate for 48 h (110 and 220 mM; 70 and 140 mM for RKO and HCT-15 cells, respectively) (**b**) Immunoblot analysis of LC3-I/II in HCT116, RKO and HCT-15 cell lines. Cells were maintained in complete medium or incubated in HBSS medium (starvation) for 6 h in the presence and absence of 20 nM bafilomycin A1 (Baf. A1). The HCT116 cell line exhibited the strongest autophagic response to nutrient limitation (starvation) and thus was chosen to address the effect of acetate on starvation-induced autophagy. (**c**) LC3-II/actin ratios of HCT116, RKO and HCT-15 cells were determined using Quantity One software (Bio-Rad manufacturer). (**d**) The autophagic flux was determined by subtracting the normalized LC3-II levels in the presence of Baf. A1 from the corresponding levels obtained in the absence of Baf. A1. (**e**) Levels of LC3-I/II and (**g**) of Beclin-1 and Atg5 in HCT116 cells exposed to acetate. Cells were left untreated (Blank) or treated with 100, 150 or 200 mM acetate for 42 h. Later, cells were maintained in complete medium or HBSS medium (starvation) with acetate for another 6 h in the presence or absence of 20 nM Baf. A1. These data demonstrate that acetate decreases the autophagic flux in CRC cells in a dose-dependent manner, as shown by the decrease in LC3-II delivery to the lysosome, and in Beclin-1 and Atg5 protein levels. (**f**) The LC3-II/actin ratios of HCT116 cells were determined using Quantity One software (Bio-Rad manufacturer). Actin was used as a loading control. Values are mean ± S.E.M. of three independent experiments. Representative immunoblots are shown in (**a**, **b**, **e** and **g**). One-way ANOVA followed by Bonferroni's test, $^{\#\#}P \leq 0.01$ and $^{\#\#\#}P \leq 0.001$ compared with control cells treated with HBSS (starvation medium)

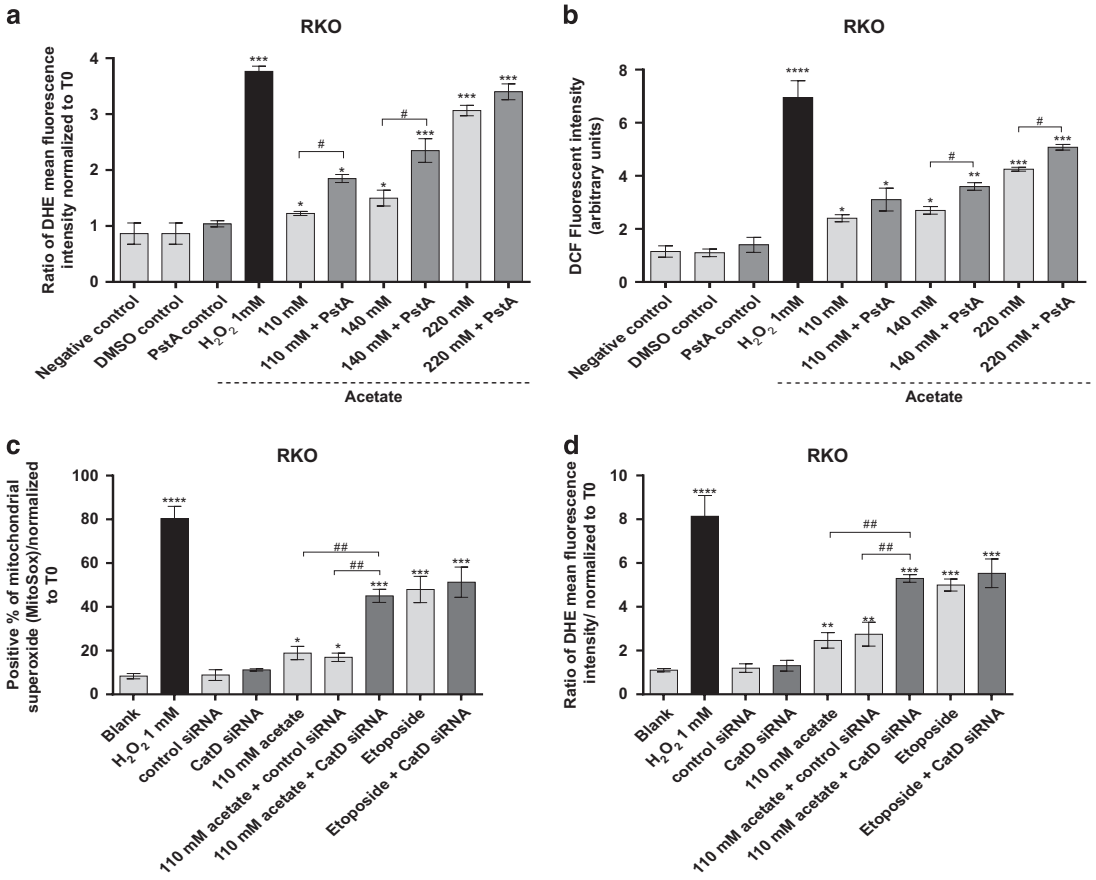

Figure 3 CatD protects CRC cells from oxidative stress. (**a** and **b**) Effect of PstA, a specific inhibitor of CatD catalytic activity, on ROS levels of RKO cells treated with acetate. PstA (100 μM) was preincubated for 16 h and then coincubated with acetate for 24 h. (**a**) Accumulation of superoxide anion (O_2^-) in RKO cells was detected with dihydroethidium (DHE) and quantified by flow cytometry. (**b**) Accumulation of H_2O_2 in RKO cells was detected with H_2DCF-DA using a fluorescence microplate reader. (**c** and **d**) Effect of CatD silencing on mitochondrial superoxide and total superoxide production in RKO cells treated with acetate (110 mM) or etoposide (50 μM) for 48 h. As controls, RKO cells were not transfected (blank), transfected with scrambled control siRNA or transfected with CatD siRNA. H_2O_2 (1 mM) was used as a positive control for ROS production. (**c**) Accumulation of mitochondrial superoxide in RKO cells was detected with MitoSOX and quantified by flow cytometry. (**c**) Downregulation of CatD induces a significant increase in mitochondrial superoxide production normalized to time 0 (*T0*) in RKO cells treated with 110 mM acetate and (**d**) a significant accumulation of total superoxide, normalized to *T0* cells. Values represent mean ± S.E.M. of at least three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ compared with control cells. $^{\#}P \leq 0.05$ and $^{\#\#}P \leq 0.001$ comparing acetate treatment with acetate/PstA or acetate treatment with acetate/CatD siRNA

fermentation, namely by Propionibacteria in the colon into SCFAs (acetate, propionate and butyrate).[1,6,15,16,28] Indeed, many studies suggest these SCFAs protect against carcinogenesis, as they reduce human colon cancer cell growth and differentiation and stimulate apoptosis in CRC cells.[4,6,27,29] Acetate is one of the most important SCFAs, but has been less investigated than propionate and butyrate. Nonetheless, previous studies proposed that acetate inhibits proliferation and induces apoptosis in colon cancer cells[3–6] and that acetate-induced apoptosis in CRC cells involves different biochemical events, including mitochondrial alterations.[3–5] We have also previously shown that acetate induces apoptosis in CRC-derived cells in a dose-dependent manner, characterized by DNA fragmentation, caspase-3 activation, phosphatidylserine exposure to the outer leaflet of the plasma membrane and the appearance of a sub-G1 population[5]. In addition, we showed that this process is associated with lysosomal alkalinization, LMP and CatD release into the cytosol.[5]

It is well established that partial LMP followed by a release of lysosomal hydrolases into the cytosol can activate intrinsic caspase-dependent apoptosis or a caspase-independent alternative cell death pathway.[7,8] Of these, CatD has emerged as an important molecular target in cancer therapy, as it is overexpressed and secreted by cells of various tumor types, including CRC.[30,31] CatD has a vital role in extracellular matrix degradation and cancer cell survival, and actively participates in the invasion of carcinoma cells during both local invasion and metastasis formation.[32,33] However, its role in apoptosis depends on cellular types, specific contexts, stimulus and catalytic activity.[10,33] Our data indicate that CatD has a protective role in acetate-induced apoptosis in CRC cells,[5] as we had shown for its ortholog Pep4p in *Saccharomyces cerevisiae*.[13] As SCFAs, and in particular acetate, have garnered increased interest as potential prevention/therapeutic agents in CRC, we sought to further understand the mechanism underlying the antiapoptotic role of this lysosomal protease under this specific cellular and stimulus context. Based on data from our group and others, as well as on the

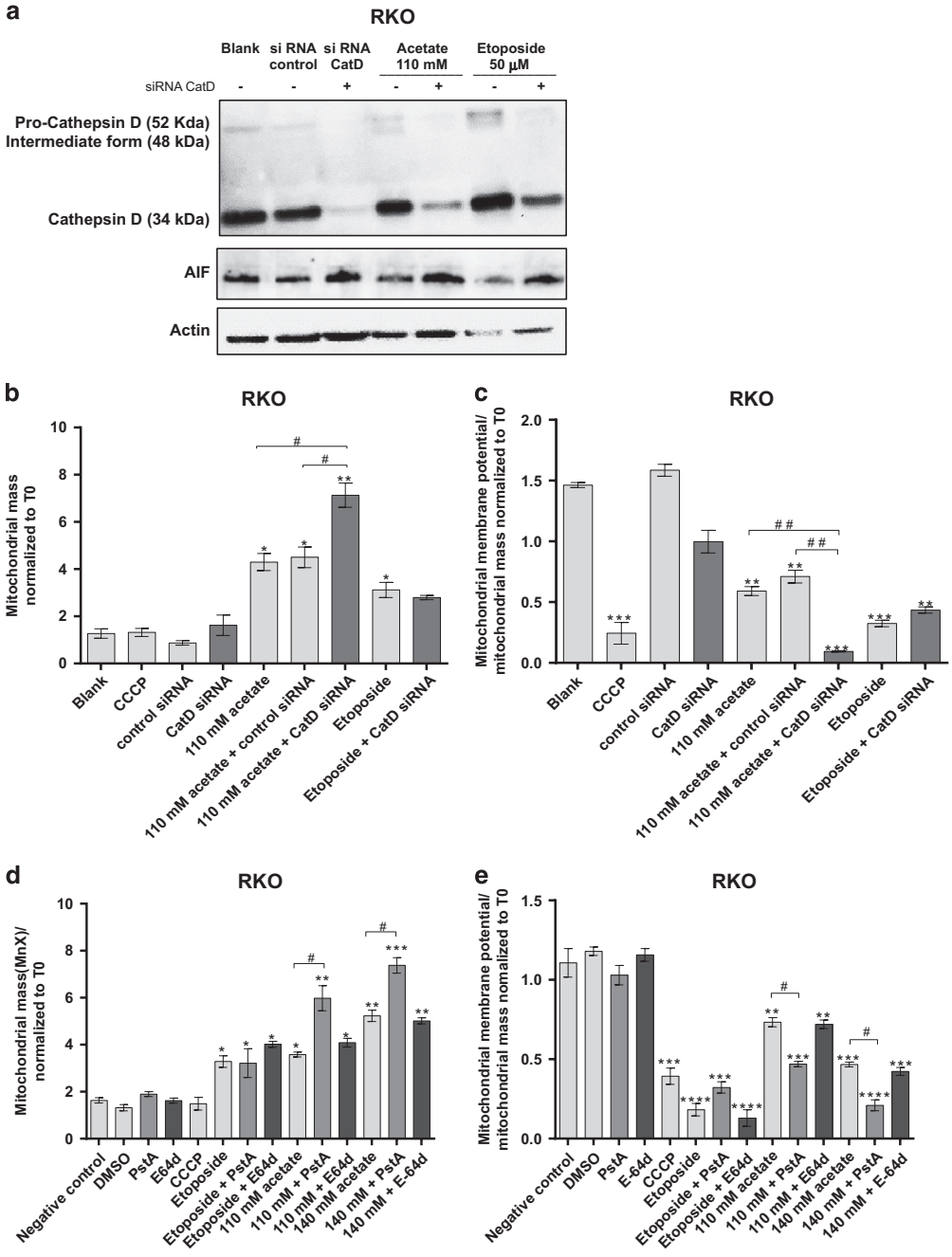

Figure 4 CatD is involved in autophagy-independent mitochondrial degradation in CRC cells. (**a**) Western blot of CatD isoforms (Pro-CatD, intermediate form and mature CatD) and AIF with or without CatD silencing in the absence or presence of acetate (110 mM) or etoposide (50 μM). As controls, RKO cells were not transfected (blank), transfected with scrambled control siRNA or transfected with CatD siRNA. Actin was used as a loading control. (**b** and **c**) Effect of CatD silencing on mitochondrial dysfunction in RKO cells treated with acetate (110 mM) or etoposide (50 μM) for 48 h. (**b**) Downregulation of CatD induces a significant increase in mitochondrial mass normalized to time 0 (*T*0) in RKO cells treated with 110 mM acetate and (**c**) a significant mitochondrial membrane depolarization normalized to mitochondrial mass, in relation to *T*0. For each bar, the mean value for at least three independent experiments is represented. Bonferroni's test, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ compared with negative control cells. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ comparing acetate treatment with acetate/CatD siRNA. (**d** and **e**) Effect of the specific CatD inhibitor (PstA) and the CatB and CatL inhibitor (E-64d) on mitochondrial dysfunction in RKO cells treated with acetate (110 and 140 mM) or etoposide (50 μM) for 48 h. E-64d (10 μM) and PstA (100 μM) were pre-icubated for 1 or 16 h, respectively, and then coincubated with acetate or etoposide for 48 h. Cells were incubated with fresh complete medium or with 50 μM CCCP as a negative and positive control, respectively. Cells were then double stained with 400 nM MitoTracker Green and 200 nM MitoTracker Red CMXRos for 30 min at 37 ºC in the dark and analyzed by flow cytometry. Inhibition of CatD activity with PstA induces an increase in mitochondrial mass (**d**) and membrane depolarization normalized to mitochondrial mass in relation to *T*0 in RKO cells (**e**). For each bar, the mean value for at least three independent experiments is represented. Bonferroni's test $^{*}P \leq 0.05$; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ compared with negative control cells. $^{\#}P \leq 0.05$ comparing acetate treatment with acetate/PstA

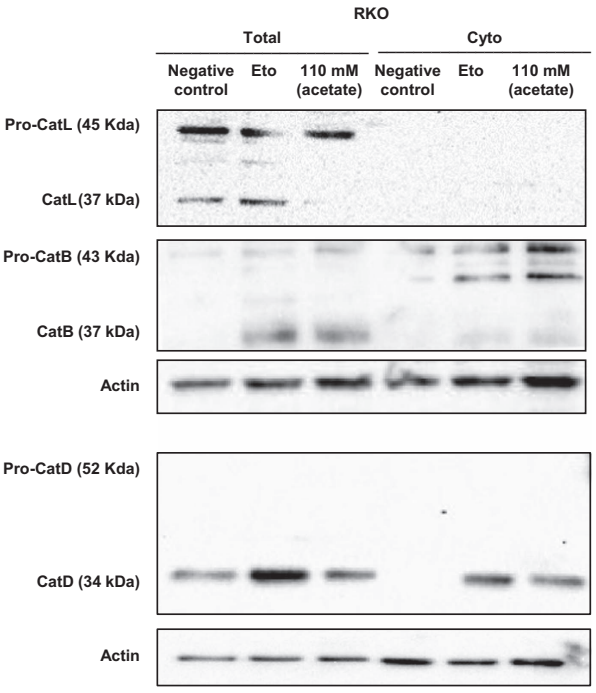

Figure 5 Acetate induces specific CatD release to the cytosol in CRC cells. Effect of acetate on the expression and release of CatL, CatB and CatD to the cytosol in RKO cells. Cells were treated with 110 mM acetate or 50 μM etoposide for 48 h, or with fresh medium as a negative control. Total and cytosolic fractions (Cyto) are shown. Actin was used as a loading control

present study, we propose that the lysosome and mitochondria communicate during acetate-induced apoptosis in CRC cells through permeabilization of both organelles and selective leakage of proteins. Indeed, we show that acetate triggers release of CatD to the cytosol, but not of CatL, and only a small amount of CatB, which does not seem to have major consequences. Besides lysosomal alterations, acetate triggered mitochondrial dysfunction in a dose- and time-dependent-manner, characterized by increased mitochondrial ROS, changes in $\Delta\Psi m$ and an increase in mitochondrial mass, which were enhanced when CatD was inhibited. This mitochondrial dysfunction is in agreement with that reported during apoptosis induction by acetate and a mixture of acetate and propionate produced by *Propionibacterium freudenreichii* in other CRC cells (HT-29), including increased ROS and $\Delta\Psi$m dissipation, as well as swelling in isolated mitochondria.[4] However, the role of CatD or the lysosome in that context was not evaluated.

The increase in mitochondrial mass observed after exposure of cells to acetate led us to investigate whether the presumed decrease in mitochondrial turnover was associated with modulation of autophagy by this SCFA. Previous studies demonstrated that apoptosis triggered by low doses (1–10 mM) of propionate and butyrate can be delayed because autophagy is also induced, which can potentially impair the therapeutic efficacy of SCFAs in colon cancer.[15–17] Indeed, autophagy has been implicated in cancer progression, used by cells for autophagic degradation of damaged organelles, long-lived proteins and pathogens, and in this way maintain homeostasis.[20,29,34] Further assays are required to

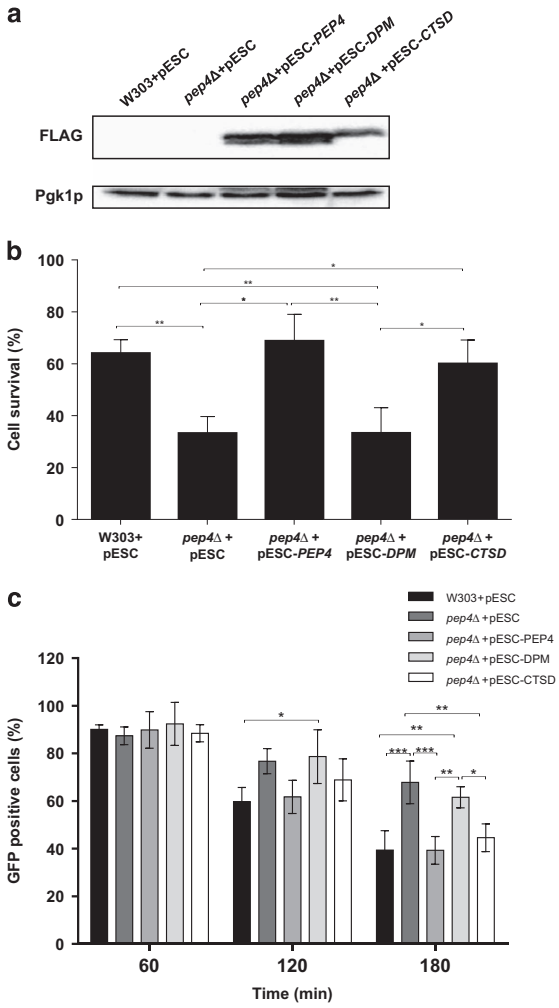

Figure 6 Cell survival and mitochondrial degradation in *S. cerevisiae* cells during acetic acid treatment. The W303 strain transformed with the empty vector (pESC) and *pep4*Δ strains, transformed with the empty vector (pESC), pESC-*PEP4* (expressing WT-Pep4p), pESC-*DPM* (expressing DPM-Pep4p) or pESC-*CTSD* (expressing human CatD), were incubated with 120 mM acetic acid for up to 180 min. (**a**) Immunoblot analysis of whole-cell extracts of *pep4*Δ cells expressing FLAG-tagged WT-Pep4p, FLAG-tagged DPM-Pep4p, FLAG-tagged CatD and the corresponding empty vector after 20 h of growth. Pgk1p was used as a loading control. (**b**) Cell survival at time 180 min was determined by standard dilution plate counts and expressed as a percentage of colony-forming units (CFU) in relation to time 0. Data represents means ± S.D. ($n = 3$). (**c**) Mitochondrial degradation was assessed by measuring the percentage of cells without loss of mtGFP fluorescence (100% corresponds to the number of GFP positive cells at time 0). Data represent means ± S.D. ($n = 4$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

ascertain whether low concentrations of acetate can cause a similar adaptive response in CRC cells. However, we previously showed that autophagy is not induced during acetic acid-induced apoptosis in yeast.[13] In the present study, we demonstrate that acetate impairs the autophagic flux of HCT116 cells and decreases Beclin-1 levels in all three cell lines. Therefore, we show that concentrations of acetate normally present in the human intestine have a previously uncharacterized effect in CRC cells: inhibition of autophagy, most likely through impairment of autophagosome and lysosome fusion during acetate-induced apoptosis.

It is known that malfunctioning mitochondria are selectively targeted for autophagic degradation.[16] However, it is conceivable that cells use alternative pathways to clear damaged mitochondria when autophagy is inactive. We therefore investigated if CatD is involved in mitochondrial degradation, similar to Pep4p in yeast, possibly having a prosurvival role through elimination of dysfunctional mitochondria. We show that CatD protects RKO cells from acetate-induced oxidative stress and mitochondrial depolarization, as its inhibition with PstA or downregulation with siRNA cause a significant increase in the levels of H_2O_2 and O_2^- and a decrease in $\Delta\Psi m$. Hah *et al*.[10] have previously suggested that CatD has a protective role in H_2O_2-induced cell death in M059J glioblastoma cells through its involvement in autophagy, which enhances cell survival under oxidative stress.[10] Accordingly, we had shown that Pep4p deletion increased the sensitivity of yeast cells to acetic acid associated with increased ROS accumulation,[14] although autophagy was not induced.[13] These data, together with our results, support the notion that reduced CatD activity leads to increased ROS accumulation and mitochondrial depolarization. We therefore proceeded to determine whether CatD is involved in the degradation of damaged mitochondria, as we presumed to be the case during acetate-induced apoptosis in CRC cells. We found that downregulation of CatD in acetate-treated RKO cells increased mitochondrial mass, as well as the cellular pool of AIF. These findings corroborate our hypothesis that CatD is involved in the degradation of damaged mitochondria during acetate-induced apoptosis through an autophagy-independent process. Although not sufficiently efficient to maintain mitochondrial mass homeostasis, as an acetate-induced increase in mitochondrial mass is still observed, this alternative degradation process allows the cell to dispose of dysfunctional mitochondria and delay cell death. It will be interesting in the future to determine if CatD is also released and is involved in mitochondrial degradation in response to other SCFAs, either directly or through downstream substrates. Inhibition of CatD with PstA mimicked the results obtained with CatD siRNA, indicating the catalytic activity of CatD is required for this function, as we previously found for the antiapoptotic role of both yeast and human CatD.[5,14] We now also show that the role of yeast CatD in acetic acid-induced mitochondrial degradation depends on its proteolytic activity and can be complemented by human CatD, indicating this mechanism is conserved through evolution.

As discussed above, partial LMP followed by a release of lysosomal hydrolases into the cytosol can activate intrinsic caspase-dependent apoptosis or a caspase-independent alternative cell death pathway.[7,8] Thus, disabling lysosome function is under investigation as an adjuvant therapeutic approach to sensitize cells to apoptosis-inducing agents.[7] Destabilization of the lysosomal membrane has been proposed as a promising strategy, as it would promote apoptosis through initiation of the lysosomal pathway in cancer cells.[8] However, our results show that the release of lysosomal proteases, in particular of CatD,[5] may enable a degradation process alternative to autophagy with a similar protective role in cell survival, thus counteracting activation of the lysosomal death pathway. This would negatively impact the efficacy of acetate, and probably of other therapeutic compounds that trigger LMP and impair autophagy, and would require inhibiting CatD in combination treatments.

In summary, our findings based on the clues provided by the yeast system unveiled a novel prosurvival function of CatD in autophagy-independent mitochondrial degradation, which can enhance cell survival in CRC cells undergoing acetate-induced apoptosis. These results offer new perspectives in the intricate regulation of life and death, and suggest using CatD inhibitors as adjunct therapy with SCFAs for prevention/therapy of CRC.

Materials and Methods

Cell lines and culture. We used three cell lines derived from human CRC, namely RKO^{BRAFV600E}, HCT-15^{KRASG13D} and HCT116$^{KRASG13D;\ PI3KCA}$. Cells were maintained at 37 °C under a humidified atmosphere containing 5% CO_2. HCT-15 cells were grown in RPMI (Biowest, Nuaillé, France) medium with L-glutamine and HEPES. RKO cells were grown in DMEM (Biowest) with high glucose and supplemented with 1 mM sodium pyruvate and 1.5 g/l sodium bicarbonate. HCT116 cells were grown in McCoy's 5 A (Biowest) medium. All culture media were supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. For autophagy studies, we used HBSS (Gibco, Paisley, UK) medium supplemented with 7.5% sodium bicarbonate.

Cell treatments with acetate. Cells were seeded and adhered onto appropriate sterile plates for 24 h before treatments in all experiments. We previously determined the IC_{50}, IC intermediate (IC inter) and 2× IC_{50} of acetate after 48 h of treatment: 70, 100 and 140 mM for HCT-15; 110, 140 and 220 mM for RKO cell lines, respectively.[5] The IC_{50}, IC inter and 2× IC_{50} values for HCT116 (100, 150 and 200 mM, respectively) were calculated from the mean values of sulforhodamine B reduction as described in Marques *et al*.[5] (Supplementary Figure S3). For each bar, the mean value of at least three independent experiments is represented. Bonferroni's test: $^*P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ compared with negative control cells.

RNA interference-mediated inhibition of CatD. RKO cells were plated in 6-well plates at a density of 3×10^4 cells per well. After 24 h, about 70% confluence was confirmed before transfection with small interfering RNA (siRNA) oligonucleotides. Cells were transfected with 100 nM on-target plus SMART pool siRNA against CatD (A-003649-16; Thermo Fisher Scientific, Lafayette, CO, USA). Transfection performance was monitored using a validated Silencer Select Negative Control (scrambled siRNA control, no. 4390843; Life Technologies, Carlsbad, CA, USA). Transfection was performed with 6 μl Lipofectamine 2000 (Invitrogen Corp., Paisley, UK). After 14 h, the transfection mixture was removed and cells were left untreated (blank) or treated with acetate (110 mM) or etoposide (50 μM) and incubated for a further 48 h in fresh medium. MitoSOx, DHE and double staining with MitoTracker Green and Mitotracker Red CMXRos were performed as described below. The experiments were carried out in three replicates, and CatD levels were monitored by western blotting.

Determination of intracellular ROS

Dichlorofluorescein assay: Cells were seeded in a 12-well plate at a density of 1.4×10^5 and 1.8×10^5 cells per well (RKO and HCT-15 cells, respectively). When indicated, cells were preincubated with PstA (Sigma-Aldrich, St Louis, MO, USA) for 16 h and then coincubated with PstA without and with different acetate concentrations for 12, 24 and 48 h. After treatment, cells were washed two times with 1x PBS and H_2DCF-DA (Sigma-Aldrich) was added to the culture plates at a final concentration of 100 μM. Plates were incubated at 37 °C for 30 min in the dark. Then, cells were lysed with 500 μl of 90% DMSO/10% PBS (lysis solution) for 10 min at room temperature in the dark, with agitation. H_2O_2 (500 μM or 1 mM for HCT-15 or RKO cell lines, respectively) was used as a positive control. H_2DCF-DA fluorescence intensity was detected with emission wavelength at 538 nm and excitation wavelength at 485 nm using a fluorescence microplate reader (Fluoroskan Ascent FL; Thermo Fisher Scientific Inc., Waltham, MA, USA). Values are expressed as the mean fluorescence intensity normalized to the cell number in each condition at the end of the experiment. Three independent experiments were performed.

DHE assay: Non-treated cells (negative control) or cells exposed to different acetate concentrations for 12, 24 and 48 h were analyzed. When used, cells were preincubated with PstA for 16 h and then coincubated with PstA without and with different acetate concentrations for 24 h. Inhibition of CatD in RKO cells was performed as described above. Approximately 1×10^6 floating and attached cells were collected, washed with 1× PBS, centrifuged at $1500 \times g$ for 5 min and incubated with 150 nM DHE (Molecular Probes, Eugene, OR, USA) (30 min, 37 °C, in the dark) to detect superoxide anion ($O_2^{\cdot-}$). Fluorescence emission of oxidized DHE was analyzed by flow cytometry. H_2O_2 (500 μM or 1 mM for HCT-15 or RKO cell lines, respectively) was used as a positive control. Values are expressed as the mean of fluorescence intensity normalized to *T*0 (control for ROS level before the treatment). Three independent experiments were carried out and data were analyzed using the Flowing software (version 2.5.1, Turku Centre for Biotechnology, Turku, Finland).

MitoSOX assay: MitoSOX Red (Molecular Probes) was used to detect mitochondrial superoxide. Approximately 1×10^6 floating and attached cells were collected, washed with 1x PBS, centrifuged at $1500 \times g$ for 5 min and incubated with 2.5 μM MitoSOX Red for 30 min at room temperature. Fluorescence emission of oxidized MitoSOX Red was analyzed by flow cytometry. H_2O_2 (1 mM) was used as a positive control. Values are expressed as the percentage of cells with positive staining normalized to *T*0 (control for ROS level before the treatment).

Analysis of mitochondrial alterations

Mitochondrial mass and ΔΨm analysis: MitoTracker Green FM (Molecular Probes) was used to analyze the relative mitochondrial mass. MitoTracker Green is a green fluorescent dye that localizes to the mitochondrial matrix regardless of the ΔΨm and covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues. MitoTracker Red CMXRos (Molecular Probes) was used simultaneously, to monitor the changes in ΔΨm. MitoTracker Red CMXRos is a red-fluorescent dye that stains mitochondria in live cells through accumulation in mitochondria in a membrane potential-dependent manner. Untreated cells (negative control), cells exposed to etoposide (50 μM), an inducer of LMP, or to different acetate concentrations were analyzed at different time points. When used, cells were preincubated with 10 μM (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E-64d; Sigma-Aldrich) (a CatB and CatL inhibitor) or 100 μM PstA for 1 and 16 h, respectively, and then co-incubated with different acetate concentrations or etoposide for 48 h. Both floating cells and attached cells were collected, washed with 1x PBS, centrifuged at $1500 \times g$ for 5 min and incubated with 400 nM MitoTracker Green FM and 200 nM MitoTracker Red CMXRos (30 min, 37 °C, in the dark). The uncoupling agent CCCP (50 μM, 30 min) was used as positive control for mitochondrial membrane depolarization. Fluorescence emission was analyzed by flow cytometry. The values of mitochondrial mass for each time point were expressed as the ratio between the mean green fluorescence intensity and the one correspondent to time zero (*T*0). The values of the ΔΨm for each time point were expressed as the ratio between the mean red fluorescence intensity and the mean green fluorescence intensity normalized to the correspondent one at *T*0. Three independent experiments were carried out.

Fluorescence microscopy studies: Cells were seeded in 6-well plates containing glass coverslips and exposed to acetate for 48 h. At the end of the experiment, cells were washed with 1x PBS and incubated with 20 nM $DiOC_6(3)$ (Molecular Probes) or with 400 nM MitoTracker Green and 200 nM MitoTracker Red CMXRos for 30 min, at 37 °C, in the dark. CCCP (50 μM, 30 min) was also used as a positive control for ΔΨm loss. Coverslips were mounted in 1x PBS and immediately observed under the fluorescence microscope (Leica DM 5000B; Leica Microsystems, Wetzlar, Germany). Three coverslips were prepared for each experimental condition and representative images are shown.

Flow cytometry analysis: Samples were analyzed in a flow cytometer (Epics XL; Beckman Coulter, Miami, FL, USA) equipped with an argon-ion laser emitting a 488-nm beam at 15 mW. Detection of red fluorescence was performed using FL-4 (488/620 nm) and detection of green fluorescence was performed using FL-1 (488/525 nm). A total of 30 000 cells were acquired *per* sample and data were analyzed using the Flowing software (version 2.5.1, Turku Centre for Biotechnology).

Assessing autophagy in CRC cell lines: Cell lines untreated (Blank) or treated with IC_{50}, IC inter or 2× IC_{50} acetate concentrations were incubated for 42 h in complete medium. Later, cells were incubated in HBSS medium for 6 h in the presence or absence of 20 nM Baf. A1 (Acros Organics, Geel, Belgium), in the absence or presence of acetate.

Cell survival and mitochondrial degradation assays in yeast: *Saccharomyces cerevisiae* strain W303-1A was used as the wild-type strain. The *pep4*Δ mutant was constructed in W303-1A by homologous recombination using a *PEP4*::*kanMX4* disruption cassette amplified from the respective Euroscarf deletion strain by PCR. Correct integration of the cassette was confirmed by PCR. To construct $CatD^{FLAG}$, the insert was amplified by PCR from the plasmid pJP1520-*CTSD* (containing human CatD cDNA) and integrated by homologous recombination into the pESC-His vector. Correct integration was verified by sequencing. The W303-1A strain was transformed with the empty vector (pESC) and the *pep4*Δ strain was transformed with the empty vector (pESC), pESC-*PEP4*, pESC-*DPM* and pESC-*CTSD* plasmids for expression of WT-Pep4, double-point mutant (DPM-Pep4p) or human CatD, respectively. The strains were transformed with the pGAL-CLbGFP (see Okamoto *et al.*[35]) vector for mitochondrial degradation studies. Strain growth conditions, acetic acid treatments and mitochondrial degradation assays were performed as described previously.[14]

Preparation of total/cytosolic protein extracts and western blotting: Cell lysis of mammalian cells, protein sample preparation (total and cytosolic) and western blotting were carried out as described previously[5] with 25 μg of total or cytosolic extracted proteins applied per lane before SDS-PAGE. Total yeast extracts were prepared by suspending ~2×10^6 cells in 0.5 ml of water and adding 50 μl of a mixture of 3.5% β-mercaptoethanol in 2 M NaOH. After a 15 min incubation on ice, proteins were precipitated with 50 μl of 3 M trichloracetic acid for 15 min on ice. After a rapid centrifugation, the pellet was resuspended in Laemmli buffer for SDS-PAGE.

The primary antibodies used were anti-Beclin-1 (1 : 3000; Cell Signaling Technology, Leiden, Netherlands), anti-LC3-II (1 : 5000; Sigma-Aldrich), anti-Atg5 (1 : 3000; Sigma-Aldrich), anti-CatD (1 : 100; Calbiochem, San Diego, CA, USA), anti-CatB (1 : 300; Abcam, Cambridge, UK), anti-CatL (1 : 1000; Abcam), anti-AIF (internal) rabbit polyclonal antibody (1 : 500; Chemicon International, Billerica, MA, USA), anti-VDAC1 (1 : 2000; MitoSciences, Eugene, OR, USA), anti-Tom22 (FL-145) (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-actin (Santa Cruz Biotechnology, Santa Cruz), anti-FLAG (1 : 5000; Sigma-Aldrich) and anti-yeast phosphoglycerate kinase (PGK1) antibody (1 : 5000; Molecular Probes). Secondary antibodies used were peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) and horseradish peroxidase-labeled goat anti-mouse immunoglobulin IgG (Jackson ImmunoResearch). Subsequent chemiluminescence detection was performed using the ECL detection system (Amersham, Biosciences, Buckinghamshire, UK) and a molecular imager by Chemi-Doc XRS system (Bio-Rad, Laboratories Inc., Hercules, CA, USA). When performed, densitometry analysis of protein bands was performed using the Quantity One software (version 4.6.9, Bio-Rad manufacturer, Hercules, CA, USA) and levels of actin used as a normalization control for protein loading.

Statistical analysis: Data are expressed as mean ± S.D. of three independent experiments. Statistical significance analysis was determined by one-way ANOVA followed by Dunnett or Bonferroni's test for multiple comparisons with the control using Prism software (GraphPad, La Jolla, CA, USA). The differences were considered significant for *P*-values lower than 0.05.

Conflict of Interest

The authors declare no conflict of interest.

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Yeast as a tool to explore cathepsin D function

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ABSTRACT **Cathepsin D has garnered increased attention in recent years, mainly since it has been associated with several human pathologies. In particular, cathepsin D is often overexpressed and hypersecreted in cancer cells, implying it may constitute a therapeutic target. However, cathepsin D can have both anti- and pro-survival functions depending on its proteolytic activity, cellular context and stress stimulus. Therefore, a more detailed understanding of cathepsin D regulation and how to modulate its apoptotic functions is clearly needed. In this review, we provide an overview of the role of cathepsin D in physiological and pathological scenarios. We then focus on the opposing functions of cathepsin D in apoptosis, particularly relevant in cancer research. Emphasis is given to the role of the yeast protease Pep4p, the vacuolar counterpart of cathepsin D, in life and death. Finally, we discuss how insights from yeast cathepsin D and its role in regulated cell death can unveil novel functions of mammalian cathepsin D in apoptosis and cancer.**

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Abbreviations:
CRC - colorectal cancer,
EGFP - enhanced green fluorescent protein,
LMP - lysosomal membrane permeabilization,
RCD - regulated cell death,
ROS - reactive oxygen species,
VMP - vacuolar membrane permeabilization.

CATHEPSINS

Cathepsins are members of a large protease family, which can be subdivided according to their structure and active-site amino acid into cysteine (cathepsins B, C, F, H, K, L, O, S, V, W, and X), serine (cathepsins A and G), and aspartic cathepsins (cathepsins D and E). While cathepsins B, L, H, C and D are ubiquitously expressed in human tissues, expression of cathepsins A, G, K, S, V, X and W is tissue and cell type specific [1-4]. In general, cathepsins are found in acidic cellular organelles, lysosomes and endosomes. Initially, their function was thought to be limited to bulk degradation of proteins delivered to the lysosome by endocytosis or autophagocytosis. However, it was later demonstrated that cathepsins possess highly specific and directed proteolytic activity, and that they can be found in other cellular compartments [5-10]. Numerous physiological functions of cathepsins have been uncovered, including a role in hormone and antigen processing, bone and tissue remodeling, growth factor and proenzyme activation and, more recently, in the immune response [5, 6, 11-13]. Cathepsins also participate in apoptosis and are translocated from the lysosomal lumen to the cytosol of mammalian cells through lysosomal membrane permeabilization (LMP) in response to a variety of apoptotic signals [14-16]. These lysosomal proteases can also be secreted from the cell and degrade extracellular matrix proteins such as collagen, fibronectin, proteoglycans and laminin [17].

In addition to their physiological function, cathepsins have also been associated with several pathologies such as cardiovascular diseases, osteoporosis, rheumatoid arthritis, atherosclerosis and cancer [6, 11, 17-19]. Elucidating the mechanisms underlying the involvement of cathepsins in the pathogenesis of these diseases, and how they can be modulated to develop new prevention and therapeutic strategies, has therefore taken center stage. Among cathepsins, cathepsin D (CatD) has attracted increased attention in recent years due to its importance in the mediation of lysosomal cell death pathways and in cancer. In this review, we will concentrate on both physiological and pathological functions of CatD, as well as on yeast as a model system to study CatD pathophysiology.

ROLE OF CATHEPSIN D IN CELLULAR PHYSIOLOGY AND PATHOLOGY

CatD is a soluble aspartic endopeptidase found in the lysosomes of most mammalian cells. Like other cathepsins,

CatD is activated by proteolytic cleavage of the synthetized inactive zymogen (preproCatD), which is composed of an N-terminal signal peptide, a propeptide, and a catalytic domain [20-22]. The signal peptide directs the nascent chain to the endoplasmic reticulum, where it is cleaved in the lumen. ProCatD is then N-glycosylated and transported to the Golgi, where the N-glycan structures acquire mannose-6phosphate (Man-6P) residues that can bind to Man-6P receptor(s) (Man-6PR), and the complex is directed to the lysosomal compartment [23]. In the acidic milieu, proCatD (52 kDa) undergoes further proteolytic processing by cleavage of the proregion, resulting in the 48 kDa single chain intermediate active form. Finally, this chain is processed into mature active CatD, composed of heavy (34 kDa) and light (14 kDa) chains linked by non-covalent interactions [24-26]. It has been shown that CatD processing involves cysteine cathepsins [26, 27] and, more recently, that it is independent of its own catalytic function and auto-activation but requires CatL and CatB [28]. Although proCatD and CatD are mostly intracellular, they can also localize in the extracellular matrix and synovial fluid of cartilage [29-31]. ProCatD/CatD are also found in human, bovine and rat milk [32-34], serum, sweat and urine [35, 36], and extracellularly in macrophage-rich regions of atherosclerotic lesions [37]. ProCatD secretion by human keranocytes [38], mammalian epithelial cells [39] and different types of cancer cells [18, 40] was also demonstrated.

It is widely accepted that the major function of CatD is its involvement in general protein degradation and turnover within the lysosomal compartment. However, CatD has also emerged as an important regulator and signaling molecule with numerous physiological functions. These include activation of enzymatic precursors, prohormones and growth factors, processing of brain-specific antigens, tissue homeostasis, and participation in apoptosis [18, 41]. CatD has also been associated with different pathological scenarios such as cancer progression and metastasis, Alzheimer's disease, atherosclerosis and inflammatory disorders [11, 12, 40, 42], and found to be a specific biomarker for several pathologies. The involvement of CatD in both physiological and pathological processes has been addressed in multiple studies, some of which are summarized in Table 1 [38, 43-62]. A more detailed description of the role of CatD in cancer is given below.

THE ROLE OF CATHEPSIN D IN CANCER

Numerous reports have demonstrated that CatD is overexpressed in several cancer types [18, 40, 42, 63-65], often correlating with poor prognosis. In particular, CatD is considered an independent prognostic marker in breast cancer associated with metastatic risk [66-68] and in colorectal cancer (CRC) [69, 70]. Mechanistically, the majority of reports attribute its role in cancer to overexpression of proCatD. As an example, transfection of rat tumor cells with human proCatD cDNA leads to increased proliferation, invasion and metastasis *in vitro* and *in vivo* [71]. Accordingly, anti-proCatD antibodies can inhibit tumor growth both *in vitro* and *in vivo* [72-74]. Overexpressed proCatD escapes normal targeting routes and is hypersecreted to the extracellular milieu, where it can act in multiple fashions. On

TABLE 1. Cellular roles of cathepsin D in physiological and pathological processes.

Role	Model	References
Limited proteolysis of proteins regulating cell growth and/or tissue homeostasis	*In vivo*: CatD-deficient mice	[43]
Postnatal tissue homeostasis including tissue renewal, remodeling, aging and RCD	*In vivo*: CatD-deficient mice; CatD-mutant mice	[44-47]
Neuronal ceroid lipofuscinosis in both animals and humans characterized by severe neurodegeneration, developmental regression, visual loss and epilepsy	*In vivo*: CatD-deficient mice; CatD-mutant mice Human pathologies Animal diseases	[45, 48-51]
Wound healing, epidermal differentiation and pathological conditions such as psoriasis	*In vivo:* CatD-deficient mice *In vitro*: normal and psoriatic keranocytes Human patients	[52-54]
Proliferation and regeneration in keratinocytes and possibly in skin regeneration	*In vitro:* keratinocyte cell line HaCaT	[38]
Processing of proteins involved in Alzheimer disease pathogenesis, such as apolipoprotein E (apoE) and Tau protein	Human patients Recombinant protein	[55, 56]
Post-partum cardiomyopathy resulting in heart failure	*In vivo*: mutant mice	[57]
Autism pathogenesis	Autistic subjects	[58]
Innate immune responses and Parkinson disease	*In vivo*: CatD-deficient mice Human patients	[59, 60]
Intracellular metabolism, transport of phospholipids and cholesterol	Human patients	[61]
Atherosclerotic lesions associated with proCatD release from monocyte-derived macrophages	Atherosclerosis patients *In vitro*: cultured atherosclerotic plaques	[62]

one hand, it can exert an autocrine effect, inducing cancer cell growth by interacting with cell surface receptors [72, 75-77]. This autocrine role has so far been observed in breast, prostate, ovarian and lung cancer cells [72-74, 78]. In addition, proCatD can play a crucial paracrine role in the tumor microenvironment by stimulating fibroblast outgrowth and tumor angiogenesis [71, 79], as well as inhibiting anti-tumor responses [80]. When in the tumor microenvironment, proCatD may also affect stromal cell behavior and/or degrade components from the extracellular matrix [81, 82], including the release of growth factors [83]. Although it has been suggested that proCatD can be processed in the acidic extracellular space to catalytically active CatD [84], the enzymatic activity of CatD is reportedly not required for its mitogenic role. Indeed, a proteolytically inactive mutant of CatD (D231N) is still mitogenic for fibroblasts [85], as well as for cancer cells both *in vitro*, in three-dimensional matrices, and in athymic nude mice [71, 86]. Similarly, proCatD stimulates angiogenesis in tumor xenografts of athymic nude mice independently of its catalytic activity [85], also suggesting that CatD can signal through protein-protein interactions.

Though less extensive, there are also examples of CatD roles in cancer cells that are not attributed to proCatD. For instance, intracellular CatD can stimulate cancer cell growth by inactivating secreted growth inhibitors [87, 88]. Moreover, mature CatD released into the cytosol as a consequence of the reportedly higher susceptibility of cancer cells to LMP [15, 89] may interact with and/or degrade pro- and anti-apoptotic proteins, modulating cell death [41].

Targeting CatD is a promising strategy in the clinic, but requires further detailed elucidation of its mechanisms of action. In the following section, we focus on the role of CatD in the apoptotic process, which is of particular relevance for cancer research. These studies may however also offer clues into the function of CatD in other physiological and pathological scenarios.

OPPOSING FUNCTIONS OF CATHEPSIN D IN APOPTOSIS

In recent years, multiple studies have shown that CatD is a central player in the apoptotic response, both under physiological and pathological conditions. In fact, depending on the cell type and context, CatD can induce or inhibit apoptosis, acting through different mechanisms [41]. On one hand, CatD can directly induce apoptosis triggered by several stimuli such as staurosporine [90], etoposide, 5-fluorouracil and cisplatin [91], as well as resveratrol [92] and others, possibly mediated by intrinsic or extrinsic pathways [41]. In the intrinsic pathway, the role of CatD is linked to the release of mature 34 kDa CatD into the cytosol and cleavage of Bid to form tBid, triggering insertion of the pro-apoptotic protein Bax into the mitochondrial membrane [15]. Subsequent mitochondrial outer membrane permeabilization leads to the release of pro-apoptotic molecules such as cytochrome *c* and apoptosis inducing factor (AIF) to the cytosol [15]. For instance, it has been shown that CatD mediates cytochrome *c* release and caspase activation in human fibroblasts undergoing staurosporine-induced apoptosis [90], and cleaves Bid and promotes apoptosis via oxidative stress-induced LMP in human neutrophils [93]. In addition, Pepstatin A and/or knockdown of CatD expression by RNA interference prevent resveratrol toxicity, impeding Bax oligomerization, mitochondrial membrane permeabilization, cytochrome *c* release and caspase 3 activation in DLD1 and HT29 CRC cell lines [92]. One study also reports that CatD mediates selective release of AIF in T lymphocytes entering the apoptosis early commitment phase through activation of Bax in a Bid-independent manner [94]. This shows that CatD can be involved in caspase-independent apoptosis by activating Bax independently of Bid cleavage. Other studies strongly suggest that cytosolic CatD may have an additional role involving protein-protein interactions. As examples, it has been shown that overexpression of either catalytically active or inactive CatD by cancer cells enhances apoptosis-dependent chemo-sensitivity [95], and that stress-induced apoptosis is not affected in fibroblasts synthesizing a catalytically inactive CatD [96]. Additionally, microinjection of inactive proCatD into the cytosol of both human fibroblasts and HeLa cells induces apoptosis [97]. Interestingly, one report also indicates that cytosolic mature CatD may reach the nucleus during cell death [98].

In contrast with the multiple studies showing CatD is pro-apoptotic, other studies describe an anti-apoptotic function of CatD. Most of these suggest it plays an anti-apoptotic role in cancer cells. For example, CatD down-regulation sensitizes human neuroblastoma cells to doxorubicin-induced apoptosis, while CatD overexpression has the opposite effect [99]. Accordingly, inhibition of CatD with pepstatin A induces caspase-dependent apoptosis in neuroblastoma cell lines [100]. Moreover, overexpression of intracellular CatD in mouse xenografs using rat-derived cell lines inhibits apoptosis [71], and expression of wild type or a catalytic mutant of CatD promotes survival and invasive growth of CatD-deficient fibroblasts [85]. Another study in glioblastoma cells proposes that CatD stimulates autophagy induction, inhibiting apoptotic cell death under genotoxic conditions [101]. More recently, we showed that inhibition of CatD in CRC cells with small interfering RNA (siRNA) or pepstatin A enhances acetate-induced apoptosis associated with a decrease in mitochondria degradation independently of autophagy [102, 103]. An anti-apoptotic role of CatD has also been described under physiological conditions using CatD-deficient mice [43-45]. Indeed, mutant mice developed apoptosis in the thymus, thalamus and retina.

In summary, it is well documented that CatD plays an important role in apoptosis regulation, both with and without involvement of its proteolytic activity. However, the exact role of CatD in apoptosis, particularly what determines whether this protease plays an anti- or pro-apoptotic function remains poorly understood. In this regard, a simpler model system would be particularly useful to offer additional clues into this dichotomy.

YEAST VACUOLAR PROTEASES

The versatility of the yeast *Saccharomyces cerevisiae* to study several conserved cellular functions such as cell metabolism, cell cycle, cell death and organelle biogenesis has justified the attractiveness of this system to study more complex mammalian physiological and pathological processes [104-108]. Like other organelles, the yeast vacuole is functionally similar to its higher eukaryote counterpart, the lysosome. It harbors seven characterized proteases, namely three aminopeptidases, three serine proteases and one aspartyl protease. Among these, two are endopeptidases: proteinase A (Pep4p), ortholog to human CatD, and proteinase B (Prb1p). Five are exopeptidases: carboxypeptidase Y (CPY), ortholog to human CatA, carboxypeptidase S (CPS1), aminopeptidase I (Ape1) and Y (Ape3), and dipeptidylaminopeptidase B (Dap2).

More recently, Hecht *et al.* reported an eighth vacuolar protease, a transmembrane metalloprotease (Pff1) [109], but although evidence of Pff1 vacuolar localization was shown, its proteolytic activity has yet to be demonstrated.

The endopeptidases are responsible for the majority of bulk protein degradation, including of plasma membrane proteins. They are also fundamental for activation of the vacuolar proteolytic cascade, particularly Pep4p, since it is involved in proteolytic activation of Prb1p, CPY and Ape1 [110, 111]. Prb1p, in turn, participates in the activation of Pep4p, CPY, CPS1, Ape1 and Ape3. Both carboxypeptidases and Ape1 are involved in peptide and glutathione degradation, respectively, but are not required for zymogen activation [111, 112].

Substrates for the vacuolar proteases are mostly imported via endocytosis (extracellular and cell surface proteins) or autophagy (cytoplasmic material and organelles). Autophagy is activated under nutrient deprivation conditions, and both Pep4p and Prb1p are implicated in the dissolution of autophagic bodies [113, 114].

In addition, vacuolar proteases play a role in sporulation. While absence of Prb1p activity alone results in partial reduction of sporulation, absence of Prb1p activity in a mutant lacking both CPY and CPS1 leads to almost complete loss of sporulation ability [115]. In addition to ensuring protein homeostasis under physiological conditions, vacuolar proteolysis therefore also appears to be a stress-responsive process, particularly under nutrient stress conditions and during sporulation. However, additional roles for vacuolar proteases have emerged in recent years, in particular for Pep4p.

Pep4p PROTEASE - THE YEAST CATHEPSIN D

Yeast CatD (Pep4p), like its lysosomal counterpart, is synthesized as an inactive zymogen, traveling via the endoplasmic reticulum and Golgi to the acidic vacuoles, where it is activated through proteolytic removal of the inhibitory propeptide [116]. Although Pep4p is mainly located in the vacuole, different cell death stimuli can lead to its release to the cytosol, involving a selective vacuolar membrane permeabilization (VMP) typical of apoptotic death.

Mason *et al*. were the first to report that Pep4p translocates from the vacuole to the cytosol [117]. These authors observed an increase in nuclear permeability associated with increased accumulation of reactive oxygen species (ROS) during H_2O_2-induced cell death, and found that Pep4p is released into the cytosol and degrades nucleoporins during this process. However, Pep4p did not affect resistance to H_2O_2-induced cell death, probably because it migrates out of vacuoles after cells are effectively unviable. They further showed that the release of a Pep4p-EGFP (Enhanced Green Fluorescent Protein) fusion from the vacuole in H_2O_2-treated cells was not associated with major rupture of the vacuolar membrane, as cells maintained a vacuolar lumen morphologically distinct from the cytosol. Other authors reported that Pep4p is involved in protein degradation and removal of oxidized proteins during H_2O_2-induced oxidative stress, but also did not ascribe a role for this protease in cell death induced by H_2O_2 [118].

Another study showed that stabilization of the actin cytoskeleton caused by lack of the actin regulatory protein End3p leads to loss of mitochondrial membrane potential, accumulation of ROS, increase in VMP and consequent migration of Pep4p to the cytosol, as well as apoptotic cell death [119]. In that study, Pep4p-EGFP was visualized exclusively in the vacuole lumen in wild type cells, but distributed throughout the entire cell in an *END3*-deficient strain. Again, no role was attributed to this protease in actin-stabilized dying cells.

Pep4p is also involved in programmed nuclear destruction during yeast gametogenesis [120]. Using cells co-expressing Pep4p-mCherry and Vma1-GFP, a GFP-tagged vacuolar membrane protein, Pep4p was shown to translocate from the vacuole into the ascal compartment of early postmeiotic cells during sporulation, with preservation of vacuolar integrity.

These observations show that VMP seems to mimic LMP in human cells. However, they do not indicate whether yeast vacuolar proteases play a role in cell survival and regulated death.

In this regard, it has been shown that Pep4p has a pro-survival role during chronological aging, since a Pep4p-deficient mutant has a shortened lifespan associated with higher levels of carbonylated proteins [118]. Carmona-Gutiérrez *et al*. further showed that deletion of *PEP4* results in both apoptotic and necrotic cell death during chronological aging [121]. Using a panel of Pep4p mutants, they conclude that Pep4p plays a dual pro-survival role composed of both anti-apoptotic and anti-necrotic functions, conferred by its proteolytic activity and its proteolytically inactive propeptide, respectively. We also previously found that Pep4p-EGFP translocates to the cytosol during acetic acid-induced apoptosis involving selective VMP in *S. cerevisiae* W303 cells, with preservation of both vacuolar and plasma membrane integrity [122]. Moreover, we demonstrated that Pep4p is required for increased cell survival and for efficient autophagy-independent mitochondrial degradation in response to this acid in a manner depending on its catalytic activity [122, 123]. This suggests that VMP associated with Pep4p release may act as an

alternative mitochondrial degradation process, delaying cell death. In contrast, we recently demonstrated that absence of *PEP4* resulted in increased resistance to acetic acid in *S. cerevisiae* BY4741 cells [124]. This prompted the hypothesis that Pep4p plays a dual function in acetic acid-induced cell death depending on the genetic background, providing an interesting tool to explore the molecular determinants of CatD function.

YEAST AS A TOOL TO EXPLORE THE ROLE OF CATHEPSIN D IN APOPTOSIS AND CANCER

It is widely established that the process of regulated cell death (RCD) involves a genetically encoded molecular machinery [125]. Core components of this machinery are conserved in yeast, which can undergo RCD exhibiting typical markers of apoptosis, autophagy and necrosis [126-128]. Thus, this eukaryotic organism has been used extensively to study the molecular mechanisms of RCD pathways, reviewed elsewhere [126-129]. These studies encompass not only analysis of yeast endogenous death pathways but also heterologous expression of human proteins involved in apoptosis, such as caspases, Bcl-2 family proteins, PKC isoforms and the p53 tumor suppressor protein [130, 131].

As discussed above, the role of the lysosome-like vacuole in the regulation of RCD has been investigated in yeast, where it has been shown to play a role similar to lysosomes [132, 133]. However, the use of this model organism to study lysosomal cell death pathways in general and cathepsin function in particular is still underexplored. So far, only translocation of Pep4p to the cytosol during yeast apoptosis has been clearly demonstrated by different authors [117, 119, 122]. One other study shows that the RNase T2 family member Rny1p is also released from the vacuole into the cytosol during oxidative stress, with preservation of vacuolar membrane integrity, directly promoting cell death [134]. The need for a comprehensive analysis of the VMP process and the vacuolar proteins released in response to different stimuli is therefore evident.

Another approach that has not been sufficiently exploited is the heterologous expression of cathepsins in yeast. Two studies have shown that rat cathepsin L and D precursor polypeptides are recognized by mechanisms similar to those involved in the intracellular sorting of vacuolar proteins in yeast cells [135, 136]. We therefore sought to further explore this tool to understand the function of human CatD. As mentioned above, we previously

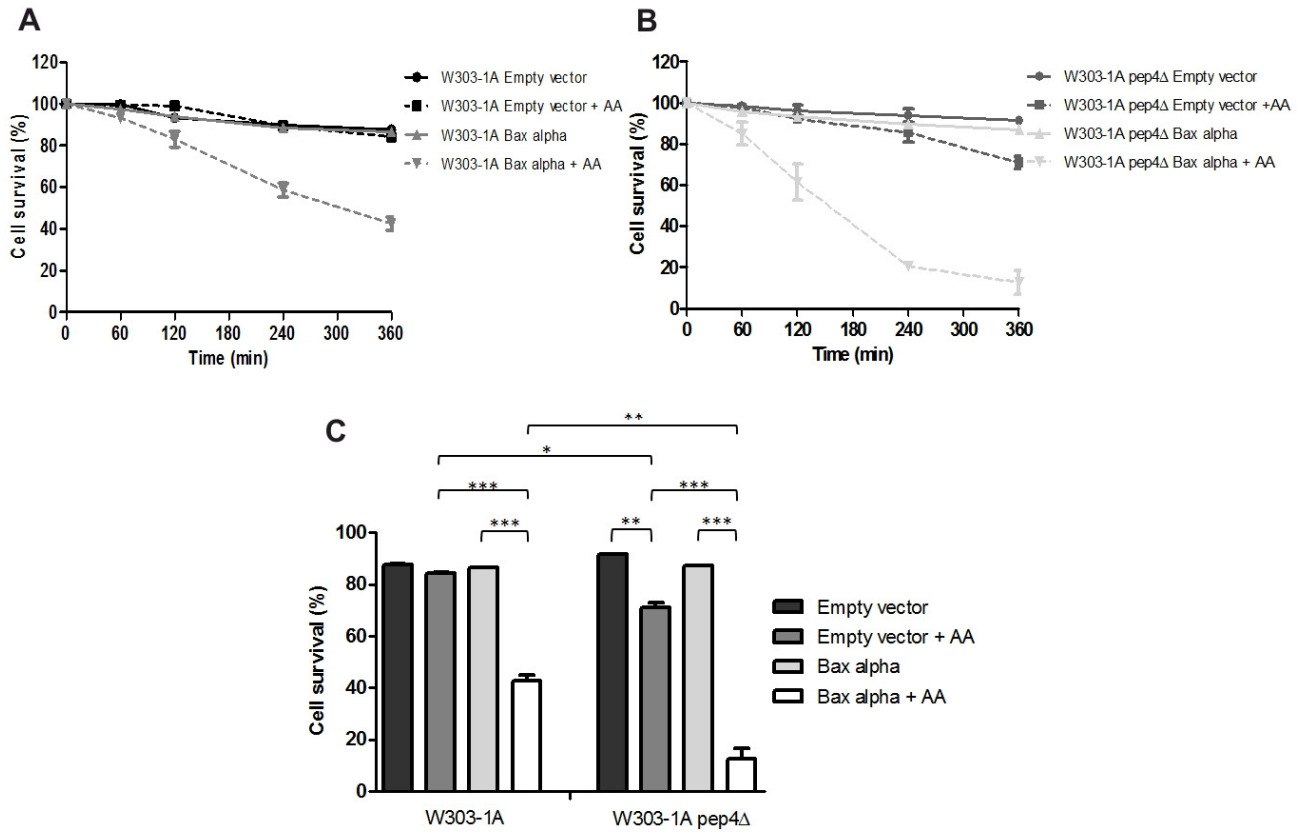

FIGURE 1: Survival of *S. cerevisiae* cells expressing Bax during acetic acid treatment. The wild type W303-1A and *pep4*Δ mutant strains transformed with the empty vector (PYES2) and PYES2-Bax alpha were incubated with 120 mM acetic acid for up to 360 min. **(A)** Cell survival of W303-1A strain and **(B)** W303-1A *pep4*Δ strain for up to 360 min was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. Data represents means ± S.D. (*n*=2). **(C)** Cell survival at time 360 min was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. Data represents means ± S.D. (*n*=2). *P < 0.05, **P < 0.01, ***P < 0.001.

showed the parallel between the role of human and yeast CatD in acetate/acetic acid-induced apoptosis and in the degradation of damaged mitochondria, which render CRC/yeast cells more resistant to apoptosis induced by acetate/acetic acid [102, 122]. We now found that heterologous expression of human CatD in yeast *PEP4*-deficient cells reverts their sensitivity to acetic acid-induced apoptosis and delays mitochondrial degradation [103], as previously observed for wild type Pep4p [122, 123]. These results provide evidence that the role of CatD in both apoptosis and mitochondrial degradation is conserved through evolution. Further elucidation of the molecular mechanisms underlying the involvement of CatD in apoptosis and in mitochondrial degradation will now be crucial to develop novel strategies to specifically inhibit this protease in apoptosis deficiency-associated diseases, such as cancer.

Taking into account the multiple functions of CatD, one caveat of using CatD inhibitors could be a negative effect on Bax activation, release of cytochrome *c* and downstream caspase activation. To address this question, we exploited the well-established system of heterologous expression of Bax in yeast, which lacks obvious orthologs of the Bcl-2 family, and allows studying how absence of yeast CatD affects Bax activity without interference from other Bcl-2 family members. Using yeast cells heterologously expressing a cytosolic inactive form of human Bax, which was activated by exposure to acetic acid, we could discard this hypothesis since absence of Pep4p enhanced Bax-induced cell death (Figure 1). It will be interesting to further exploit this system with heterologous co-expression of Bax and human CatD, in order to dissect the role of this lysosomal protease in the regulation of Bax activity independently of Bid.

As a final conclusion, it becomes apparent that the approaches with yeast have already provided and can further offer new perspectives for an increased understanding of the role of CatD in mammalian apoptosis, and its implications in cancer. Indeed, studies with yeast further reinforce the use of this eukaryotic organism as a valuable model to identify and characterize novel RCD processes, and open the door to new clinical opportunities, with a substantial impact in public health.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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The role of acetate on monocarboxylate transporter (MCT) expression and glucose metabolism in colorectal cancer cells: therapeutic implications

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Abstract

Acetate is a short-chain fatty acid relevant in the regulation of colorectal cancer (CRC) cells survival. Monocarboxylate transporters (MCTs) have gained increased interest in cancer contributing to their metabolic phenotype and to the uptake of drugs such as 3-bromopyruvate (3BP). The mechanism by which acetate enters CRC cells is not well understood. Here, we aimed to investigate the mechanism of acetate transport as well as the role of acetate on MCTs expression and glucose metabolism. We further wanted to explore the use of 3BP and acetate as a possible therapeutic approach for CRC.

Our results show that the transport of acetate occurs through the sodium dependent monocarboxylate transporter (SMCT-1) and by passive diffusion via aquaporins. MCT-1 is also involved in acetate uptake, indeed acetate increased the expression of MCT-1, MCT-4 and of the highly glycosylated CD147 chaperone, promoting membrane re-localization of MCT-1 and changes in glucose metabolism. We further verified that 3BP potentiates acetate-induced apoptosis in CRC cells. Altogether we provide new evidences for the role of acetate in the regulation of MCTs expression and glycolytic profile, which influence acetate transport and increases the sensitivity of CRC cells to 3BP therapy.

1. Introduction

Colorectal cancer (CRC) is the most common malignancy and cause of cancer death in developed countries [1]. Dietary fiber and the gut microbial-derived short chain fatty acids (SCFA), namely acetate, propionate and butyrate, exert multiple beneficial effects on the colon energy metabolism [1-3]. SCFA are produced by colonic microbiota in a molar ratio of approximately 60:20:20 of acetate > propionate > butyrate respectively [4]. These microbial metabolites are a major source of energy for colonocytes, having the ability to prevent and inhibit colon carcinogenesis *in vivo* [1, 2]. We, and others, previously established that SCFA exert a role in the regulation of CRC cells survival [3, 5-8]. Indeed we showed that acetate inhibit cell proliferation, induce apoptosis and promote lysosomal membrane permeabilization with release of cathepsin D, which seems to protect CRC cells through an autophagy-independent degradation of damaged mitochondria [5, 8]

Cancer cells, including CRC, exhibit profound metabolic adaptations, including the hyper-glycolytic phenotype which is characterized by production of high amounts of lactate, contributing to the acidification of the tumor microenvironment [9, 10]. MCTs (monocarboxylate transporters) have a central role in the maintenance of the cancer cell glycolytic metabolism, as lactate transporters as well as pH regulators [11, 12]. The MCT family is composed of 14 members [13], but only the first four (MCT-1, MCT-2, MCT-3 and MCT-4) have been demonstrated to catalyze the proton-linked transport of metabolically important monocarboxylates [11, 13]. MCT-1, MCT-2 and MCT-4 have been shown to be overexpressed in CRC cases [9, 14, 15], while SMCT-1 (sodium-linked) is usually silenced [16-19]. Interestingly MCT-1 and MCT-4, require the presence of a chaperone, CD147, which needs to be glycosylated to reach the plasma membrane [13, 20].

SCFA exist almost entirely in their dissociated form in the colon and their transport across cell membranes may be dependent on a specific carrier protein [21]. Some mechanisms for SCFA absorption across the plasma membrane of colonocytes in various species have been postulated [7, 21-23]. SCFA seem to enter intestinal epithelial cells through passive diffusion of protonated SCFA or the transmembrane transport is mediated by monocarboxylate transporters (MCTs), especially SMCT-1 and MCT-1 [1, 23]. Studies regarding the

involvement of MCTs in the transport of SCFA in CRC cells are scarce and contradictory. For instance MCT-1 was suggested to play a major role in transport of butyrate [1, 24, 25] but information on the transport of acetate or propionate is missing. Thus, additional studies are needed to understand the role of MCTs in SCFA transport, namely in the transport of acetate in CRC cells.

The relevance of SCFA to normal human colon function has been well established namely as major energy source for colonocytes, as regulators of intracellular pH and in functions associated with ion absorption and gut motility of intestinal epithelial cells [23, 26]. Once absorbed, SCFA are converted by colonocytes into glucose, ketone bodies, and amino acids [24, 27]. They can also be used for *de novo* synthesis of lipids and glucose [2, 27, 28], but how SCFA, especially acetate, affect glucose metabolism of CRC cells is still elusive.

MCTs are attractive targets in cancer therapy, as their elevated expression in cancer cells can mediate selectively the entry of chemotherapeutic agents into cancer cells [12]. 3-Bromopyruvate (3BP) is an anticancer compound that targets cancer cell metabolism presenting a high selectivity. 3BP activity is potentiated by butyrate in breast cancer cells through the increase of its uptake as consequence of an increase of MCTs expression [29, 30]. We described recently that 3BP uptake is mediated by MCT-1 in the presence of its glycosylated chaperone CD147, and that its selectivity towards cancer cells was dependent on the pH of acidic tumor microenvironment [31].

Since acetate is the most relevant SCFA produced in the colon, we aimed to identify the mechanisms involved in its transport across the plasma membrane and the engagement of MCTs in such process. We further aimed to understand the role of acetate in the expression and localization of MCTs in CRC cells and in the glycolytic metabolism. We also intended to explore the combination of 3BP with acetate as a therapeutic strategy for CRC cells.

2. Material and Methods

2.1 Material

2.1.1 Reagents

Acetic acid and $HgCl_2$ were purchased from Merck. Radiolabelled [^{14}C] acetate, with a specific activity of 55.2 mCi/mmol, was purchased from PerkinElmer. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), monensin, valinomycin and α-cyano-4-hydroxycinnamic acid (CHC) were purchased from Sigma. 4,4'-Di-isothiocyano-2,2'-stilbenedisulfonic acid (DIDS) was obtained from Santa Cruz Biotechnology and AR-C155858 was a gift from AstraZeneca.

2.1.2 Cell lines

Human colorectal cancer (CRC) cell lines, RKO^{BRAFV600E} and HCT-15 ^{KRASG13D} were cultured at 37 ºC under a humidified atmosphere containing 5% CO_2. HCT-15 cells were grown in RPMI (Biowest) medium with L-glutamine and HEPES. RKO cells were grown in DMEM (Biowest) with high-glucose and supplemented with 1 mM sodium pyruvate and 1.5 g/l sodium bicarbonate. All culture media were supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. Cells were seeded and adhered onto appropriate sterile plates for 24 hours before treatments in all experiments.

2.2 Methods

2.2.1 Measurement of acetate uptake

The protocol for [^{14}C] acetate uptake was adapted from Kobayashi et al. [32] as described in Azevedo-Silva et al. [31]. Briefly, cells were seeded in 24-well plates at 2–3×10^5 cells/well, incubated overnight at 37◦C with 5% CO_2, washed twice and incubated with HEPES buffer (pH 7.4) for 10min at 37ºC. HEPES buffer was removed and cells were incubated in MES buffer (pH 6.0) with the radiolabelled [^{14}C] acetate (4000 d.p.m./nmol) for the appropriate period of time at 37◦C. Afterwards, the [^{14}C] acetate solution was removed, the plate was incubated on ice and the cultures washed with ice-cold HEPES buffer, pH 7.4. Cells were then solubilized with 1% SDS/0.2 M NaOH and mixed with scintillation liquid for measuring radioactivity in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer with d.p.m. correction. For normalization, protein of all samples was quantified using a BCA Protein Assay Kit (Pierce). As [^{14}C] acetate

uptake was linear up to 5 min, 3 min of incubation of the cells with the radiolabelled substrate was adopted in the present study. The effect of distinct inhibitors AR-C155858, CHC, DIDS, CCCP, monensin, valinomycin and $HgCl_2$ was evaluated in cells incubated for 3 min with each compound in MES buffer, pH 6.0, prior to incubation with 1.0 mM [^{14}C] acetate for 3 min.

2.2.2 Preparation of total protein extracts and Western blotting

Cells were seeded in 6-well plates and exposed to acetate for 48 hours: 45 mM, 70 mM for HCT-15 and 75 mM, 110 mM for RKO cells (IC_{30} and IC_{50} acetate doses for both cell lines). As negative control, cells with fresh medium were used. Cell lysis, total protein preparation and Western blotting were carried out as previously described [5] with 25 µg of total extracted proteins applied per lane before SDS-PAGE.

The primary antibodies used were anti-MCT-1 (1:500, Santa Cruz Biotechnology), anti-MCT-2 (1:200, Santa Cruz Biotechnology), anti-MCT-4 (1:500, Santa Cruz Biotechnology), anti-SMCT-1 (1:250, Santa Cruz Biotechnology), anti-CD147 (1:500, Santa Cruz Biotechnology), anti-GLUT 1 (1:200, Abcam) and anti-actin (1:5000, Santa Cruz Biotechnology). Secondary antibodies used were peroxidase conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) and horseradish peroxidase-labeled goat anti-mouse immunoglobulin IgG (Jackson ImmunoResearch). Subsequent chemiluminescence detection was performed using the ECL detection system (Amersham, Biosciences, Buckinghamshire, UK) and a molecular imager by Chemi-Doc XRS system (Bio-Rad, Laboratories Inc., Hercules, CA, USA). When performed, densitometry analysis of protein bands was performed using the Quantity One software (version 4.6.9) and levels of actin used as a normalization control for protein loading.

2.2.3 Immunofluorescence microscopy analysis

Cells were seeded in 12-well plates containing glass coverslips and exposed to acetate for 48 hours (IC_{30} and IC_{50} acetate doses for both cell lines). As negative control, cells with fresh medium were used. At the end of the experiment, cells were washed with 1x PBS and fixed with 4% paraformaldehyde for 15 min at RT. Then, coverslips were washed in 1x PBS and permeabilised with 0,1% Triton X-

100 in 1x PBS for 2 min. After this, coverslips were washed in 1x PBS and blocked with 5% BSA for 30 min. Coverslips were incubated with anti-MCT-1 (1:250, Santa Cruz Biotechnology) or anti-MCT-4 (1:500, Santa Cruz Biotechnology) or anti-CD147 (1:250, Santa Cruz Biotechnology) diluted in 5% BSA, overnight. At the end, coverslips were washed twice with 1x PBS and incubated with fluorescence anti-rabbit (for MCT-4, 1:500, Alexa Fluor® 488, Invitrogen A-11008) and fluorescence anti-mouse (MCT-1 and CD147, 1:250, Alexa Fluor® 594, Invitrogen A-11032) for 1 h. So, the coverslips were washed twice with 1x PBS and finally mounted on Vectashield Mounting Medium with DAPI and immediately observed under the fluorescence microscope (Olympus BX61 fluorescence microscope, Tokyo, Japan). Three coverslips were prepared for each experimental condition and representative images are shown.

2.2.4 Glucose consumption and lactate production assays

Cells cultured in 48-well plates were pre-incubated in glucose-free media for 2 h. Then, cells were washed with 1x PBS and incubated with the doses of acetate - IC_{30} and IC_{50}, as mentioned before). Non-treated cells (negative control) or exposed to acetate concentrations were analyzed. After incubation, conditioned medium was collected at 3, 6, 12, 24 and 48 hours. Glucose consumption and extracellular lactate were measured by the enzymatic colorimetric kits: Glucose Assay Kit (Roche, Mannheim, Germany) and Lactate Assay kit (SpinReact, Sant Esteve de Bas, Girona, Spain), following the manufacturer's instructions. Glucose and lactate fluorescence intensity were detected with absorbance emission at 490 nm using a fluorescence microplate reader. Values are expressed as the mean of fluorescence intensity normalized to T0 (control for glucose and lactate levels before the treatment with acetate) and the cell biomass for each condition (by Sulforhodamine (SRB) assay). The SRB protocol was performed as previously described [5].

2.2.5 Cell treatments with acetate and 3-bromopyruvate (3BP)

Cells were treated during 48 hours, using the concentration (IC) of acetate previously determined [5]: 45 mM, 70 mM, for HCT-15 and 75 mM, 110 mM for RKO cell lines (values for the IC_{30} and IC_{50}, respectively). The concentrations of 3-bromopyruvate (3BP) used (17,5 µM, 35 µM and 75 µM, 150 µM; IC_{25} and IC_{50},

respectively for HCT-15 and RKO cells) in the assay were calculated from the mean values obtained in the SRB assays. For the assays with acetate and 3BP, the 3BP was co-incubated 16 hours before completing 48 hours of acetate treatment.

2.2.6 Cell proliferation assay using Sulforhodamine (SRB)

Cells were seeded in 24-well before the treatments. Cells were incubated 48 hours with 70 mM and 110 mM (IC_{50} acetate for HCT-15 and RKO cells, respectively) with and without 16 h of 3BP co-incubation. In these assays, the 3BP IC_{25} and IC_{50} values were used for each cell line (as described above). H_2O_2 (500 μM or 1 mM for HCT-15 or RKO cell lines, respectively) was used as positive control. The protocol preparation was performed as previously described [5]. All samples were measured in triplicates and the values were expressed relative to the negative control (cells incubated only with fresh completed medium).

2.2.7 Assessment of cell proliferation by colony formation assay (CFA)

Cells were seeded in 6-well plates at 600 cells/mL, 300 cells/mL (respectively for HCT-15 and RKO cell lines) and incubated 24 h at 37º C with 5% CO_2 before the treatments. Cells were treated as described for the SRB assay (the same conditions). After 48 hours of treatment, the medium was replaced by fresh medium twice *per* week during 14 days. To evaluate the colony numbers formed for each condition, the cells were washed with 1x PBS and then fixed with 6% glutaraldehyde/0.5% crystal violet solution for 30 min, at room temperature (RT). The cells were then washed with water and air-dried.

2.2.8 Analysis of apoptosis by flow cytometry

Cells were seeded in 6-well plates and the percentage of cells undergoing apoptosis after 48 h of acetate treatment was determined using Annexin-V FITC (AV) (BD Biosciences) and Propidium iodide (PI) (Sigma Aldrich). Both CRC cell lines, with and without 16 h of 3BP co-incubation, were exposed to acetate (IC_{50}). As negative control we used cells incubated with fresh medium and H_2O_2 as positive control (500 uM and 1 mM for HCT-15 and RKO cell lines, respectively). Both floating and attached cells were collected and prepared as described

previously by us [5] and then analyzed by flow cytometry. Cell viability and cell death was assessed by double staining with Annexin-V FITC/ PI. Unstained and stained cells were classified as follows: viable cells (AV-/PI-), early apoptotic cells (AV+/PI-), late apoptotic cells (AV+/PI+) or necrotic cells (AV-/PI+).

2.2.9 Statistical analysis

Kinetic parameters were determined using GraphPad for the non-linear regression of the values of the initial uptake rates of acetate as a function of the acid concentration. Other statistical significance analyses were determined by two-way ANOVA or by one-way ANOVA followed by Dunnett or Bonferroni's test for multiple comparisons using Prism software version 6 (GraphPad La Jolla, CA, USA). All results are presented as mean ± standard deviation (SD) of three independent experiments. Differences were considered significant for *P* values lower than 0.05.

Conflict of interest

The authors declare no conflict of interest.

3. Results

3.1 Kinetics and energetics of acetate uptake across the plasma membrane of CRC cells

The initial uptake rates of [^{14}C] acetate were evaluated at pH 6.0 in HCT-15 and RKO cell lines **(Figure 1a and b)**. The non-linear regression analysis shows that HCT-15 cells transport acetate follows a second order kinetics with an affinity constant (K_m) of 1.97 ± 0.57 mM and a transport capacity (V_{max}) of 62 ± 9 nmol/mg of protein/min. In RKO cells, acetate follows a first order kinetics with a diffusion constant of 5.19 ± 0.16 µL/mg of protein/min.

To evaluate the energetics of [^{14}C] acetate transport we tested the influence of agents known to disrupt different ion membrane electrochemical potentials **(Figure 1c)**, such as CCCP (a protonophore which disrupts both the pH and the electric potentials), monensin (an ionophore that specifically disrupts a Na^+ gradient) and valinomycin (an ionophore which preferentially affects a K^+ gradient and, to a lesser extent, a Na^+ gradient across biological membranes). We also tested the inhibition of acetate transport using monocarboxylate transporters (MCTs) inhibitors such as CHC (inhibitor of MCT-1 and SMCT-1), DIDS (inhibitor of MCTs and anion exchangers) and AR-C155858 (specific inhibitor of MCT-1 and MCT-2). We also used $HgCl_2$, which is described as an inhibitor of aquaporin activity [33, 34]. Our results showed that CCCP inhibited significantly acetate transport in both HCT-15 (71%) and RKO (52%) cells; monensin also inhibited acetate uptake in HCT-15 (78%) and RKO (36%) and valinomycin presented only a small inhibitory effect in both cell lines (22% inhibition in HCT-15 and 21% in RKO cells). Regarding MCT inhibitors, CHC inhibited both HCT-15 (30%) and RKO cells (35%) but DIDS and AR-C155858 did not prevent acetate uptake. Interestingly, $HgCl_2$ (aquaporin inhibitor) inhibited acetate uptake in HCT-15 (51%) and RKO (56%) cells indicating the contribution of aquaporins in acetate diffusion into CRC cells.

We further investigated if this inhibition was independent of the inhibition detected for CCCP or monensin. HCT-15 and RKO cells were used to evaluate the inhibition of acetate uptake using double combinations of CCCP (1 µM), $HgCl_2$ (5 µM) and monensin (20 µM) **(Figure 2a and b).** However, in these assays lower concentrations than described above were used. We verified that in HCT-15 cells CCCP inhibited 34%, $HgCl_2$ 37% and monensin 69% of acetate transport.

Combination of CCCP and $HgCl_2$ resulted in a 56% inhibition of acetate uptake, $HgCl_2$ and monensin in 76%, and CCCP with monensin inhibited 75%. In RKO cells acetate uptake was inhibited in 37% with CCCP, 28% with $HgCl_2$ and 68% with monensin. Combination of CCCP with $HgCl_2$ and monensin with $HgCl_2$ resulted in 46% inhibition of acetate transport while CCCP with monensin inhibited 81%.

In conclusion, these results point for the involvement of at least two mechanisms of transport in CRC cells: a secondary active transport dependent on electrochemical Na^+ gradient and diffusion mediated by aquaporins.

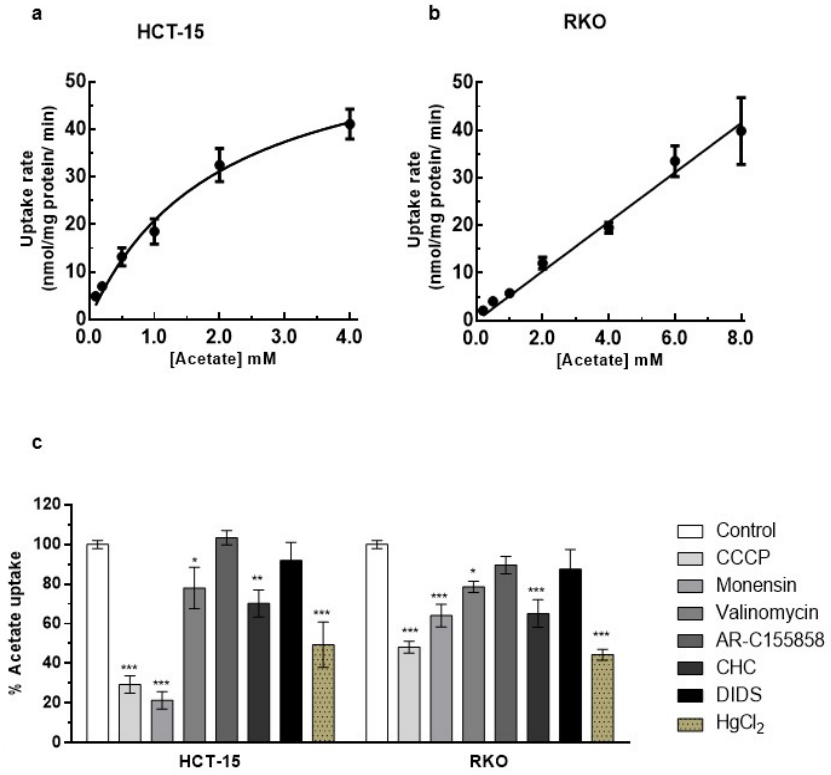

Figure 1: Characterization of acetate uptake in CRC cells. (a) and (b) Plots of the initial uptake rates of labeled acetate, as a function of the acid concentration at pH 6.0 in HCT-15 and RKO, respectively. Kinetic parameters, as affinity constant (K_m) and transport capacity (V_{max}) or Diffusion constant (K_d) for the uptake of acetate were based on the non-linear regression for the Michaelis-Menten equation f [V]=(Vmax x [Acetate])/(Km + [Acetate]) and Passive diffusion equation f[V] = Kd.[Acetate]. In HCT-15 a transporter system exists with a K_m of 1.97 ± 0.57 mM and a V_{max} of 62 ± 9 nmol/ mg of protein/ min. In RKO acetate enters cells by passive diffusion with a K_d of 5.19 ± 0.16. (c) Effect of CCCP (100 µM), Monensin (100 µM), Valinomycin (100 µM), AR-C155858 (1 µM), CHC (10 mM), DIDS (1 mM) and $HgCl_2$ (100 µM) in the uptake of 1 mM of acetate. Statistical analysis was performed by two-way ANOVA: ***, ** and * indicate significant differences with a respective P-values of <0.001, <0.01 and <0.05 (n=3).

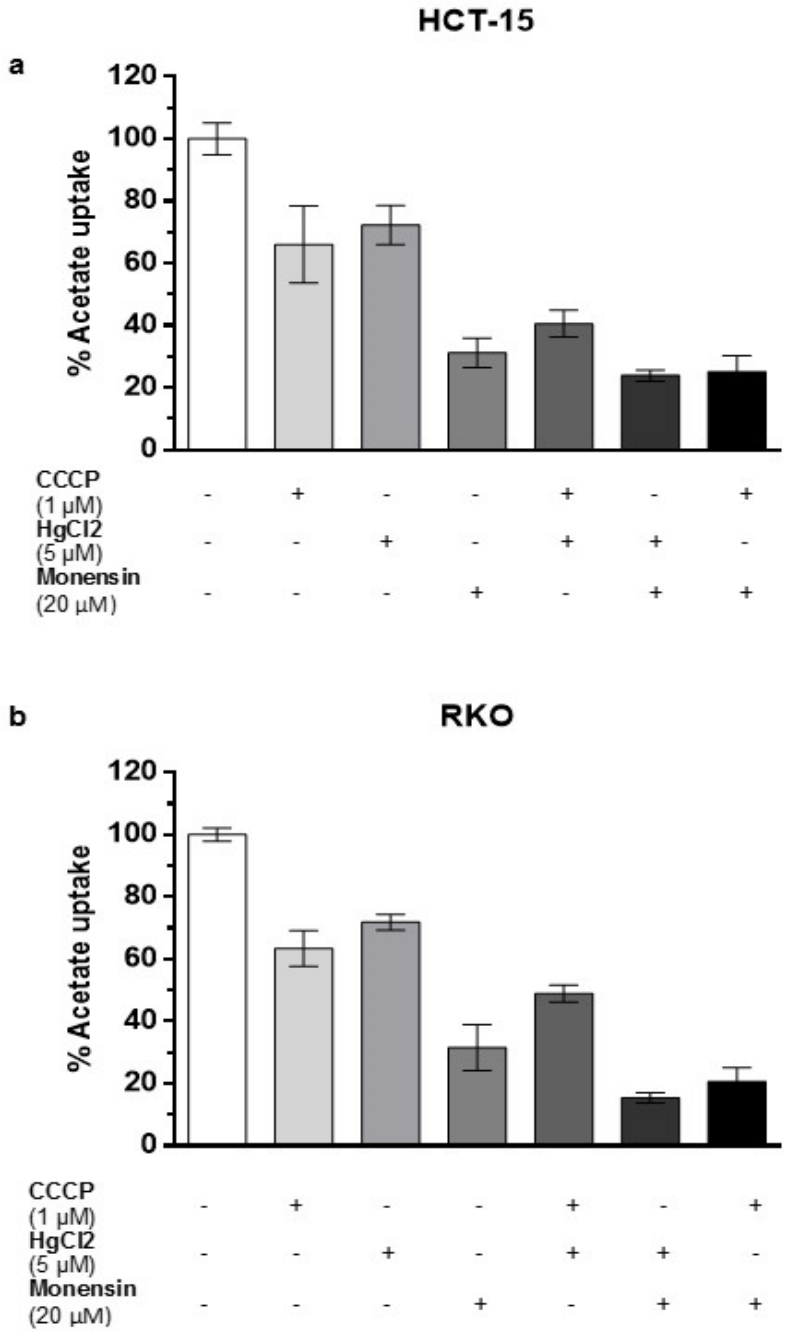

Figure 2: Inhibition of 1 mM of acetate uptake by CCCP (1 µM), $HgCl_2$ (5 µM) and Monensin (20 µM) alone or in combination in HCT-15 (a) and RKO (b) cells. Statistical analysis was performed by two-way ANOVA: ***, ** and * indicate significant differences with a respective P-values of <0.001, <0.01 and <0.05 (n=3).

3.2 Acetate pre-treatment alters the kinetics and the energetics of acetate uptake by CRC cells

To evaluate if acetate treatment could influence the kinetics and energetics of acetate, we tested the influence of CCCP, monensin, AR-C155858 and $HgCl_2$ in the uptake of 1 mM of acetate in cells treated with IC_{30} of acetate for 48h **(Figure 3)**. Untreated cells of HCT-15 and RKO cell lines behaved as expected with acetate transport being inhibited with CCCP (HCT-15: 54%; RKO: 62%), monensin (HCT-15: 60%; RKO: 59%) and $HgCl_2$ (HCT-15: 51%; RKO: 56%) but not with AR-C155858. Cell treated with the IC_{30} of acetate for 48 hours also presented inhibition for acetate uptake using CCCP (HCT-15: 52%; RKO: 53%), monensin (HCT-15: 60%; RKO: 38%) and HgCl2 (HCT-15: 60%; RKO: 55%). Acetate transport in HCT-15 (29%) and RKO (23%) cells treated with acetate was significantly inhibited by AR-C155858. These results led us to conclude that in cells treated with acetate, MCT-1, besides other transport mechanisms described above, is also involved in acetate transport.

3.3 Acetate upregulates MCT-1, MCT-4 and CD147 expression in CRC cells

We wanted to study if acetate influences the expression of MCTs as it was already reported by us for butyrate in breast cancer cells [30, 31]. We assessed the expression profile for the SMCT-1, MCT-1, MCT-2, MCT-4 and the chaperone CD147 (an important protein for MCT activity) in response to acetate by western blot analysis of cell extracts **(Figure 4)**. We analyzed CRC cells treated with both IC_{30} and IC_{50} acetate doses (45 mM and 70 mM; 75 mM and 110 mM, respectively for HCT-15 and RKO) after 24 and 48 hours of incubation. We found that both CRC cell lines express SMCT-1 and MCT-2 but no difference in expression after acetate treatment was observed in both cell lines. We found an elevated expression of MCT-1 and MCT-4 after 48 hours but not after 24 hours acetate treatment in both cell lines compared to the negative control **(Figure 4)**. The increase of MCT-1 and MCT-4 expression was accompanied by an increased expression of the chaperone cluster of differentiation (CD)147, important for MCT-1 and MCT-4 activity. The chaperone CD147, was present in both fully (FG) and core-glycosylated forms in both CRC cell lines after 48 hours of acetate treatment compared to the negative control.

A higher level of CD147 expression was observed in RKO compared to HCT-15 cells **(Figure 4)**. We also evaluated the expression of GLUT-1 after acetate treatment. GLUT-1 is responsible for basal glucose transport across the plasma membrane into the cytosol in many cancer cells [35]. We observed that both cells expressed GLUT-1 transporter, but no difference in the expression of this transporter was observed after acetate treatment. Our results suggest that acetate regulates MCT-1, MCT-4 and CD147 expression in CRC cells.

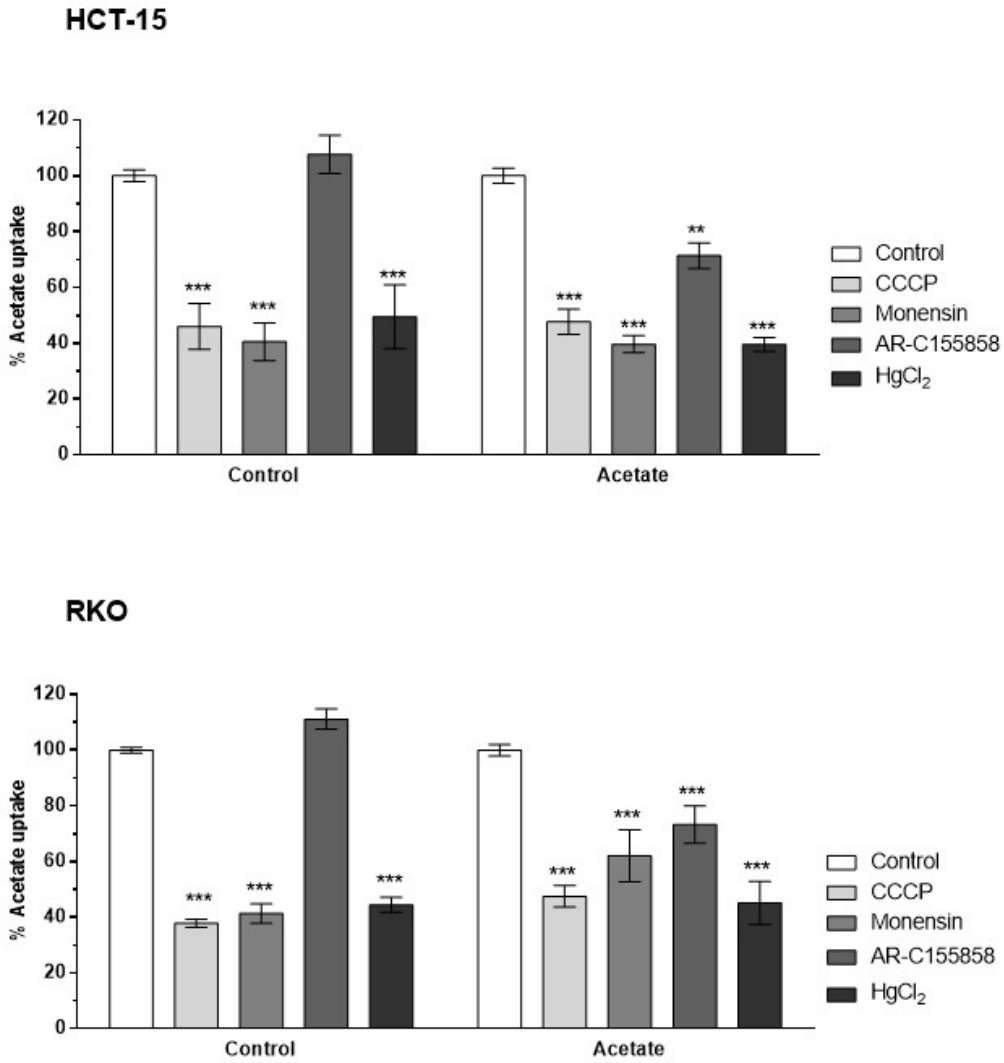

Figure 3: Inhibition of acetate (1 mM) uptake in HCT-15 and RKO cell lines treated with IC_{30} of acetate for 48h with CCCP (100 µM), Monensin (100 µM), AR-C155858 (1 µM) and $HgCl_2$ (100 µM). Statistical analysis was performed by two-way ANOVA: ***, ** and * indicate significant differences with a respective P -values of <0.001, <0.01 and <0.05 (n=3).

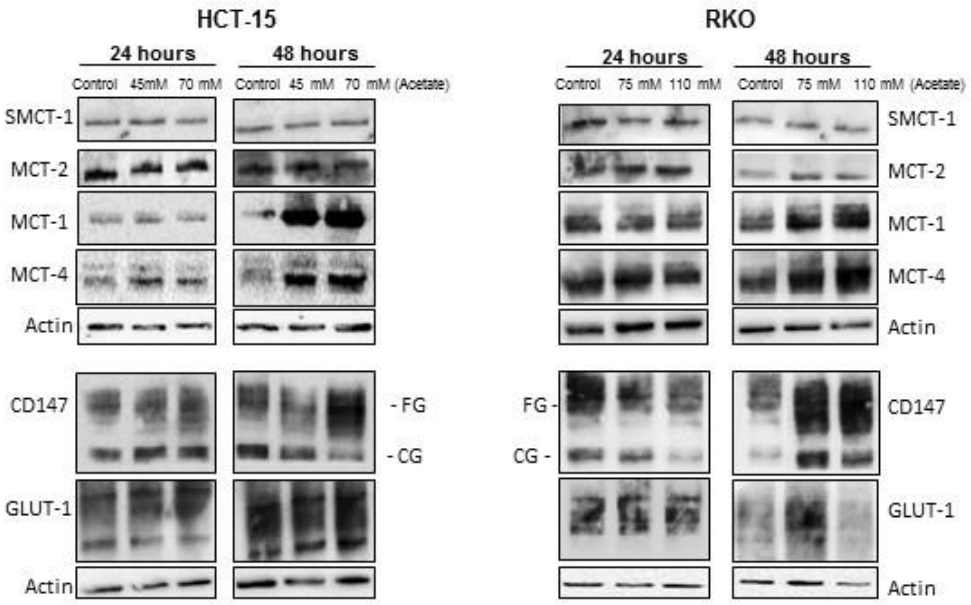

Figure 4: Characterization of MCTs expression and other glycolytic markers in CRC cells after acetate treatment. HCT-15 and RKO cells were incubated with acetate (45 mM and 70 mM for HCT-15 cells; 75 mM and 110 mM for RKO cells, respective doses for the IC_{30} and IC_{50}) or with fresh medium (as negative control) for 24 and 48 hours. Western blotting images of the SMCT-1, MCT-1, MCT-2, MCT-4, CD147 and GLUT-1 expression. β-actin was used as loading control. A representative experiment of at least three independent experiments is shown.

3.4 Acetate induces MCT-1 plasma membrane localization in CRC cells

We analyzed by immunofluorescence the cellular localization changes induced by acetate in MCT-1, MCT-4 and CD147. CRC cells were treated with acetate IC_{30} and IC_{50} doses during 48 hours, as described above for immunodetection. In agreement with the western blot analysis we could observe that acetate treatment leads to an elevated expression of these transporters and an increase in MCT-1 localization at the plasma membrane in comparison to the cytoplasm in both cell lines **(Figure 5)**, being more evident in HCT-15 than in RKO cells. Moreover, we found no differences between MCT-4 and CD147 co-localization after acetate treatment. Our results suggest that acetate treatment (in physiological concentrations) induces MCT-1 plasma membrane localization, which might explain that the plasma membrane transport of acetate also occurs by MCT-1 in CRC cells.

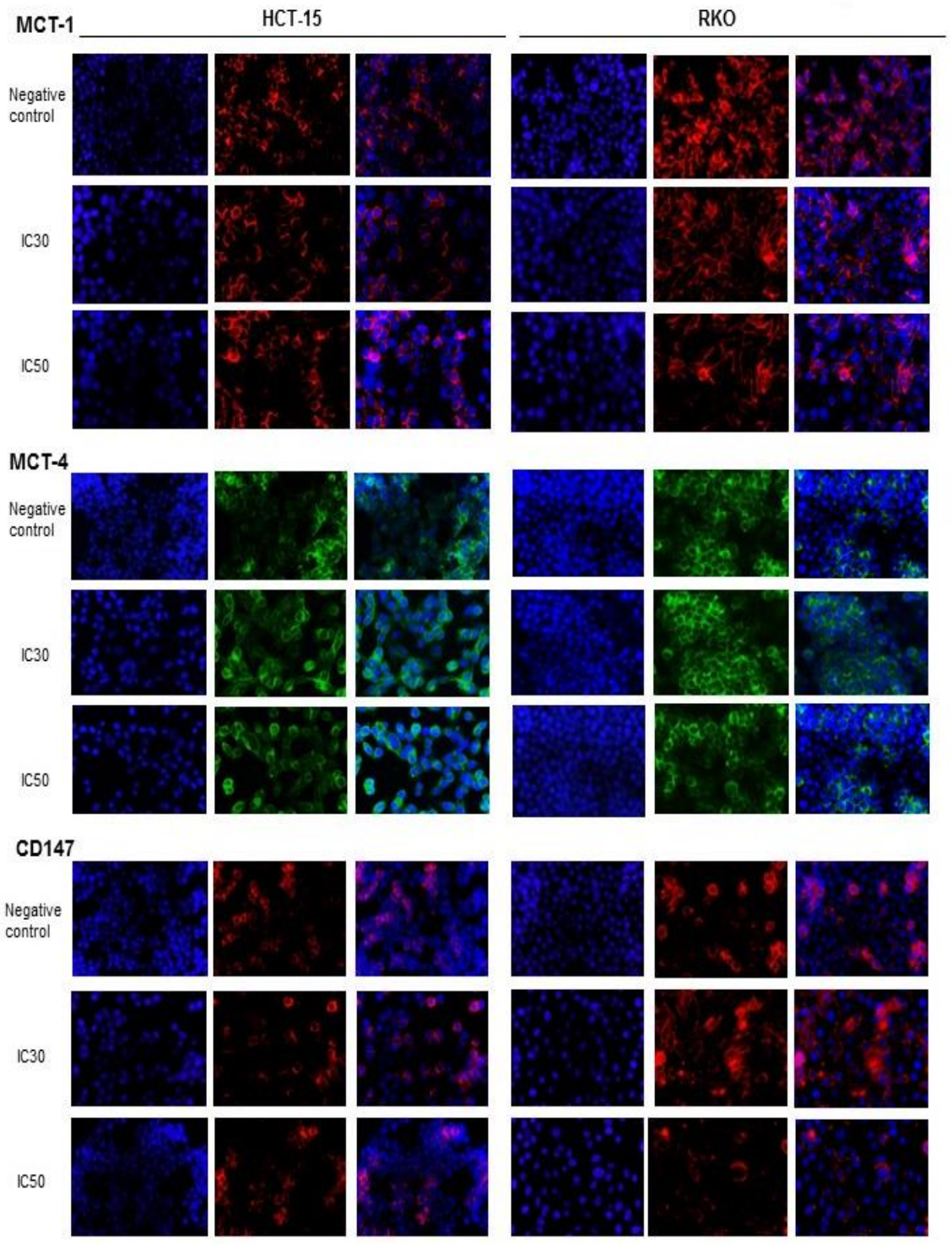

Figure 5: Localization of MCT-1, MCT-4 and CD147 in CRC cells after acetate treatment. HCT-15 and RKO cells were incubated with acetate (45 mM and 70 mM for HCT-15 cells; 75 mM and 110 mM for RKO cells, respective doses for the IC_{30} and IC_{50}) or with fresh medium (as negative control) for 48 hours. Representative images of immunofluorescence are shown (400× magnification).

3.5 Acetate treatment induces changes in the glycolytic metabolic profile in CRC cells

MCTs are known to be overexpressed in cancer cells to maintain high glycolytic rates by performing lactate efflux from glycolysis as well as pH regulation by the co-transport of protons [9]. Herein, we showed that acetate increases MCT-1 localization at the plasma membrane upon acetate treatment being accompanied by an increase in MCT-1, MCT-4 and CD147 expression. We therefore wonder if acetate might induce metabolic changes like glucose consumption and lactate production. To test this hypothesis, CRC cell lines were exposed to IC_{30} and IC_{50} doses of acetate and the relative rate of glucose consumption and extracellular lactate production were measured at 3, 6, 12, 24 and 48 hours in triplicate and normalized to T0 (amount of glucose and lactate at time 0) and to cell biomass (by SRB assay). We found that CRC cells in response to acetate, exhibited a higher consumption of glucose ($p < 0.05$) and lactate production ($p < 0.05$; $p < 0.01$) up to 24 hours in a dose-dependent manner and then significantly decreased at 48 hours **(Figure 6)**. RKO cells appear to be more glycolytic than HCT-15 cells since they exhibited a higher consumption of glucose ($p < 0.01$) and lactate production up to 12 hours after acetate treatment.

Glycolytic metabolism is a well-known characteristic of cancer cells which contributes to their aggressive phenotype, such as increased proliferation, suppression of anti-cancer immune response, invasion, angiogenesis and metastasis [36, 37]. Our results indicate that there is an increase in the glycolytic metabolism in response to short incubation periods of acetate, although after 48 hours of treatment, acetate induces a significant decrease in the glycolytic metabolism of CRC cells.

3.6 3BP potentiates acetate-induced apoptosis

It has been demonstrated that cancer cells exhibit the Warburg effect switching their metabolism to anaerobic glycolysis, characterized by glucose conversion into lactate even in the presence of oxygen [38]. Regarding our results on the metabolic profile and the elevated expression of MCT-1 and MCT-4 in CRC cells after acetate treatment we decided to study the effect of 3-bromopyruvate (3BP) in combination with acetate. 3BP is a small molecule analogue of lactate and pyruvate that acts as a glycolysis inhibitor and which is transported by MCTs in

cancer cells [29, 31, 39]. It was previously described by us the capability of butyrate to sensitize breast cancer cells to 3BP by increasing the expression and activity of MCTs [30, 31]. Our present results indicate that acetate have a similar effect in CRC cells regarding the expression of MCTs and thus we hypothesize that acetate could also sensitize these cells to 3BP.

To test this hypothesis, we used the colony formation assay (CFA) to analyze CRC cells treated with IC_{25} and IC_{50} values for the 3BP (17,5 µM, 35 µM and 75 µM, 150 µM, respectively for HCT-15 and RKO cells) alone or in combination with acetate treatment (only with the IC_{50} value for acetate: 70 mM, for HCT-15 and 110 mM for RKO cell lines) after 48 hours of incubation. We found that the combined treatment (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate) decreases cell proliferation (number of colonies formed) in both CRC cell lines in a 3BP dose-dependent manner in comparison to the negative control and to the same dose of 3BP or acetate alone **(Figure 7a)**. The effect of the combined treatment was more evident in RKO cells compared to HCT15 cells.

Analysis of cell proliferation by SRB assay **(Figure 7b)** showed that IC_{25} and IC_{50} of 3BP reduced proliferation of HCT-15 and RKO cell being only significant with IC_{50} of 3BP. In addition, we found that combination of IC_{25} of 3BP/ IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate also reduced cell proliferation in both cell lines with a significant difference compared to the negative control. We also observed a significant difference between acetate treatment alone compared with the 3BP+acetate combination in both CRC cell lines ($p < 0.01$ and $p < 0.0001$), being more evident in RKO cells as reported previously.

We next analyzed cell death through AV/PI staining **(Figure 7c)** and found that the number of apoptotic (early and late) cells stained with AV (AV + PI - plus AV + PI +) were 11%, 13% and 7% for IC_{25} of 3BP, IC_{50} of 3BP and IC_{50} of acetate, respectively for HCT-15 cells in comparison to 3% in the negative control. In RKO cells the levels of apoptotic cells stained with AV were 4%, 13% and 18% for IC_{25} of 3BP, IC_{50} of 3BP and IC_{50} of acetate, respectively in comparison to 2% in the negative control. In addition, the treatment with 3BP alone induces a significant increase ($p < 0.05$ and $p < 0.01$) in cells stained with AV – PI + in a dose-dependent manner in both CRC cells. We could observe that the combined treatment of 3BP and acetate (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate) potentiates apoptosis, as the number of cells stained with AV + PI - and

AV + PI + showed a significant increase comparing with acetate alone ($p < 0.01$ and $p < 0.001$) in both CRC cells, being more evident in RKO cells. In summary, our results show that the combination treatments (acetate in physiologic concentration + 3BP) potentiate apoptosis in CRC cells.

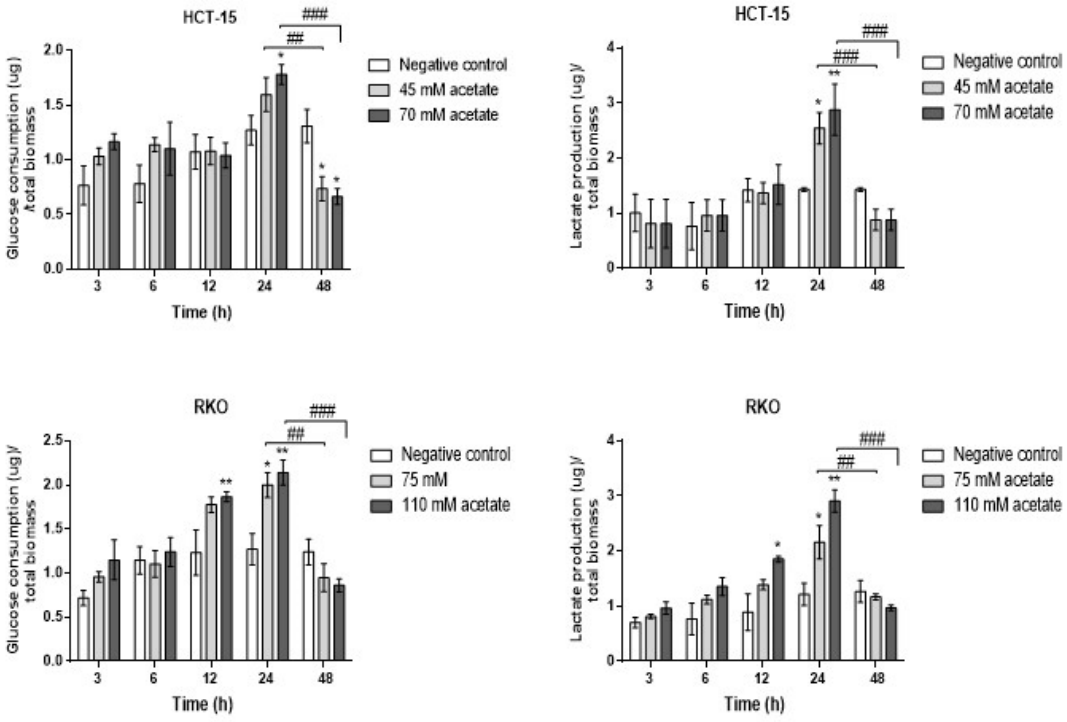

Figure 6: Acetate induces metabolic changes in CRC cells. HCT-15 and RKO cells were incubated with acetate (45 mM and 70 mM for HCT-15 cells; 75 mM and 110 mM for RKO cells, respective doses for the IC_{30} and IC_{50}) or with fresh medium (as negative control). Extracellular amounts of glucose consumption and lactate production overtimes 3, 6, 12, 24 and 48 hours are shown. Values are expressed as mean ± SD of at least three independent experiments. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 compared with control cells and # P ≤ 0.05, ## P ≤ 0.01 and ### P ≤ 0.001 comparing acetate treatment at 24 h with acetate treatment at 48 h.

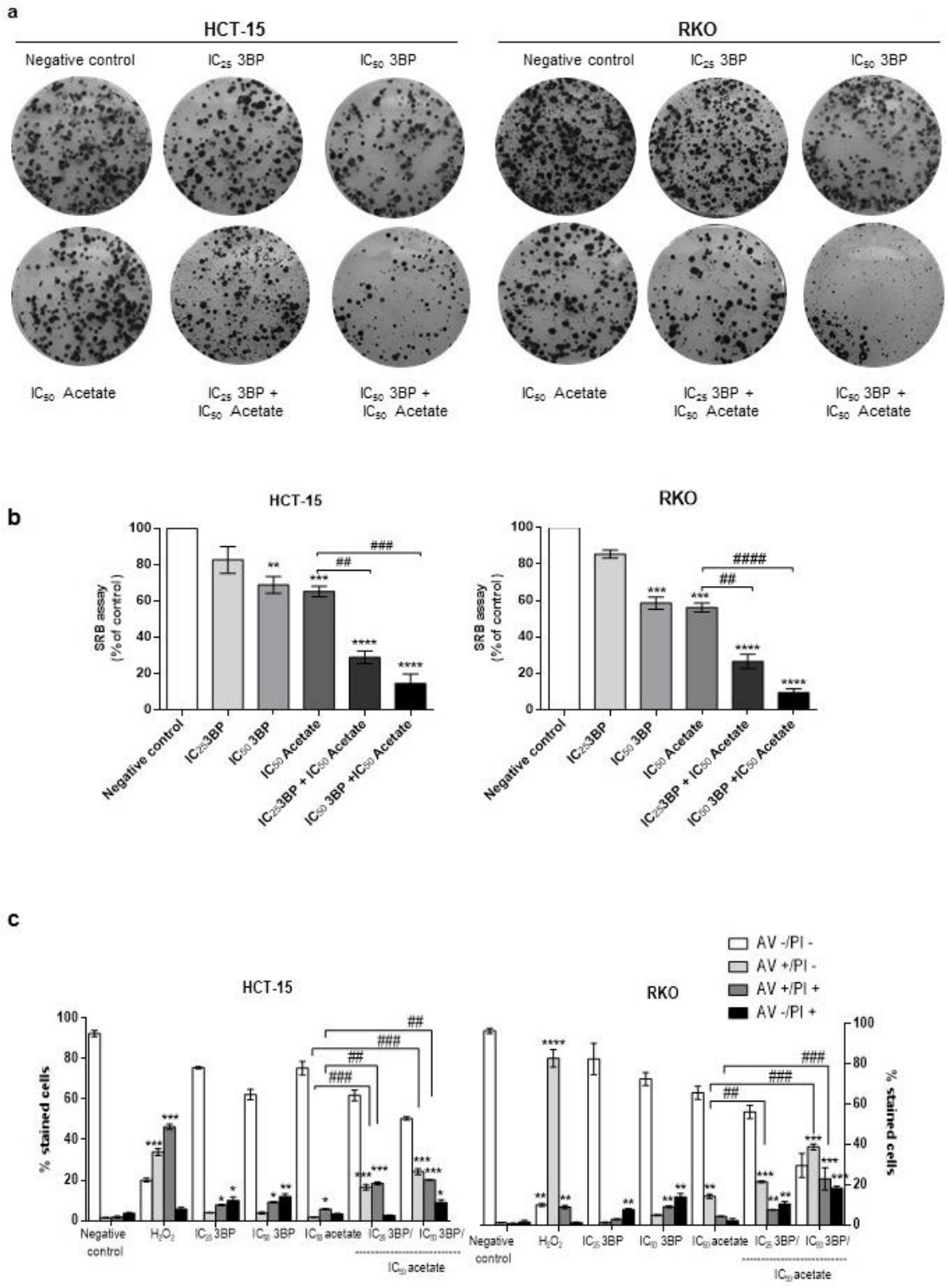

Figure 7: Effect of acetate with 3-bromopyruvate (3BP; a glycolysis inhibitor) in CRC cells. (a, b and c) CRC cells were treated with IC_{25} and IC_{50} values for the 3BP (17,5 uM, 35 uM and 75 uM, 150 uM, respectively for HCT-15 and RKO cells). The treatments were performed with 3BP alone or with acetate treatment combination (only the IC_{50} value for the acetate: 70 mM for HCT-15 and 110 mM for RKO cell line) after 48 h of incubation. 3BP was added 16 hours before to complete 48 hours of the treatment. As negative control, cells were treated with fresh medium. H_2O_2 (500 uM or 1 mM for HCT-15 or RKO cell lines, respectively) was used as positive control. **(a)** Colony formation assay shows that the combined treatment (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate) decreases cell proliferation (number of colony formed at the end of

the assay) in both CRC cell lines in a dose-dependent manner compared to the negative control and with the same dose of the 3BP or acetate alone. **(b)** Sulforhodamine B (SRB) assay analyzes the cell proliferation of the same conditions. Values represent mean ± SD of at least three independent experiments. **P ≤ 0.01, ***P ≤ 0.001 and ***P ≤ 0.0001 compared with negative control cells and ## P ≤ 0.01 and #### P ≤ 0.0001 comparing acetate alone with combined treatment (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate). **(c)** Quantitative analysis of AV/PI staining in HCT-15 and RKO cells. Values represent mean ± SD (AV – PI -, AV + PI -, AV + PI + and AV – PI +) of each condition (n=3). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ***P ≤ 0.0001 compared with negative control cells and ## P ≤ 0.01 and #### P ≤ 0.0001 comparing acetate alone with combined treatment (IC_{25} of 3BP/IC_{50} of acetate and IC_{50} of 3BP/IC_{50} of acetate).

4. Discussion

Acetate is a monocarboxylic acid, which figures among the most abundant SCFA in the human organism and is absorbed at the intestinal level being able to reach distant organs [2, 40]. Acetate transport across biological membranes in the colon model is not well understood, thus here we deepen the study of the kinetics and energetics of acetate transport across CRC cells. Our results indicate the participation of sodium dependent transporters such as SMCT-1 or anion-exchangers (AE-1 for example) in the uptake of acetate in CRC cells. These results are in accordance with several reports showing that SMCT-1 transport monocarboxylic acids such as butyrate, 3BP and dichloroacetate in cancer cells [18, 19, 41] and it has also been described that anion-exchangers might be involved in acetate transport in HT29-18-C1, a CRC cell line [42].

Our results also show that at pH 6.0 (similar to the gut environment) acetate transport across CRC cells membrane occurs preferentially via a sodium-dependent mechanism possibly by SMCT-1. Indeed this is in agreement to the fact that monocarboxylate transport family members (MCT 1-4) are proton symporters, involved in the uptake and/or efflux of pyruvate, lactate, ketone bodies and SCFA through the plasma membrane [9, 23]. The literature on acetate transport is scarce and little is known about SCFA transport mediated by MCTs in CRC cells, being that the majority of the studies use butyrate as model to study SCFA transport, both in normal intestinal epithelial and CRC cells [1, 16, 24]. In this regard, some studies demonstrated that butyrate can be transported into colon cancer cells mainly by MCT-1 [1, 24, 25]. It has also been reported that MCT-1 is responsible for acetate transport in mouse cancer cells with an affinity

constant of 3.7 mM [43]. Herein we showed for the first time that MCT-1 participates actively in the transport of acetate into CRC cells.

Interestingly our data suggest that other transport mechanisms such as passive diffusion through aquaporins might also contribute to acetate uptake. Aquaporins are important membrane proteins for cellular homeostasis, being known to be upregulated in different types of cancer including CRC [44], suggesting their capacity to transport not only water but other compounds. To the best of our knowledge, this is also the first report suggesting that aquaporins might play a novel role in acetate transport in colorectal cancer.

Acetate is an important messenger in the symbiotic relationship between intestinal microbiota and colonocytes and is thus predictable that may play an important role in the regulation of transporters expression and metabolism of colon human cells [2, 40]. These issues are poorly understood and needed further understanding namely in CRC cells. Our data suggest that acetate might interfere with MCT-1 expression and with its cellular localization influencing the total acetate uptake/transport. Accordingly, it has been reported that butyrate and other SCFA, may modulate cell behavior by activation of several specific proteins depending of concentration and cell models used [2, 24, 45].

Despite the controversy in the literature concerning MCT expression in CRC, it has been shown that MCTs (especially MCT-1, MCT-2 and MCT-4) are usually upregulated in CRC tumors compared to normal epithelium samples [9, 14, 15]. We have previously shown that butyrate led to an increase in MCT-1, MCT-4 and CD147 expression with an increased localization of MCT-1 expression at the plasma membrane in breast cancer cells [30, 31]. Our results on CRC cells demonstrated that acetate is also capable of inducing the expression of MCT-1, MCT-4 and CD147 with an increase in plasma membrane localization of MCT-1. Our observations suggest that MCT-1 might be involved in acetate transport across the membrane by regulating MCT-1 expression and activation at the plasma membrane.

The mechanisms involved in the regulation of MCTs in CRC cells in response to SCFA (especially acetate), are not well clarified, being the majority of the reports focused in the role of butyrate in CRC cells [1, 16, 24, 25]. Some studies have reported that MCT-1, MCT-4, MCT-5 and MCT-6 isoforms are also expressed in CRC cells [24]. In this regard, it has been shown that upon butyrate treatment,

MCT-1 is the most abundant MCT isoform expressed in Caco-2, a CRC derived cell line [24]. Furthermore, some investigators have shown that SMCT-1 was silenced by DNA methylation in CRC cells [1, 16, 18, 19], which conferred a selective advantage to escape butyrate-induced cell death [16, 17]. Taking into account our results, we cannot exclude the involvement of SMCT-1 as the Na^+-linked symporter for acetate in CRC cells, since SMCT-1 was expressed after acetate treatment in CRC cells. To the best of our knowledge, this is the first report showing that the SCFA acetate leads to an increase in MCTs expression and to a cellular re-localization of MCT-1 in CRC cells. Our results put forward a possible regulatory role of acetate at both MCT-1 expression and localization in CRC cells, similarly to what was described by us with butyrate in breast cancer cells [31].

Our data also show that MCT-1 re-localization to the plasma membrane is associated with increased expression of MCT-1 and CD147 in response to acetate treatment. Indeed it has been described that MCT-1 and MCT-4 require the presence of CD147, for proper localization and function [13].

MCT-4 expression has been associated with highly glycolytic tissues (including CRC), where it is essentially responsible for lactate efflux [9, 12]. We observed an elevated MCT-4 expression after 48 hours of acetate treatment that can be related with the increased glucose consumption and lactate production upon 24h of acetate treatment in CRC cells. Our findings are in agreement with Matthews and co-workers, which described that butyrate and propionate, alone or in combination, significantly increased glucose consumption at 24 hours with a considerable decrease 48 hours after treatment [6]. The authors explained the increased rate in glucose consumption as a way to provide energy efficiently and then the decreased rate in glucose consumption associated with the use of butyrate as second source of energy in CRC cells [6].

It is known that CRC cells use 30 to 40 times more glucose for energy metabolism than normal colon cells [25]. As glucose becomes the primary source in CRC cells, we also evaluated the expression of GLUT-1, the main glucose transporter detected in colorectal cancer tissues [25, 36, 46]. Interestingly, we found that both CRC cells express GLUT-1 but the levels remain unchanged after acetate treatment over the time. Therefore the decrease in glucose consumption is not due to a decrease uptake and further studies are required to assess whether it

relates to inhibition of the glycolytic flux as already reported in yeast in response to acetic acid [47].

It is known that cancer cells usually express high levels of MCTs to maintain a highly glycolytic profile and an appropriate pH environment essential for tumor growth. MCTs became attractive targets in cancer therapy, especially in cancer with a hyper-glycolytic phenotype [11, 12]. Furthermore, MCTs can also be seen as Trojan horses and recent reports have demonstrated that MCTs are also involved in the transport of drugs such as 3BP [31, 39]. In this case, the elevated MCT expression is used to mediate the entry of the chemotherapeutic agent 3BP into the cells and then selectively kill cancer cells [12, 31]. 3BP is an anti-cancer agent that targets energy metabolism of cancer cells [31]. Our results showed that the combination of acetate and 3BP potentiates the effect of acetate or 3BP alone in cell proliferation inhibition and apoptosis induction in CRC cells. This result is in agreement with our previous data showing that butyrate also sensitize breast cancer cancer cells to 3BP treatment [30, 31].

Summing up, here we show that acetate uptake involves at least two mechanisms of transport in CRC cells: mediated by MCTs and diffusion by aquaporins. Indeed, acetate induces changes in glycolytic metabolism, upregulation of MCT-1, MCT-4 and CD147 and plasma membrane localization of MCT-1. These findings may provide a novel strategy for CRC therapy based on the elimination of CRC cells in the presence of acetate through their sensitization by 3BP or other glycolytic metabolism inhibitor whose transport is mediated by MCTs.

5. Acknowledgments

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The role of diet related short-chain fatty acids in colorectal cancer: prevention or therapeutic strategy?

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Abstract

In Western societies, colorectal cancer (CRC) is a major cause of cancer-related death, the majority being CRC sporadic cases. These cases are rapidly increasing in developed countries, especially among populations that are adopting Western-style diets. Additionally, the environment, a sedentary lifestyle, medication, stress and intestinal diseases also influence the incidence of sporadic CRC. These risk factors are associated with alterations in the intestinal microbiota, accompanied by an increase of the colon pH due a decreased production of short chain fatty acids (SCFAs), influencing colorectal carcinogenesis. In this regard, many epidemiological studies have suggested the manipulation of colon microbiota, through the use of probiotics. These agents be used in the colorectal cancer prevention/treatment due to their ability to produce SCFAs, known as anti-neoplastic agents in CRC cells. In this review, we will describe the normal colon microbiota environment and its changes in CRC, as well as the concentration of SCFAs and the physiological effects these metabolites in the normal colon. Furthermore, we will focus on the anti-neoplastic role of SCFAs, especially butyrate, propionate and acetate in CRC cells to discuss how a probiotic rich diet could improve the amount of SCFAs in the colon and provide one strategy to prevent or treat CRC.

1. Normal colon microbiota and short-chain fatty acids

The human intestine harbours as many as 10^{12} microorganisms from 500 - 1000 different bacterial species, described by their variation in 16S ribosomal RNA genes (Sobhani, Amiot et al. 2013; Zeng, Lazarova et al. 2014; Cipe, Idiz et al. 2015). Several studies have proposed that the normal human microbiota is formed mainly by strict anaerobes, such as *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Fusobacterium*, *Peptostreptococcus*, and *Atopobium* (Neish 2009; Tiihonen, Ouwehand et al. 2010; Liu, Cao et al. 2013), representative of 2 main divisions: *Bacterodeites* and *Firmicutes* (Neish 2009; Tiihonen, Ouwehand et al. 2010; Holmes, Li et al. 2011; Liu, Cao et al. 2013; Zeng, Lazarova et al. 2014). *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* were also found, but in minor proportions (Liu, Cao et al. 2013; Zeng, Lazarova et al. 2014). It is known that the intestinal microbiota is a very complex system with numerous beneficial roles to human health, including protection against pathogens, immune system maturation, degradation of toxic substances, digestion of complex carbohydrates and production of short-chain fatty acids (SCFAs) (Holmes, Li et al. 2011; Russell, Hoyles et al. 2013).

In this case, although the main SCFAs (namely acetate, propionate and butyrate) exert multiple beneficial effects in the normal colon physiology, their role as cancer-preventing/treating agents has received special attention by several researchers (Scheppach, Bartram et al. 1995; Kim, Park et al. 2014; Kasubuchi, Hasegawa et al. 2015). These microbial compounds are produced by anaerobic microorganisms able to ferment polysaccharides, oligosaccharides, proteins, peptides and glycoproteins in the colon, including *Firmicutes*, *Clostridium*, *Bifidobacterium, Propionibacterium, Bacteroides* and *Lactobacillus* (Neish 2009; Adom and Nie 2013; Layden, Angueira et al. 2013; Kim, Park et al. 2014). The ability to produce butyrate requires a specific enzymatic process via butyryl-CoA: acetate CoA-transferase (mainly by *Eubacterium*, *Roseburia, Anaerostipes* and *Faecalibacterium prausnitzii* species) or via butyrate kinase (in certain *Clostridium* and *Coprococcus* species) (Russell, Hoyles et al. 2013; Kim, Park et al. 2014; Zeng, Lazarova et al. 2014). *Firmicutes* species are able to produce butyrate by both pathways (Russell, Hoyles et al. 2013; Kim, Park et al. 2014; Zeng, Lazarova et al. 2014).

Propionate is generally produced by *Bacteroides, Firmicutes* and *Propionibacterium* species which involve the succinate, acrylate and propanediol pathways (Hosseini, Grootaert et al. 2011; Russell, Hoyles et al. 2013; Kim, Park et al. 2014), while acetate is produced by acetogenic bacteria such as *Acetobacterium* species, *Clostridium aceticum* and *Propionibacterium,* able to perform reductive acetogenesis from formate (Russell, Hoyles et al. 2013; Kim, Park et al. 2014). Normally, these bacteria exist in a mutually beneficial symbiotic relationship in the human colon, providing the proper amount of these SCFAs to help maintain colon homeostasis (Cipe, Idiz et al. 2015).

Indeed, it has recently been shown, that some changes in SCFAs production is directly associated with alterations in the intestinal microbiota modulated by numerous extrinsic factors such as diet, age, medication, treatment (drugs, radiation, surgery), stress and diseases (Zhu, Michelle Luo et al. 2011; Cipe, Idiz et al. 2015). These changes in the normal symbiotic state (normobiosis) can be associated with many intestinal disorders, such as obesity, inflammatory bowel disease and colorectal cancer (Chen, Liu et al. 2012; Di Mauro, Neu et al. 2013; Sobhani, Amiot et al. 2013; Zeng, Lazarova et al. 2014). In this regard, the main challenge is to characterize the differences between a “healthy” intestinal microbiota and a CRC microbiota and its consequences on SCFAs production. This study would facilitate future studies towards the development of novel strategies for the diagnosis, treatment, and prevention of colorectal cancer (Cipe, Idiz et al. 2015).

2. Intestinal microbiota and short-chain fatty acids in colorectal cancer patients

Several factors (genetic and environmental) are involved in the transformation of normal colonic epithelium to adenoma and CRC (Keku, Dulal et al. 2015). It has been demonstrated that specific changes in human intestinal microbiota (dysbiosis) can also be associated with CRC development, especially in sporadic cases (Sobhani, Tap et al. 2011; Sobhani, Amiot et al. 2013; Gao, Guo et al. 2015). These changes support the hypothesis that “colon cancer may be

a bacteria-related disease" (Sobhani, Amiot et al. 2013; Gao, Guo et al. 2015), and that specific bacterial species can be involved in CRC pathogenesis (Ohigashi, Sudo et al. 2013; Geng, Song et al. 2014; Cipe, Idiz et al. 2015; Gao, Guo et al. 2015) (Figure 1).

The majority of studies have shown a significant difference between the bacteria genera associated with cancerous and non-cancerous intestinal tissue or fecal samples (Sobhani, Amiot et al. 2013; Gao, Guo et al. 2015; Keku, Dulal et al. 2015). The intestine microbial composition of CRC patients is usually increased in the following genera: *Lactococcus*, *Fusobacterium, Prevotella, Streptococcus, Bacteroides* (Sobhani, Tap et al. 2011; Gao, Guo et al. 2015; Keku, Dulal et al. 2015). It has been demonstrated that some bacteria have a complex arsenal of virulence factors which allow them to colonize and persist in the intestine, inducing chronic inflammation, accumulated mutations by DNA damage through superoxide radicals, genotoxin formation, increased T-cell proliferation and biosynthesis of procarcinogenic compounds that interfere with cell cycle regulation contributing to colorectal carcinogenesis (Yang and Jobin 2014; Cipe, Idiz et al. 2015; Gao, Guo et al. 2015; Leung, Tsoi et al. 2015; Nistal, Fernandez-Fernandez et al. 2015).

Escherichia coli (*E. coli*, a member of Enterobacteriaceae family) and *Fusobacterium nucleatum (F. nucleatum*) are the main bacteria associated with CRC cases and commonly over-represented in many tumor tissues from CRC patients (Sobhani, Amiot et al. 2013; Yang and Jobin 2014; Gao, Guo et al. 2015; Keku, Dulal et al. 2015; Leung, Tsoi et al. 2015). Interestingly, in healthy individuals, Enterobacteriaceae constitute only a small fraction (less than 1%) of the gut microbiota, but *E. coli* in particular becomes dominant in the gut microbiota of individuals with inflammatory bowel disease (IBD), in several animal models of gut inflammation and in CRC cases (Yang and Jobin 2014). An interesting fact is that, *E. coli* strains isolated from individuals with IBD and CRC are often adherent and invasive, displaying several pathogenic properties which modulate host cells and mediate carcinogenesis, and are in this way classified as inflammation drivers of the CRC formation (Leung, Tsoi et al. 2015). Beyond that, *F. nucleatum* is an opportunistic bacterium able to induce chronic inflammation and interact directly or indirectly with colonocytes leading to uncontrolled cell proliferation, dysbiosis and intestinal tumorigenesis (Kostic,

Chun et al. 2013; Leung, Tsoi et al. 2015). Recently, *F. nucleatum* has been considered as an indicator of disease progression and tumor severity (Leung, Tsoi et al. 2015). Likewise, it has also been shown that *Streptococcus gallolyticus*/*bovis* (involved with intestinal inflammatory disorders) is present in 20-50% of CRC and in less than 5% of normal colon (Gao, Guo et al. 2015).

In contrast, patterns that preserve microbial intestinal homeostasis, such as *Bifidobacterium*, *Lactobacillus*, *Clostridium* and *Ruminococcus* are under-represented in CRC patients compared to healthy individuals (Sobhani, Tap et al. 2011; Chen, Liu et al. 2012; Sobhani, Amiot et al. 2013; Nistal, Fernandez-Fernandez et al. 2015).

Changes in the intestinal microbial community of CRC patients affect the amount and types of metabolites produced by colonic microbes. In this regard, some studies have reported that changes in colon microbiota are associated with a decrease in SCFA concentrations accompanied by an elevated pH in patients with CRC compared to healthy individuals (Sobhani, Tap et al. 2011; Ohigashi, Sudo et al. 2013; Sobhani, Amiot et al. 2013) (Figure 5). However, it is not yet understood if the changes in colon microbiota are a cause or a consequence of CRC development. In this context, it is important to characterize the possible over- and under-represented bacteria, exploring the relationship between changes in the colon microbiota composition with the production of bacterial metabolites as well as the tumorigenesis process of CRC. The relationship between diet, intestinal microbiota, SCFA metabolism and colorectal cancer is very complex and remains an important area of research. In the following sections, the role of SCFAs in normal conditions is discussed (their concentrations, metabolism, beneficial effects, transporters and receptors), as well as how these microbial metabolites, namely butyrate, propionate and acetate, can be useful in the prevention and/or treatment of CRC.

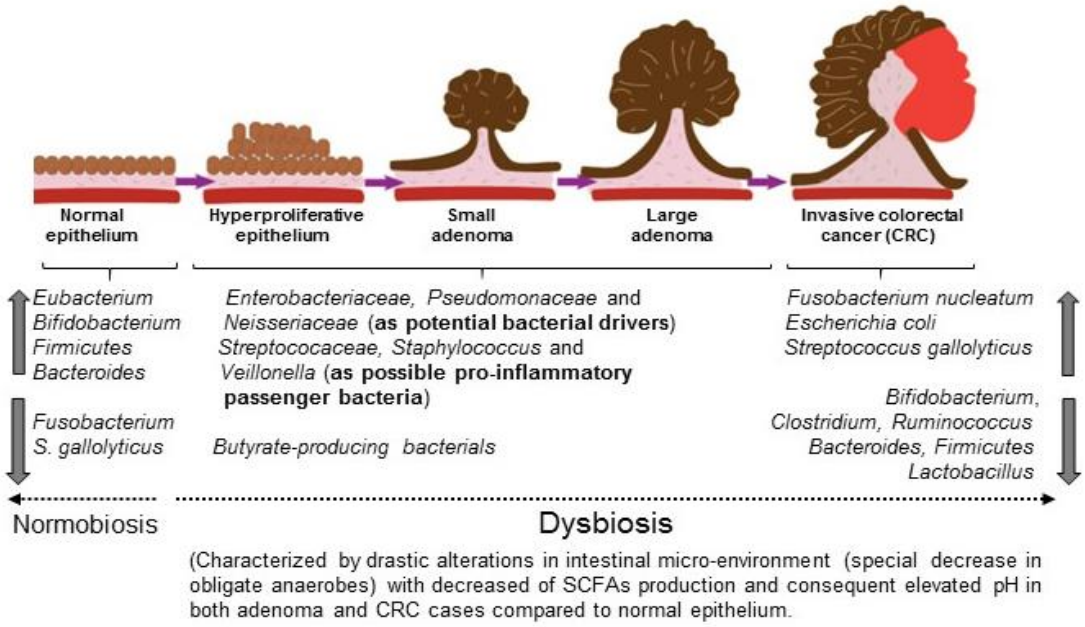

Figure 1: Schematic representation of the most important changes in the microbiota from normal to colorectal cancer (CRC).

3. Short-chain fatty acid concentrations in colon

Short chain fatty acids (SCFAs) are the major products of bacterial fermentation of undigested dietary fiber and of starches resistant to digestion in the human colon (Adom and Nie 2013; Zeng, Lazarova et al. 2014). Indeed, a diet rich in fiber, resistant starches and complex carbohydrates leads to increases the levels of colonic SCFAs (Adom and Nie 2013; Layden, Angueira et al. 2013).

SCFAs are carboxylic acids with aliphatic tails ranging from formic (C1), acetic (C_2), propionic (C_3), butyric (C_4), valeric (C_5) and caproic (C_6) acid (Layden, Angueira et al. 2013). SCFAs constitute approximately two-thirds of the colonic anions (~150 mM). At physiological pH, they predominate in a dissociated form, specifically acetate, propionate, butyrate, valerate and caproate (Nedjadi, Moran et al. 2014). The rate and amount of SCFAs produced depend on diet composition (which determines the type of the substrate fermented), on the microbiota present in the colon and on gut transit time (Mortensen and Clausen 1996; Macfarlane and Macfarlane 2012; Zeng, Lazarova et al. 2014). SCFAs are usually generated by carbohydrate fermentation according to the following equation (Zeng, Lazarova et al. 2014) :

59 $C_6H_{12}O_6$ + 38 H_2O = 60 CH_3COO^- (Acetate) + 22 CH_5COO^- (Propionate) + 18 CH_7COO^- (Butyrate) + 96 CO_2 + 256 H^+ + heat + gases

Acetate, propionate and butyrate are the three major colonic SCFAs. They are found in the colon at considerably high concentrations, ranging from 40–80 mM to 10–25 mM, respectively (Scheppach, Bartram et al. 1995; Alles, Hartemink et al. 1999; Jenkins, Kendall et al. 1999; Topping and Clifton 2001). Although not constant, the ratio of SCFA concentrations in the colonic lumen is approximately 60% acetate, 25% propionate and 15% butyrate (Scheppach, Bartram et al. 1995; Canani, Costanzo et al. 2011). In addition, the concentration of acetate, propionate and butyrate vary along the human intestine as follows: approximately 130 ± 9 mmol/L in the cecum (where the amounts of fermentable substrates are higher), ~ 80 ± 11 mmol/L in the descending colon and ~ 13 ± 6 mmol/L in the rectum (Cummings, Pomare et al. 1987; Du, Shi et al. 2014). This variation in acid concentration is linked with pH differences along the human colon, namely: ~ 5.5-6.8 in the cecum and ~ 6.6-7 in the descending colon (Figure 2). The higher acidity is associated with more active carbohydrate fermentation in the cecum than in the sigmoid/rectum, where the pH is more alkaline (Cummings, Pomare et al. 1987).

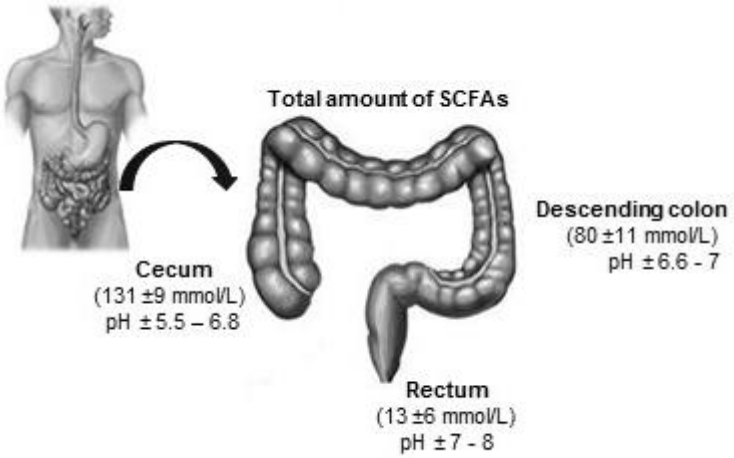

Figure 2: Regions of the colon and rectum with respective total amount of SCFA concentrations and pH.

4. Metabolism of short-chain fatty acids in the colon

It has been demonstrated that SCFAs derived from microbial metabolism in the gut play a central role in colon homeostasis (den Besten, Lange et al. 2013; den Besten, van Eunen et al. 2013). SCFAs are substrates for energy metabolism by the epithelial cells, converted by colonocytes into glucose, ketone bodies, and amino acids (Hadjiagapiou, Schmidt et al. 2000; den Besten, Lange et al. 2013; Kasubuchi, Hasegawa et al. 2015). Previous studies showed a preferential utilization of SCFAs in the order of butyrate > propionate > acetate by colonocytes (Adom and Nie 2013). Butyrate is the major energy source for colonocytes, but also for other cells from liver and muscle tissues (Ahmad, Krishnan et al. 2000; Adom and Nie 2013; Russell, Hoyles et al. 2013; Zeng, Lazarova et al. 2014). Butyrate is usually metabolized to acetyl-CoA by colonocytes (Donohoe, Collins et al. 2012) while propionate and acetate can modulate lipogenesis and gluconeogenesis (Comalada, Bailon et al. 2006). It has been demonstrated that propionate is largely metabolized in the liver (Russell, Hoyles et al. 2013) and also acts as substrate for gluconeogenesis inhibiting cholesterol synthesis in hepatic tissue (Adom and Nie 2013; Scott, Gratz et al. 2013). Acetate can be oxidized in the tricarboxylic acid (TCA) cycle, used as a substrate for the synthesis of cholesterol, long-chain fatty acids or as a co-substrate for glutamine and glutamate synthesis by colonocytes (Adom and Nie 2013; den Besten, Lange et al. 2013; Zeng, Lazarova et al. 2014). This compound also enters systemic circulation and can be used by many tissues, including heart, adipose, kidney, liver and muscle (den Besten, Lange et al. 2013; Scott, Gratz et al. 2013).

In contrast to normal colonocytes, CRC cells primarily undergo aerobic glycolysis instead of oxidative metabolism (Donohoe, Collins et al. 2012). In this case, it has been proposed that the preference of CRC cells for glucose metabolism instead SCFAs as an energy source explains the opposite effects on the growth of normal *versus* cancerous colonocytes (Donohoe, Collins et al. 2012). However, this paradox is still poorly understood and more studies are needed to understand how the metabolic differences between normal and cancerous colonocytes can be used to inhibit tumorigenesis in CRC.

5. Short-chain fatty acid transporters and receptors

Production of total colonic SCFAs is difficult to determine because more than 95% of these SCFAs are water soluble and rapidly absorbed and metabolized by colonocytes (Roy, Kien et al. 2006; Kim, Park et al. 2014; Zeng, Lazarova et al. 2014). Only a small amount of these SCFAs leave the colon intact and can be transported via the blood circulation to other organs (Roy, Kien et al. 2006; Ganapathy, Thangaraju et al. 2013; Kim, Park et al. 2014).

Though distinct mechanisms for SCFAs absorption across the plasma membrane of colonocytes in various species have been postulated (Hijova and Chmelarova 2007; Nedjadi, Moran et al. 2014), most studies on SCFAs transporters both show in intestinal epithelial cells and CRC cells used butyrate transport as a model (Hadjiagapiou, Schmidt et al. 2000; Ganapathy, Thangaraju et al. 2013; Goncalves and Martel 2013) and information concerning other SCFAs like propionate or acetate transport is still rare.

SCFAs enter intestinal epithelial cells through passive diffusion of protonated SCFAs or mediated transport by monocarboxylate transporters (MCTs) (Goncalves and Martel 2013). The MCT family comprises 14 members, however, only the first four (MCT-1 - 4) are known to mediate the proton-coupled transport of monocarboxylic acids across the plasma membrane (Pinheiro, Longatto-Filho et al. 2012; Halestrap 2013). The main function of MCT has been associated with the uptake or efflux of monocarboxilic acids through the plasma membrane, according to the cell metabolic needs, characterized by a high affinity transport for L-lactate, pyruvate, acetate, propionate, D,L-β-hydroxybutyrate and acetoacetate (Halestrap and Meredith 2004).

SMCT-1 and MCT-1 have been identified as the main monocarboxylate transporters, responsible for the uptake of SCFAs across the membrane of intestinal cells (den Besten, Lange et al. 2013; Goncalves and Martel 2013; Kim, Park et al. 2014). SMCT-1 is a Na^+-coupled transporter for a variety of SCFAs, especially butyrate (Canani, Costanzo et al. 2011; Ganapathy, Thangaraju et al. 2013), while MCT-1 is a H^+-coupled transporter for SCFAs and related organic acids, which transports these molecules across the plasma membrane depending on the H^+ eletrochemical gradient (Kim, Park et al. 2014). MCT-1 is expressed widely in many different cell types and has been characterized as the

primary butyrate transporter in colon epithelial cells (Canani, Costanzo et al. 2011; Fung, Cosgrove et al. 2012).

MCT-1 also exports lactate to the extracellular milieu, which is a potentially cytotoxic metabolic by-product of glycolysis, indicating that it plays a dual role to enhance CRC cell survival (Fung, Cosgrove et al. 2012). In contrast, it has been shown that CRC cells silence SMCT-1 by DNA methylation (Hadjiagapiou, Schmidt et al. 2000; Thangaraju, Cresci et al. 2008; Babu, Ramachandran et al. 2011; Ganapathy, Thangaraju et al. 2013; Goncalves and Martel 2013) which confers a selective advantage to escape butyrate-induced cell death through limitation of butyrate uptake (Li, Myeroff et al. 2003; Ganapathy, Thangaraju et al. 2013).

In addition to SCFA transporters, their receptors constitute a new and rapidly growing field of research, as more functions of SCFA receptors have been discovered. SCFAs are able to activate cells through several cell-surface G-protein-coupled receptors (GPRs), especially GPR41 and GPR43 (Kim, Kang et al. 2013; Kuwahara 2014), involved in immune response regulation (Kim, Kang et al. 2013). These two GPRs are expressed not only by intestinal epithelial cells where SCFAs are produced, but also at multiple other sites considered to be metabolically important such as adipose tissue and pancreatic islets (Layden, Angueira et al. 2013). Recent studies have identified the cell-surface receptors GPR41, GPR43 and GPR109A as essential for the biologic effects of SCFAs in the colon (Ganapathy, Thangaraju et al. 2013; Kim, Kang et al. 2013). GPR41 (also called free fatty acid receptor 3; FFA3) expressed in colon cells is activated by SCFAs, primarily by butyrate and propionate (Layden, Angueira et al. 2013; Kuwahara 2014), while GPR43 (free fatty acid receptor 2; FFA2) has a more potent affinity for propionate and acetate (Ganapathy, Thangaraju et al. 2013; Layden, Angueira et al. 2013). In addition, GPR109A (hydroxycarboxylic acid receptor 2), a receptor for niacin and vitamin B3, is also a receptor for butyrate in colon cells (Kim, Park et al. 2014; Kuwahara 2014). Nevertheless, the regulation of SCFA transporters and receptors in CRC cells is still poorly understood, and more studies are needed to grasp how these metabolites can influence the regulation of their transporters and receptors in CRC cells.

6. Physiological effects of short-chain fatty acids in normal colon

It is known that SCFAs play a significant role in maintaining the normal physiological functions of the colonic mucosa, serving as regulators of intracellular pH, cell volume and other functions associated with ion absorption and gut motility of intestinal epithelial cells (Kim, Park et al. 2014; Ohtani 2015). SCFAs are the major energy source for colonocytes and can also be used for *de novo* synthesis of lipids as referred before (Scharlau, Borowicki et al. 2009; Kim, Kang et al. 2013; Kasubuchi, Hasegawa et al. 2015).

In addition, it has been demonstrated that SCFAs have a suppressive role in inflammation and cancer of the intestine (Kim, Park et al. 2014; Keku, Dulal et al. 2015). Though the anti-inflammatory mechanism of SCFAs is still not adequately clarified, a recent study showed that, once absorbed by intestinal epithelial cells and/or immune cells, SCFAs (especially butyrate and propionate) inhibit the activity of histone deacetylases (HDACs), promoting the hyperacetylation of histones and trans-activating gene expression, which results in downregulation of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and IL-12 (Ohtani 2015).

Interestingly, it has been demonstrated that total SCFAs and/or local differences in SCFA concentrations along the intestinal tract might be implicated in diseases of the colon, especially gastrointestinal disorders and colorectal cancer (Wong, de Souza et al. 2006). For example, a decreased level of butyrate in the environment of colonocytes has been suggested to contribute to the genesis of ulcerative colitis (UC) and to a significantly increased risk of colon cancer (Huda-Faujan, Abdulamir et al. 2010). Therefore, increased production and greater distal delivery of SCFAs may result in a protective effect against these diseases (Wong, de Souza et al. 2006).

SCFAs, especially butyrate, are involved not only in the regulation of expression of several genes, cell proliferation, differentiation and apoptosis to promote colonic wellbeing in epithelial cells, but in the prevention of colon cancer by promoting growth inhibition, cell-cycle arrest and apoptosis in transformed colonocytes (Sakata 1987; Comalada, Bailon et al. 2006; Fung, Cosgrove et al. 2012; Ganapathy, Thangaraju et al. 2013; Kim, Park et al. 2014). Furthermore, as described in more detail below, it has been shown that SCFAs exhibit anti-

cancer activities acting through different mechanisms to induce cell death in CRC cells (Comalada, Bailon et al. 2006; Lan, Lagadic-Gossmann et al. 2007; Zhang, Zhou et al. 2010; Adom and Nie 2013; Marques, Oliveira et al. 2013; Oliveira, Pereira et al. 2015).

7. Mechanisms of action of short-chain fatty acids in colorectal cancer cells: prevention and therapeutic implications

A variety of biological effects of SCFA have been reported, and a vast number of experimental studies keep showing new aspects of these molecules. Most notably, it has been shown that the SCFAs butyrate, propionate and acetate induce apoptosis in CRC cells but not in normal cells (Sakata 1987; Comalada, Bailon et al. 2006; Sauer, Richter et al. 2007; Zhang, Zhou et al. 2010; Tang, Chen et al. 2011; Imbernon, Whyte et al. 2014). The anti-cancer effect of SCFAs is also supported by epidemiological studies suggesting an inverse relation between the level of dietary fibers and the incidence of CRC (Scheppach, Bartram et al. 1995; Sengupta, Muir et al. 2006; Zeng, Lazarova et al. 2014). These dietary components influence the risk of human colon cancer through diverse mechanisms, which include activation of cell death through different processes depending on the concentration, pH and cell type. The mechanisms by which these SCFA are metabolized and regulate cell proliferation, differentiation, cell death and their role in inflammation in CRC cells will be discussed below.

7.1 Butyrate

Butyrate is considered the most potent of the SCFAs regarding prevention and inhibition of colon carcinogenesis, and its antitumor effects have been the most studied (Scheppach, Bartram et al. 1995; Comalada, Bailon et al. 2006; Scharlau, Borowicki et al. 2009; Zhang, Zhou et al. 2010; Matthews, Howarth et al. 2012; Goncalves and Martel 2013). Butyrate's protective effects against human colon cancer cells involve inhibition of cell differentiation, promotion of cell-cycle arrest, apoptosis and of inhibition of histone deacetylases (HDACs)

(Table 1) (Hinnebusch, Meng et al. 2002; Comalada, Bailon et al. 2006; Zhang, Zhou et al. 2010; Donohoe, Collins et al. 2012; Fung, Cosgrove et al. 2012; Matthews, Howarth et al. 2012).

Previous studies in CRC cell lines showed that induction of apoptosis and cell cycle arrest in G0-G1 or G2-M by butyrate could occur *via* p53-dependent or p53- independent pathways (Archer, Meng et al. 1998; Mariadason, Velcich et al. 2001; Matthews, Howarth et al. 2012). Zhang and co-workers analyzed the cytotoxicity mechanism of butyrate (0-40 mM) in human colon cancer (RKO cells) and showed that it exhibited a strong growth inhibitory effect against RKO cells, inducing the intrinsic apoptosis pathway characterized by DNA fragmentation and activation of caspase-9 and caspase-3 (Zhang, Zhou et al. 2010). The expression of anti-apoptotic protein Bcl-2 decreased, whereas the apoptotic protein Bax increased in a dose-dependent manner during butyrate-induced apoptosis (Zhang, Zhou et al. 2010).

Critical tumorigenesis signaling pathways that regulate cell proliferation, cell migration and apoptosis, including extracellular signal-regulated protein kinase 1/2 (ERK1/2), C-Jun N-terminal kinase (JNK) and p38 MAPK (p38) were also studied (Zhang, Zhou et al. 2010). An important point is that a high levels of ERK1/2 activation/expression are associated with cell proliferation and survival in various cancer cells, including CRC (Davido, Richter et al. 2001; Zhang, Zhou et al. 2010). However, it was demonstrated that butyrate-induced growth inhibition occurs with inactivation or downregulation of ERKs in RKO and HT-29 cells, respectively (Davido, Richter et al. 2001; Zhang, Zhou et al. 2010). Moreover, activation of the JNK MAPK pathway played an important role in butyrate-induced apoptosis in RKO cells (Zhang, Zhou et al. 2010).

Matthews *et al* 2012, showed that butyrate (5 mM) induced apoptosis and G2-M arrest mediated by alterations in the oxidative pentose pathway, reduction in glutathione availability and glucose consumption, and increased levels of reactive oxygen species (ROS) (Matthews, Howarth et al. 2012). He *et al* (2007) observed that downregulation of GLUT-1 expression was associated with apoptosis-induced by butyrate (5 mM) in HT-29 cells, also correlated with an increase in the expression and activity of MCT-1 as a mechanism to maximize intracellular availability of butyrate (He, Li et al. 2007). Some authors state that butyrate inhibits CRC cell proliferation and induces cell death due to its

inefficient metabolism associated with the Warburg effect and nuclear accumulation in transformed colonocytes, where it acts as a HDAC inhibitor (Shao, Gao et al. 2004; Wong, de Souza et al. 2006; Donohoe, Collins et al. 2012). Robert Li and co-workers (2006) confirmed accumulation of acetylated histone 3 (H3) as a reslt of butyrate treatment in CRC cells. Butyrate further contributes to hyperacetylation through conversion to acetyl-CoA and stimulation of histone acetyltransferase (HAT) activity (Barshishat, Polak-Charcon et al. 2000; Li and Li 2006). However, the metabolic state of the cell influences the amount of intranuclear butyrate and acetyl-CoA levels determining whether butyrate functions as a HDAC inhibitor or stimulate HATs, thus epigenetically regulating the expression of different target genes (Mariadason, Velcich et al. 2001; Donohoe, Collins et al. 2012). Deregulation of the expression or activity of HATs and HDACs may lead to alterations in gene expression profiles, associated with reactivation or silencing of important genes for cancer progression, differentiation and apoptosis (Sambucetti, Fischer et al. 1999; Iacomino, Tecce et al. 2001; Marchion and Munster 2007). In this context, Archer *et al* 1998, showed that butyrate (0-20 mM) increased p21 expression through a process involving histone hyperacetylation, and that p21 was required for butyrate-mediated growth arrest in colon cancer cells (HT-29 and HCT116). Furthermore, compared to propionate and acetate, butyrate has strong anti-inflammatory properties, and this effect is mediated mainly by inhibition of TNF-α production, nuclear factor-kappa B (NF-κB) activity and IL-8, 10 -12 expression in immune and colonic epithelial cells (Tedelind, Westberg et al. 2007; Kim, Park et al. 2014; Zeng, Lazarova et al. 2014). Yet, little is known about the role of butyrate on inflammation in CRC cells.

The main conclusion is that the exposure of the human colon to butyrate might protect against CRC by reducing survival and inducing cell death in CRC cells by several mechanisms discussed above. It is worth noting that cells that metabolize butyrate at a higher rate, are usually less susceptible to its apoptosis-inducing effects. This may explain why normal colonocytes are unaffected by high levels of this SCFA in the colon, since they preferentially use butyrate as an energy source, in contrast with CRC cells which seem to prefer glucose.

Table 1 - Effects of SCFA butyrate in colorectal cancer cells.

Butyrate			
Human colorectal cancer cell lines	**Concentration (mM)**	**Effects**	***Reference***
HCT116 *HT-29*	0 - 20	p21 is required for butyrate-mediated growth arrest	*Archer et al, 1998*
Caco-2 *SW620*	2 - 10	Cell cycle arrest and apoptosis more effective in undifferentiated cells, whereas differentiated cells were essentially resistant to butyrate effects	*Mariadason et al, 2001*
HT-29		Growth inhibition by the downregulation of ERK1/2	*Davido et al, 2001*
HCT116 *HT-29*	1 5	Cell growth arrest, differentiation, apoptosis, induction of histone H4 hyperacetylation	*Hinnebusch et al, 2002*
HT-29	1 - 8	Inhibition of cell proliferation, induction of differentiation and apoptosis	*Comalada, 2006*
HT-29	5 - 40	Modulation of histone acetylation	*Kiefer et al, 2006*
HT-29	5	Downregulation of the GLUT-1 expression mediates apoptosis	*He et al, 2007*
LT92 *HT-29*	0 - 50	Induction of GSTs as a possible mechanism of chemoprevention	*Scharlau et al, 2009*
RKO	0 - 40	Inhibition of cell proliferation involving inactivation of ERK MAPK, induction of apoptosis via activation of caspase-9 and caspase-3	*Zhang et al, 2010*
Caco-2	5	Induction of apoptosis, G2-M arrest, alterations in the oxidative pentose pathway	*Matthews et al, 2012*
RAW264.7[*]	0 – 1.200 µmol/L	Decrease of proinflammatory factors with an increase in the antiinflammatory cytokine	*Liu et al, 2012*

*Murine macrophage cell line

7.1 Propionate

Though propionate exerts an anti-neoplastic effect in colorectal cancer cells and has a mechanisms of action similar to butyrate, there are less published studies with this SCFA than with butyrate (Kiefer, Beyer-Sehlmeyer et al. 2006; Hosseini, Grootaert et al. 2011) (Table 2).

Jan and co-workers showed that propionate (10- 40 mM) induced typical signs of apoptosis in human CRC cell lines, with loss of mitochondrial membrane potential ($\Delta\Psi m$), generation of ROS, cytochrome c release, caspase-3-processing and nuclear chromatin condensation (Jan, Belzacq et al. 2002). Matthews and co-workers demonstrated that at lower doses (5 mM) propionate also induced apoptosis, characterized by an elevated ROS production and decreased glucose oxidation after 48 h of treatment in the Caco-2 colon cancer cell line (Matthews, Howarth et al. 2012). Tang *et al*. showed that propionate treatment (3 -10 mM) induces ROS generation and loss of mitochondrial membrane potential ($\Delta\Psi m$) with autophagy induction to degrade damaged mitochondria in HCT116 cells, showing in this case that propionate-triggered autophagy serves as an adaptive strategy to delay mitochondria-mediated cell death in CRC cells (Tang, Chen et al. 2011).

It has been demonstrated that propionate, like butyrate, also acts as an inducer of histone acetylation in CRC cells, which is possibly associated with a modulated growth response of tumorigenic lesions in the gut (Kiefer, Beyer-Sehlmeyer et al. 2006; Hosseini, Grootaert et al. 2011; Kim, Park et al. 2014). In this regard, Kiefer *et al* showed that propionate alone (2.5 – 40 mM) or in combination with butyrate or acetate induced significant histone acetylation in HT-29 cells. In addition, another study demonstrated that propionate-induced growth inhibition, like butyrate, involves downregulation of ERK1/2 in HT-29 cells (Davido, Richter et al. 2001).

Furthermore, it has been shown that propionate has anti-inflammatory properties in the gut, comparable to those already described for butyrate (Tedelind, Westberg et al. 2007; Liu, Li et al. 2012; Kim, Park et al. 2014). Tedelind *et al* 2007, observed that the anti-inflammatory activity of propionate (0.3 - 30 mM) involves the inhibition of the NF-κB pathway and suppression of IL-6 release in Colo320DM cells.

Taken together, these findings suggest that propionate could be effective in the prevention and treatment of some colon alterations, including CRC. Nevertheless, further studies both *in vitro* and *in vivo* are required to understand its real role alone and in combination with other SCFAs, as discussed below.

Table 2- Effects of SCFA propionate in colorectal cancer cells.

Propionate

Human colorectal cancer cell lines	**Concentration (mM)**	**Effects**	***Reference***
HT-29		Growth inhibition by the downregulation of ERK1/2	*Davido et al. 2002*
HT-29 *Caco-2*	0 - 40	Decrease of viability/apoptosis with mitochondrial alterations	*Jan et al. 2002*
HT-29	20 - 40	Modulation of histone acetylation	*Kiefer et al, 2006*
HT-29	2 - 16	Antiproliferative effect	*Comalada, 2006*
HT-29	30	Apoptosis at pH 7.5 Necrosis at pH 5.5	*Lan et al, 2007*
Colo320DM	0,3 - 30	Inhibition of the NF-$_{k}$B pathway	*Tedelind et al, 2007*
HCT116	0 - 10	Mitochondrial defects and autophagy	*Tang et al, 2011b*
HCT116 *SW480*	0 - 3	Two types of programmed cell death (autophagy and apoptosis)	*Tang et al, 2011a*
Caco-2	5	Induction of apoptosis, G2-M arrest, alterations in the oxidative pentose pathway.	*Matthews et al, 2012*
RAW264.7[*]	0 – 1.200 µmol/L	Decrease of proinflammatory factors with an increase in the antiinflammatory cytokine	*Liu et al, 2012*

*Murine macrophage cell line

7.2 Acetate

Acetate has been the least studied of the three most relevant SCFAs (table 5). Initially, Jan and co-workers showed that acetate (0-40 mM) decreased the viability and induced typical signs of apoptosis in the colon adenocarcinoma cell line HT-29, including loss of mitochondrial membrane potential, generation of ROS, caspase-3 processing and nuclear chromatin condensation (Jan, Belzacq et al. 2002).

Studies in the yeast species *Saccharomyces cerevisiae* first demonstrated that acetic acid induced a mitochondria-mediated apoptotic process (Pereira, Silva et al. 2008; Guaragnella, Zdralevic et al. 2012) with several features similar to apoptosis mediated by SCFAs in CRC cells. Indeed, alterations in mitochondria were identified in yeast, including production of ROS, mitochondrial swelling, decrease of $\Delta\Psi m$ (Ludovico, Rodrigues et al. 2002), mitochondrial fragmentation/degradation (Fannjiang, Cheng et al. 2004), mitochondrial outer membrane permeabilization (MOMP) with consequent release of pro-apoptotic factors like cytochrome *c*, yeast apoptosis inducing factor 1 (Aif1p) and Nuc1p (yeast ortholog of EndoG) (Ludovico, Rodrigues et al. 2002; Wissing, Ludovico et al. 2004; Kroemer, Galluzzi et al. 2007). The yeast orthologs of the mammalian VDAC (voltage-dependent anion channel) and ANT (adenine nucleotide transporter) were shown to play a role in MOMP and cytochrome *c* release during acetic acid-induced apoptosis in yeast (Pereira, Camougrand et al. 2007) Later, vacuolar membrane permeabilization (VMP) and release of Pep4p, yeast cathepsin D (CatD), from the lysosome-like vacuole to the cytosol, were observed in yeast cells exhibiting apoptotic cell death induced by acetic acid (Pereira, Chaves et al. 2010). In that study, it was also shown that, once in the cytosol, Pep4p played an important role in mitochondrial degradation through an autophagic-independent process, which protected yeast cells from acetic acid-induced apoptosis. Recently, it was demonstrated that both the protective function of Pep4p and its role in mitochondrial degradation during acetic acid-induced apoptosis in yeast depend on Pep4p proteolytic activity, and is complemented by heterologous expression of human CatD (Pereira, Azevedo et al. 2013; Oliveira, Pereira et al. 2015).

Taking into account the aforementioned results, we therefore hypothesized if similar events could occur in response to acetate in CRC cells (Figure 3). We showed that acetate treatment in CRC cells (0 - 140 mM and 0 - 220 mM, respectively to HCT-15 and RKO cells) decreased cell proliferation and induced apoptosis in CRC cells (Marques et al., 2013). We also demonstrated that the process was characterized by DNA fragmentation, caspase-3 activation and phosphatidylserine exposure to the outside leaflet of the plasma membrane with appearance of a sub-G1 population (Marques et al., 2013) (Figure 3).

We also found that acetate induced lysosome membrane permeabilization (LMP) with cathepsin D (CatD) release to the cytosol. In this context, we showed that CatD, but not CatB and CatL (also overexpressed in CRC cells), has an anti-apoptotic role in acetate-induced apoptosis (Marques et al., 2013). We next showed that acetate induced many mitochondrial dysfunctions as well as ROS (total and mitochondrial) production and an increase in mitochondrial mass accompanied by mitochondrial membrane depolarization (Oliveira et al., 2015).

Additionally, we found an increase in the levels of mitochondrial proteins, namely the apoptosis inducing factor (AIF), the voltage dependent anion channel (VDAC1) and a subunit of the outer mitochondrial membrane translocator (TOM22) in CRC cells after acetate treatment (Oliveira et al., 2015). We could demonstrate that, like acetic acid in yeast, acetate-induced apoptosis is not associated with autophagy induction in CRC cells, but inhibition of CatD (with siRNA or pepstatin A) enhanced apoptosis associated with higher mitochondrial dysfunction and increased mitochondrial mass (Oliveira et al., 2015). This effect seems to be specific of CatD, since inhibition of Cathepsins B and L (using E-64d, a well-known inhibitor of CaB and CatL) had no effect, nor were these proteases significantly released to the cytosol during acetate-induced apoptosis (Oliveira et al., 2015).

The protective role of CatD demonstrated by our data can explains partly the CatD overexpression in some CRC clinical cases in comparison to normal colon mucosa. In summary, our group provided a novel CatD function in the degradation of damaged mitochondria when autophagy is impaired, which protects CRC cells from acetate-induced apoptosis and that CatD inhibitors could therefore enhance acetate-mediated cancer cell death, presenting a novel strategy for prevention or therapy of CRC. More recently, and aiming to get an

overall picture of the effects of acetate on CRC cells, we focused the work in the transport mechanisms of acetate across the plasma membrane and its effects on the MCT expression and localization, as mentioned before (unpublished results) (Figure 3).

As already referred for butyrate and propionate, the mechanism through which acetate mediates cell death and its anti-inflammatory effects are currently poorly understood. However, it has been shown that acetate also reduces the production of pro-inflammatory factors, including TNF-α, IL-1β, IL-6, while enhancing the production of the anti-inflammatory cytokine IL-10 (Tedelind, Westberg et al. 2007; Liu, Li et al. 2012; Kim, Park et al. 2014) and induces inhibition of the NF-κB pathway in CRC cells (Tedelind, Westberg et al. 2007). Although it has been demonstrated that acetate can affect the immune response in the colon, more studies are necessary in order to understand the precise role of this SCFA and its interaction with intestinal microbiota, host immune cells, colonocytes and CRC cells. In conclusion, as we previously reported, the idea of acetate, like butyrate and propionate, as chemopreventive agent should be also considered. However, more studies, especially using SCFA combinations, that mimic their relative concentrations in the colon, are necessary to support their combined use as a new strategy to potentiate their effects in CRC cells.

Table 3 - Effects of SCFA acetate in colorectal cancer cells.

Acetate

Human colorectal cancer cell lines	Concentration (mM)	Effects	Reference
HT-29 Caco-2	0 - 40	Decrease of viability/Apoptosis with mitochondrial alterations	Jan *et al*, 2002
HT-29	4 - 32	Ineffective in these concentrations	Comalada, 2006
HT-29	80	Does not induce histone acetylation	Kiefer *et al,* 2006
Colo320DM	2,4	Inhibition of the NF-$_{k}$B pathway	Tedelind *et al,* 2007
HT-29	15 mM	Apoptosis at pH 7.5 Necrosis at pH 5.5	Lan *et al,* 2007
RAW264.7*	0 – 1.200 µmol/L	Decrease of proinflammatory factors with an increase in the antiinflammatory cytokine	Liu *et al,* 2012
HCT-15 RKO	0 - 140 0 - 220	Induction of apoptosis/Inhibition of proliferation and partial lysosome permeabilization with CatD release to the cytosol	Marques *et al*, 2013
HCT-15 RKO HCT116	0 - 140 0 - 220 0 - 200	Mitochondrial dysfunction/ inhibition of autophagy and active CatD involved in mitochondrial degradation independent of autophagy	Oliveira *et al,* 2015
HCT-15 RKO	0 - 70 0 - 110	Acetate transport is mediated by SMCT-1 or diffusion by aquaporins. Acetate treatment is involved with MCTs regulation and co-localization associated with changes in glucose metabolism. Acetate effects are potentiated by the combined treatment with 3BP (a well-known inhibitor of glycolysis)	Oliveira et al (unpublished results)

*Murine macrophage cell line

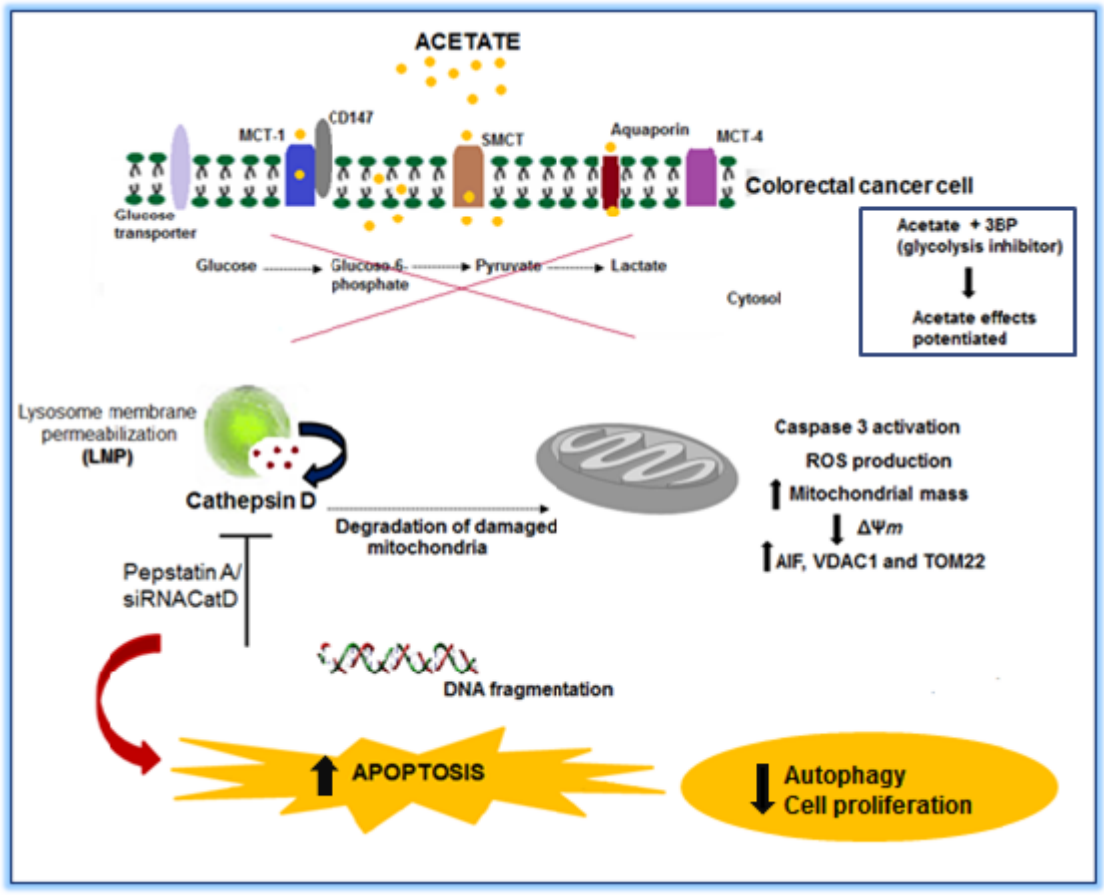

Figure 3: Schematic representation of different cell processes involved in apoptosis triggered by acetate in colorectal cancer cells. Different transporter systems which may mediate acetate uptake by CRC cells are represented. Acetate transport can be mediated by mocarboxylate transporters (MCT), such as SMCT-1 and MCT-1 (only in acetate pre-treated cells) or via passive diffusion by aquaporins. Acetate induces an increase in the expression of MCT-1, MCT-4 as well as of CD147 that after glycosylation leads to plasma membrane re-localization of MCT-1 and glycolysis perturbation. Acetate also induces partial lysosome membrane permeabilization (LMP) with specific release of cathepspin D (CatD) into the cytosol, which protects cells from acetate-induced apoptosis. DNA fragmentation and mitochondrial dysfunctions (accumulation of reactive oxygen species ROS; increase of mitochondrial mass, decrease of mitochondrial membrane potential, $\Delta\Psi m$; caspase 3 activation, and increase in the levels of different mitochondrial proteins such as apoptosis inducing factor, AIF; voltage dependent anion channel, VDAC1 and outer mitochondrial membrane translocator, TOM22) are other cellular events associated with acetate-induced apoptosis. Under these conditions autophagy is not induced and the inhibition of CatD (with pepstatin A or siRNA) enhanced acetate-induced apoptosis associated with a higher mitochondrial dysfunction. CatD plays a role in the degradation of damaged mitochondria when autophagy is impaired in CRC cells. Combined treatment (acetate + 3-bromopyruvate, 3BP, a well-known glycolysis inhibitor) potentiates the acetate effects, decreasing cell proliferation and inducing elevated rate of apoptosis.

7.4. Combined effects of SCFAs

Neither butyrate, propionate or acetate are available as the only metabolite present in the human colon, and therefore their combined effect should be taken into consideration. Although there are few studies on the combined effect of SCFAs performed in CRC cells, Tang *et al* 2011a, using a combination of butyrate and propionate (1-3 mM), showed for the first time that SCFAs orchestrate two types of programmed cell death in colon cancer cells (HCT116 and SW480). Butyrate and propionate treatment induced autophagy to dampen apoptosis, whereas inhibition of autophagy potentiated SCFA-induced apoptosis (Tang, Chen et al. 2011). Matthews and co-workers demonstrated that the combination (butyrate and propionate: 5 mM) induced cell cycle arrest in G2-M associated with a rapid and extensive apoptosis with changes in redox state and D-glucose metabolism in Caco-2 cells, compared to single treatments (Matthews, Howarth et al. 2012). In addition, Kiefer *et al*. showed that mixtures of acetate, butyrate and propionate (at molar ratios 75: 11 : 14, 69 : 16 : 15 and 43 : 24 : 33, respectively) which mimic the relative concentrations occurring in the gut, modulated histone acetylation. However, they concluded that the histone acetylation was mainly due to additive effects of butyrate and propionate, but not due to acetate (Kiefer, Beyer-Sehlmeyer et al. 2006). Lan and co-workers showed that acetate and propionate (15 mM and 30 mM, respectively) produced by *Propionibacterium freudereichii* decreased proliferation and induced cell cycle arrest in G2/M, followed by a sequence of cellular events characteristic of apoptosis at pH 7.7, but induced necrosis at pH 5.5 in colon cancer cells (HT-29) (Lan, Lagadic-Gossmann et al. 2007). This study demonstrated for the first time the impact of the extracellular pH prevailing within the colon (from 5.5 to 7.5) on the mode of cell death triggered by propionibacteria-produced SCFA in CRC cells (Lan, Lagadic-Gossmann et al. 2007). In this regard, our group has recently characterized the potential of *P. freudenreichii* subsp. *freudenreichii* DSM 20271 as a producer of SCFAs (propionate and acetate) using several culture media to simulate the digestive stress process and the composition of digested products present in the colon (Marta *et al* unpublished results). Pure SCFAs, as well as the bacterial fermented broth, inhibited CRC cell proliferation and increased cell death. The

results showed that co-culture of *P. freudenreichii* and CRC cells is possible and favourable for the bacteria and that *P. freudenreichii* could potentially be used as a probiotic in CRC prevention and/or treatment through its ability to produce SCFAs (Marta *et al* unpublished results).

Although studies on the combined effects of SCFAs are still few, it seems that they have a greater effect in colorectal cancer cells compared with treatment with each SCFA separately. In summary, there is increasing evidence of the anti-neoplastic effects of SCFAs in CRC cells, which may be modulated by several aspects, namely the cell type, SCFA concentration/availability, extracellular pH, time of exposure to SCFAs, their absorption and metabolism.

8. Final considerations

The potential use of SCFAs in prevention and/or as adjuvants to conventional chemotherapy regimens of colorectal cancer is currently well supported by the data in the literature. Since the concentrations of SCFAs in colon cancer seem to be low, the generation of SCFAs in the colon could be improved with specific nutritional diet such as the intake of fibers and appropriate probiotics like Propionibacteria. It can be speculated that these microorganisms are able to metabolize complex fibers and produce high levels of SCFAs which function *in vivo* as tumor suppressor agents, resulting in the elimination of CRC cells and hence in the inhibition of colon tumor progression.

In addition, as we reported previously that CatD protects CRC cells from acetate exposure, mediating a mitochondrial degradation process alternative to autophagy, suggesting that this could impact negatively the efficacy of acetate and probably of other therapeutics compounds. In this case, the use of CatD inhibitors in combination with strategies to increase acetate (or other SCFAs) concentrations in the colon, namely nutraceuticals, emerges as a novel strategy for prevention/ therapy of CRC.

9. Conflict of interest

The authors declare no conflict of interest.

10. Acknowledgement

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