HISTONE DEACETYLASES IN INFLAMMATORY BOWEL DISEASES - INFLUENCE ON THE ANTIMICROBIAL BARRIER

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Abbreviations

AMP	Antimicrobial Peptide
AP1	Activator protein-1
C	Control
CD	Crohn's disease
CMV	Cytomegalovirus
DMEM	Dulbecco's Modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EcN	*E. coli* Nissle 1917
ER	Endoplasmatic reticulum
FCS	Fetal calf serum
FFPE	Formalin fixed paraffin embedded
GALT	Gut associated lymphatic tissue
GIT	Gastrointestinal tract
hBD	Human beta defensin
HD	Human defensin
HDAC	Histone deacetylase
HDACi	HDAC inhibitor/HDAC inhibition
HE	Hematoxylin and eosin
Hgep	Human gingival epithelial cells
HNP	Human neutrophil peptide
IBD	Inflammatory bowel diseases
IFN	Interferon
IL	Interleukin
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
Myd88	Myeloid differentiation primary response gene
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD2	Nucleotide-binding oligomerization domain containing 2
NEAA	Non-Essential Amino Acids
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PRR	Pattern recognition receptor
PTM	Posttranslational modification

RPMI	Roswell Park Memorial Institute medium
RSPO	R-spondin
mRNA	Messenger ribonucleic acid
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
sPLA2	Phospholipase A2 group IIA
TCF	Transcription factor (T-cell specific, HMG-box)
TLR	Toll like receptor
TNF	Tumor necrosis factor
TSB	Tryptic soy broth
UC	Ulcerative colitis

Summary

The development and clinical behavior of the two major inflammatory bowel diseases (IBD) subgroups Crohn's disease (CD) and ulcerative colitis (UC), are determined by multiple underlying factors leading to an impaired antimicrobial barrier and chronic inflammation. Contributing environmental influences on the onset of the diseases such as the intestinal microbiota, antibiotics use, smoking, or nutrition have more and more entered the limelight of the field. The increasing incidence of IBD, the big variance in disease progression, and the discrepancy between monocygotic twins urgently impose the question how exactly environmental factors might impact on IBD risk and progression. The emerging field of epigenetics offers a mechanistic framework to dissect the present interplay between environment and genome in the context of IBD and will allow a better understanding of disease pathology.

Histone deacetylases (HDACs) are important epigenetic factors implicated in intestinal tissue homeostasis. They deacetylate histones but also a vast number of non-histone proteins, e.g. the transcription factor NF-κB, thereby impacting on transcriptional regulation on different levels. A role for HDACs in the epigenetically-mediated modulation of hBDs has been demonstrated in a number of studies, underscoring a potential involvement of HDACs in the β-defensin-related defects found in colonic IBD. In addition, inhibiting HDACs has been proposed as an IBD intervention, making more detailed studies on the role of HDACs in gut antimicrobial barrier function indespensible.

Firstly, in this work, a systematic overview of class I HDAC mRNA intestinal expression has been performed in a large cohort of IBD patients. First insights on the expression pattern on the protein level are also given.

Secondly, this study aimed to contribute to the small body of knowledge on HDAC-mediated epigenetic control of antimicrobial peptide (AMP) expression; focusing on human β-defensin 2 (hBD2). Herein, emphasis has been laid on the therapeutically relevant probiotic *E. coli* Nissle 1917 (EcN) as a potent hBD2-inducing factor in addition to the pro-inflammatory cytokine IL1β and the bacterial membrane component LPS. *In vitro* HDAC inhibition (HDACi) in colonic epithelial cells generated a strong, NF-κB-dependent enhancement of EcN- and LPS-induced hBD2 expression, but also of the pro-inflammatory cytokine IL8. For IL1β-induced hBD2, the observed augmentation seems to be mediated by additional transducing factors and conditions depending on which HDACs are inhibited. In the case of IL1β, the enhancing effect of HDACi on hBD2 could be evinced in a second colonic epithelial cell line.

In an effort to closer mimic the *in vivo* situation, an *ex vivo* human colonic biopsy culture has been established with almost identical culture conditions using IBD and healthy control

tissue. This was aimed to allow a comparison to the *in vitro* results, since it is of substantial importance to learn how a more complex, non-tumorous human tissue compound reacts to HDACi. Strikingly, in this context, an opposing impact of HDACi on EcN-stimulated hBD2 expression was observed. Inhibiting HDAC function using pan-HDAC inhibitors hindered hBD2 expression instead of enhancing it, but did not impede IL8 expression. Whether these contrary results could be due to the malignant nature of the *in vitro* cell lines was investigated using *ex vivo* treated human colorectal cancer tissue showing the same response as non-cancerous intestinal biopsies. In addition, first insights into the reactivity of a primary, non-transformed gingival epithelial cell line towards HDACi showed a comparable hBD2 response upon IL1β-stimulation as did the cancerous intestinal cell lines. Furthermore, *ex vivo* fold induction levels of hBD2 in CD patients have been reduced hinting towards a disturbance in hBD2 inducibility in response to EcN.

In this study, differential intestinal mRNA expression patterns have been unveiled for class I HDACs. Importantly, a strong regulatory influence of HDACs on the expression of hBD2 could be demonstrated. The simultaneous upregulation of IL8 in epithelial cells and the missing downregulation of the same in biopsy culture under HDACi, advises caution in considering HDACi as therapeutic in IBD. Furthermore, a dependency of the HDACi-induced hBD2 enhancement on NF-κB could be found. Utilizing different culture approaches, the obtained results argue for a cellular context-dependent modulation of the epigenetic regulation of hBD2 expression by HDACs. Overall, this work promotes the understanding of epigenetics as the conjoining integrative mechanism between genome and environment, bridging the way to answering many yet elusive questions in the pathogenesis of IBD.

Zusammenfassung

Der Pathogenese und der klinischen Präsentation der beiden chronisch entzündlichen Darmerkrankungen (CED), Morbus Crohn (MC) und Colitis Ulcerosa (CU) liegt eine Vielzahl bedingender Faktoren zu Grunde. Diese führen letztlich zu einer Beeinträchtigung der intestinalen, antimikrobiellen Abwehr und zu chronischer Entzündung. Diverse Umwelteinflüsse, wie zum Beispiel die intestinale Mikrobiota, die Einnahme von Antibiotika, Rauchen oder die Ernährung, sind maßgeblich an der Krankheitsentwicklung von CED beteiligt. Die stiegende Inzidenz von CED, die große Variabilität im Krankheitsverlauf und die Diskordanz zwischen eineiigen Zwillingen drängt die Frage danach auf, wie genau verschiedene Umweltfaktoren auf das Erkrankungsrisiko und den Krankheitsverlauf wirken. Das wachsende Forschungsfeld der Epigenetik kann dabei helfen diese Frage zu klären und aufzeigen, wie genau sich Umweltfaktoren auf die Interpretation der Gene im Kontext von CED auswirken und somit zu einem besseren Verständnis dieser Erkrankungen beitragen.

Histon-Deacetylasen (HDACs) sind wichtige epigenetisch wirkende Enzyme, die unter Anderem eine Rolle in der Aufrechterhaltung der intestinalen Homöostase spielen. Sie deacetylieren Histone, aber auch eine Reihe von nicht-Histon-Proteinen wie beispielsweise den Transkriptionsfaktor NF-κB, und beeinflussen so die Regulation der Transkription auf verschiedenen Ebenen. Eine Reihe von Studien beschreibt eine Rolle für HDACs in der epigenetisch-vermittelten Modulation der humanen β-Defensin-Expression. Sie unterstreichen somit eine mögliche Beteiligung dieser Enzyme an den Defekten in der β-Defensin-Expression, die bei Morbus Crohn des Dickdarms gefunden wurden. HDACs in ihrer Wirkweise zu inhibieren wurde zudem als mögliche therapeutische Intervention für CED vorgeschlagen. Vor diesem Hintergrund ist es unverzichtbar die genaue Rolle, welche HDACs für die Funktion der antimikrobiellen Darmbarriere spielen, detaillierter zu untersuchen.

In dieser Arbeit wurde daher zum einen ein systematischer Überblick über die mRNA-Expression von Klasse I HDACs in einer großen Kohorte von CED Patienten erstellt. Des Weiteren gibt sie Einblicke in die HDAC Expression auf Proteinebene.

Zum anderen sollte mit dieser Studie die epigenetische, HDAC-vermittelte Regulation der Expression antimikrobieller Peptide (AMPs) genauer untersucht werden; im Speziellen die des humanen β-Defensin 2 (hBD2), da dessen Induzierbarkeit bei MC des Dickdarms beinträchtigt ist. Besonderes Augenmerk wurde bei den Untersuchungen auf das therapeutisch relevante Probiotikum *E. coli* Nissle 1917 (EcN) gelegt, von dem gezeigt wurde, dass es hBD2 in Dickdarmepithelzellen induziert. Des Weiteren wurden das pro-inflammatorische Zytokin IL1β und der bakterielle Zellmembranbestandteil LPS als hBD2 stimulierende Faktoren mit einbezogen. Die Inhibition von HDACs *in vitro* führte in Kolon-Epithelzellen zu einer enormen,

NF-κB-anhängigen Verstärkung der EcN- und LPS-induzierten hBD2 Expression und der des pro-inflammatorischen Zytokins IL8. Bei IL1β-induziertem hBD2 scheint der Verstärkungseffekt durch zusätzliche Transduktionsmoleküle vermittelt zu werden, zudem abhängig davon, welche spezifischen HDACs inhibiert werden. Der durch HDACi verursachte Verstärkungseffekt konnte für IL1β-induziertes hBD2 in einer zweiten Kolon-Epithelzelllinie gezeigt werden.

Um die *in vivo* Situation genauer nachbilden zu können, wurde ein *ex vivo* Ansatz etabliert, in dem Dickdarmbiopsien von Gesunden sowie von CED-Patienten unter nahezu identischen Kultur-Bedingungen behandelt werden können, um einen möglichst guten Vergleich zu den *in vitro* Ergebnissen ziehen zu können. Diese *ex vivo* Kultur ermöglicht die Untersuchung der Effekte von HDACi in einem komplexeren, nicht-tumorösen menschlichen Gewebeverband. Bemerkenswert ist, dass in diesem Kontext ein völlig gegensätzlicher Effekt von HDACi auf die EcN-vermittelte hBD2 Induktion beobachtet wurde. Bei Verwendung von Pan-HDACi wurde die Expression von hBD2 vollständig verhindert anstatt sie zu verstärken. Jedoch wurde die IL8-Expression nicht reduziert. Ob diese, zu den *in vitro* Ergebnissen gegensätzlichen Effekte, auf den tumorösen Charakter der Zelllinien zurückzuführen sind, wurde mit Hilfe der Verwendung kolorektaler Tumorbiopsien untersucht. Diese zeigten jedoch dieselbe Reaktion, wie die nicht-malignen Kolonbiospien. Zudem wurde in einem ersten Pilotexperiment mit nicht-transformierten primären humanen Mundschleimhaut-Epithelzellen, eine ähnliche Verstärkung der IL1β-induzierten hBD2-Expression beobachtet. Des Weiteren wurde in den *ex vivo* hBD2-Stimulationsexperimenten mit EcN beobachtet, dass Proben von MC-Patienten niedrigere Induktionslevel für hBD2 aufwiesen, was eine Störung der hBD2-Induzierbarkeit in MC Patienten bestätigt.

In dieser Arbeit wurden unterschiedliche mRNA-Expressionsmuster von Klasse I HDACs zwischen Gesunden und Patienten mit CED aufgedeckt. Außerdem wurde ein starker regulatorischer Einfluss von HDACs auf die Expression von hBD2 gezeigt. Die simultane Hochregulation von IL8 *in vitro* und fehlende Herunterregulation desselben *ex vivo* verlangt nach Vorsicht bei der Evaluation von HDACi als Therapeutikum in CED. Darüber hinaus wurde in Teilen eine NF-κB-Abhängigkeit für die Verstärkung der hBD2 Induktion unter HDACi gezeigt. Die Ergebnisse aus dem Vergleich unterschiedlicher Kulturansätze erlaubt die Spekulation, dass die HDAC-vermittelte epigenetische Regulation von hBD2, Zell-Kontext-anhängig zu sein scheint. Zusammenfassend fördert diese Studie das Verständnis von integrativen, epigenetischen Mechanismen zwischen Umwelt und Genom und die Beantwortung vieler noch ungelöster Fragen in Bezug auf die Pathogenese von CED.

1 Introduction

1.1. Epigenetics – bridging genes and environment

There is more to our story than our genes can tell. Our genes are not solely responsible for who and how we are. They also do not on their own define our susceptibility to disease and whether or not and when we might sicken. If the nucleotide sequences of our genes, of our DNA, are the words our story is written in, something else resembling grammar and punctuation rules must exist to enable differential interpretation of those plain words. Those rules convey the different and individual meanings in particular situations and contexts by ensuring the right words are used and read with the right emphasis and accentuation. This becomes especially obvious when we look at monozygotic twins. They are natural clones in respect to their DNA sequence- their words are the same. Yet monozygotic twins can still be quite different individuals. They do not exactly look alike and they can be discordant in respect to the diseases they might develop. Similar observations have been made in cloned animals. Despite carrying identical donor DNA, their phenotypes differ from one another and from their donor (Rideout III, 2001). Classic genetics does not fully explain these phenomena. So, there must be more to the story.

In 1942, Conrad H. Waddington coined the term *epigenetics* for studies on the mechanisms and processes at work during development from genotype to phenotype (Waddington 1942). Since then, the definition of the term epigenetics has changed and quite substantial research has been undertaken in the field. Especially within the last two decades, it has picked up tremendous speed. Even though, a great deal of exciting discoveries is still to be made, the current knowledge carves the following definition. Epigenetics describes the study of mechanisms leading to mitotically but also potentially meiotically heritable changes in gene expression and associated changes in phenotypes. Such changes do not include alterations in the primary DNA sequence (Berger et al., 2009; Dupont et al., 2009; Portela and Esteller, 2010). Epigenetics describes how chemical marks are assigned to DNA and chromatin, but also to a wide range of non-histone proteins, and how these impact on gene transcription and the functionality of the genome in general (Esteller, 2008). Altogether, genome-wide molecular modifications that are functionally relevant make up the epigenome of an organism (Fofanova et al., 2016).

Epigenetic mechanisms have been shown to act in a developing organism and during cellular differentiation (Li, 2002), when cells start to run different sets of genes departing from the toti- or pluripotent state. While those different sets of genes originate from the same genome, epigenetic mechanisms silence or activate them. This reprograming of the genome in

embryogenesis and for tissue-specific gene expression leads to different cellular functionalities and identities (Boland et al., 2014; Salts and Meshorer, 2016). Stability of differentiated cellular phenotypes is supported by an 'epigenetic memory' that is passed on through cell divisions (Nashun et al., 2015; Shipony et al., 2014). Thereby, epigenetics helps answering questions like, how stem cells differentiate but also how an epithelial cell gives rise to new epithelial daughter cells.

Besides the modulation of cell type-specific transcriptional profiles, epigenetics also plays an important role in X-chromosome inactivation (Heard et al., 2004), genomic imprinting (Ferguson-Smith, 2011), or chromatin-organization by regulating histone modifications (Gardner et al., 2011; Henikoff and Shilatifard, 2011). Unsurprisingly, the importance of the correct function of epigenetic features becomes obvious in many diseases. The best studied example is cancer, where globally occurring perturbations in DNA methylation and histone marks but also changes in the expression profiles of chromatin remodeling enzymes have been reported, leaving cancer cells with an extensively disturbed epigenome (Esteller, 2007; Portela and Esteller, 2010). Among other conditions, autoimmune disorders (Javierre et al., 2010) and inflammatory conditions, such as inflammatory bowel diseases (IBD) have been associated with changes epigenetic landscaping (Bayarsaihan, 2011; Scarpa and Stylianou, 2012; Shanmugam and Sethi, 2013).

A common feature of these diseases is the epigenetic contribution to disease onset, penetrance and progression (Portela and Esteller, 2010; Scarpa and Stylianou, 2012). During an individual's lifetime, exogenous impact on epigenetic variation naturally increases, as epigenetic modifications occur as cellular responses to environmental stimuli. This epigenetic adaptation to an individual's environment can be witnessed very impressively in monozygotic twins whose epigenetic drift increases by age and life style differences (Esteller, 2008). Most strikingly, diseases with an underlying genetic susceptibility, such as IBD, have shown to be strongly influenced by environmental cues via epigenetic mechanisms which impact on disease susceptibility and presentation (Esteller, 2008; Scarpa and Stylianou, 2012).

Taken together, epigenetics may be considered as a bridge between genome and environment - internal as well as external, that can help explain complex phenotypes, phenotypic discordance in monozygotic twins, disease variabilities and sporadic incidence (Petronis, 2010; Scarpa and Stylianou, 2012).

1.1.1. Epigenetic mechanisms and their molecular tools

There are several distinct epigenetic mechanisms that integrate intrinsic signals and also allow the embedding of external environmental cues into genetic interpretation. They operate interconnectedly on diverse levels and can conceptually be grouped into the following

categories: DNA methylation, histone modification, nucleosome positioning and RNA interference.

1.1.1.1. DNA methylation

DNA methylation is an intensely studied epigenetic mechanism regulating gene transcription. It describes the covalent addition of methyl groups to cytosines (Hotchkiss, 1948; Sinsheimer, 1955) in the relatively sparsely occurring (only about 1% in the genome) cytosine-guanine dinucleotides (CpG) but also of cytosines in non-CpG sequences (Ramsahoye et al., 2000; Ratel et al., 2006; Woodcock et al., 1987). Those concentrate in certain areas of the genome called "CpG islands". These islands are defined as sequences of 200 bases of DNA containing more than 50% CpG dinucleotides and an observed-to-expected CpG ratio greater than 0.6 (Portela and Esteller, 2010). CpG islands are mainly found near transcriptional start sites and within promotor regions of genes (Saxonov et al., 2006). The borders of these islands are termed "CpG shores", contain less CpG dinucleotides and make up about 2 kb upstream of CpG islands. Both, methylation of CpG islands as well as CpG shores are associated with gene silencing or transcriptional inactivation either e.g. via preclusion of transcription factor binding or the recruitment of histone modifiers and chromatin-remodeling complexes (Portela and Esteller, 2010). Tissue-specific methylation patterns seem to mostly occur in CpG shores (Doi et al., 2009). The case of DNA methylation of gene bodies, on the other side, has been found to facilitate transcription and correlates positively with gene expression (Hellman and Chess, 2007; Laurent et al., 2010).

The addition of methyl groups from S-adenosyl methionine to DNA is catalyzed by the family of DNA methyltransferases (DNMTs), which in mammals consists of 5 known members- DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. However, only DNMT1, 2a and 3b possess methyltransferase activity (Jeltsch, 2006). The *de novo* DNMTs (DNMT3a and 3b) are abundant in embryonic stem cells and seem responsible for the establishment of the DNA methylation pattern during embryonic development, but are reduced in differentiated cells. The maintenance DNMT1 has a strong preference for hemi-methylated DNA and is especially active during DNA replication and cell division, but also has been shown to have *de novo* activity (Portela and Esteller, 2010). Furthermore, DNMTs are coordinated and controlled on several levels, via different pathways and a great number of posttranslational modifications (PTM) (Denis et al., 2011).

A big question of the field is how and when the DNA methylation machinery is guided to its target sequences. Many reports suggest interactions of DNMTs with other epigenetic marks and factors, including non-coding RNAs (Jeong et al., 2009; Portela and Esteller, 2010; Zhao et al., 2009). Moreover, it is under discussion whether DNA methylation rather has to be considered a secondary event to gene silencing via other mechanisms, leading to stably silenced genes (Bird,

2002; Clark and Melki, 2002). DNA methylation processes have been found to be crucial in regulating cellular differentiation, organ development, and cell type-specific gene expression. DNA Methylation is a key player, for example, in genetic imprinting (Ferguson-Smith, 2011; Kacem and Feil, 2009) and for X chromosome inactivation in females (Reik and Lewis, 2005). Furthermore, aberrations in DNA methylation resulting in decreased DNA methylation or hypermethylation of certain gene promotors that are normally unmethylated have been associated with a range of diseases (Robertson, 2005) such as IBD and cancer (Esteller, 2007; Heyn et al., 2016; Karatzas et al., 2014; Sandoval and Esteller, 2012; Scarpa and Stylianou, 2012; Ventham et al., 2013).

1.1.1.2. Histone modifications

Histones are alkaline predominantly globular proteins present in all eukaryotic cell nuclei. There they act as scaffolding for chromatin, organizing DNA into its basic structural units called nucleosomes. These are made up of two of each of the four core histones (H2A, H2B, H3 and H4) forming an octamer which is wrapped round by a 147 base pair (bp) containing DNA segment. The unstructured N terminal tails of the core histones which extend from the surface of the nucleosomes, are decorated with a great number of different PTMs, including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and more than 50 others (Tan et al., 2011) but also the globular domains can be modified (Kouzarides, 2007). Histone modifications play an important role in transcriptional regulation but also in many other cellular processes such as DNA repair (Huertas et al., 2009), the regulation of alternative splicing (Luco et al., 2010), or chromatin condensation (Kouzarides, 2007). In general, histone PTMs lead to changes in chromatin structure via alterations in the electrostatic charge of the histone proteins thereby affecting DNA accessibility but also acting as recruitment platforms for transcription factors (TFs) and transcriptional co-regulators (Gardner et al., 2011). A wide range different patterns of histone modifications can occur at different sites simultaneously that have been shown to be potentially interdependent and in constant cross-talk (Rando, 2012; Suganuma and Workman, 2011; Wang et al., 2008). Whether or not different patterns of histone modifications constitute a "histone code" that might be fully capable of predicting downstream biological effects, as does the genetic code, is a highly and controversially debated issue (Henikoff and Shilatifard, 2011; Jenuwein and Allis, 2001; Rando, 2012).

Condensed chromatin, where DNA is tightly wrapped around nucleosomes and packed into a higher order-chromatin structure, is termed heterochromatin and transcriptionally inactive. Euchromatin, on the other side, is more relaxed and open and can be actively transcribed. Low levels of histone acetylation and methylation of specific residues are, for example, associated with heterochromatin whereas high levels of trimethylation of other sites and highly acetylated histone tails are characteristic for euchromatin (Li et al., 2007; Portela and Esteller, 2010). Additionally, different levels of histone modifications have been found to be

predictive for gene expression (Karlić et al., 2010). Histone PTMs do furthermore interact with other epigenetic factors, such as DNMTs, directing de novo DNA methylation to specific sites (Ooi et al., 2007) – and vice versa, DNA methylation can orchestrate histone modifications to specific sequences (Fuks et al., 2003).

Overall, multiple histone-modifying enzyme families have been described that are responsible for the establishment or removal of their designated histone PTMs (Bhaumik et al., 2007; Dillon et al., 2005; Kouzarides, 2007; Marmorstein and Roth, 2001; Selvi et al., 2010; Seto and Yoshida, 2014). Interestingly, while histone methyltransferases, demethylases and kinases are relatively specific to certain histone subunits and residues (Chi et al., 2010; Kouzarides, 2007), histone acetyltransferases (HATs) or histone deacetylases (HDACs) are rather unspecific targeting several residues (Portela and Esteller, 2010). However, histone substrate specificity of HAT enzymes is being discussed (Marmorstein and Zhou, 2014) . Taken together, histone modifications represent a diverse epigenetic toolbox allowing the complex and dynamic changes in the landscape of DNA accessibility necessary to enable cellular reactivity to internal as well as external environmental cues (Klemm et al., 2019).

Histone deacetylation and inhibitors of histone deacetylases

An evolutionary very old protein modification is the acetylation of lysine residues as it has even been described in bacteria (Gardner et al., 2006). In 1969, histones have been the first substrates to be described for lysine deacetylases (KDACs) (Inoue and Fujimoto, 1969), the enzymes responsible for removing acetyl groups from proteins, leading to the term histone deacetylases (HDACs) in eukaryotes (Yang and Seto, 2008) even though a large number of non-histone protein targets has been identified in the meantime (Seto and Yoshida 2014). Histone or protein acetylation at the ε-amino group of lysine residues is mediated by histone/lysine acetyltransferase enzymes (HATs) (Marmorstein and Zhou, 2014) and can be reversed by HDACs/KDACs. For convenience, as from now the term HDAC will be used when talking about histone/lysine deacetylases. HDACs are amidohydrolases and comprise an ancient enzyme family that exists in mammals, plants, fungi and bacteria. The first mammalian HDAC enzyme, HDAC1, was isolated in 1996 by Taunton and colleagues, and after that the field of histone deacetylation has rapidly expanded (Taunton et al., 1996). In humans, HDACs are grouped into four classes (class I, II, III, also called sirtuins and IV), based on their sequence homology to corresponding enzymes in yeast and their sequence similarities among each other. Homologues to reduced potassium dependency 3 (Rpd3) in yeast constitute class I HDACs (HDAC1, 2, 3 and 8), class II HDACs (class IIa: HDAC4, 5, 7 and 9; class IIb: HDAC6 and 10) are homologues to yeast histone deacetylase-A 1 (hda1), class III (SIRT1-7) corresponds to yeast silent regulator 2 (Sir2) and the single HDAC constituting class IV (HDAC11) is much less homologous to either Rpd2 or hda1 enzymes (Yang and Seto, 2008). Class I, II and IV HDACs are also called classical

HDACs and their enzymatic activity is dependent on a Zn^{2+} ion as a cofactor, whereas Sirtuins are NAD^{+}-dependent (de Ruijter et al., 2003). Concerning their subcellular localization, class I HDACs have long been described as located in the nucleus, but can also be found in the cytoplasm and certain organelles of the cell. Class II HDACs have all to some extend been described as located to the cytoplasm, but also stimulus-dependent shuttling between nucleus and cytoplasm has been observed (Chawla et al., 2003).

As posttranslational modifiers of histones but also of a vast number of non-histone protein substrates, such as transcription factors, signaling molecules, DNA binding and repair molecules, or chaperone proteins, HDACs function as regulators and modulators of a large number of vital cellular processes, most notably, cell cycle and transcriptional regulation (Chen et al., 2002; Glozak et al., 2005; Luo et al., 2000; Seto and Yoshida, 2014; Yang and Seto, 2003). More than 3600 acetylation sites have been found on more than 1700 proteins, many of which have been shown to be affected by HDACs (Choudhary et al., 2009). Via deacetylating histones, HDACs unmask the positive electrostatic charges of the core histones that have been neutralized by the acetyl-groups, leading to an increased affinity of the negatively charged backbone of the DNA to the histones and thereby to more condensed chromatin. Consequently, transcription is sterically hindered (Figure 1). Therefore, HDACs are referred to as epigenetically acting transcriptional (co-)repressors.

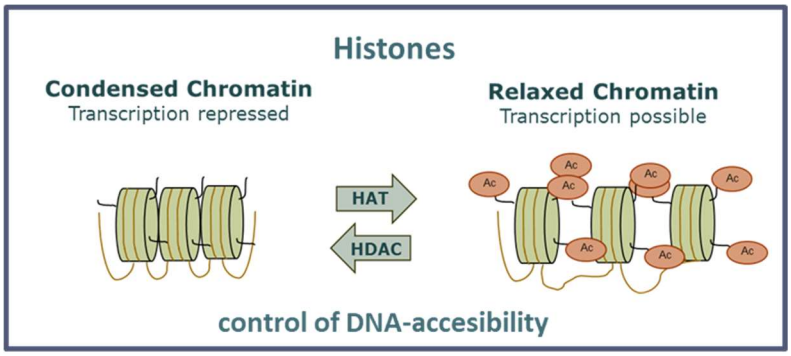

Figure 1: Schematic of histone (de)acetylation and the effects on chromatin structure

HDAC enzymes themselves are regulated by several mechanisms at transcriptional, translational and posttranslational levels (Segré and Chiocca, 2011), but also via subcellular localization (Schlumm et al., 2013) and protein-protein interactions (Zhang et al., 2005). Furthermore, class I HDACs have been found to be part of and form large multi-subunit protein complexes to exert their transcriptional regulation. Class I HDACs have been shown to be integral parts of corepressor complexes. HDAC1 and 2, when isolated show low enzymatic activity, interact with each other and are found together as the catalytic core in the Sin3, the nucleosome remodeling deacetylase (NuRD), and the co-repressor of REST (repressor element 1 silencing transcription factor (CoREST)) complexes (Ahringer, 2000; Ayer, 1999; Denslow and Wade, 2007), whereas

HDAC3 has been found as the only class I HDAC as a component of endogenous nuclear receptor co-repressor (NCoR)/ silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) complexes (Kao et al., 2000; Li et al., 2000). Interaction of HDAC3 with the NCoR/SMRT complex has been shown to be necessary for HDAC3 catalytic activity (Emmett and Lazar, 2018; Guenther et al., 2001).

A large number of naturally occurring (Bassett and Barnett, 2014) and synthetic compounds capable of inhibiting HDAC function have been characterized and can be categorized upon their chemical properties as hydroxamic acid derivates (e.g. Trichostatin A (TSA) or SAHA), benzamides (e.g. MS-275), short-chain fatty acids (e.g. butyrate and valproate) cyclic peptides (e.g. depsipeptide) (Miller et al., 2003; Minucci and Pelicci, 2006). Most histone deacetylase inhibitors (HDACi) bind to the Zn^{2+} ion in the catalytic pocket of HDACs and have been shown to induce cell cycle arrest and differentiation in transformed cells (Johnstone, 2002). Besides the investigation of pan-HDACi, such as TSA or SAHA, more selective inhibitors are being developed (Bantscheff et al., 2011; Olzscha et al., 2016). This is important, since inhibiting HDAC function does not only result in an overall, "global" reduction of the deacetylation status of core histones of nucleosomes which affects chromatin structure in general, but also has an effect on non-histone protein acetylation (Olzscha et al., 2016; Seto and Yoshida, 2014). Since HDACs display aberrant activity and are overexpressed in many cancers, HDACi are promising anticancer drugs (Li and Seto, 2016; Ropero and Esteller, 2007), but are also being discussed for the treatment of many other conditions related to epigenetic abnormalities including inflammatory diseases such as IBD (Felice et al., 2014; Glauben and Siegmund, 2011).

1.1.1.3. Nucleosomes and chromatin remodeling

The positioning of nucleosomes and chromatin structure is dynamically regulated and continuously remodeled by diverse mechanisms such as the aforementioned histone modification and the process of nucleosome positioning. The packaging of DNA into nucleosomes presents a barrier for the transcription machinery and is therefore a crucial mean of epigenetic regulation of gene expression (Längst and Manelyte, 2015; Li et al., 2007). Specialized multiprotein chromatin remodeling complexes are diverse and highly abundant in the cell and ATP-dependently slide, rotate, or eject nucleosomes around and from gene regulatory sequences (Rippe et al., 2007). They have also been shown to exchange canonical histones with histone variants that can alter the properties of the affected nucleosome and chromatin (Papamichos-Chronakis et al., 2011). Chromatin remodelers recognize different histone modifications (Wysocka et al., 2006), DNA sequences or RNA signals (Yoo et al., 2009) which target them to specific genes. The known chromatin remodeling complexes can be grouped into four enzyme families (SWI/SNF, ISWI,CHD and INO80) that hydrolyze ATP to induce protein conformational changes in order to regulate nucleosome positioning as an epigenetic mechanism of gene expression regulation (Längst and Manelyte, 2015).

1.1.1.4. microRNAs

Lastly, non-coding RNAs (ncRNAs) play an important role as epigenetic regulators of gene expression. They emanate from intergenic transcription and comprise for example small non-coding RNAs, mid-size RNAs, and long non-coding RNAs (Cech and Steitz, 2014). Small RNAs loom large at RNA degradation, translational repression, chromatin modification and the regulation of gene expression via RNA interference (RNAi) (Holoch and Moazed, 2015). MicroRNAs (miRNAs) are about 22 nucleotide long RNAs that post-transcriptionally downregulate the translation of mRNAs (Bartel, 2018) by targeting the partially complementary 3' untranslated region of mRNAs leading to gene silencing. Hundreds of miRNAs have been identified in the human genome that seem to target and regulate the majority of the human genes (Friedman et al., 2009). Furthermore, there is substantial crosstalk between ncRNAs and the afore described epigenetic mechanisms of DNA methylation, histone modification and chromatin formation (Mochizuki et al., 2002; Parodi et al., 2016; Taguchi, 2015; Volpe et al., 2002). Unsurprisingly, the disruption of non-coding RNAs is relevant in many diseases (Esteller, 2011).

1.1.2. Environmental influences on the intestine

Throughout our whole life, we are exposed to a multitude of environmental stimuli influencing the homeostasis of our cells and our whole organism. Besides the skin, the gastrointestinal tract also represents an organ system with a very large surface of about 32 m^2 (Helander and Fändriks, 2014) that is under constant internal and external influences. Right after birth it is gradually being colonized by an enormous number of microorganisms (Dominguez-Bello et al., 2010; Huang et al., 2013), while it is simultaneously exposed to a myriad of nutrients, breakdown products thereof and bacterial metabolites, but also to potentially toxic compounds such as pharmaceuticals. Infections but also stress and other psychological factors also impact on the balanced physiology of the intestinal surface (Legaki and Gazouli, 2016). Especially, the single cell layer of the intestinal epithelium is in constant, close contact to the external environment (Zilbauer and Kraiczy, 2017). The epigenetic toolbox represents an important mean enabling intestinal microbiota and other environmental cues to leave their signature on epithelial gene expression (Alenghat et al., 2013; Dai and Wang, 2014; Kelly et al., 2018) which is why epigenetic mechanisms are increasingly recognized as key contributors of pathologies of the intestines such as IBD (Fofanova et al., 2016; Legaki and Gazouli, 2016; Zilbauer and Kraiczy, 2017).

1.2. The human gastrointestinal tract

The digestive or gastrointestinal (GI) tract comprises an interconnected organ system of about nine meters in length (Hounnou et al., 2002) consisting of mouth, esophagus, stomach, followed by the small and large intestines or small and large bowels, and rectum and anus. Its purpose is to digest food that has been taken in, absorb nutrients and energy and excrete the solid leftover waste as feces. An immense number of microbes is constantly living inside the lower intestinal tract (small and large intestines) that are summarized as the commensal intestinal microbiota. This symbiotic relationship between host and resident microbiota starts right after birth (Dominguez-Bello et al., 2010) and affects a multitude of processes in the host starting from a healthy development of the Gi tract, the development of the immune system and immune tolerance towards microbiota but also food-derived proteins (Chistiakov et al., 2015; Hooper et al., 2012), up to metabolism and the shaping of the epigenetic landscape (Kelly et al., 2018). Needless to say, however, microorganisms always also represent a potential threat that needs to be balanced and held under tight control by the host immune system. Owed to its task to digest food and absorb nutrients, the small intestine represents the largest part of the GI tract in terms of surface area (Helander and Fändriks, 2014). In the subjacent following large bowel, water is reabsorbed and remaining feces are stored before defecation. The general histology of the GI tract comprises four concentric layers combining into the gastrointestinal wall (Paxton et al., 2003). The outermost layer consists of connective tissue and is called *serosa* or *adventitia*. Subjacent lies a layer of smooth muscles, the *muscularis propria*. This is followed by the submucosa, a layer of loose connective tissue traversed by large blood vessels, lymphatics and nerves and serves to support the mucosa. The innermost layer of the GI tract, the *tunica mucosa*, is composed of an underlying muscle layer (*lamina muscularis mucosae*), followed by the *lamina propria mucosae*, a layer of loose connective tissue containing nerves and small blood and lymph vessels, and the top luminal layer of epithelial cells, the *lamina epithelialis mucosae.* The single cell layer of the intestinal epithelium is responsible for nutrition uptake and for building up a physical and biological border to restrict harmful substances and microorganisms from entering. About every four days, it is completely renewing itself via cell division, differentiation and migration and shedding of cells.

1.2.1. The small and large intestine

Between the stomach and the large intestine lies the tubular structure of the small intestine. It begins with the duodenum followed by the jejunum and ends with the ileum and the ileocecal valve, the sphincter muscle separating the terminal ileum from the colon. Adapted to its function of digestion and nutrition and mineral uptake, the mucosal surface area is strongly enlarged to

approximately 30 m^2 (Helander and Fändriks, 2014). This is realized via in- and evaginations of the mucosa called crypts of Lieberkühn (*Glandulae intestinalis*) and villi, respectively, and even more increased by the protrusions of the cellular membranes of the enterocytes called microvilli. Partly digested food enters the duodenum coming from the stomach. The duodenum further receives pancreatic and hepatic juice to promote digestion, breaking down proteins and other macromolecules and emulsifying fats. Along the way to the terminal ileum, epithelial enterocytes are responsible for the resorption of the products of digestion. With increasing distance from the stomach, specialized cells residing at the bottom of the crypts, so called Paneth cells, are increasing in number (Sekirov et al., 2010). They produce and secrete different antimicrobial compounds making them important players in antimicrobial host-defense and immunity.

The demarcation of the small intestine from the large intestine is provided by the ileocecal valve. The large intestine, also referred to as colon, is about 1.5 m long (Hounnou et al., 2002) and has a much smaller mucosal surface area of about 2 m^2 than the small intestine, also owed to the fact, that while there are crypts there are no villi in the colon. There are also no Paneth cells in the colonic mucosa, but the number of goblet cells for the production of a thick protective mucus layer covering the epithelium, is much higher than in the small intestine. This mucus layer serves as a physical barrier against the invasion of microbiota into the mucosa and also stores antimicrobial peptides (Dupont et al., 2014). The large intestine can be subdivided into the cecum with the attached appendix, which is a small finger-like pouch, that possibly serves as a storage place for normal, healthy gut bacteria and has a potential immune function in diarrheal illness (Laurin et al., 2011). The cecum is then followed by the ascending, transverse, descending and sigmoid colon, rectum, and anus.

The colon mainly functions in reabsorbing salt and water from the chyme and solid waste of the digestive process. It furthermore represents a reservoir for the highest density of bacteria and the bacteria-assisted fermentation of unused carbohydrates and protein and indigestible dietary fiber (Blaut and Clavel, 2007; Edwards and Rowland, 1992). The large number of bacteria residing within the colonic lumen is also reflected by the fact that about 60% of the fecal dry mass are made up by bacteria (Guarner and Malagelada, 2003).

1.2.2. Microbiota of the intestines

The number of microbial cells in the gut has been reported to be higher than the total amount of human cells in the body of the host (Sender et al., 2016) and comprising a biomass of up to 2 kg. The intestinal microbiota represent an extremely diverse ecosystem (Eckburg et al., 2005), which supports an optimal nourishment of the host, but is also essential for the development of the GI tract and the enteric immune system (Hooper et al., 2012; Huang et al., 2013). It can

furthermore, mainly via microbial metabolites, even influence remote organs, such as the cardiovascular system or the brain, and adipose tissue (Koeth et al., 2013; Lee et al., 2006; Schroeder and Bäckhed, 2016; Stilling et al., 2014). On the other hand, an individual's genetic background, age, lifestyle, usage of antibiotics, geographical location and especially dietary habits influence on the dynamic and diverse composition of the intestinal microbiome of each individual (Voreades et al., 2014; Yatsunenko et al., 2012). Furthermore, niche competition between different bacterial species for nutrients and habitat but has an impact, also in terms of protection against potentially pathogenic bacteria (Fukuda et al., 2011). However, despite of all these strong influencers, the individual microbiota in adults is relatively stable and changes occur rather in the abundances of different species and not so much in species composition itself (Lozupone et al., 2012; Rajilić-Stojanović et al., 2013).

The microbial density gradually increases from the proximal to the distal segments of the intestine and the local microbial composition differs among the different segments, mostly due to distinct physiologic conditions within the compartments (Human Microbiome Project Consortium, 2012). The passage through the acidic environment of the stomach is deadly for most of the ingested bacteria and therefore the stomach and the following duodenum are barely populated. The highest microbial density and diversity is found in the colon. There are five prevalent phyla present in the intestine- Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia, however single species can vary considerably (Forster et al., 2019; Schroeder and Bäckhed, 2016). For the characterization of the microbiota of the different intestinal segments, and depending on whether luminal or mucus-adherent bacteria are subject to the investigation, different sampling procedures are required, making it generally difficult to create a detailed description of a healthy microbiota. In general, the luminal content of the distal colon is easily accessible via stool sampling, whereas small intestinal microbiota has to be accessed via more invasive procedures such as colonoscopy and biopsy sampling. Intestinal tissue data revealed that the mucosal microbiota of the ileum is especially abundant in Bacteroidetes and Firmicutes (Bacillus and Clostridiales species), as opposed to the colon where Bacillus species are strongly diminished and mainly Bacteroidetes and Firmicutes/Clostridiales are prevalent (Peterson et al., 2008). Alterations in composition of the gut microbiota are associated with a range of diseases affecting all kinds of different organs (Schroeder and Bäckhed, 2016; Sekirov et al., 2010). Conditions as diverse as obesity, type 2 diabetes, arteriosclerosis, autism spectrum disorders and especially IBD have been linked to dysbiosis, an imbalance or maladaptation of the intestinal microbiome (Bäckhed et al., 2004; Koeth et al., 2013; Matsuoka and Kanai, 2015; Morgan et al., 2012; Perry et al., 2016; Schroeder and Bäckhed, 2016; Wang et al., 2012). Study design, sampling method and analysis strategy are however pivotal factors for the reliability of the deduced associations between the microbial effector and the disease (Hanage, 2014; Walters et al., 2014). The reported associations for IBD, where

mainly a generally reduced diversity accompanied with a reduction in Firmicutes and an elevation of Proteobacteria and Actinobacteria were found, have however been consistent across several studies together with a high effect size (Frank et al., 2007; Gophna et al., 2006; Papa et al., 2012; Peterson et al., 2008; Walker et al., 2011; Walters et al., 2014).

1.2.3. Immunity – the intestinal barrier

While the immense surface of the intestine constitutes an optimization for the resorption of nutrients, at the same time, it represents an interface for microbial-mucosal contact and a potential attack surface. Therefore, an arsenal of defense mechanisms is needed for the protection of the host against invasion by microorganisms but also to shape the composition of the microbiota. An effective intestinal antimicrobial barrier consists of two basic defense lines – the physical or mechanical barrier composed of the epithelium and the overlaying mucus, and the second defense line, a biochemical barrier, involving antimicrobial peptides (AMPs) and the gut associated lymphatic tissue (GALT).

1.2.3.1. Physical barrier – epithelium and mucus

A single cell layer, the epithelium, is lining the intestines towards/from the lumen. Adjacent epithelial cells are connected via tight junctions, forming a continuous, sealed cellular barrier (Peterson and Artis, 2014). Specialized epithelial cells, the mucus-producing goblet cells, secrete highly glycosylated mucins into the lumen. The main component is mucin 2 (Rousseau et al., 2004), which is also essential for the two-layered organization of the mucus in the colon (Johansson et al., 2011).

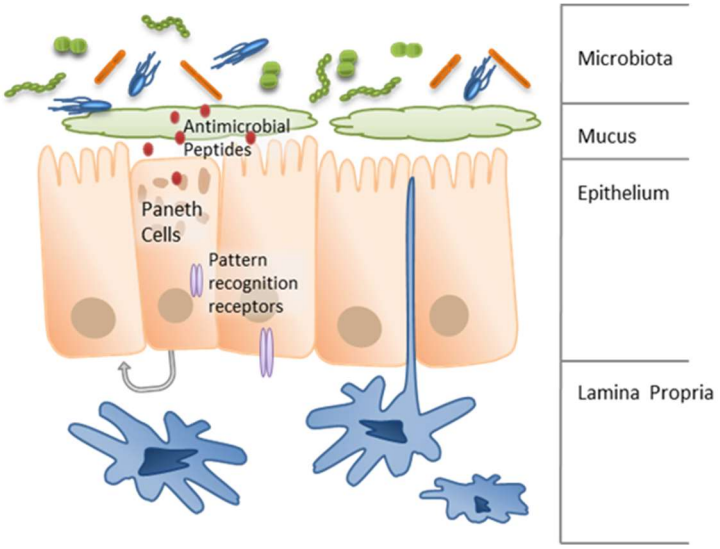

Figure 2: Composition of the small intestinal barrier
Figure was kindly provided by Dr. Lioba Courth

While in the small intestine the mucous layer is only loosely attached to the epithelium (Figure 2), and can be transported onward by the peristalsis of the gut, mucus is double-layered in the large intestine. The inner layer is rather compact and impenetrable for bacteria. The outer layer is assumed to emerge from the inner layer through degradation and is only loosely layered above building the habitat for commensal bacteria (Johansson et al., 2011). The tight structure of the epithelium together with the mucus layer, which furthermore serves as a reservoir for antimicrobial peptides secreted from epithelial cells, represent a strong mechanical barrier against bacterial invasion (Johansson et al., 2013; McGuckin et al., 2011; Sansonetti, 2004).

1.2.3.2. Biological barrier – Innate immune factors of the intestines

The biological component of the innate intestinal antimicrobial barrier is provided by immune cells like dendritic cells and macrophages and the production and secretion of an arsenal of antimicrobial peptides by the intestinal epithelium. Cells of the adaptive immune system present in the lamina propria of intestine will be discussed in Chapter 1.2.3.3 Adaptive immunity in the intestine.

Antimicrobial peptides

Many epithelial cells, immune cells and a number of specialized cells in the human body produce diverse small AMPs (Ostaff et al., 2013) with an amphipathic character, containing cationic and hydrophobic residues (Cederlund et al., 2011; Zasloff, 2002). These peptides are conserved from plants to humans and present in all eukaryotes (Zasloff, 2002). Concerning their modes of antimicrobial action, different mechanisms have been reported for different types of AMPs. Many AMPs target structures of the bacterial cell wall, as e.g. lysozyme enzymatically degrades bacterial peptidoglycan (Ganz, 2003), or the secreted phospholipase sPLA2, which invades the cell wall and also hydrolyses phospholipids of cellular membranes (Koprivnjak and Peschel, 2011). Others build up pores in the bacterial surface disrupting the membrane potential (Hill et al., 1991; Kagan et al., 1990; Mukherjee et al., 2014). Human α-defensin 6 (HD6) has been reported to form nanonets *in vivo,* thereby entrapping bacteria (Chu et al., 2012). Furthermore, immuno-chemotactic properties have been shown for AMPs, e.g. human α-defensin 5 (HD5) or human β-defensin 2 (hBD2) (Grigat et al., 2007; Röhrl et al., 2010).

The Paneth cell of the small intestine is a specialized secretory epithelial cell residing in the stem cell niche at the bottom of the crypts, providing large amounts of AMPs, thereby contributing on a big scale to the intestinal antimicrobial barrier. It expresses lysozyme, the lectin Reg-3γ, the secreted phospholipase sPLA2 and the α-defensins HD5 and HD6 (Bevins and Salzman, 2011), which are among the most abundant AMPs in the small intestine(Kübler et al., 2009). Further AMPs, such as the cathelecidin LL-37 or β-defensins, are produced by other epithelial cells of the intestine (Pazgier et al., 2006). An important human group of AMPs are defensins. Structural characteristics of defensins are richness in β-sheets and the occurrence of

three disulfide bridges between six cysteine residues (Ganz, 2003; White et al., 1995). Depending on the arrangement of these disulfide bridges, defensins are classified into α-, β-, or θ-defensins, the latter of which, the cyclic defensins, are only found in Rhesus monkeys (Ganz, 2003; Mukherjee et al., 2008). Interestingly, most genes encoding human defensins are clustered on chromosome 8p23 (Harder et al., 1997a; Linzmeier et al., 1999; Sparkes et al., 1989). Furthermore, the antimicrobial spectrum of defensins includes all types of microorganism (bacteria, fungi, viruses and protozoa) (Selsted and Ouellette, 2005; Wilson et al., 2013; Zasloff, 2002).

α-defensins

So far, in humans, six distinct α-defensins have been identified. The α-defensins 1-4, the Human Neutrophil Peptides 1-4 (HNPs), have first been described in neutrophils but they are also expressed in monocytes and natural killer cells (Selsted and Ouellette, 2005). HD5 and HD6 are only expressed by Paneth cells of the small intestine, presumably constitutively. The regulatory elements of HD5 and HD6 genes display great similarities, e.g. both contain Wnt-response elements (Andreu et al., 2005; Wehkamp et al., 2007). Accordingly, the Wnt pathway has been shown to be a regulator of HD5 and HD6 expression via the Wnt transcription factors TCF-1 and TCF-4 (Beisner et al., 2014; Wehkamp et al., 2007). However, many further influencers and activators of Paneth cell HD5 and HD6 expression remain elusive. Recently, more light has been shed on their expressional regulation. Courth et al could show that the peripheral blood mononuclear cells (PBMC) secretome, more precisely the Wnt ligands therein, was able to induce HD5 and HD6 in *ex vivo* stimulated ileal biopsies. However, PBMC supernatants derived from Crohn's Disease (CD) patients, displayed an impaired induction capacity likely due to reduced levels of Wnt ligands (Courth et al., 2015).

HD5 and HD6 are being stored intracellularly as propeptides within vesicles and their content is secreted upon stimulation such as the detection of bacterial at the cell surface. Thereafter, the tryptic cleavage of the propetide into the active form of 32 aminoacids in length takes place in the crypt lumen (Ghosh et al., 2002). There, high concentrations these AMPs can be reached (Ghosh et al., 2002), ensuring the crypt environment to stay sterile (Ericksen et al., 2005; Ouellette and Bevins, 2001). The study by Erickson et al found partially diverting antimicrobial activities for all α-defensins, except for HD6 (Ericksen et al., 2005). They are active against bacteria, fungi and viruses (Porter et al., 1997; Wilson et al., 2013). The mechanism of action for both, HD5 and HD6, has however been under intense investigation. HD5 has been reported to form dimers (Szyk et al., 2006) and to disturb cell division in gram-negative bacteria by provoking the formation of membrane vesicles termed blebs (Chileveru et al., 2015). Furthermore, HD5 seems to hold the ability to detoxicate bacterial toxins (Kudryashova et al., 2014). In a mouse gain-of-function model transgenic for HD5, it presented activity against

Salmonella typhimurium and lead to a change in the microbiota composition (Salzman et al., 2003, 2010). Experts were long baffled by the seemingly missing antimicrobial activity of HD6 (Bevins, 2013; Ericksen et al., 2005). But within the last several years, even two distinct antimicrobial mechanisms have been reported to be inherent in HD6. It has been shown to form extracellular, bacteria-trapping nanonet structures *in vivo* (Chu et al., 2012) and been found to develop a direct antimicrobial killing activity against specific bacteria under reducing conditions like they are found in the intestine (Schroeder et al., 2014). Together, HD5 and HD6 build a broad defense arsenal for the maintenance of the gut innate antimicrobial barrier.

ß-Defensins

The evolutionary older family of β-defensins is the largest defensin family and members have been found in about all vertebrates studied so far (Zhu and Gao, 2013). Extensive gene duplications have led to about 30 β-defensin genes in humans (Rodríguez-Jiménez et al., 2003; Schutte et al., 2002) but so far mainly human β-defensins 1, 2,and 3 have been studied in greater detail (Pazgier et al., 2006). They are mainly expressed in many different epithelia, such as of the genitourinary and respiratory tract, the corneal epithelium (McDermott et al., 2003), gingival epithelial cells, the epidermis of the skin (Harder et al., 1997b, 2000; Schröder and Harder, 1999), and epithelial cells of the intestine (Bals et al., 1998; O'Neil et al., 1999), but can also be found in immune cells (Duits et al., 2002; Wah et al., 2006; Yin et al., 2010). Not much is known, however, about the intracellular storage mode of β-defensins. In keratinocytes of the skin, hBD2 is for example stored in lamellar bodies (Oren et al., 2003). Depending on the anatomical site of expression, β-defensins are either constitutively expressed or inducible (Selsted and Ouellette 2005). HBD1 is mostly constitutively expressed but its expression can be modulated (Duits et al 2002), hBD2 and hBD3 are differentially expressed and often inducible in a Toll-like receptor (TLR)- or inflammation dependent manner via pro-inflammatory cytokines (García et al., 2001; Liu et al., 1998; Selsted and Ouellette, 2005; Wehkamp et al., 2002). Among the manifold stimulants capable of inducing β-defensins at the transcriptional level are also different probiotic and pathogenic bacteria (Birchler et al., 2001; Harder et al., 2001; O'Neil et al., 1999; Schlee et al., 2007, 2008). Concerning their antimicrobial spectrum, β-defensins have been shown to be mainly effective against Gram-negative bacteria and fungi but have also been found to be active against Gram-positive bacteria and viruses ((Wehkamp et al., 2002, 2005a) Harder et al., 2001; Weinberg et al., 2012). Furthermore, immune-modulatory functions have been reported for β-defensins (Lai and Gallo, 2009), where, in different settings, they showed to be capable of recruiting immune cells (Yang et al., 1999), breaking self-DNA tolerance (Lande et al., 2015) or reducing the expression of pro-inflammatory cytokines (Donnarumma et al., 2007).

Interestingly, in the case of hBD1, the antimicrobial potential was long thought to be small. However, in 2011, it could be demonstrated that the environmental conditions are crucial

for hBD1 to unfold its antimicrobial activity (Schroeder et al., 2011). Reducing testing conditions mimicking the *in vivo* situation in the gut lead to the reduction of the intramolecular disulfide-bridges in hBD1 conferring it with a potent antimicrobial activity against commensals and fungi (Jaeger et al., 2013), but also against pathogenic bacteria (Raschig et al., 2017; Wendler et al., 2018). *In vivo*, the reduction of hBD1 can occur via the thioredoxin system (Jaeger et al., 2013). Alterations in the inducibility of β-defensins has furthermore been found in specimens of colonic CD and of ulcerative colitis (UC), highlighting their important contribution to an efficient antimicrobial defense system (Wehkamp et al., 2002, 2003, 2005a).

1.2.3.3. Adaptive immunity in the intestine

GALT is the part of the lymphatic system associated with the intestine. Along the whole gastrointestinal tract, diffuse aggregates of lymphocytes but also organized lymphoid follicles and mesenteric lymph nodes (MLNs) can be found in the mucosa and submucosa of the small and large intestine. If necessary, these effector sites function in the initiation of adaptive immune responses. In the small intestinal wall, especially the ileum, so called Peyer's patches can be found which are large aggregates of up to 50 lymphoid follicles, containing a great number of B and T cells (Mowat, 2003). Right above the Peyer's patches, specialized enterocytes, microfold cells (M cells), are scattered into the epithelium besides infiltrated B cells, T cells, dendritic cells (DCs) and macrophages (Mowat, 2003). M cells serve as detectors of invasive pathogens and other antigens and transcytose them across the epithelium. Thereafter, antigen presenting cells process and forward the information leading to activation of adaptive immunity. This includes not only the maturation and differentiation of residing naïve lymphocytes but also the recruitment of further immune cells from the blood stream into affected tissue sites (Mowat, 2003).

1.2.3.4. Recognition of bacterial components in the intestine

The intestinal epithelium, as the first defense line, is equipped with pattern recognition receptors (PRRs) enabling epithelial cells to keep track of the composition of the resident microbiota. These PRRs recognize highly conserved pathogen-associated or microbe-associated molecular patterns (PAMPs or MAMPs) (Didierlaurent et al., 2002). The term MAMP is more general since there are many PAMPs that can also be found in non-pathogenic commensals. Abundant PRRs include TLRs, which are located on the surface membrane of epithelial cells and intracellular PRRs such as Nucleotide-binding oligomerization domain containing molecules (NODs) in NOD-like receptors (NLRs). Upon activation by respective ligands, pro-inflammatory pathways involving distinct signaling components are triggered, e.g. Myeloid differentiation primary response gene (MyD) 88, mitogen-activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) with the goal to quickly eliminate the intruders or if necessary induce adaptive immune responses (Cario, 2005; Takeda and Akira,

2004). In general, TLRs and their associated signaling cascades are involved in many processes affecting the well-functioning of the intestinal barrier, such as proliferation of epithelial cells, the integrity of tight junctions and AMP production (Abreu, 2010; Santaolalla et al., 2011). One mechanism to maintain a tolerant immune microenvironment towards the abundant microbiota in the intestine is the selective spatial expression of PRRs in the epithelia, limiting specific TLRs to be localized in low levels mostly to the basolateral membrane but to be inducible upon demand in macrophages (Abreu, 2010; Cario, 2010; Hausmann et al., 2002). Furthermore, the intracellular receptor for muramyldipeptide NOD2 is for example mainly expressed in Paneth cells of small intestinal crypts (Lala et al., 2003). Last but not least, the aforementioned overlying mucus layer provides a first shield against the attachment and invasion of microbes.

1.3. Inflammatory bowel diseases and the impaired antimicrobial barrier

There are two major entities in IBD, CD and UC, which are both characterized by a chronic relapsing intestinal inflammation and infiltrating mono- and lymphocytes into the intestinal wall (Podolsky, 2002). IBD pathology is currently incompletely understood and thought to be caused by a myriad of genetic susceptibilities (Halfvarson et al., 2006; McGovern et al., 2015; Schreiber et al., 2005; Van Limbergen et al., 2014) together with environmental influences driving intestinal inflammation into a chronically activated status. Genetically and environmentally caused predispositions which seem to eventually result in a loss of mucosal tolerance towards resident microbiota and bacterial invasion leading to a continuous adaptive immune reaction and chronic inflammation (Figure 3) (Beisner et al., 2010; Ostaff et al., 2013; Wehkamp and Stange, 2010).

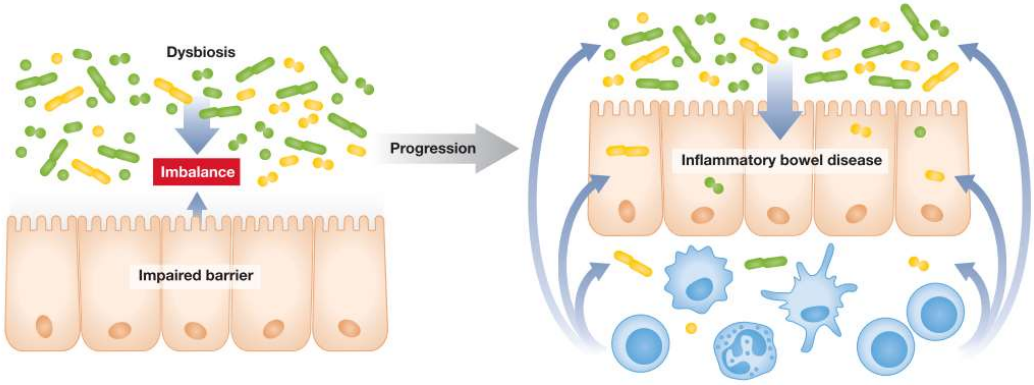

Figure 3: Proposed model for development of IBD.
Graphic from Ostaff et al., 2013, published in EMBO Mol Med 2013;5:1465-1483

Great environmental influence seems to be exerted by the bacterial communities living in the intestine, individual life styles, antibiotics use, smoking, and dietary habits (Legaki and Gazouli, 2016; Sartor, 2008). In the proposed model depicted in Figure 3, these environmental effectors act upon an initially impaired barrier, which is e.g. weakened due to reduced mucus and AMP production, within a genetically predisposed host. The resulting loss in host-microbe-homeostasis is herein thought to be primary to the consequence of an overshooting adaptive immune response (Ostaff et al., 2013; Wehkamp and Stange, 2010).

The division into the two major IBD subgroups, CD and UC, is mainly determined by differing clinical behavior. CD can affect all sites of the whole GI tract displaying a rather patchy, over time often transmural inflammation pattern. UC, on the other side is characterized by a continuous mucosal inflammation beginning in the rectum which can gradually wander up to proximal colonic segments but stays restricted to the colon. Furthermore, CD and UC differ in terms of many molecular mechanisms contributing to disease onset, progression, or severity. In total, more than 200 genetic susceptibility loci have been identified for IBD, but only a fraction are shared by CD and UC (Anderson et al., 2011; Franke et al., 2010; Jostins et al., 2012; Khor et al., 2011; de Lange et al., 2017) affecting innate and adaptive immunity pathways, including the expression of pro-inflammatory and regulatory cytokines, endoplasmatic reticulum (ER) stress, or autophagy, among others. Furthermore, non-genetic factors are thought to play a bigger role in UC than in CD, since monozygotic twins display a concordance rate of only about 15% in UC but of about 35%-50% in CD, additionally emphasizing the role of the environment in those diseases (Khor et al., 2011; Loddo and Romano, 2015; Spehlmann et al., 2008) The divergence between CD and UC is also reflected by partially differing response to therapeutical approaches. For example, the probiotic bacterium *E. coli* Nissle 1917 is effective in maintaining remission in UC patients (Mack, 2011), possibly via the induction of hBD2 in colonic mucosa (Möndel et al., 2009; Wehkamp et al., 2004a), whereas CD patients are lacking a probiotic benefit. No cure has been found so far, wherefore most therapies only treat the symptoms. Upregulated pro-inflammatory cytokines in IBD, e.g. TNF-α, IL-17, or IL-23 mark them as targets for immunosuppressive approaches. Blocking TNF-α with anti-TNF antibodies is effective in many patients and can strongly ameliorate symptoms (Chudy-Onwugaje et al., 2018; Dahlén et al., 2015), however, targeting IL-17 lead to severe disease exacerbations (Neurath, 2014). Hence, more research is needed on the interplay of all disease influencers and the affected signaling pathways, including their interconnected crosstalks, to find a more causal therapeutic approach.

1.3.1. Crohn's Disease

Depending on the localization of the disease, which stays quite stable once it is established in a patient, CD is classified into further subdivisions according to the Vienna classification. L1

patients show ileal involvement only, L2 only colonic involvement, and L3 patients have both ileal and colonic tissue affected. If also the upper GI tract is involved the description "+L4" is added to the label. Relating to some extend to disease localization, distinct molecular mechanisms regarding defective AMP expression could be observed, reflecting their importance in a healthy functioning antimicrobial barrier.

Patients with CD localized to the small intestine, have for example a deficit in the AMP human alpha-defensin 5 and 6 (HD 5/6) due to multi-layered defects in Paneth cells (Courth et al., 2015; Koslowski et al., 2009, 2012; Wehkamp et al., 2005b, 2007, 2008). This reduced expression of HD5 and HD6, which is independent of the inflammatory status (Bevins et al., 2009), renders patients with decreased antimicrobial activity (Wehkamp et al., 2005b). Underlying defects in the expressional regulation could be uncovered. The HD5 and HD6 controlling Wnt pathway transcription factors TCF-1 and TCF-4 have been found to be diminished in small intestinal CD (Beisner et al., 2014; Perminow et al., 2010; Wehkamp et al., 2007). Furthermore, ileal CD could be associated with mutations in the gene regulatory sequence of TCF-4 (Koslowski et al., 2009). Concerning the age of disease onset, a variant of the Wnt ligand co-receptor LRP6 has been found to be linked to an early onset (Koslowski et al., 2009). Another interesting discovery concerning the induction of HD5 and HD6 could recently be made. It has been found that the secretome of stimulated PBMCs contains Wnt ligands and is capable of inducing the two α-defensins in ileal biopsies ex vivo. However, in contrast to PBMC supernatant from healthy individuals, CD derived PBMCs failed to do so which was concomitant with a lower expression of Wnt ligands in patient PBMCs (Courth et al., 2015). Further genetic defects affecting Paneth cell physiology have been reported. A single-nucleotide polymorphism (SNP) in the calcium-activated potassium channel protein (KCNN4) has been associated with ileal CD (Simms et al., 2010). This channel is involved in the process of granule secretion, thereby potentially affecting the availability of HD5 and HD5 in the intestinal lumen (Bevins and Salzman, 2011). In addition, genetic variants of the PRR NOD2, which is abundantly expressed in Paneth cells (Lala et al., 2003) have been identified as a strong risk factor for small intestinal CD (Economou et al., 2004; Hugot et al., 2001; Ogura et al., 2001) and could furthermore also be linked to reduced HD5 and HD6 expression (Wehkamp et al., 2004b, 2005b). So far, this correlation lacks profound causal connection, it still delivers a further conjunction between the sensing microbes and antimicrobial defensins in CD. Other susceptibility genes for ileal CD are the gene encoding a component of the ER stress response, XBP1, which has been reported to lead to an augmentation in Paneth cell apoptosis (Adolph et al., 2013; Kaser et al., 2008) and ATG16L1, an autophagy protein involved in the exocytosis of granules (Cadwell et al., 2008; Hampe et al., 2007). All the above described defects in Paneth cells unequivocally make these cells gain center stage not only for the upkeeping of the mucosal antimicrobial barrier as well in the pathogenesis of ileal CD.

Patients with colonic CD on the other hand, but also UC (which will be discussed in the next chapter), produce a reduced amount of the constitutively expressed hBD1 in their large bowel epithelia (Wehkamp et al., 2003) and are furthermore impaired in their redox-activation of the same peptide (Jaeger et al., 2013). Furthermore, colonic CD is associated with an attenuated upregulation of the hBD2, but also hBD3 and hBD4 and other AMPs like Elafin and LL37 are diminished (Fahlgren et al., 2004; Schmid et al., 2007; Wehkamp et al., 2003, 2008), accompanied by reduced mucosal antimicrobial activity (Nuding et al., 2007). HBD2 has been shown to be upregulated in colonic mucosa in response to probiotic bacteria (Möndel et al., 2009; Schlee et al., 2008; Wehkamp et al., 2004a), possibly underlying the beneficial effect of probiotics on intestinal barrier function. The impaired inducibility of this important defense molecule in colonic CD under inflammatory or infectious conditions may, however, help explain why these patients are more susceptible to a bacterially induced chronic inflammation; and furthermore why probiotics show no efficiency in prolonging colonic CD remission (Mack, 2011). Underlying causes of the defects in AMP expression have, however, so far only been scarcely investigated and are not yet fully understood. While predisposing genetic factors have been reported in the context of reduced Paneth cell mediated epithelial defense in the small intestine (Wehkamp and Stange, 2010), not much has been reported regarding the genetics of epithelial antimicrobial immunity in colonic CD. Data on hBD2 copy number variations have been inconclusive (Aldhous et al., 2009; Fellermann et al., 2006; Groth et al., 2010; Hollox, 2010) and so far only limited data exists on a hBD1 promoter SNP association with colonic CD (Lakatos et al., 2008).

1.3.2. Ulcerative colitis

In the colonic mucus layer of UC, adherent bacteria has been found (Swidsinski et al., 2002). Likewise to the situation in colonic CD, these patients also show diminished levels of hBD1 expression and functional redox-activation thereof also seems to be impaired (Jaeger et al., 2013; Schroeder et al., 2011; Wehkamp et al., 2003). UC is additionally also characterized by a defective goblet cell maturation and a thinned mucus layer on the surface of the colon, especially during inflammation (Gersemann et al., 2009; Johansson et al., 2013; Pullan et al., 1994; Strugala et al., 2008). These defects indicate that an impairment in the antimicrobial barrier is a key factor in this disorder aswell. Although other β-defensins, such as hBD2, hBD3 and hBD4 are readily upregulated in colonic mucosal epithelium of UC (Fahlgren et al., 2003, 2004; Wehkamp et al., 2003), this potentially compensatory effort to repair the antimicrobial defense likely hazards the consequences of an increase in inflammation caused by the pro-inflammatory and immunomodulatory effects of these AMPs (Lai and Gallo, 2009; Niyonsaba et al., 2005, 2007; Yang et al., 1999). An additional negative influence on disease progression might be, that the

defective mucus layer is also less effective in binding the secreted AMPs and keeping them in close proximity to the epithelium where they could exert their antimicrobial killing activity (Ostaff et al., 2013). Hints for this have been obtained in a number of mouse models, including models of dextran sulfate sodium (DSS)-induced colitis (Fu et al., 2011; Petersson et al., 2011). Overall, however, the causal mechanisms for the dysregulations in AMP expression remain largely elusive at this point.

1.3.3. Epigenetics in IBD

The development and clinical behavior of CD and UC is determined by multiple underlying factors as has been described above. Among the most well studied ones are a growing number of genetic susceptibility loci (Halfvarson et al., 2006; McGovern et al., 2015; Schreiber et al., 2005; Van Limbergen et al., 2014) but also contributing environmental influences such as antibiotics use, smoking, or nutrition have found their way into the fields' focus (Legaki and Gazouli, 2016). The increasing incidence of IBD (Molodecky et al., 2012), the big variance in disease progression, localization, severity, the age of onset, and the discrepancy between monozygotic twins (Loddo and Romano, 2015; Spehlmann et al., 2008) however urgently impose the question how exactly environmental factors might impact on IBD risk and progression. The rapidly growing field of epigenetics could help to further dissect how environmentally induced changes occur in the epigenome affecting the interpretation of different genes in an individual in the context of IBD to allow a more wholistic understanding of disease pathology (Scarpa and Stylianou, 2012; Aleksandrova et al., 2017; Fogel et al., 2017; Wawrzyniak and Scharl, 2018; Zilbauer and Kraiczy, 2017; Fofanova et al., 2016).

Gloria and colleagues were among the first to investigate epigenetic features in IBD, who found global DNA hypomethylation in UC rectal mucosa while exploring the role in associated cancer development (Glória et al., 1996). Later on, IBD pathogenesis was linked to mutations in the DNA methylation enzyme DNMT3a (Franke et al., 2010). Furthermore, for ileal CD, methylation changes in proximity to susceptibility loci including NOD2 have been identified (Nimmo et al., 2012). Others have investigated differences between the methylation profiles of inflamed and non-inflamed tissue in IBD and in comparison to healthy individuals (Cooke et al., 2012; Häsler et al., 2012). Despite these and further interesting findings affecting the methylome in IBD and considering the plausible rationale to investigate epigenetics in these disorders, detailed mechanistic studies remain relatively scarce. Also still sparsely examined are changes in histone modifications in the field of IBD (Wawrzyniak and Scharl, 2018). However, there are several elegant mouse studies, closer describing the role of HDACs and histone methylation in the context of intestinal homeostasis and inflammation. Murine models of colitis have demonstrated the efficacy on inhibiting HDACs (Glauben et al., 2006; Turgeon et al., 2014).

Furthermore, Turgeon and colleagues have shown recently, that a double knockout of HDAC1 and HDAC2 in intestinal epithelial cells lead to a more severe DSS colitis and to higher inflammatory gene expression. However, single depletion of HDAC2 protected against DSS colitis and resulted in increase in colonic antimicrobial expression (Turgeon et al., 2013). Another very interesting finding has been made, where HDAC3 has been found to act as an integrator of signals derived from intestinal commensal microbiota for the correct regulation of the host-microbe relationship (Alenghat et al., 2013). In a very recent report, Kelly et al further report potential mechanism linking genetic suscepitbility and environment in mice and IBD patients. They describe how the microbiome-host interplay might effect on the epigenetic histone methylation landscape potentially priming the epithelium into future inflammation (Kelly et al., 2018)). In general, the question how microbiome and especially diet influence on epigenetics in IBD is gaining more and more center stage in the field (Aleksandrova et al., 2017). Not least because a large number of HDACi have been identified to occur as natural food components or emerge from bacterial metabolism, e.g. short chain fatty acids (Bassett and Barnett, 2014; Bhat and Kapila, 2017; Furusawa et al., 2013).

Concerning the impaired AMP expression in IBD, while genetic studies are still insufficient in explaining the changes in colonic defensin expression, some first insights on potential involvement of epigenetics in hBD regulation have been gained in non IBD related studies. One epigenetically mediated mechanism might be exerted by HDACs. Recently, Yin and Chung found HDACs to be important regulators of the TLR-dependent induction of the antimicrobial peptide hBD2 in gingival epithelial cells (Yin and Chung, 2011). HDAC1 has been shwon to be involved in the transcriptional regulation of hBD1 in human lung epithelial cells (Kallsen et al., 2012). Besides these interesting reports, *in vitro* experiments could also show that treatment with a dietary HDACi of histone deacetylation leads to an upregulation of hBD2 expression in intestinal epithelial cell lines (Schwab et al., 2008). A further study by Fischer and colleagues, that has been conducted in parallel to this one, investigated colonic epithelial cells and colonic organoids, also highlighting HDACs as regulators of hBD2 (Fischer et al., 2016). Overall, these findings sharply point towards the importance of HDACs in β-defensin regulation and consequently in antimicrobial defense.

1.4. Aim of the study

In IBD, the innate antimicrobial barrier has been shown to be severely compromised – for a big part due to different defensin deficiencies. Data on the genetics of epithelial antimicrobial immunity concerning colonic CD but also UC remain scarce. Especially the underlying mechanisms of the attenuated inducibility of hBD2 in colonic CD (Wehkamp et al., 2003) are largely unknown so far. On the contrary, UC patients show strongly increased levels of hBD2 expression, especially in inflamed tissue (Fahlgren et al., 2003; Wehkamp et al., 2003). Despite the large number of identified genetic susceptibility loci in IBD (Khor et al., 2011; de Lange et al., 2017), the discordance found in monozygotic twin studies emphasizes the role of environmental factors as crucial contributors to and modulators of these diseases (Loddo and Romano, 2015). Therefore, a feasible rationale for the investigation of epigenetic mechanisms in the context of IBD is given since it could help to gain more insight into the crosstalk between environment, epigenome and genome (Aleksandrova et al., 2017; Fofanova et al., 2016; Scarpa and Stylianou, 2012). A role for HDACs in the epigenetically-mediated modulation of hBDs has been demonstrated in a number studies (Fischer et al., 2016; Kallsen et al., 2012; Schwab et al., 2008; Yin and Chung, 2011) underscoring a potential involvement of HDACs in the β-defensin related defects found in colonic IBD.

Prompted by these findings, I aimed at studying HDAC expression in the epithelium of IBD patients by determining the mRNA and protein levels of class I HDACs in a large cohort including healthy controls and individuals with active as well as inactive ileal and/or colonic CD or UC via RT-PCR and western blotting. Furthermore, immunohistochemistry for those HDACs were performed on intestinal biopsy sections. The strategic analysis should allow first insights into the overall deacetylation capacity of the intestinal tissue in IBD based on the inflammatory status, as well as in comparison to overall healthy tissue and open up promising new research avenues regarding epigenetic involvement in disease pathology and could pinpoint potential new targets for therapeutic intervention.

While HDACi have been proposed as therapeutics in IBD (Felice et al., 2014; Glauben and Siegmund, 2011), their at most safe appliance demands a profound understanding of the exact role of HDACs in intestinal antimicrobial barrier function and inflammatory processes. Thus, it is vital to extend the existing body of knowledge on HDAC-mediated epigenetic regulation of the inducible hBD2, an IBD-associated, important player in both gut barrier function and inflammation. I therefore planned on studying the effects of HDACi on hBD2 expressional regulation in colonic epithelial cells *in vitro*, focusing on the therapeutically relevant probiotic EcN as a potent hBD2-inducing factor (Möndel et al., 2009) in addition to IL1β and LPS. Since our working group could recently successfully study small intestinal innate immune regulation in an *ex vivo* ileal biopsy culture (Courth et al., 2015), I established a human colonic biopsy culture

surviving in a culture medium almost identical to that used for the *in vitro* experiments presented herein to potentially better mimic and refelct the *in vivo* situation and to integrate the more complex tissue context. A substantial cohort was assembled, including IBD-derived and healthy control biopsies as well as several originating from colorectal cancer tissue. The latter served to examine and dissect the potential impact of the cancer nature inevitably present in transformed cell lines on the observed effects *in vitro*.

Finally, mechanistic investigations had to be focused on *in vitro* settings, due to the rare and valuable nature of human biopsy material. Since hBD2 has been shown to be, at least in part, regulated NF-κB-dependently (Wehkamp et al., 2004a) and NF-κB has been reported to be a non-histone protein target of HDACs (Ashburner et al., 2001; Chen et al., 2001; Singh et al., 2010), I aimed to investigate the potential role of NF-κB in HDAC-mediated hBD2 expressional modulation using pharmacological inhibition of NF-κB in addition to employing differentially mutated reporter gene constructs of the hBD2 promotor region.

2 Material and Methods

2.1. Material

Non-listed equipment, consumable items, plastics, or chemicals were conform of laboratory standards.

2.1.1. Equipment

Equipment	Manufacturer
ABI Prism™310 Sequencer	Applied Biosystems, Germany
Agilent 2100 bioanalyzer	Agilent, USA
BX63 microscope	Olympus, Germany
DP80 camera	Olympus, Germany
cellSens Dimension imaging software	Olympus, Germany
Enspire® Multimode Plate reader	PerkinElmer, USA
LightCycler® 480	Roche, Germany
NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA
ChemiDoc™ MP	Bio-Rad, USA

2.1.2. Software

Program	Manufacturer
EnSpire software	PerkinElmer, USA
Geneious Pro 5.1.7	Biomatter Ltd., New Zeeland
GraphPad Prism Version 7.03	GraphPad Software, Inc., USA
Leica SCN400 software	Leica, Germany
LightCycler Software V. 3.5	Roche, Germany
Image Lab Software 5.1	Bio-Rad, USA

2.1.3. Consumables

Item	Manufacturer
LightCycler® 480 Multiwell Plate 96	Roche, Germany
OptiPlate™ 96 (for Enspire)	PerkinElmer, USA
Columbia Agar plates with 5% Sheep Blood	Becton Dickinson, USA

2.1.4. Chemicals, kits and antibodies

2.1.4.1. Chemicals

Chemical / Reagent	Manufacturer
100bp DNA Ladder	Invitrogen, USA
1kb Plus DNA Ladder	Invitrogen, USA
Asparagine	Merck Millipore, Germany
CnT-Prime (CnT-PR)	CELLnTEC, Switzerland
CnT-Prime-D (CnT-PR-D)	CELLnTEC, Switzerland
$CaCl_2$	Sigma-Aldrich, Germany
DMEM	Life Technologies, USA
EcoRI	NEB, USA
Eosin	Sigma-Aldrich, Germany
FCS	Life Technologies, USA
Hepes Buffer, 1M	Invitrogen, USA
Helenalin	Enzo Life Sciences, Switzerland
Hematoxylin	Merck, Germany
HotStar Taq DNA-Ploymerase	Qiagen, Germany
Human IL-1ß	PeproTech, Germany
Human TNF-α	Sigma-Aldrich, Germany
L-Lactat Dehydrogenase	Roche, Germany
LPS *E. coli* serotype O111:B4	Sigma-Aldrich, Germany
MS-275 (Entinostat)	Selleckchem, USA
Non-Essential Amino Acids Solution 100x	Life Technologies, USA
Passive lysis buffer	Promega, USA
Penicillin/Streptomycin (Pen/Strep)	Life Technologies, USA
PIC complete Ultra Tablets, EDTA-free	Roche, Switzerland
RNAlater	Qiagen, Germany
Sodium-butyrate	Sigma-Aldrich, Germany
Sodium-pyruvate (100 nM)	Life Technologies, USA
Suberoylanilide hydroxamid acid (SAHA; Vorinostat)	InvivoGen, USA
Triton X	Sigma-Aldrich, Germany
TRIzol	Life Technologies, USA
Trypsin-EDTA	Roth, Germany
Tryptic Soy Broth media (TSB)	Becton Dickinson, USA
TurboFect Transfection Reagent	Thermo Fisher Scientific, USA

2.1.4.2. Kits and antibodies

Kit / Antibody	Manufacturer
AMV Reverse Transcription System	Promega, USA
Anti-HDAC1 antibody (ab109411)	Abcam, UK
Anti-HDAC2 antibody (ab12169)	Abcam, UK
Anti-HDAC3 antibody (ab7030)	Abcam, UK
Anti-HDAC8 antibody (HPA048560)	AtlasAntibodies, Sweden

Big Dye Terminator V.1.1 Cycle Sequencing Kit	Applied Biosystems, USA
Cytotoxicity Detection Kit (LDH)	Roche, Germany
Dako REAL™ EnVision™ Detection System	Dako, Denmark
Direct-zol RNA Miniprep Kit	ZymoResearch, USA
Dual-Luciferase® Reporter Assay System	Promega, USA
HotStar Taq DNA-Polymerase	Qiagen, Germany
Human Beta-Defensin2 (hBD2) ELISA Kit	Phoenix Pharmaceuticals, USA
iScript™ cDNA Synthesis Kit	Bio-Rad, USA
LightCycler®480 SYBR Green I Master Kit	Roche, Switzerland
MTT	Sigma-Aldrich, Germany
QIAprep Spin Miniprep Kit	Qiagen, Germany
QIAquik PCR Purification Kit	Qiagen, Germany
Quick RNA Miniprep Kit	ZymoResearch, USA
RNA 6000 Nano Assay	Agilent, USA
RNeasy Kit	Qiagen, Germany
RNeasy Mini Kit	Qiagen, Germany
TOPO TA Cloning Kit	Invitrogen, USA

2.1.5. Media and buffer

2.1.5.1. Cell culture

CaCo2/HCT116 cultivation media

DMEM	435 ml
FCS	50 ml
Sodium Pyruvate	5 ml
NEAA	5 ml
Pen/Strep	5 ml

Colonic biopsy cultivation media

DMEM	430 ml
FCS	50 ml
Sodium Pyruvate	5 ml
NEAA	5 ml
Pen/Strep	10 ml

Hgep cultivation media

CnT-PR	
CnT-PR-D (+/-Ca^{2+})	
Ca^{2+}	1.2 mM

2.1.5.2. Other

TAE Buffer (50x)

Tris	242 g
Acetic acid	57,1 ml
0,5M EDTA pH 8,0	100 ml
H_2O	up to 1 l

TBS (10x)

NaCl	400 g
KCl	10 g
Tris-Base	150 g
HCl	set to pH 7,4
H_2O	up to 5 l

TBS-T

TBS	1x in H_2O
Tween20	1%

FA Gel-buffer (10x)

MOPS	41.85 g
Sodium acetate	4.1 g
EDTA	1.86 g
HCl/NaOH	set to pH 7
H_2O, RNAse-free	up to 1l

Protein lysis buffer/PIC

50 mM Tris-HCl	3,94 g
0,25 mM NaCl	7,30 g
0,1 TritonX 100	500 µl
5 mM EDTA	0,73 g

Add 1 PIC (Roche) tablet to 25 ml lysis buffer

1% Agarose gel

Agarose	1 g
10x FA Gel-buffer	10 ml
H_2O	1.86 g
HCl/NaOH	set to pH 7
H_2O, RNAse-free	87 ml

boil and cool down to 50 °C

Formaldehyde (37%)	3 ml

2.1.6. Plasmids and sequences

Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany), High Purity Salt Free purified.

Table 1: Primer for real-time PCR

TARGET	SENSE (5'-3')	ANTISENSE (5'-3')
Human		
hBD2	ATC AGC CAT CAG GGT CTT GT	GAG ACC ACA GGT GCC AAT TT
hBD1	GGC CTC AGG TGG TAA CTT TCT	TTC TTC TGG TCA CTC CCA GC
IL8	ATG ACT TCC AAG CTG GCC GTG GC	TCT CAG CCC TCT TCA AAA ACT TC
Ki67	GAG AAG AAC CTC TGC TCC CCA	TTT GCT GCA TTC TGT GCA CTG
β-Actin	GCC AAC CGC GAG AAG ATG A	CAT CAC GAT GCC AGT GGT A
HDAC1	CCA AGT ACC ACA GCG ATG AC	TGG ACA GTC CTC ACC AAC G
HDAC2	TGA AGG AGA AGG AGG TCG AA	GGA TTT ATC TTC TTC CTT AAC GTC TG

HDAC3	GAG TGG CCG CTA CTA CTG TC	ATT CAA CGC ATT CCC CAT GC
HDAC8	GCT GGT CCC GGT TTA TAT CT	TGC AGT GCA TAT GCT TCA ATC

Table 2: Plasmids

Name	Description	Source
hBD2-2338-luc	Luciferase Reporter: 2,3kB of hBD2 promoter region in pGL3-Basic vector	Prof. Jürgen Harder, Kiel; (Harder et al., 2000; Wehkamp et al., 2004a)
NF-κB-mut1-luc	Luciferase Reporter: 2,3kB of hBD2 promoter region in pGL3-Basic vector, NF-κB-binding site mutated at position -205 to -186; see Figure 31	Prof. Jürgen Harder, Kiel; (Harder et al., 2000; Wehkamp et al., 2004a)
NF-κB-mut2-luc	Luciferase Reporter: 2,3kB of hBD2 promoter region in pGL3-Basic vector, NF-κB-binding site mutated at position -596 to -572; see Figure 31	Prof. Jürgen Harder, Kiel; (Harder et al., 2000; Wehkamp et al., 2004a)
NF-κB-mut1+2-luc	Luciferase Reporter: 2,3kB of hBD2 promoter region in pGL3-Basic vector, both NF-κB-binding sites mutated; see Figure 31	Prof. Jürgen Harder, Kiel; (Harder et al., 2000; Wehkamp et al., 2004a)
AP1-mut-luc	Luciferase Reporter: 2,3kB of hBD2 promoter region in pGL3-Basic vector, AP1-binding site mutated at position -127 to -121; see Figure 31	Prof. Jürgen Harder, Kiel; (Harder et al., 2000; Wehkamp et al., 2004a)
NF-κB-mut1+2+AP1-luc	Luciferase Reporter: 2,3kB of hBD2 promoter region in pGL3-Basic vector, both NF-κB-binding sites and the AP1-binding site mutated; see Figure 31	Prof. Jürgen Harder, Kiel; (Harder et al., 2000; Wehkamp et al., 2004a)
pNF-κB-luc	Luciferase Reporter: tandemly repeated NF-κB binding sites in pGL3-Basic vector	Clontech BD Biosciences, USA
pAP1-luc	Luciferase Reporter: tandemly repeated AP1 binding sites in pGL3-Basic vector	Clontech BD Biosciences, USA
pGL3 Basic	Luciferase Reporter vector: includes Luciferase gene without vector	Promega, USA
Renilla CMV	Renilla reporter gene with CMV-promoter	Promega, USA

2.1.7. Cell lines and bacteria

2.1.7.1. Cell lines

The majority of experiments has been conducted with the human colonic epithelial adenocarcinoma cell line CaCo2 subclone TC7 (Chantret et al., 1994; Sambuy et al., 2005) which were received from Oliver Burk, IKP Stuttgart, Germany. In general, CaCo2 cells undergo spontaneous enterocytic differentiation (Chantret et al., 1988). The TC7 subclone has been isolated from the parental line at a late passage (Fogh et al., 1977) and represents a more homogenic and faster growing subculture with specific characteristics in terms of sucrase-isomaltase expression, transport function, and differentiation (Chantret et al., 1994). In our experiments, cells were used at an internal early passage of about 25 - 40.

Additionally, HCT116 cells (Brattain et al., 1981) have been used as an additional colorectal carcinoma cell line with an epithelial morphology. They have been kindly provided by Heiko van der Kuip, IKP Stuttgart, Germany.

Furthermore, human primary gingival epithelial cells (hgeps; CELLnTEC, Switzerland) have been utilized in a pilot experiment to study the observed effects from cancerous cell lines in a normal epithelial setup.

2.1.7.2. Bacteria

Table 3: Bacteria used

Species	Serotype	Characteristics	Source
E. coli Nissle 1917 (DSM6601)	O6:K5:H1	Apathogen, pharmaceutical strain	ACS

ACS: Ardeypharm Collection of Strains, Herdecke, Germany

2.1.8. Patients

All patients and controls included in this study gave their written and informed consent after they were informed about the study purpose, sample procedure, and potential adjunctive risks before being endoscoped because of diagnostic reasons. The study protocol was previously approved by the ethical committee of the University Hospital Tübingen, Germany and all experiments were conducted in accordance with the relevant guidelines and regulations. All tissue samples used for mRNA analysis were part of the Stuttgart cohort which has been continuously collected at the Robert-Bosch Hospital, Stuttgart, Germany, since 2001. Diagnosis was performed according to standard criteria including clinical, radiological, endoscopic, and histopathological results. The standard Vienna classification based on the disease localization in Crohn's disease patients was applied to subgroup them into L1 (ileal involvement), L2 (colonic involvement), and L3 (ileal and colonic involvement). Further subgrouping of included patients into inflamed or non-inflamed was done based upon clinical phenotype data. Samples for western blot and immunohistochemical analyses were obtained in the same way as for the mRNA experiments. Biopsies for *ex vivo* culture studies were sampled from the sigmoidal colon of healthy controls, patients with Crohn's disease, and patients with ulcerative colitis during routine colonoscopy at the Robert-Bosch Hospital, Stuttgart, Germany and the University Hospital Tübingen, Germany. Biopsies from colorectal tumors were obtained within 90 minutes after resection of the tumors at the Marienhospital, Stuttgart, or at the University Hospital Tübingen, Germany and were treated right away. The detailed numbers of patient biopsies used for different experiments are specified in the according figure legends and under 2.1.8.1 and the following (Stebe-Frick et al., 2018).

2.1.8.1. *Ex vivo* biopsy cohort

Group	N [total]	Gender		Age [mean±SEM]
		f	m	
L1 Patients	15	47 %	55 %	47.4 ± 3.34
L3 Patients	14	29 %	71 %	38.29 ± 2.93
UC Patients	14	36 %	64 %	39.92 ± 2.7
Controls	13	53 %	47 %	53.69 ± 3.4
Colonic carcinoma	4	25 %	75 %	66.33 ± 9.5

2.1.8.2. *In vivo* ileal biopsy cohort (numbers taken from the HDAC2 mRNA measurement)

Group	N [total]	Gender		Age [mean±SEM]	Localisation	
		f	m		L1	L3
Patients	60	52 %	45 %	40 ± 1.7	47 %	53 %
Controls	25	56 %	44 %	51 ± 4		

2.1.8.3. *In vivo* colonic biopsy cohort (numbers taken from the HDAC2 mRNA measurement)

Group	N [total]	Gender		Age [mean±SEM]	Localization			Inflammation (UC)	
		f	m		L2	L3	UC	+	-
Patients	100	53 %	47 %	37 ± 1.2	22%	33%	45%	40%	60%
Controls	24	58 %	42 %	46 ± 4.2					

2.1.8.4. Patient samples for western blotting and immunohistochemistry

For western blot analysis of ileal tissue, five ileal specimens of healthy controls and five ileal specimens of uninflamed L3 CD patients were analyzed. For western blot analysis of colonic tissue, five colonic specimens of healthy controls and uninflamed L3 CD patients, as well as 4 uninflamed UC specimens were used for HDAC1 and 2 detection. Five further samples of all three groups (healthy controls, L3 and UC) have been used for HDAC3 analysis. All patients were between 18 and 73 years old and female and male numbers were relatively equal in distribution.

Sampling has been conducted in the same way as for all the other intestinal biopsies used in this study. Exemplarily shown immunohistochemical stainings from intestinal tissue slices and those used for the semiquantitative analysis of HDAC1 in the human ileum (cf. Figure 10) had been sampled from patients that were mostly also included in the *ex vivo* biopsy cohort (see Chapter 2.1.8.1) and therefore fit into the same age range and gender distribution.

2.2. Methods

2.2.1. Cell culture methods

2.2.1.1. Cell line cultivation

CaCo2/TC7 and HCT116 cells were cultured in CaCo2/HCT116 cultivation media in a humidified atmosphere at 37°C with 5% CO_2. For maintenance, cells were passaged every 3-4 days. Both cell lines were used for a maximum of 40 passages. Experiments were performed in FCS- and Pen/Strep-free medium in 12-well-plates on cellular monolayers at about 80-90 % confluency. After a total treatment duration of 20 hrs mRNA and protein was subsequently analyzed (Stebe-Frick et al., 2018).

Hgep cells were first cultured in CnT-PR media until they nearly reached confluency. Then they were switched to CnT-PR-D medium containing 1.2 mM Ca^{2+} to stimulate differentiation for either 48 or 72 hrs. Cells were then treated as depicted in 2.2.1.3 Treatment of cells and biopsies.

2.2.1.2. *Ex vivo* biopsy cultivation

Freshly collected colonic biopsies were utilized to study the expression of hBD2 in a more complex *ex vivo* tissue context as compared to mono-layered cell culture. After receiving the specimen, they were immediately washed several times with ice-cold PBS containing 10% (v/v) Pen/Strep, transferred into wells of a 24-well-plate with 1 ml of designated media consisting of DMEM containing 2% (v/v) Pen/Strep to minimize bacterial overgrowth and incubated at 37°C with 5% CO_2 for a total treatment duration of 20 hrs. Thereafter, biopsies were put into RNAlater until RNA isolation and the supernatant was collected for subsequent quality control. The integrity of total RNA was controlled using the RNA 6000 Nano Assay. The viability of the samples was tested via the supernatants using LDH-ELISA (Stebe-Frick et al., 2018).

For the hematoxylin and eosin staining of biopsy sections, biopsies were either directly fixed with 4% formalin or after 20 hrs of treatment (Stebe-Frick et al., 2018).

Biopsies which were used for immunohistochemcial analysis of class I HDAC protein expression were put into 4% formalin. After incubation overnight, the biopsies were fixed in paraffin. Slides with a thickness of 7 µm were prepared and used for immunohistochemistry (Stebe-Frick et al., 2018).

2.2.1.3. Treatment of cells and biopsies

Cells or biopsies were pretreated with suberoylanilide hydroxamid acid (SAHA; Vorinostat), Pyridin-3-ylmethyl *N*-[[4-[(2-aminophenyl)carbamoyl]phenyl]methyl] carbamate (MS-275; Entinostat) (both dissolved in Dimethyl sulfoxide (DMSO)), or sodium-butyrate (SB) (dissolved in H_2O) for 2 hrs prior to the start of the stimulation with either heat-inactivated *E. coli* Nissle 1917, Interleukin 1β (IL1β), or lipopolysaccharide (LPS) from *E. coli* serotype O111:B4 which

took place in parallel to HDAC inhibition for additional 18 hrs adding up to a total of 20 hrs of treatment. Treatment with the NF-κB inhibitor Helenalin took place for 1h right at the beginning of the 20 hrs and was then removed to avoid cytotoxic effects (Stebe-Frick et al., 2018).

The non-toxic character of the treatment reagents was confirmed via MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-tests which are based on reduction of the tetrazolium salt (Berridge and Tan, 1993; Mosmann, 1983) performed by a technician and by stratifying for DMSO effects on cells with concentrations of up to 1% DMSO which showed to have no effects on target of interest mRNA expression.

2.2.1.4. Transient transfection, luciferase assay and promoter activity

CaCo2/TC7 cells seeded in 24 well plates were transfected with 500 ng DNA using 1 µl Turbofect transfection reagent according to manufacturer's protocol at about 70% confluency. HBD2 reporter plasmids used in this work (hBD2-2338-luc, NF-κB-mut1-luc, NF-κB-mut2-luc, NF-κB-mut1+2-luc, NF-κB-mut1+2+AP1-luc, AP1-mut-luc) have been described previously (Harder et al. 2000, Wehkamp et al. 2004) and are specified in Table 2. Briefly, these plasmids carry differentially mutated 2338 bp of the wildtype hBD2 promoter affecting positions in NF-κB or AP1 transcription factor binding sites. CMV-Renilla plasmid was used as an internal standard to which firefly luciferase activity was normalized. Promoterless pGL3basic firefly luciferase vector (Promega, USA) being the backbone of the aforementioned hBD2 reporters, was used as control. Used cultivation media was the above described DMEM used for cell culture but without FCS and Pen/Strep. 24 hrs post transfection, treatment with indicated media or agents was started as described above in "treatment of cells and biopsies". After 20 hrs of treatment cells were lysed for measurement of luciferase activity with the Dual Luciferase Reporter Assay- adding 20 µl of firefly luciferase reagent (LARII) and after measuring the luminescence adding 20µl of Stop and Glow reagent to quench the firefly luciferase reaction and initiate Renilla reaction. Bioluminescences was measured using an Enspire PlateReader. Firefly luciferase signals were normalized to Renilla activities, the latter representing the transfection efficiency. Each transfection was performed in triplicates in three or more independent experiments (Stebe-Frick et al., 2018).

2.2.2. Bacterial methods

Cultivation and inactivation of bacterial cultures

E. coli Nissle 1917 (EcN) has been heat-inactivated for all experiments as has been described before (Wehkamp et al., 2004a). Heat-inactivated bacteria have been used, since it has been shown that they show the same effect as living EcN (Wehkamp et al., 2004a). EcN has been grown overnight in trypticase soy broth (TSB) medium at 37°C under constant shaking. Anaerobic bacteria (Bifidobacteria) were first cultured on blood agar, on the next day

transferred to TSB media and then grown in anaerobic jars for additional 24 hrs. The next morning, 100 µl of the bacterial suspension were diluted in 10 ml of TSB medium to keep bacteria in a linear growth phase and grown under shaking conditions at 37°C. After about 3 hrs, EcN were heat killed in a water bath at 65°C for 45 min and then diluted to a concentration of 3×10^8 cells/ml in FCS-free and Pen/Strep-free DMEM cell culture medium (Stebe-Frick et al., 2018).

2.2.3. Nucleic acid methods

2.2.3.1. RNA isolation and cDNA synthesis

RNA isolation from biopsies- cohort and ex vivo

Acidic phenol/chloroform partitioning of RNA into an aqueous supernatant was carried out to isolate RNA from cohort biopsies. For this, TRIzol reagent was used according to the manufacturer's protocol (Life technologies). Frozen biopsies were pestled and TRIzol, containing protein denaturating (including RNAses) guanidinthiocyanate, was added. The acidic phenol dissolves DNA and protein.. In the next step, chlorophorm was added to create different layers. The uppermost aqueous phase containing the RNA was carefully removed into a fresh tube and precipitated with isopropanol. Ethanol washing steps were followed by RNA dissolution in DEPC-H_2O and storage at -80°C.

From *ex vivo* treated biopsies RNA was isolated using the Direct-zol RNA Miniprep Kit (ZymoResearch) according to the manufacturer's protocol. The method is based on the same principles using TRIzol reagent as described above.

RNA isolation from cell culture experiments was done using the RNeasy Mini Kit (QIAgen) or the Quick RNA Miniprep Kit (ZymoResearch) based on the company's protocols. The gained pellet was resolved in 25-50 µl DEPC-H_2O and stored at -80°C.

RNA quantification and quality control

RNA quantity was determined by a NanoDrop spectrophotometer. UV light (λ=260 nm) stimulation of RNA samples leads to some absorption of the light by the nucleic acids, proportional to their quantity which is shown in OD. Furthermore, possible contamination can be detected due to the fact that proteins, phenol and other components absorb λ=280 nm. To determine contaminations the ratio of 260/280 nm for RNA is calculated, which should be around 2.0 for RNA.

To ensure good RNA quality for real-time PCR, it was evaluated using the Agilent RNA 600 nano kit and the Agilent 2100 bioanalyzer (Agilent) according to the manual. 18S and 28S ribosomal RNA bands were analysed to assure that the RNA was not degraded. Samples with degraded RNA were excluded from the study.

Reverse transcription – cDNA synthesis

Since DNA is necessary as a template for real-time PCR, RNA had to be reversely transcribed into cDNA. For biopsy cohort RNA samples, this was performed using the AMV reverse transcriptase kit with oligo-dT primers to provide amplification only of mRNA by using the polyA$^+$-tail as template. For cell culture and *ex vivo* treated biopsies RNA the iScript™ cDNA Synthesis Kit (Bio-Rad) was utilized following manufacturer instructions. A final concentration of 10 ng/µl RNA was used as template in each experimental setup.

2.2.3.2. Cloning (TA cloning, primer design)

Real-time PCR analyses (based on SYBR Green) were performed in absolute quantification. Specific primers targeting the desired DNA sequence and plasmids had to be generated. Primers were designed using Geneious software and NCBI Primer Blast to ensure there were no off-target sequences and to receive primer locations spanning exons and about 20 bp leading to an amplification of a product of between 80 and 250 bp. All primers were double checked and their sequences are specified in Table 1.

Construction of plasmid standards for absolute mRNA quantification of specific products was achieved using the TA cloning technique according to TOPO TA Cloning Kit procedures. Therefore, designed primers were utilized in a PCR reaction amplifying cDNA from human intestinal tissue. Achieved amplicons with TA-overhangs created by the HotStarTaq DNA polymerase were separated by an agarose gel, purified with the QIAquick PCR purification kit and ligated into an open pCR 2.1-TOPO-Vector, which has thymidine (T') and adenine (A') overhangs for simple ligation. The ligated product was transformed into competent Top10 *E. coli* by a 45 second heat-shock and plated on an agar plate coated with ampicillin and X-Gal. Bacteria containing the plasmid plus the insert exhibit a white phenotype and were picked for further cultivation in LB-media. Purification of plasmids was carried out using the QIAprep spin Miniprep Kit and verification was achieved by a control digestion using the EcoRI restriction enzyme. All plasmids were then controlled by sequencing using the Big Dye terminator 3.1 kit based on the the chain-terminator method according to Sanger (Sanger et al., 1977) in an ABI 3500 DX sequencer according to manufacturer's protocol.

2.2.3.3. Real-time PCR

The achieved plasmid standards described above were used for quantitative real-time PCR measurements. Quantification of specific DNA molecules is achieved by adding the fluorescent dye SYBR Green that can intercalate with double-stranded DNA and the measurement of the melting curves. In each PCR cycle, emitted fluorescence is determined which rises with the increasing amount newly synthesized amplicons creating a direct proportionality between fluorescence and the amount of target in the samples. Standard DNA plasmid samples whose concentration was determined at a NanoDrop spectrophotometer with the appropriate insert

were used for absolute quantification, as they allowed the generation of a calibration curve. For this, plasmid standards were diluted from 1ng in a 1:10 manner till a concentration of 10-7ng was achieved. Real-time PCR was performed using indicated primers specified in Table 1 and measured in a Roche Light Cycler 480 as given in the manufacturer's protocol. Thereby the SYBR® Green master mix contains all PCR components plus the SYBR Green dye. In general, the PCR started with a first denaturation step and subsequently 40 amplification cycles. The annealing temperature was specific for each assay and PCR details are specified in Table 4. Each run was analyzed for efficiency and quality using the Light Cycler software.

Table 4: Real-Time PCR program

Target	Denaturation	Amplification	Melting curve
IL-8; Ki67;	96°C – 5 min.	96°C – 10 s **62°C** – 5 s 72°C – 10 s	95°C – 1 s 60°C – 15 s 95°C – ∞
hBD2	96°C – 5 min.	95°C – 10 s **60°C** – 5 s 72°C – 10 s	95°C – 1 s 58°C – 15 s 95°C – ∞
HDAC1, HDAC3, HDAC8; hBD1	97°C – 5 min.	96°C – 10 s **62°C** – 5 s 72°C – 10 s	95°C – 1 s 60°C – 15 s 99°C – ∞
HDAC2	96°C – 5 min.	96°C – 10 s **60°C** – 5 s 72°C – 10 s	95°C – 1 s 58°C – 15 s 95°C – ∞
ß-Actin	95°C – 10 min.	95°C – 15 s **60°C** – 5 s 72°C – 10 s	95°C – 1 s 65°C – 15 s 95°C – ∞

2.2.4. Protein methods

2.2.4.1. LDH test

The viability of treated biopsies was tested via their supernatants using lactate dehydrogenase (LDH) -ELISA (Roche) according to manufacturer's protocol. Lactate dehydrogenase (LDH) is a soluble, cytosolic enzyme which is released by every dying cell whose membranes lose their integrity during the process of apoptosis or necrosis. This way, LDH concentration in cell or tissue culture supernatants can be used as a marker for cell death. The cytotoxicity detection kit (LDH) from Roche serves to measure a colorimetric change happening when LDH reduces NAD^+. Here, the supernatants of all treated biopsies were tested and measured in a plate reader. DMEM culture media was the negative control and biopsies treated with 2% TritonX served as positive controls. Samples with LDH values higher than 200 at 37° after 15 mins were excluded from the study.

2.2.4.2. Immunohistochemistry

Immunohistochemical stainings were performed based on the EnVision™ technique by Dako according to their protocol.

Class I HDACs were detected in ileal (n=29) and colonic (n=23) biopsy tissue from patients with Crohn's disease (ileum n=12, colon n=13) or ulcerative colitis (ileum n=10, colon n=7) and from healthy controls (ileum n=7, colon n=3).

Dewaxing, antigen retrieval and endogenous blocking, was performed according to standard protocols. Descending alcohol concentrations, a 30 min steam treatment in target retrieval solution was used at pH 6 (S1699, 1:10; Dako) for HDAC2 and HDAC3 and at pH 9 for HDAC1 and HDAC8. Furthermore, an endogenous peroxidase blocking solution (S2023, Dako), was applied Primary antibodies were used in the following dilutions at 4 °C overnight: 1:500 (HDAC1), 1:10.000 (HDAC2), 1:250 (HDAC3), and 1:10 (HDAC8), all in antibody diluent (S2022, Dako). After washing with TBST, a horse reddish peroxidase (HRP) conjugated secondary antibody was applied for 30 min at room temperature. Detection of the secondary antibody was accomplished using the kit DAB solution. Counterstaining of cell nucleii was done using hematoxylin Gill II 25 % for 7 seconds.

Ex vivo biopsy tissue integrity after cultivation was evaluated via a hematoxylin and eosin staining of biopsy sections. Tissue slides were 7 µm thick and used for standard staining procedures with hematoxylin for 7 mins and eosin for 3 mins.

2.2.4.3. Protein quantification

To measure the amount of hBD2 protein in CaCo2/TC7 cell lysates, an enzyme-linked immunosorbent assay (Human Beta-Defensin2 (hBD2) ELISA Kit; Phoenix Pharmaceuticals, USA) was performed according to the manufacturer's protocol. For this, cells were pelleted at 4°C and washed twice with ice cold PBS. Pellets were shock-frozen in liquid nitrogen until final usage. Lysis of cells was achieved by adding 30-50 µl of whole protein lysis buffer containing a protein inhibitor cocktail (PIC) to the pellet, incubating for 30 mins on ice under occasional vortexing. After that, lysed cells were centrifuged at 4 °C/10000g for 25 mins. Protein concentration was determined out of the supernatants using the Bradford method (Stebe-Frick et al., 2018).

2.2.4.4. Western blotting

Western blotting of intracellular proteins to determine the relative amounts of class I HDACs in human intestinal tissue of patients with IBD and healthy controls and to investigate the level of acetylation at histone level after usage of HDACi in CaCo2/TC7 cells was done with and by the Lab of Dr. Dr. Sascha Venturelli, Tübingen. For the description of the method, also see (Venturelli et al., 2013). Cellular proteins were separated on 10% SDS-polyacrylamide gels (for the detection of HDAC1, HDAC2, HDAC3, HDAC8 and vinculin as control) or 12% SDS-

polyacrylamide gels (for the detection of H3, acetylated H3 and vinculin) under reducing conditions and then transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked for 1 h in Tris-buffered saline (TBS, 150 mmol/l NaCl, 13 mmol/l Tris, pH 7.5) containing 5% non-fat dry milk powder. Next, the membranes were incubated with anti-vinculin (1∶5,000, Sigma-Aldrich, Germany), anti-HDAC1(1:500, Abcam plc, Cambridge, UK), anti-HDAC2 (1:10,000, Abcam plc), anti-HDAC3 (1:1000, Abcam plc), anti-HDAC8 (1:200, Santa Cruz Biotechnology, Dallas, USA), anti-H3 (1:10,000, Active Motif, La Hulpe, Belgium) anti-acetyl-histone H3 (1∶8,000, Millipore, USA) overnight at 4°C, then washed three times with TBS-T (TBS containing Tween 20) and incubated with peroxidase-conjugated anti-rabbit (1∶8,000, Bio Rad, USA) or anti-mouse (1∶8,000, Bio Rad) for 45 min. Membranes were washed three times in TBS-T and further detection was performed by the ECL Western blotting detection system on Hyperfilm-ECL (Amersham Biosciences). Densitometrical analysis of obtained bands was done with a ChemiDoc™ MP Imager (BioRad) using associated Software Version 5.1. To obtain images, membranes were scanned using a Lexmark CX510de office scanner.

2.2.5. Microscopy

Examination of stained tissue slides was carried out with an BX63 microscope. Picture acquisition was done using a DP80 camera and the imaging software cellSens Dimension.

Semi-quantitative analysis of HDAC1 protein expression on immunohistochemically stained ileal tissue was analyzed blinded. The approximate number of positively stained cells in either the crypt or the villus areas of was estimated separately (in %) by four different individuals.

2.2.6. Statistical methods

Statistical evaluation was done using the GraphPad Prism software. To examine the power of observed differences between groups, several statistical tests were performed. Testing of differences between two groups was done using non-parametric tests. Not normally distributed data was subjected to a Mann-Whitney test. Unpaired t-tests were conducted when data showed a Gaussian distribution. Fold changes, normalized to control treatment, were analyzed by Wilcoxon signed rank tests or Mann-Whitney tests as indicated in the corresponding figure legends. Shown are mean values + sem unless indicated otherwise.

P-values showing the statistical significance were displayed by asterisks:

$p>0.05$ = ns; $p\leq0.05$ = *; $p\leq0.01$ = **; $p\leq0.001$ = ***; $p<0.0001$ = ****

3 Results

3.1. Differential expression of class I histone deacetylases in the gastrointestinal tract

The current understanding of IBD pathology includes genetic susceptibilities (Schreiber et al., 2005; Halfvarson et al., 2006; Jostins et al., 2012; McGovern et al., 2015) as well as environmental influences driving intestinal inflammation into a chronically activated status. Such environmental influences can be of microbiological nature or be derived from an individuals life style, hygene standards, dietary habits, or antibiotics use (Legaki and Gazouli, 2016). The emerging field of epigenetics could dissect the likely present interactions between environment and genome in the context of IBD and would allow a better understanding of disease pathology (Fogel et al., 2017; Legaki and Gazouli, 2016; Scarpa and Stylianou, 2012; Ventham et al., 2013). While previously a link between intestinal homeostasis, gut inflammation and HDAC function has been established, so far mainly animal studies have been done to investigate a potential role in IBD like settings (Felice et al., 2014; Glauben and Siegmund, 2011). HDACs are increasingly recognized as key factors in regulating tissue homeostasis via epigenetic control of gene expression (Alenghat et al., 2013; Turgeon et al., 2013). In the context of chronic intestinal inflammation, a role of HDAC activity has been suggested in murine models of colitis (Glauben et al., 2006; Turgeon et al., 2014). The current body of knowledge points out an important role for class I HDACs. However, so far not much is known about their expression status in IBD patients. Therefore, the following strategic analysis of class I HDAC expression in active as well as inactive IBD allows for some first insights into the overall deacetylation capacity of the intestinal tissue in IBD based on their inflammatory status, as well as in comparison to and within overall healthy tissue.

3.1.1. Class I HDACs in the ileum and colon of healthy controls and patients with inflammatory bowel diseases

Class I HDACs, being comprised of HDAC1, 2, 3 and 8, have been investigated. For this, mRNA expression levels were determined via quantitative real-time PCR in ileal and colonic samples of healthy controls (n=24) as well as patients with active and inactive IBD including ileal CD (L1 n=28), ileocolonic CD (L3 n=32), colonic CD (L2 n=22), and ulcerative colitis (UC) (n= 45). Furthermore, western blotting was used to analyze protein levels. Immunohistochemistry

served to gain insight into the location and distribution of specific HDACs in intestinal tissue (Figure 4).

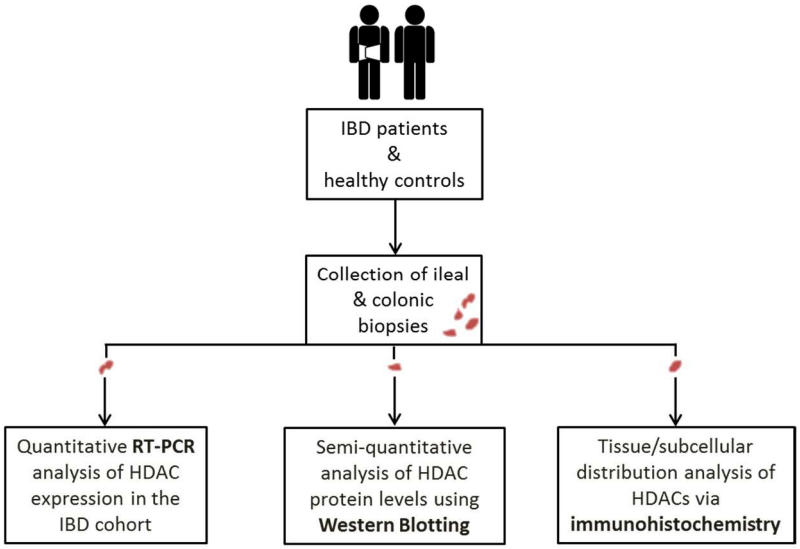

Figure 4: Experimental setup for the investigation of Class I HDAC expression in IBD
Ileal and colonic biopsies were collected from healthy individuals and patients with either a manifestation of CD or UC constituting the IBD cohort from which RT-PCR analysis was conducted. Several biopsies out of this cohort were processed to isolate whole protein to run western blot analyses. Additional biopsies were collected and subjected to immunohistochemical stainings.

3.1.1.1. mRNA analyses

First, the overall expression levels of HDAC1, 2, 3 and 8 were analyzed in ileal and colonic tissue of healthy individuals. A direct comparison of these levels can be seen in Figure 5. HDAC2 seems to be the most abundant class I HDAC in terms of mRNA expression levels in both ileum and colon with around 45.000 to 70.000 transcripts. HDAC8 shows the lowest abundancy in both tissues.

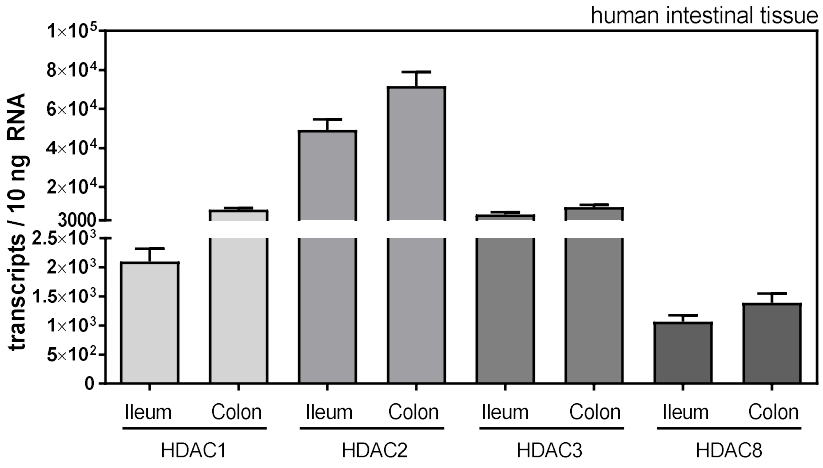

Figure 5: Class I HDAC expression in healthy human intestinal tissue

Class I HDAC mRNA expression in human ileal (n=25) and colonic (n=24) tissue of healthy controls determined by quantitative RT-PCR. Shown are absolute transcript levels.

Clear mRNA expression differences between inflamed and non-inflamed patient samples as well as in comparison to healthy controls became apparent. The strongest effects on the mRNA level were seen for HDAC2 in ileal tissue of CD and for HDAC1 and 2 in colonic tissue of CD as well as UC where they were significantly further reduced during inflammation (Figure 6). HDAC3 and HDAC8 were only reduced in UC specimens, in the case of HDAC3 this seemed to be only the case in inflamed samples. HDAC8, however, is diminished independently from inflammation in UC patient samples (Figure 6). Taken together, class I HDACs seem to be differentially expressed in IBD patients, although only reduced statuses were observed on the mRNA level.

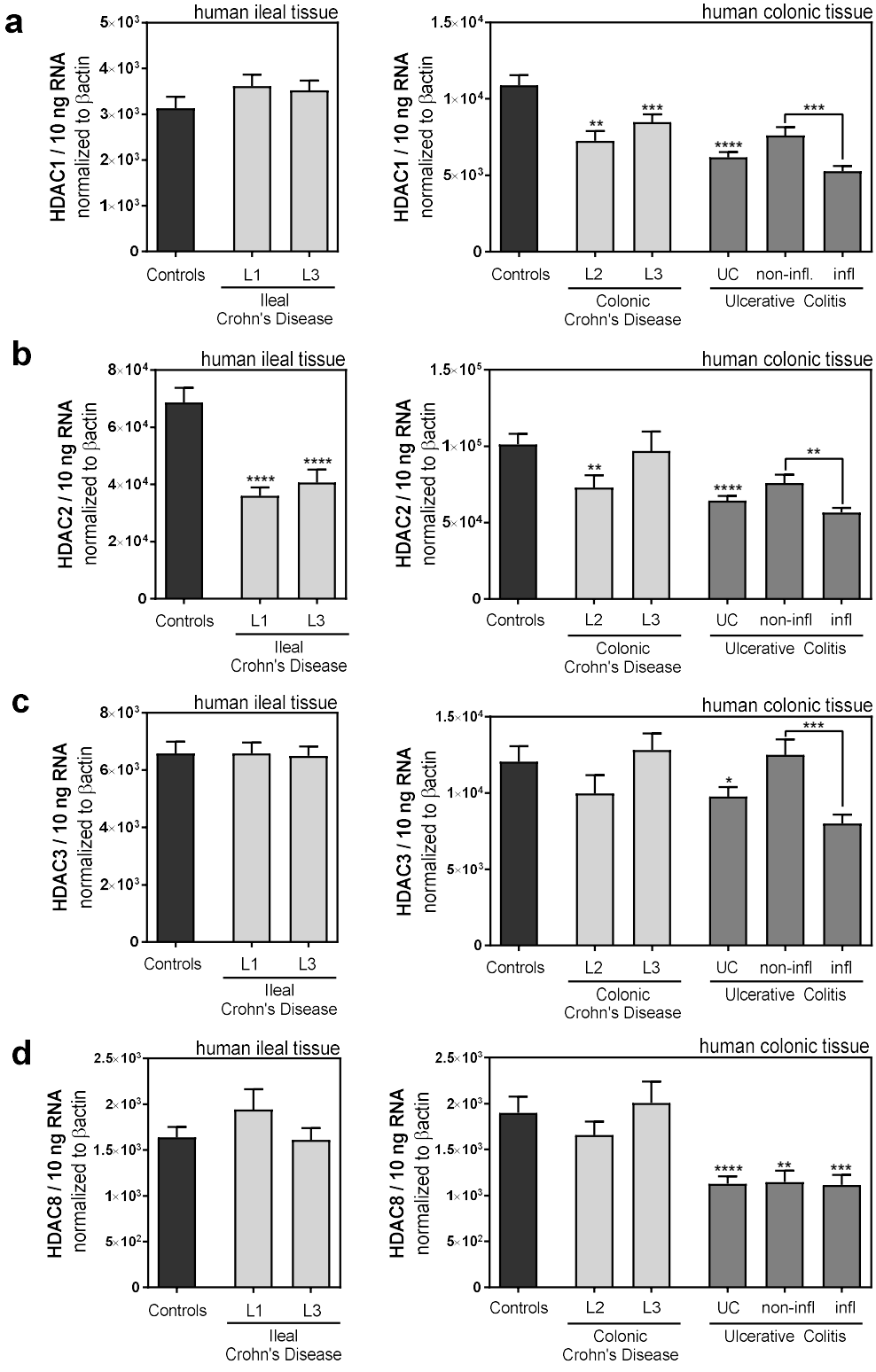

Figure 6: Differential expression of Class I HDAC mRNA in inflammatory bowel diseases

Ileal and colonic biopsies from patients biopsies (n=60 for ileal (L1 n=28; L3 n=32) and n=100 for colonic tissue (L2 n=22; L3 n=33; UC=45, inflamed n=18, UC uninflamed n=27) and controls (n=25 for ileal tissue and n=24 for colonic tissue) were analyzed for Class I HDACs 1 (a), 2 (b), 3.(c) and 8 (d) mRNA expression via RT-PCR. Shown are relative transcript levels. * $p<0.05$, ** $p\leq0.01$, *** $p\leq0.001$, **** $p<0.0001$ evaluated by Mann-Whitney u test.

3.1.1.2. Protein analyses

Statement on collaborative efforts for this chapter

Western blotting of intracellular proteins to determine the relative amounts of class I HDACs in human intestinal tissue of patients with IBD and healthy controls and to investigate the level of acetylation at histone level after usage of HDACi in CaCo2/TC7 cells was done in cooperation with and by the Lab of Dr. Dr. Sascha Venturelli, Tübingen. Patient samples from the Stuttgart IBD cohort were processed by Sabrina Stebe-Frick. Results of the Western blots were discussed and interpreted with the support of Dr. Dr. Sascha Venturelli. Final densitometrical analysis, evaluation and figure preparation was done by Sabrina Stebe-Frick.

Western Blotting

For western blot experiments intestinal biopsies from patients from the IBD cohort had to be used that had not been included in the mRNA analysis. The specimens regarded here have been sampled from further patients that had not been included in the "mRNA cohort".

For western blot analysis of the ileum, five ileal specimens of both healthy controls and uninflamed L3 CD patients were analyzed and evaluated densitometrically. Western blots of HDAC1 and 2 from ileal tissue lysates revealed generally very low levels of HDAC1 in the ileum of controls. In L3 patients without acute inflammation a tendency towards increased protein levels of HDAC 1 and 2 was observed (Figure 7). This is in contrast to the mRNA data where HDAC1 displayed no differences in the ileum of L3 patients. Similarly, HDAC2 mRNA levels were decreased in L3 patient ileum but protein seems to be augmented. HDAC3 protein showed no difference which is in line with the mRNA data (Figure 7, cf. Figure 6). Unfortunately, western blots for the detection of HDAC8 protein were unsuccessful up to date due to technical reasons and a limited amount of protein from the same patients.

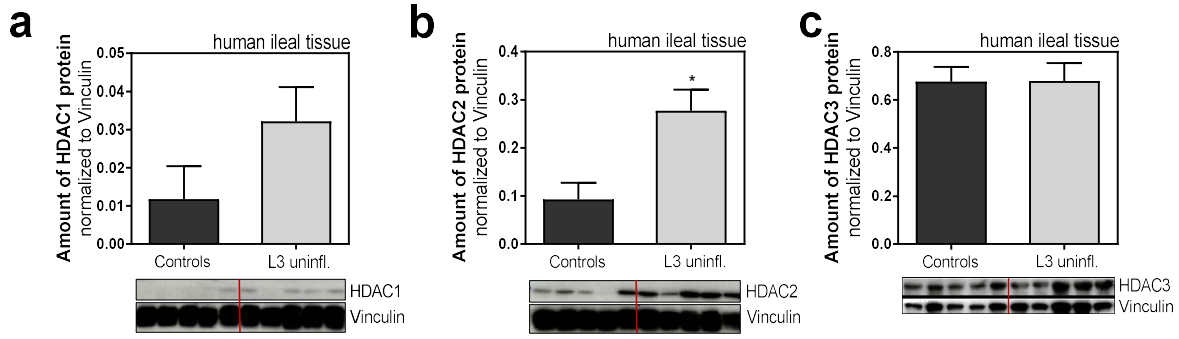

Figure 7: HDAC1, 2 and 3 protein levels in ileal tissue of CD patients

Western blots and corresponding densitometrical analyses of ileal tissue homogenate from healthy controls (n=5) and uninflamed L3 patients (n=5). Each lane on the western blots represents one patient. * $p<0.05$ evaluated by Mann-Whitney u test.

The single sample lanes of the western blots showed some interindividual variations in protein levels, especially for HDAC1 in L3 and HDAC2 in controls and L3 patients. This observation is in line with the growing knowledge on seemingly substantial inter- and even intraindividual variations in epigenotypes (Flanagan et al., 2006; Fraga et al., 2005; Petronis et al., 2003).

Western blots of HDAC1 and 2 from colonic tissue showed no difference between controls and uninflamed IBD patients, neither in L3 nor UC patients (Figure 8). However, a slight tendency towards a reduction for HDAC1 and an increase for HDAC2 in UC patients could be observed. Furthermore, the investigated UC patient samples seemed to have a diminished protein expression of HDAC3 (Figure 8). A fifth UC patient sample has also been tested for HDAC1 and 2 but had to be applied to another blot due to technical reasons and has therefore not been included in the densitometrical analysis. It is of note, however, that no HDAC1 had been detectable in that fifth sample and a similar band for HDAC2 was observed in that fifth patient sample as compared to the other four, showing a consistent trend.

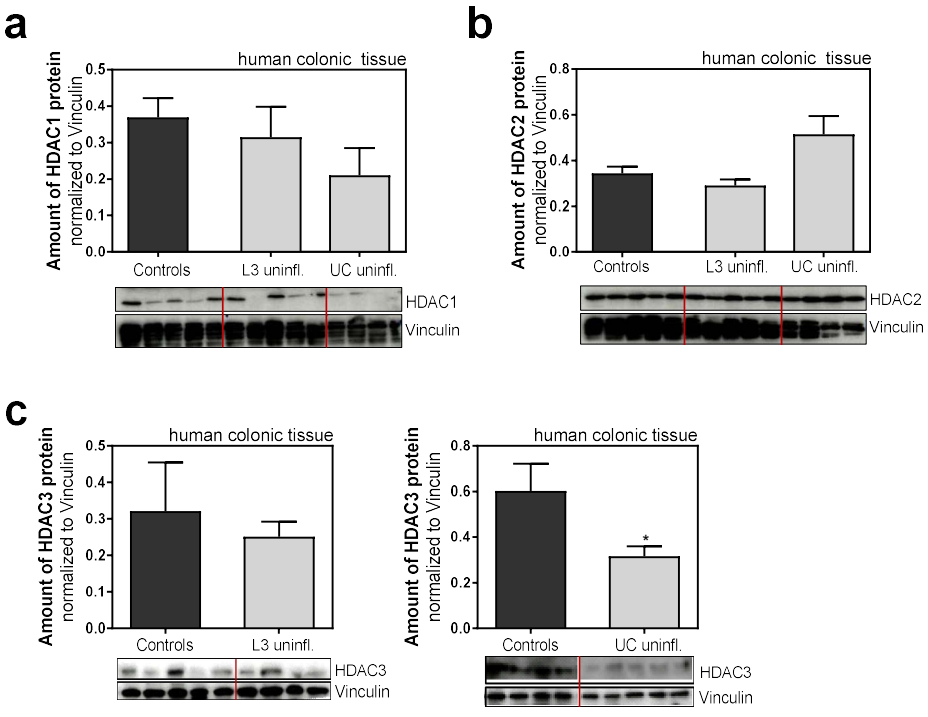

Figure 8: HDAC1, 2 and 3 protein levels in colonic tissue of CD and UC patients

Western blots and corresponding densitometrical analyses of colonic tissue homogenate from healthy controls (n=5, uninflamed L3 patients (n=5 for HDAC1 and 2; n=4 for HDAC3), and uninflamed UC patients (n= 4 for HDAC1 and 2; n=5 for HDAC3). Each lane on the western blots represents one patient. * $p<0.05$ evaluated by Mann-Whitney u test.

The tendencial reduction of HDAC1 and HDAC3 in UC colon is in line with the observed mRNA expressional changes. The slight increase of HDAC2 stands in contrast to the mRNA data (cf. Figure 6). HDAC1 mRNA in L3 patients' colonic tissue was unchanged- so was the protein level.

HDAC2 mRNA was unaltered in L3 patients and so was the protein level; HDAC3 mRNA is unchanged and so was the protein level (Figure 8, cf. Figure 6).

Drawing an interindividual comparison from the colonic specimens, distinct differences in HDAC1 protein levels became apparent within the control group as well as among L3 samples. UC colonic tissue seemed to contain a consistently low amount of HDAC1. HDAC2 showed relatively consistent interindividual levels. In the case of HDAC3, protein levels of controls and L3 patient tissue displayed slightly bigger interindividual variation than UC specimens. All in all, western blot analyses revealed interesting tendencial differences in class I HDAC protein levels between IBD patients and healthy controls as well as in part remarkable interindividual variations.

Immunohistochemistry

To gain further insight into the expression patterns of class I HDACs in intestinal tissue of IBD patients, additional immunohistochemical analyses were performed on ileal as well as colonic mucosal biopsies from healthy controls, uninflamed L3 and uninflamed UC patients. These specimens had to be collected from new patients. Herein, HDAC1 protein was found predominantly in epithelial cells of the small intestine, here with a stronger intensity than in the lamina propria compartment (Figure 9). Interestingly, ileal CD patients showed more HDAC1-positive epithelial cells in the villus compartment as compared to controls (Figure 9c and d (arrows)) where many areas of unstained villus epithelium could be found.

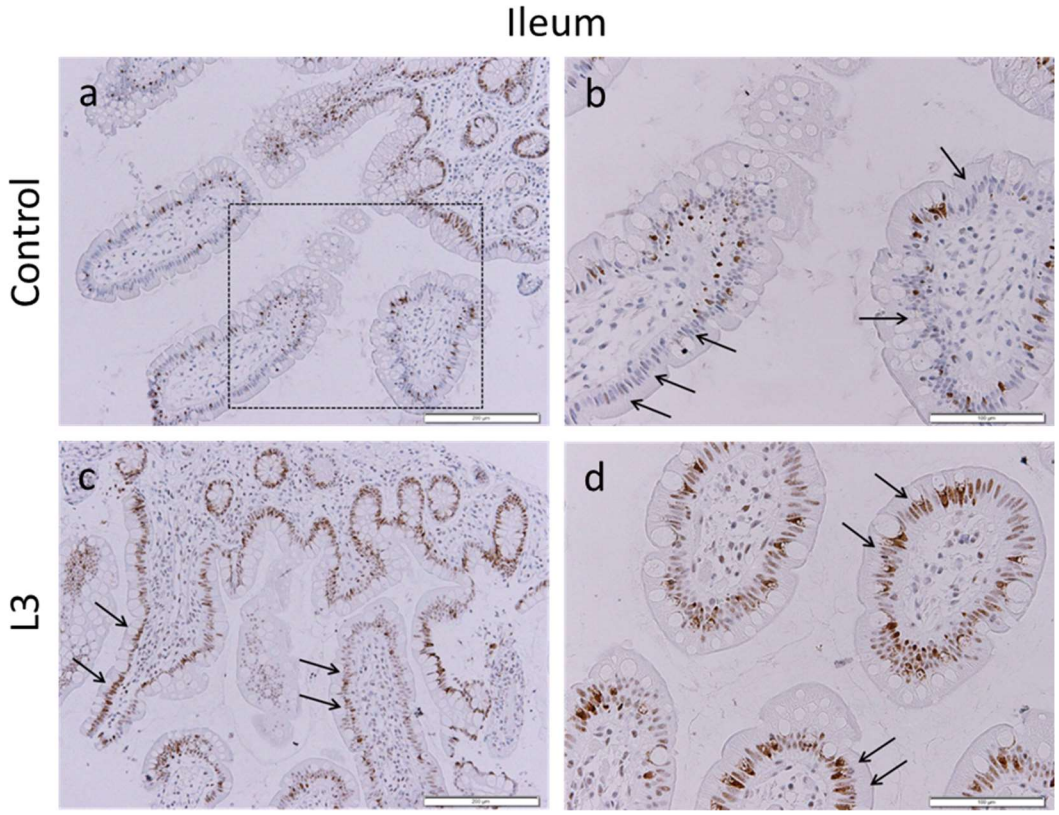

Figure 9: Expression of HDAC1 protein in ileal tissue

a-d Representative immunohistochemical staining of HDAC1 with an HDAC1-specific antibody on sections through ileal biopsies of controls (**a, b**) and patients with L3 Crohn's disease (**c, d**). b higher magnification of the boxed area in **a**. Scale bars **a, c** = 200 µm, **b, d** = 100 µm.

This finding was analyzed quantitatively and blinded by 4 independent individuals and is depicted in Figure 10**Fehler! Verweisquelle konnte nicht gefunden werden.**. The higher number in HDAC1 positive cells in L3 ileal specimen is in line with the findings for this HDAC from the western blot analysis, where it was tendencially increased in CD patients as well (cf. Figure 7).

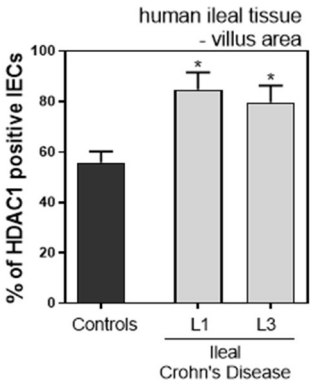

Figure 10: Increased number of HDAC1 positive cells in the villus area of CD ileal tissue

Blinded quantitative analysis by 4 independent individuals of 7 control biopsies, 3 L1 (2 uninflamed samples, 1 inflamed sample), and 8 L3 biopsies (2 inflamed, 6 uninflamed) stained with an HDAC1-spedific antibody. * $p<0.05$ evaluated by Mann-Whitney u test.

As shown in Figure 11, in the large intestine HDAC1 could be found predominantly in the nuclei of epithelial cells with a much stronger staining intensity there than in the lamina propria compartment.

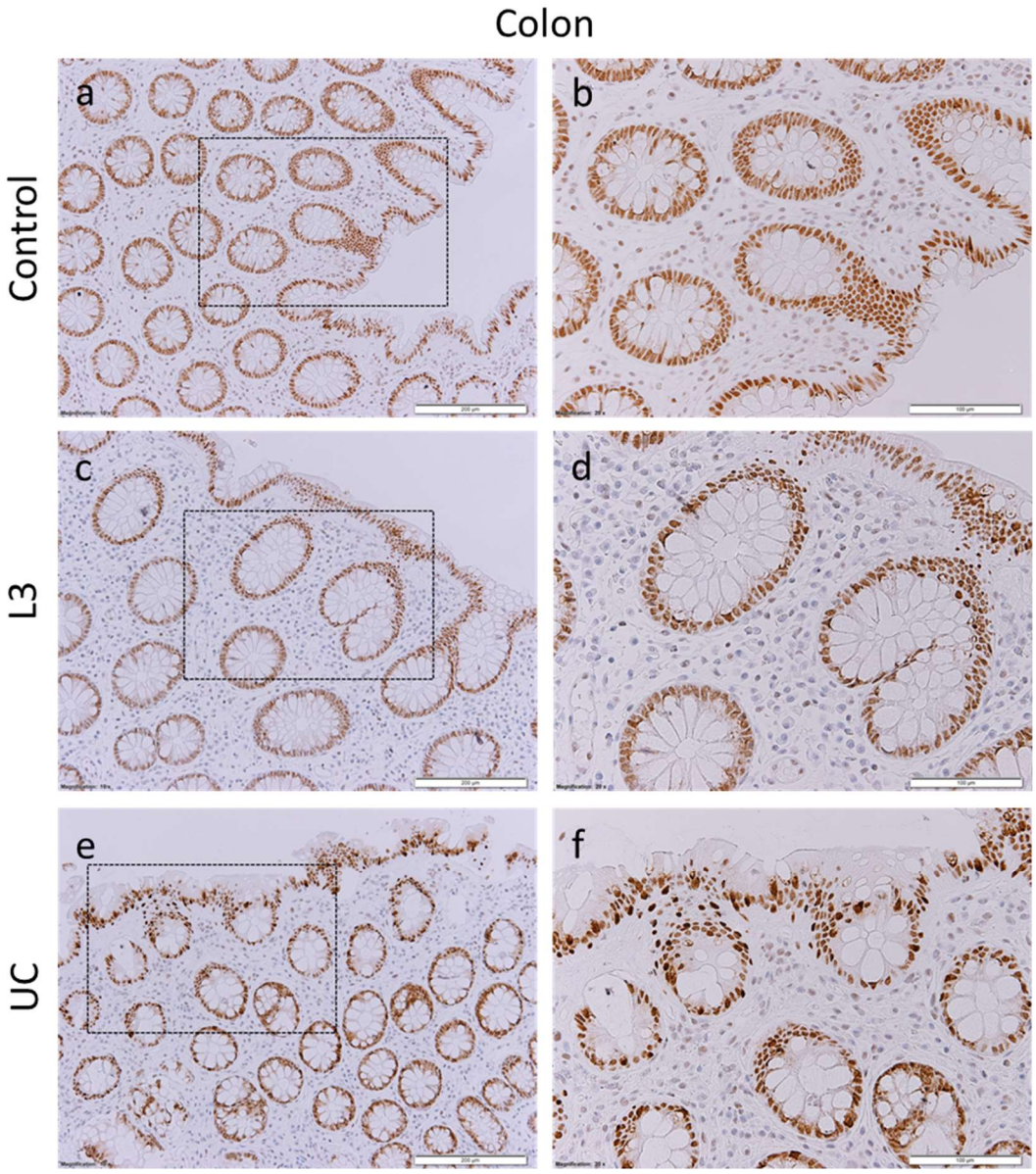

Figure 11: Expression of HDAC1 protein in human colonic tissue

a-d Representative immunohistochemical staining of HDAC1 with an HDAC1-specific antibody on sections through colonic biopsies of controls (**a, b**), patients with L3 Crohn's disease (**c, d**) and patients with ulcerative colitis (**UC**) (**e, f**). **b, d, f** Higher magnification of the boxed areas in **a, c** and **e.** Scale bars **a, c, e** = 200 µm, **b, d, f** = 100 µm.

Interestingly, HDAC2 immunohistochemical staining was found to be equally strong in epithelial and lamina propria cells in the ileal tissue of both healthy controls and L3 CD patients (Figure 12). Furthermore, the staining seemed to be restricted to the nuclei.

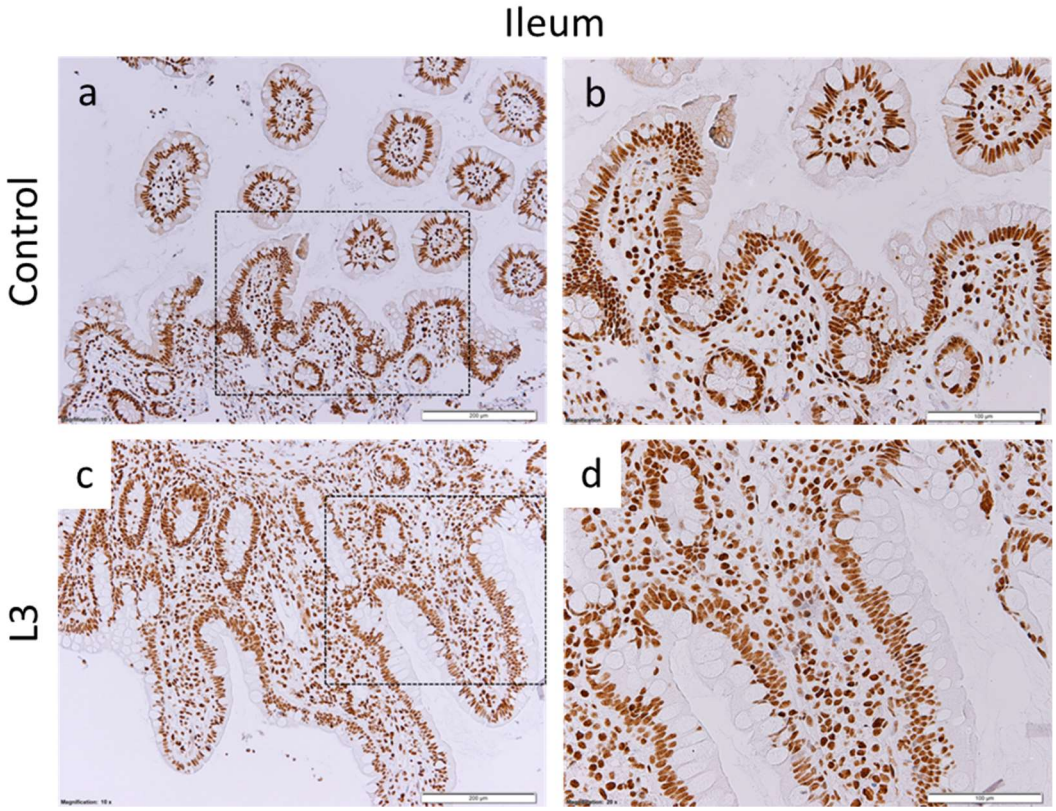

Figure 12: Expression of HDAC2 protein in ileal tissue

a-d Representative immunohistochemical staining of HDAC2 with an HDAC2-specific antibody on sections through ileal biopsies of controls (**a, b**) and patients with L3 Crohn's disease (**c, d**). b and d higher magnification of the boxed areas in **a** and **b.** Scale bars **a, c** = 200 µm, **b, d** = 100 µm.

Very similar results were observed for HDAC2 protein expression in colonic specimens of controls, L3 and UC patients without acute inflammation (Figure 13). In this tissue, the staining was also equally strong in the epithelial cells as in the lamina propria and seemed to be restricted to the nuclei.

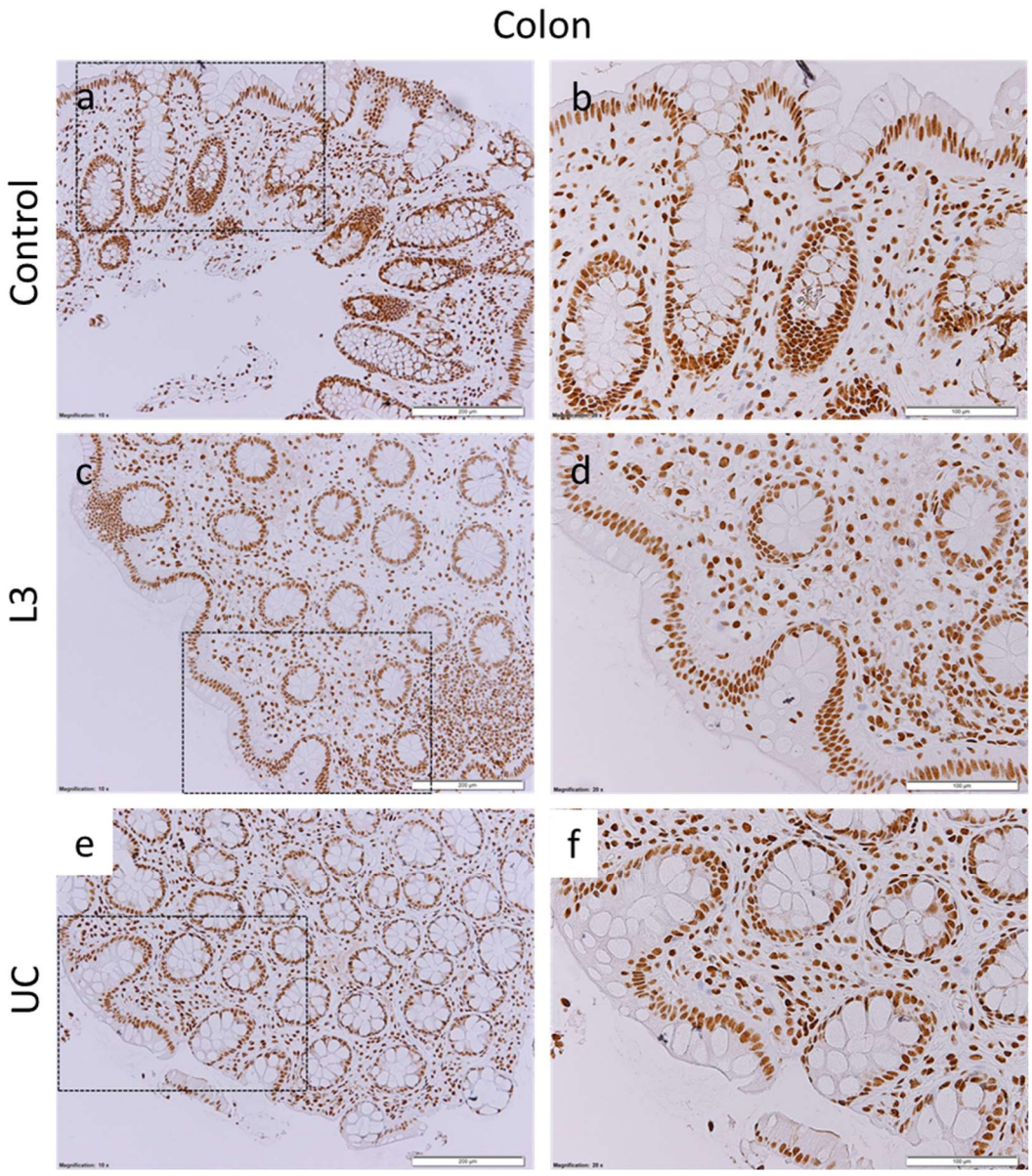

Figure 13: Expression of HDAC2 protein in colonic tissue

a-d Representative immunohistochemical staining of HDAC2 with an HDAC2-specific antibody on sections through colonic biopsies of controls (**a, b**), patients with L3 Crohn's disease (**c, d**) and patients with ulcerative colitis (**UC**) (**e, f**). **b, d, f** Higher magnification of the boxed areas in **a, c** and **e.** Scale bars **a, c, e** = 200 µm, **b, d, f** = 100 µm.

HDAC3 staining in ileal biopsies was also equally strong in epithelial and lamina propria cells, however, apical villi epithelial cells appeared to bear stronger cytoplasmic staining (Figure 14 arrows) as compared to the more basal villi areas (Figure 14 arrowheads). Thereby an

interesting pattern was observed, with the villi showing some sort of graded staining in the cytoplasmatic compartment. Overall, no differences in staining intensity between healthy controls and L3 patients was found (Figure 14).

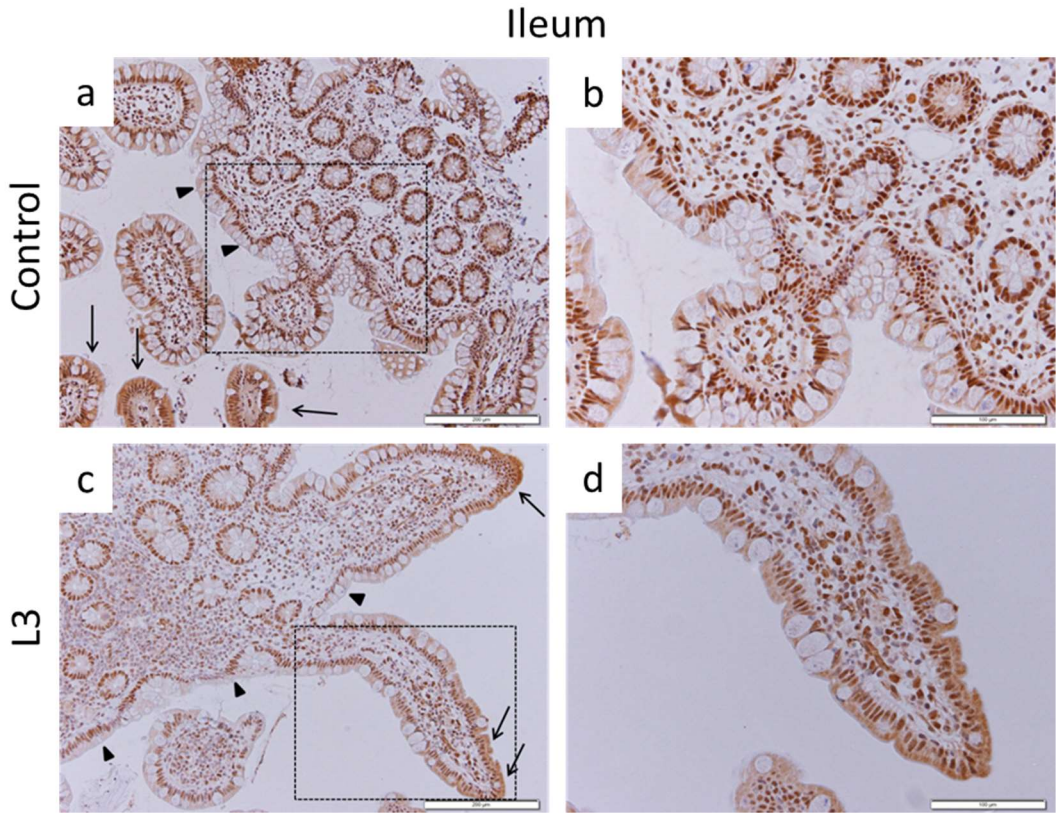

Figure 14: Expression of HDAC3 protein in ileal tissue

a-d Representative immunohistochemical staining of HDAC3 with an HDAC3-specific antibody on sections through ileal biopsies of controls (**a, b**) and patients with L3 Crohn's disease (**c, d**). **b** and **d** higher magnification of the boxed areas in **a** and **b**. Scale bars **a**, **c** = 200 µm, **b**, **d** = 100 µm.

Comparable results were found for HDAC3 staining in colonic biopsies where equally strong intensities were observed in epithelial and lamina propria cells (Figure 15). Again, there seemed to be a prevalent cytoplasmatic staining in the "villus" compartment since the cytoplasm in the colonic crypts was less intensely stained. Taken together, there was no difference in staining intensity between patients and controls.

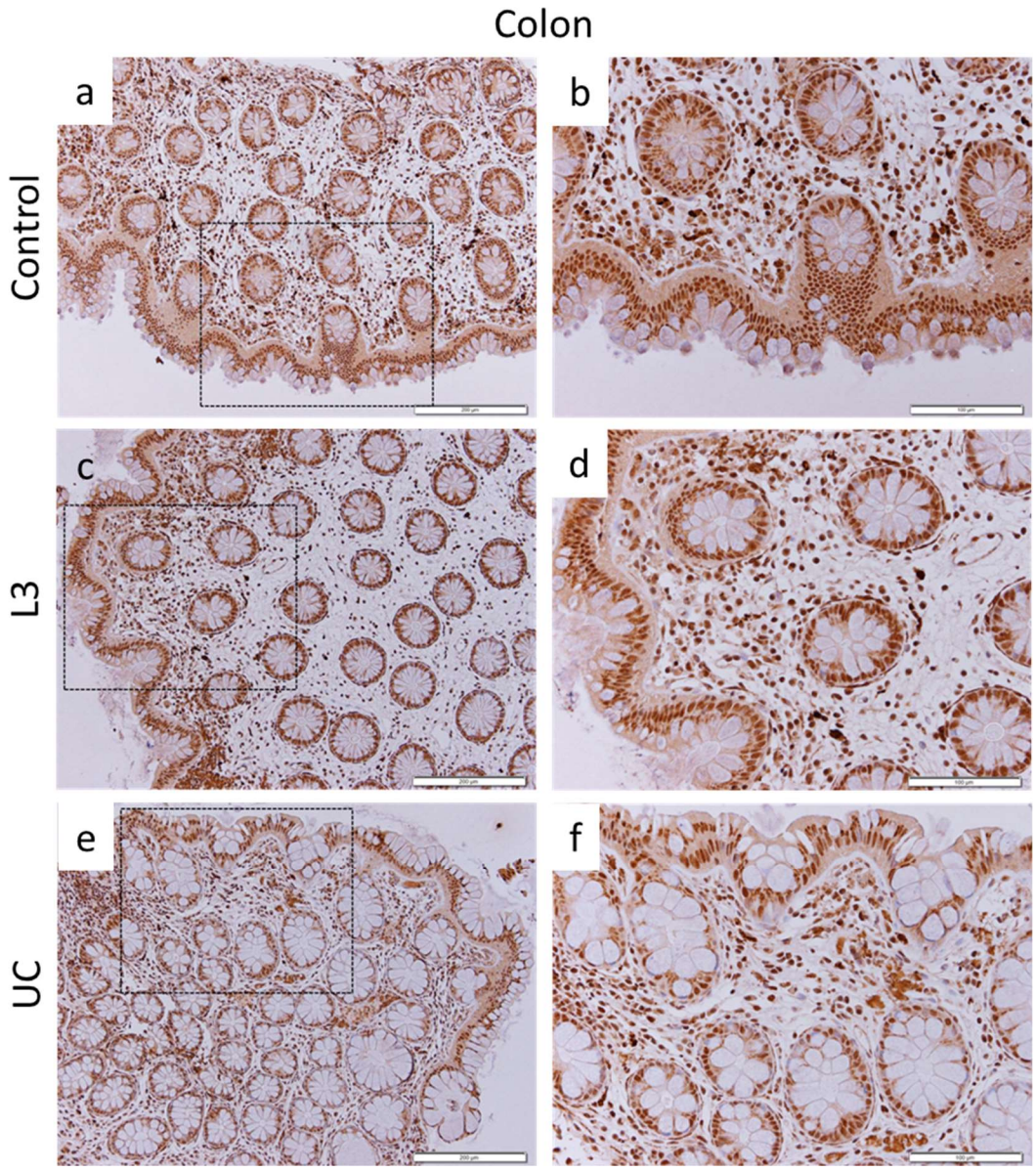

Figure 15: Expression of HDAC3 protein in colonic tissue

a-d Representative immunohistochemical staining of HDAC3 with an HDAC3-specific antibody on sections through colonic biopsies of controls (**a, b**), patients with L3 Crohn's disease (**c, d**) and patients with ulcerative colitis (UC) (**e, f**). **b**, **d**, **f** Higher magnification of the boxed areas in **a, c** and **e**. Scale bars **a**, **c, e** = 200 µm, **b, d**, f = 100 µm.

HDAC8 showed an equal pattern and staining intensity in ileal epithelial and lamina propria cells among biopsies from healthy controls (Figure 16a and b). L3 patients showed a trend towards a generally weaker HDAC8 staining in ileal epithelium and also less HDAC8-positive cells were found in the lamina propria of ileal biopsies from patients with L3 CD (Figure 16c and d).

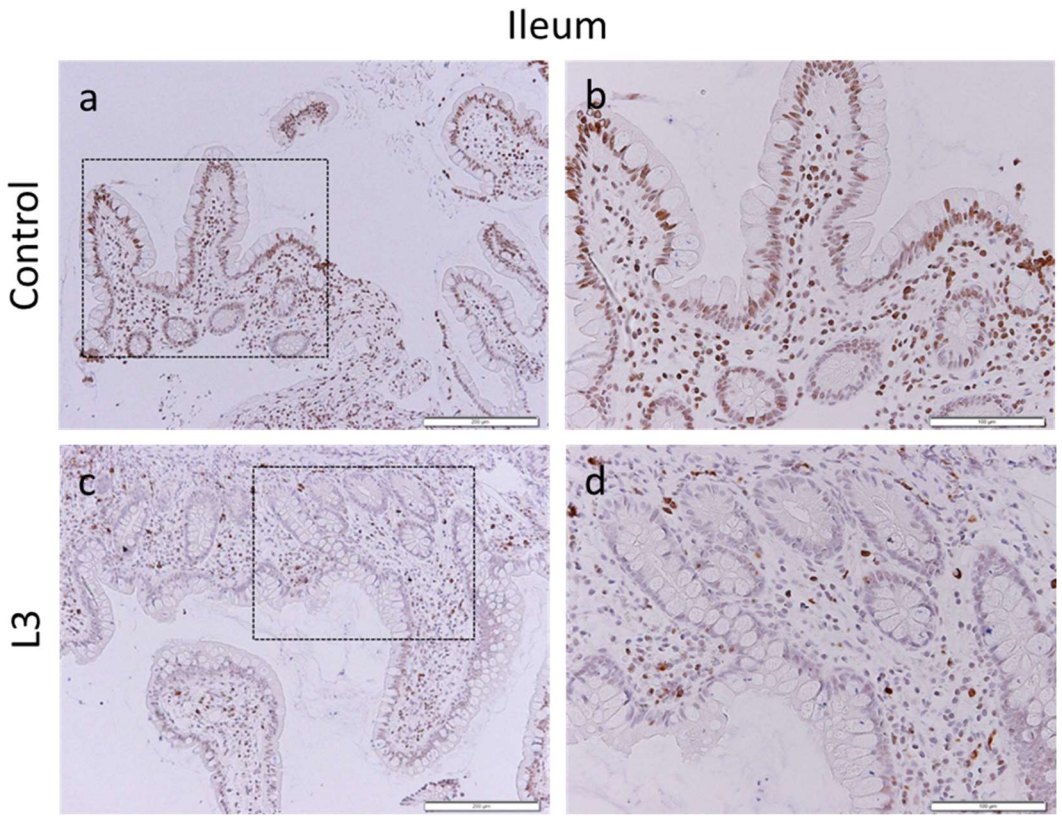

Figure 16: Expression of HDAC8 protein in ileal tissue

a-d Representative immunohistochemical staining of HDAC8 with an HDAC8-specific antibody on sections through ileal biopsies of controls (**a, b**) and patients with L3 Crohn's disease (**c, d**). **b** and **d** higher magnification of the boxed areas in **a** and **b**. Scale bars **a, c** = 200 µm, **b, d** = 100 µm.

HDAC8 staining in colonic biopsies of healthy controls also showed equal patterns and intensity in epithelial and lamina propria cells (Figure 17a and b). In tissue from L3 patients, HDAC8 staining seemed weaker and was partly absent in epithelial cells; in the lamina propria there were less HDAC8 positive cells observed (Figure 17c and d). A similar result was found for UC tissue, especially in the crypts, where the epithelium was particularly faintly stained whereas the villi epithelial cells were more distinctly positive for HDAC8 (Figure 17e and f).

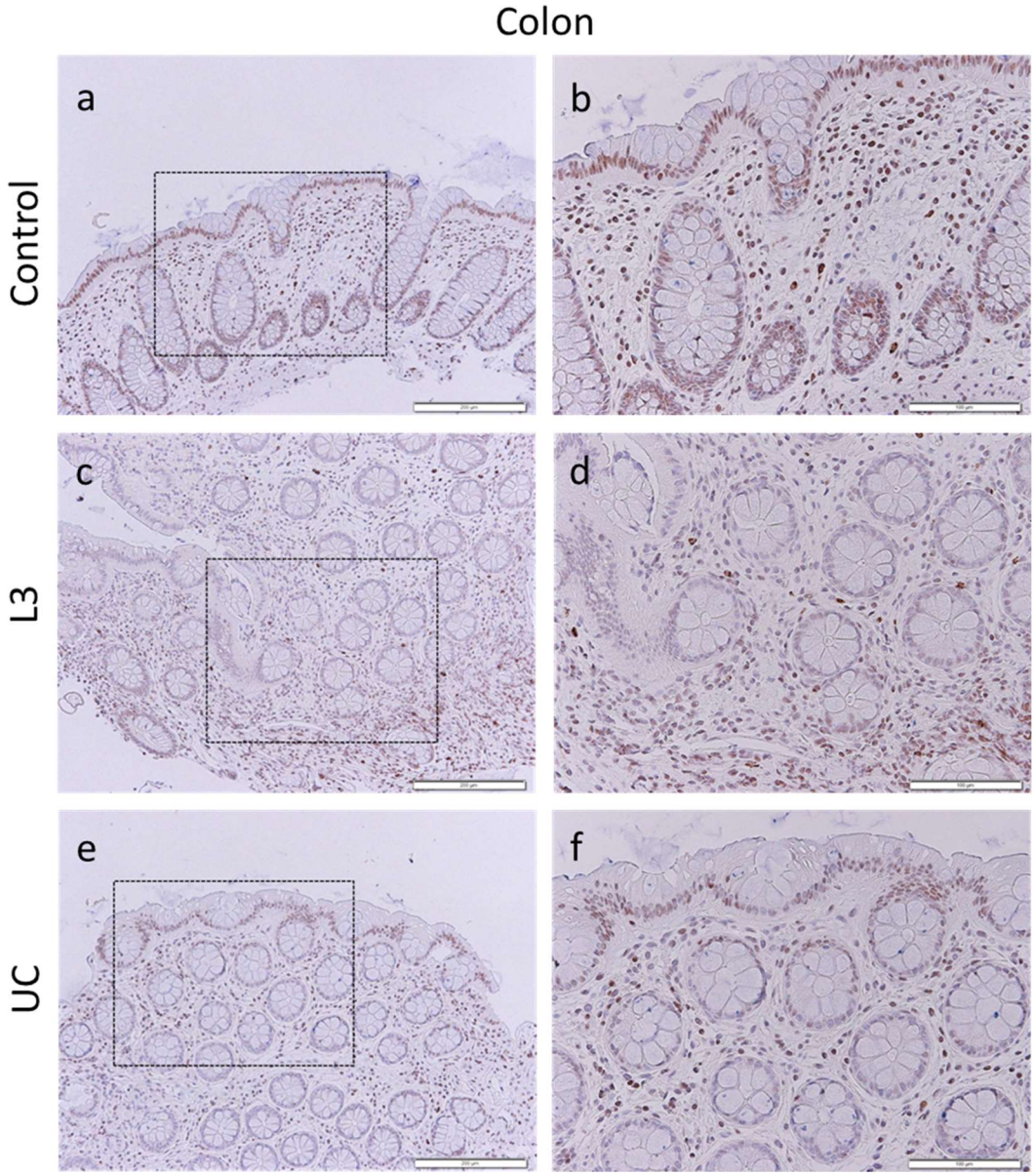

Figure 17: Expression of HDAC8 protein in colonic tissue

a-d Representative immunohistochemical staining of HDAC8 with an HDAC8-specific antibody on sections through colonic biopsies of controls (**a, b**), patients with L3 Crohn's disease (**c, d**) and patients with ulcerative colitis (UC) (**e, f**). **b, d, f** Higher magnification of the boxed areas in **a, c** and **e.** Scale bars **a, c, e** = 200 µm, **b, d, f** = 100 µm.

Overall, quite distinctive staining patterns could be detected for the different class I HDACs in intestinal tissue sections from IBD patients and healthy individuals even though no major differences could be determined between diseased and healthy tissue via immunohistochemistry. Staining was found in the nuclei with exception for HDAC3 which showed some cytoplasmic staining especially in the villus or more apical areas of the mucosa. This localization of class I HDACs to the nuclei is in line with the literature (Karagiannis and Ververis, 2012; Khochbin et al., 2001). In general, the distribution patterns of the single HDACs seemed to be quite similar for both ileal and colonic mucosal tissue. Of note, HDAC1 showed increased protein staining in the villi of CD patients and seemed to be quite restricted to the epithelium. Whereas the other class I HDACs, especially HDAC2 and 3 could be equally found in epithelial and lamina propria cells.

Comparing the findings for mRNA levels and protein from those first western blot experiments, in uninflamed L3 ileum, a possible slightly negative correspondence for HDAC1 and an even stronger negative one for HDAC2 mRNA and protein became apparent. HDAC1 and 2 protein seems to be increased whereas mRNA is unchanged, or even reduced as for HDAC2. HDAC3 protein seems to correspond positively to the amount of its mRNA- no changes in mRNA or protein level could be detected. In L3 colon, HDAC1 shows reduced mRNA but protein is rather unchanged. HDAC2 and 3 in L3 are possibly corresponding positively. In the uninflamed colon of UC patients, HDAC1 and 3 might correspond positively, whereas HDAC2 mRNA and protein levels are rather opposed to each other. In general, these findings show there seem to be rather minor changes in HDAC protein levels with a tendency towards stabilization, despite reduced mRNA levels in IBD intestinal tissue, especially in UC. It has to be kept in mind that mRNA data and protein results came from separate biopsies of different patients and can therefore be affected by possible interindividual epigenetic variabilities. Despite this limitation, a valid rationale for these investigations was still given, since these are the first-time systematic results on differential HDAC expression in human tissue samples of IBD patients. They contribute to opening up promising new research avenues regarding epigenetic involvement into the disease pathology and potentially pinpoint new targets for therapeutic intervention.

Parts of the thesis have previously been published in:

Stebe-Frick, S., Ostaff, M.J., Stange, E.F., Malek, N.P., and Wehkamp, J. (2018). Histone deacetylase-mediated regulation of the antimicrobial peptide hBD2 differs in intestinal cell lines and cultured tissue. Sci. Rep. 8, 12886.

Herein, S.S.-F. and M.J.O. contributed equally. S.S.-F. performed the experiments; S.S.-F. and M.J.O. analyzed the data and interpreted the results. M.J.O. drafted the project. S.S.-F., M.J.O. and J.W. designed the study and wrote the manuscript. E.F.S. and N.P.M. were involved in discussion and finalization of the manuscript.

3.2. HDACs in the regulation of human β-defensin 2

HBD2 is an inducible innate antimicrobial molecule present in epithelia (Harder et al., 2000; Schröder and Harder, 1999), but also in immune cells (Duits et al., 2002; Wah et al., 2006; Yin et al., 2010). Microbial and/or inflammatory stimuli lead to its upregulation and it has a crucial role in defending the host against infections. Interestingly, hBD2 has been found to be upregulated in the epithelium of the colon in response to probiotic bacteria (Möndel et al., 2009; Wehkamp et al., 2004a), which has been suggested to be one of the mechanisms via which probiotics bolster gut barrier function. It might also be why they show efficiency in remission maintenance in ulcerative colitis (UC) (Mack, 2011). The colonic subgroup of Crohn's disease have been associated with an attenuated induction of epithelial hBD2 (Wehkamp et al., 2003, 2008). A lower inducibility of this important defense molecule might partly explain why these patients are prone to chronic inflammation. It might furthermore be one reason why, different from UC patients, CD patients do not seem to benefit from treatment with probiotics during remission (Mack, 2011). While genetic studies are still unsatisfactory in explaining the changes in colonic defensin expression, several potential hints on an involvement of epigenetics in human beta-defensin regulation have been gained in non IBD related studies. One epigenetic regulatory mechanism might be implemented by HDACs. An influence of oral microbiota on the epigenetic regulation of hBD2 in connection with changes in class I HDACs 1 and 2 expression has been observed in gingival epithelial cells (Yin and Chung, 2011). Furthermore, Kallsen and colleagues demonstrated an involvement of HDAC1 in the transcriptional control of hBD1 in human lung epithelial cells (Kallsen et al. 2012). In addition to these findings, *in vitro* experiments could also show that food derived compounds with inhibitory effects on histone deacetylation processes can induce hBD2 expression in intestinal epithelial cell lines (Schwab et al. 2008). Furthermore, a recent study from Fischer et al. - that has been conducted simultaneously to this study - used colonic epithelial cell culture as well as primary cell derived organoids to demonstrate a role of HDACs in hBD2 regulation. Their data also showed that HDACi can augment hBD2 expression in response to *E.coli* K12 (Fischer et al., 2016). Taken together, these studies point towards an

importance of HDAC function in the regulation of hBDs and thereby antimicrobial defense. Consequently, they could also be involved in the hBD related defects observed in colonic IBD. As mentioned above, HDAC inhibitor use has been proposed as an IBD intervention (Felice et al., 2014; Fischer et al., 2016; Glauben and Siegmund, 2011), hence, further detailed studies on the exact role of HDACs in human gut barrier function are needed. Therefore, herein the role of HDACs in regulating hBD2 inducibility has been investigated using *ex vivo* cultured colonic biopsies serving as a more *in vivo* like approach (Stebe-Frick et al., 2018).

3.2.1. Human colonic biopsy culture

Up to now, most studies on the effect of HDACi on hBD2 inducibility and transcriptional regulation have been carried out in mono-layered cell lines *in vitro* (Fischer et al., 2016; Schwab et al., 2008). A peril of using cancerous epithelial cell lines is, however, that HDACs are likely overexpressed and otherwise deregulated in cancers (Ishihama et al., 2007; Marks et al., 2001; Wilson et al., 2006; Yang et al., 2014). This could potentially translate into diverging responses that likely differ from the reactivity in healthy non-tumorous tissue (Stebe-Frick et al., 2018). A human colonic tissue culture has been established allowing the integration of the 'multicellular-tissue-context' into the study. This biopsy culture can survive in a culture medium nearly identical to the one which has also been used for the CaCo2/TC7 assays described in the following chapter to achieve optimal comparability. Stimulation with the probiotic bacterium EcN and concurrent treatment with the HDAC inhibitors SAHA, MS-275 and SB was done exactly as in the *in vitro* setting of this study at hand.

The colonic biopsy culture has been established based on a setting that has been utilized recently by our working group to study small intestinal innate immune regulation (Courth et al., 2015). After collection during routine colonoscopy, tissue samples from healthy individuals as well as IBD patients' colonic mucosa were washed thoroughly with ice-cold PBS containing 10% antibiotics to remove remaining bacteria. Specimens were then submitted to treatment for a total of 20 hrs at 37 °C with 5% CO_2; thereof 20 hrs with different compounds of HDACi (suberoylanilide hydroxamid acid (SAHA), Pyridin-3-ylmethyl *N*-[[4-[(2-aminophenyl)carbamoyl]phenyl]methyl] carbamate (MS-275), or sodium-butyrate (SB)). For MS-275, 5 µM not 3 µM were used as in the *in vitro* experiments (cf. 3.2.2 Cell culture functional studies on hBD2 inducibility). After 2 hrs of preincubation with the HDACi, start of stimulation with heat-inactivated EcN was started and carried out for the following 18 hrs in parallel to the HDACi. After that, samples were transferred into RNAlater until RNA isolation took place. Obtained RNA underwent a RNA quality check via Agilent; specimens with degraded RNA were rejected. Furthermore, LDH assays were conducted to investigate the viability of the tissue. No negative effects were observed on the LDH level neither by EcN nor the different inhibitors,

specimen with high LDH values (compared to a TritonX-treated positive control) were excluded from the study. As shown by histochemical analyses using HE staining, the tissue was affected but still intact after 20 hrs of cultivation (Figure 18).

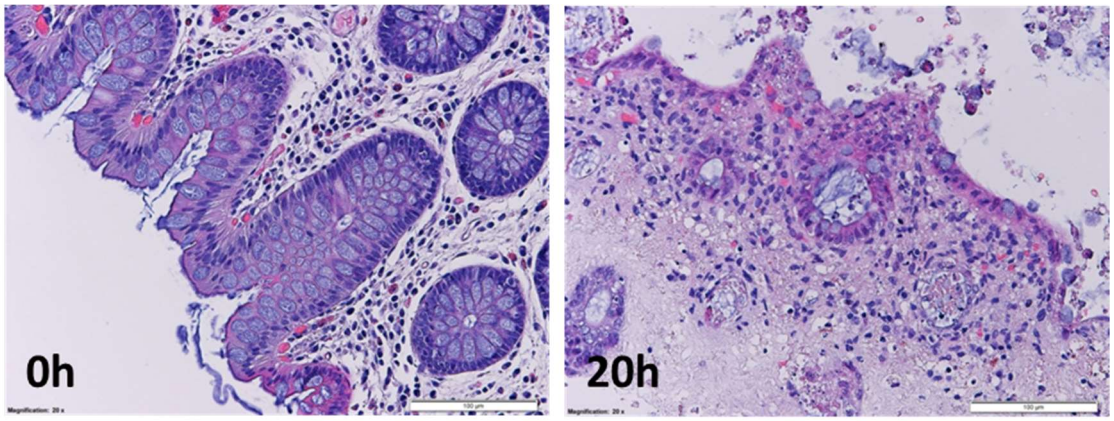

Figure 18: Colonic biopsy before and after cultivation
Hematoxylin and eosin (HE) staining of an uncultured (0h) and medium cultured (20 hrs) healthy control biopsy. Scale bars 100 µm. Figure adapted from Stebe-Frick et al., 2018.

3.2.1.1. Effect of HDAC inhibition on hBD2 expression levels in healthy individuals and IBD patients

EcN, which is efficiently used to maintain the remission in UC patients (Kruis, 2004), is a potent inducer of hBD2 in colonic CaCo2-cells (Möndel et al., 2009). Therefore, this probiotic strain was tested for its potential to induce hBD2 in *ex vivo*-stimulated colonic biopsies of healthy controls as well as IBD patients. Treatment of *ex vivo* colonic biopsies with probiotic EcN strongly induced hBD2, especially in healthy controls and patients with UC (Figure 19). The basal hBD2 induction levels observed under medium-treatment have been defined as one to allow control in comparison to this condition rather than to 'naïve', untreated samples (in addition, relative expression levels of hBD2 of all tested patient groups are depicted in Appendix Figure 1). The observed results are in line with (Lewis et al., 2016) who could also show that EHEC infection of a colonic biopsy model induced hBD2 expression, dependent on flagellin. Fold changes in EcN-stimulated hBD2 expression in the colon of patients with inactive ileal or colonic CD (Figure 19, L1 or L3 respectively), however, were lower and not significant indicating a disturbance in hBD2 inducibility in response to EcN (Stebe-Frick et al., 2018).

It was aimed at gaining insight into, whether a combinatatorial use of HDACi together with the therapeutic EcN might be a potential strategy to strengthen protective immune responses. For this, HDAC function was blocked in *ex vivo* cultured tissue using either SAHA, MS-275, or SB (Figure 19). Interestingly, SAHA and SB abolished hBD2 expression in all samples, regardless of disease type. However, the inhibition of mainly only HDACs 1 and 3 with MS-275 did not suffice to prevent hBD2 induction. Overall, these observations impressively demonstrate

that EcN-mediated induction of hBD2 in a tissue compound is strongly dependent on HDAC function (Stebe-Frick et al., 2018).

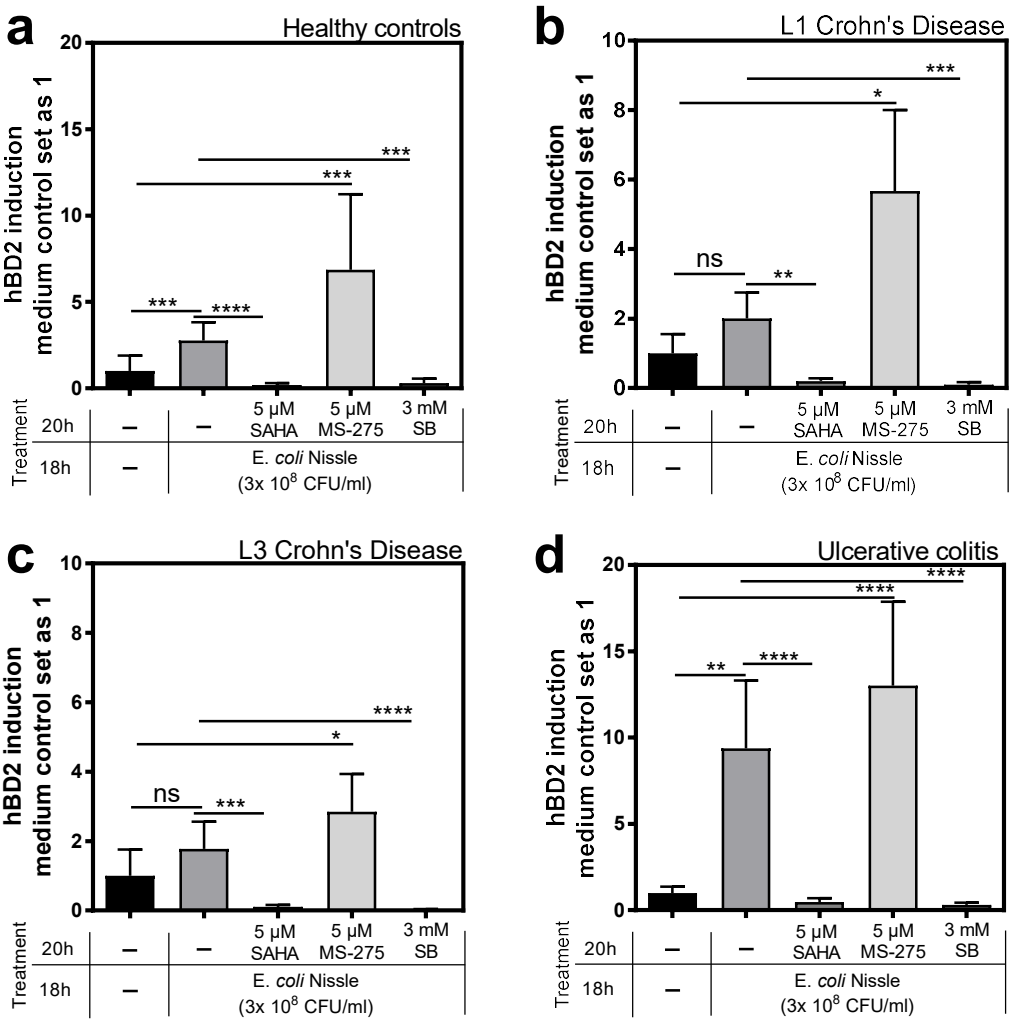

Figure 19: *E. coli* Nissle stimulates but HDACi hinders hBD2 expression in *ex vivo* cultured human colonic biopsies

HBD2 mRNA in cultured human colonic biopsies of (**a**) controls (C, n=13), (**b**) L1 (n=15) or (**c**) uninflamed L3 (n=14) CD patients, or (**d**) uninflamed UC patients (n=14) in response to 18 hrs of EcN stimulation with or without HDACi with SAHA (5 µM), MS-275 (5 µM), or SB (3 mM) shown in the right panels of each graph respectively. Shown are relative fold changes compared to medium treatment alone according to 10 ng total RNA normalized to βactin expression. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p<0.0001 evaluated by Mann-Whitney u test. Figure adapted from Stebe-Frick et al., 2018.

Tissue integrity of the biopsies was again assessed via HE staining showing no differences between medium, EcN, or treatments with HDACi after 20 hrs of cultivation (Figure 20).

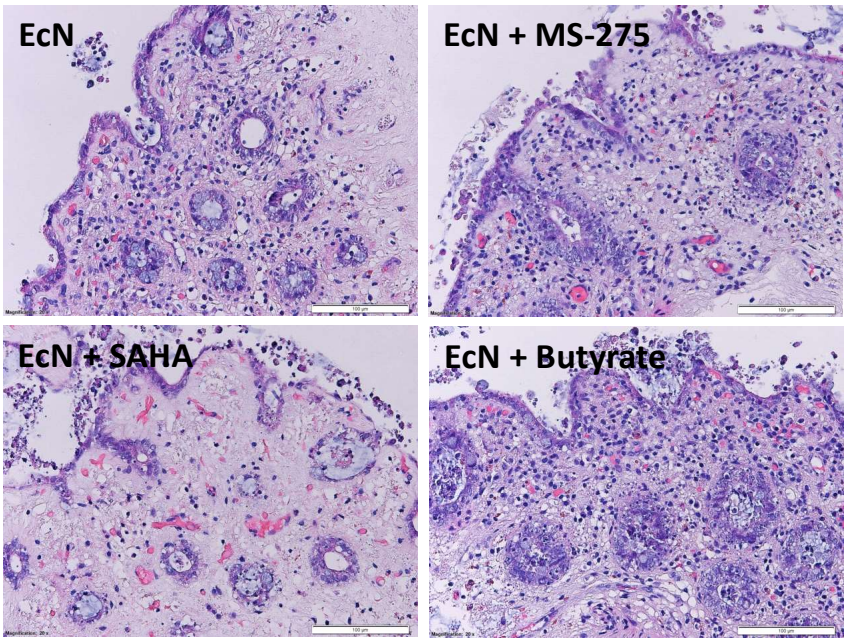

Figure 20: Colonic biopsies before and after cultivation with EcN and HDACi
HE staining of a 20 hrs medium plus EcN cultured biopsy (top left), EcN plus SAHA (bottom left), EcN plus MS-275 (top right), and EcN plus SB cultured biopsy (bottom right). Scale bars 100 µm.

3.2.1.2. IL8 expression in *ex vivo* biopsies

Furthermore it was investigated whether IL8 expression, as an inflammatory response, was affected in the same way as hBD2 by HDACi. It was observed that cultivation with medium alone cuased an induction of IL8. The different treatments, however, mostly did not augment this induction (Figure 21) (Stebe-Frick et al., 2018). Most notably, HDACi did not abolish IL8 expression as compared with hBD2, pointing towards more distinctive epigenetic regulatory mechanisms for IL8 and hBD2 on the level of HDAC function (Stebe-Frick et al., 2018).

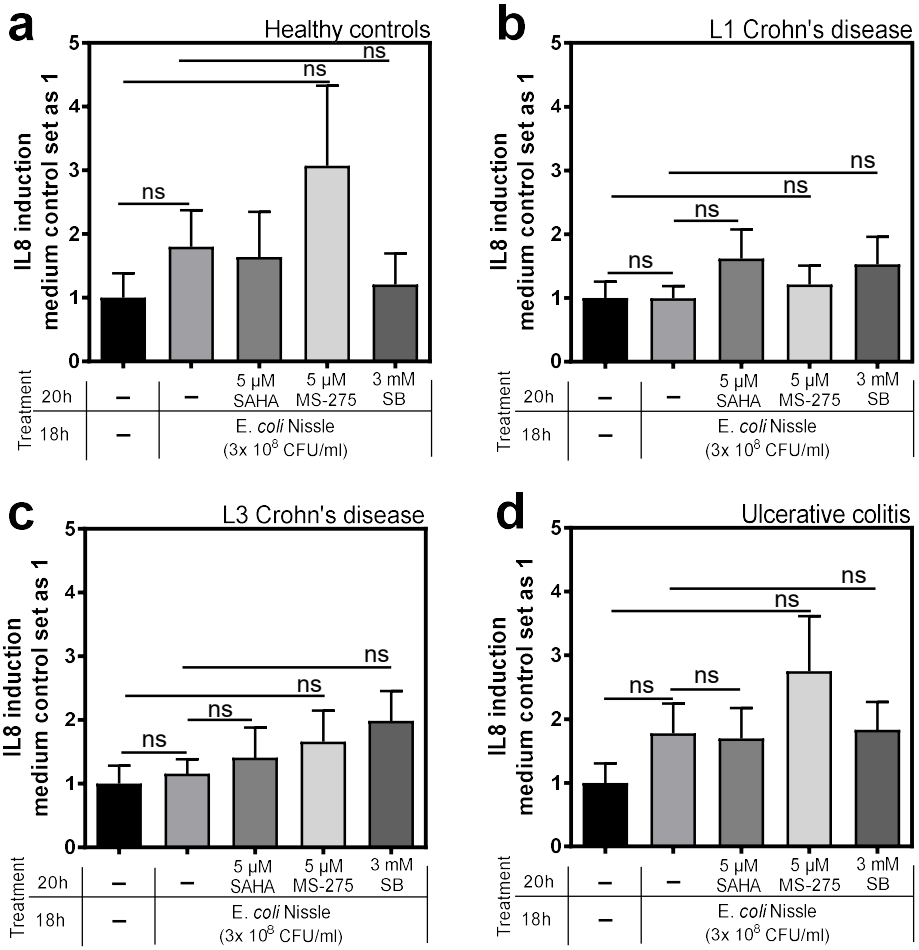

Figure 21: Inhibition of histone deacetylases (HDACs) does not abolish IL8 expression in *ex vivo* biopsies

IL8 mRNA expression in cultured colonic biopsies from healthy controls (**a**), L1 (**b**) , L3 (**c**) , and UC (**d**) patients in response to 18 hrs of EcN stimulation, alone or together with either SAHA (5 µM), MS-275 (5 µM), or SB (3 mM). Inhibitor treatment started 2 hrs prior to the stimulation with EcN, which took then place in parallel to the HDAC inhibition for another 18 hrs. Shown are relative fold changes compared to medium treatment alone according to 10 ng total RNA normalized to βactin expression. Figure adapted from Stebe-Frick et al., 2018.

3.2.1.3. hBD1 expression in *ex vivo* biopsies

To get an insight into whether a constitutively expressed defensin, such as human β-defensin 1, is affected by the treatment with EcN with or without HDACi, the expression of this defensin has also been measured. Comparable observations as with IL8 were made for the expression of hBD1- no significantly changes were induced by neither EcN alone nor the simultaneous treatment with HDAC inhibitors (Figure 22) (Stebe-Frick et al., 2018).

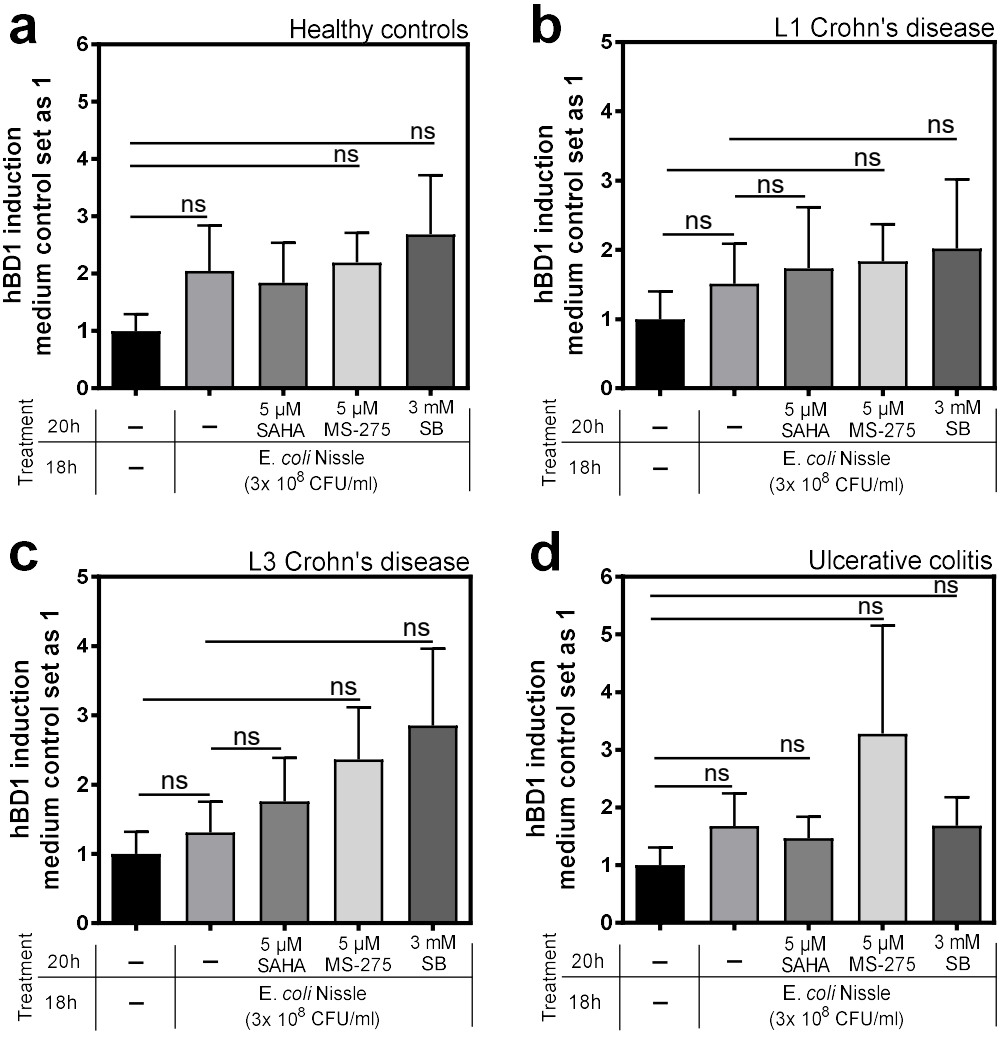

Figure 22: Effect of histone deacetylase (HDAC) inhibition on human β-defensin 1 (hBD1) expression in *ex vivo* biopsies

hBD1 mRNA expression in cultured colonic biopsies from healthy controls (**a**), L1 (**b**) , L3 (**c**) , and UC (**d**) patients in response to 18 hrs of EcN stimulation, alone or together with either SAHA (5 µM), MS-275 (5 µM), or SB (3 mM). Inhibitor treatment started 2 hrs prior to the stimulation with EcN, which took then place in parallel to the HDAC inhibition for another 18 hrs. Shown are relative fold changes compared to medium treatment alone according to 10 ng total RNA normalized to βactin expression. Figure adapted from Stebe-Frick et al., 2018.

3.2.2. Cell culture functional studies on hBD2 inducibility

Due to the limited number of biopsies that could be obtained per patient, closer functional investigations regarding the underlying mechanisms on how HDACi impacts the expression of hBD2 could not be conducted *ex vivo.* For this reason, all functional analyses had to be performed *in vitro* - using the human colonic epithelial CaCo2 subclone TC7 cells (CaCo2/TC7) to study the role of different hBD2 stimuli (heat-inactivated EcN, LPS and IL1β) without and with simultaneous inhibition of HDAC function by the aforementioned compounds SAHA, MS-275, or SB (Figure 23) (Stebe-Frick et al., 2018). Mono-layered CaCo2/TC7 cells were chosen for this endeavor since it had been shown before, that EcN is a good inducer of hBD2 in this cell line (Möndel et al., 2009).

3.2.2.1. Induction of hBD2 by different stimuli – The effect of HDAC inhibition

Cells were challenged with the stimulants for 18 hrs, which led to the induction of high basal levels of hBD2 as shown in Figure 23a (Stebe-Frick et al., 2018). The use of the inhibitors alone did not affect hBD2 expression within 20 hrs (Figure 23b). Very low transcript numbers of hBD2 per 10 ng total RNA (40 transcripts max) can be considered as negavtive. Strikingly, when HDAC function was inhibited simultaneously, a strong enhancement of the hBD2 transcription levels has been detected as shown in Figure 23a (Figure 23c-e) (Stebe-Frick et al., 2018); especially in the case of the pan-HDAC-inhibitor SAHA (Figure 23c). Employing the more specific MS-275, mainly inhibting HDACs 1 and 3 at the here used concentration (Beckers et al., 2007; Hu et al., 2003; Khan et al., 2008; Tatamiya et al., 2004) (Figure 23d), caused a comparably strong effect in combination with EcN (Figure 24) (Stebe-Frick et al., 2018). When combined with IL1β and LPS the trend was still significant but less marked (Stebe-Frick et al., 2018). The short chain fatty acid derivate butyrate - which occurs naturally in the human intestine and also has known HDAC inhibitory function - augmented hBD2 induction as well albeit to a lesser extent (Figure 23e) (Stebe-Frick et al., 2018). HBD2 intracellular protein levels mirrored the observed response on the mRNA level (Figure 25) (Stebe-Frick et al., 2018).

Summarizing, these findings also point out an importance for HDACs in the transcriptional regulation of hBD2 in intestinal epithelial cells. Seemingly, they level up hBD2 inducibility in the monolayer cellular setting. However, this is in stark contrast to the findings from the *ex vivo* experiments with colonic tissue samples where HDACi has an opposing effect. These differences could be due to either the tumorous nature of the CaCo2 cells or due to more complex tissue context of the biopsies (Stebe-Frick et al., 2018). Nevertheless, the observation from the *in vitro* approach is in line with a recent report showing similar effects, also in CaCo2/TC7 cells and additionally in human colonic organoids under the use of the inhibitors TSA and SAHA (Fischer et al., 2016).

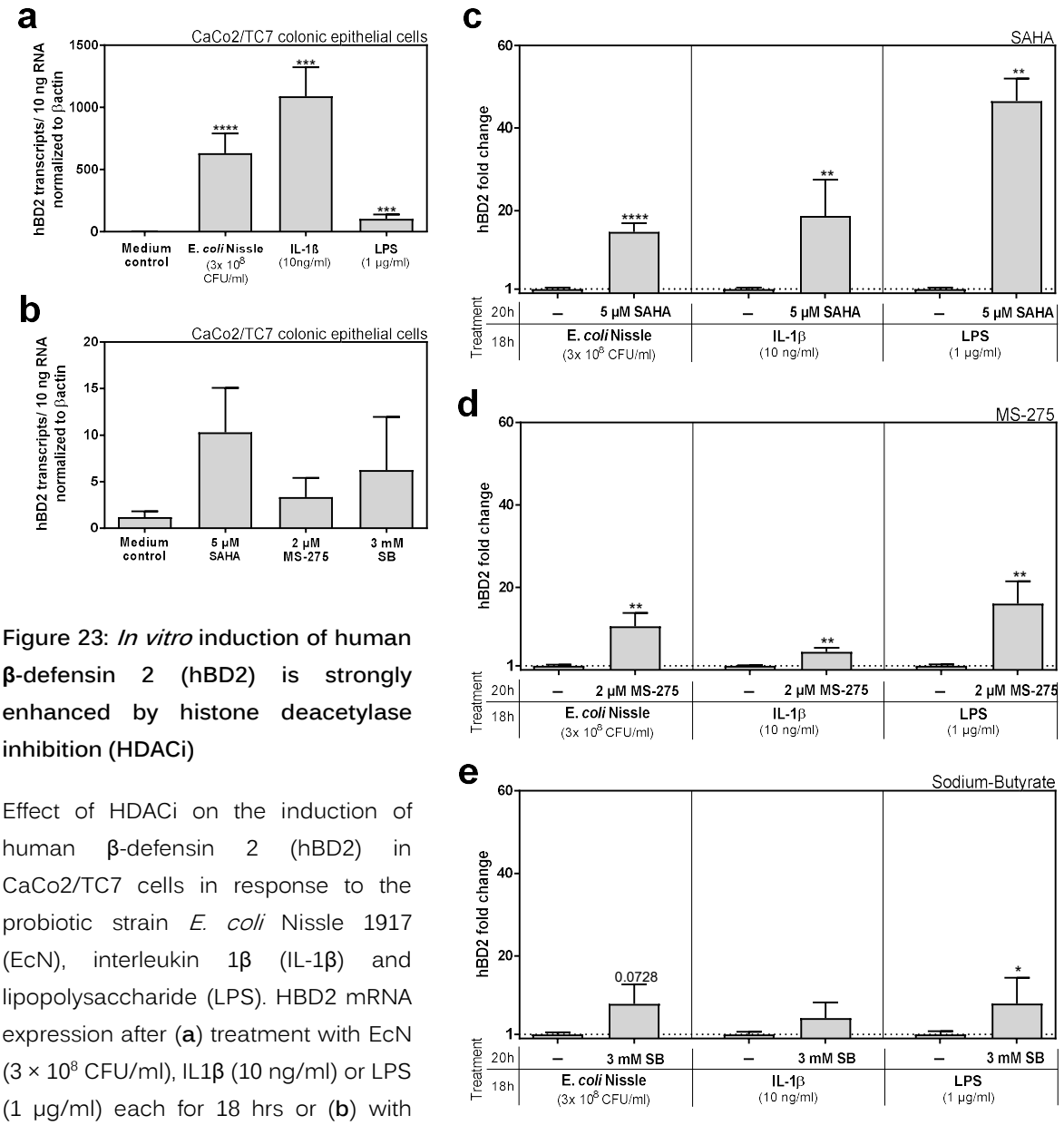

Figure 23: *In vitro* induction of human β-defensin 2 (hBD2) is strongly enhanced by histone deacetylase inhibition (HDACi)

Effect of HDACi on the induction of human β-defensin 2 (hBD2) in CaCo2/TC7 cells in response to the probiotic strain *E. coli* Nissle 1917 (EcN), interleukin 1β (IL-1β) and lipopolysaccharide (LPS). HBD2 mRNA expression after (**a**) treatment with EcN (3×10^8 CFU/ml), IL1β (10 ng/ml) or LPS (1 µg/ml) each for 18 hrs or (**b**) with HDAC inhibitors SAHA (5 µM), MS-275 (2 µM) or SB (3 mM) each for 20hrs. Shown are relative copy numbers of hBD2 per 10 ng total RNA normalized to βactin expression. c-e shows the fold change in hBD2 induction compared to each stimulation alone when co-treated with either SAHA (5 µM) (**c**), MS-275 (2 µM) (**d**), or SB (3 mM) (**e**). Here, inhibitor treatment started 2 hrs prior to the stimulations, which took then place in parallel to HDAC inhibition for another 18 hrs. Shown are results of at least three independent experiments carried out in biological triplicates. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p<0.0001 evaluated by Mann-Whitney u test. Figure from Stebe-Frick et al., 2018.

Furthermore, the comparable hBD2 enhancing effects of SAHA and MS-275 when cells are stimulated with EcN show that, in this case, HDAC1 and 3 seem to be the mainly involved HDACs in the repression of hBD2 gene expression keeping it at a basal induction level when they can exert their normal function.

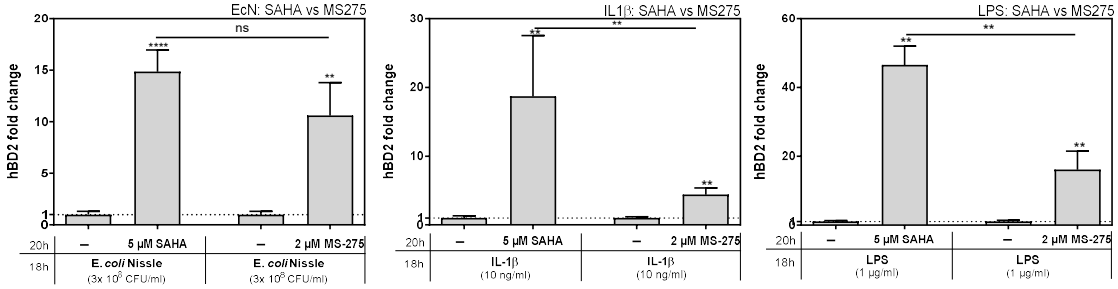

Figure 24: Comparison of the effects of SAHA and MS-275 on hBD2 expression

Inhibition of all HDACs by SAHA and only inhibiting HDAC1 and 3 via MS-275 leads to a comparable enhancement of hBD2 when it is stimulated by EcN (left). If hBD2 is induced by IL1β (middle) or LPS (right), however, inhibition of HDAC1 and 3 does not lead to the same level of augmentation as the pan-HDACi via SAHA. ** p≤0.01, **** p<0.0001 evaluated by Mann-Whitney u test.

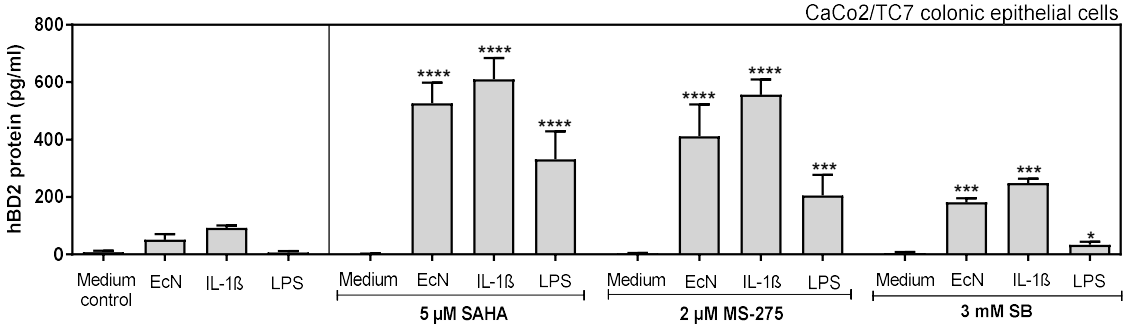

Figure 25: Intracellular hBD2 protein after stimulation with EcN, IL1β, or LPS and HDACi

HBD2 peptide levels from CaCo2/TC7 cell lysates as determined by ELISA. Treatment was carried out exactly as for mRNA analyses. Results represent three independent experiments measured as technical triplicates. * p≤0.05, *** p≤0.001, **** p<0.0001 evaluated by Mann-Whitney u test. Figure from Stebe-Frick et al., 2018.

3.2.2.2. IL8 expression under HDAC inhibition

The influence of HDACi on the inflammatory response in CaCo2/TC7 cells was examined via measuring the expression of interleukin 8 (IL8) (Figure 26). The basal induction IL8 levels evoked by the the application of the stimulants alone, are displayed in Figure 26a. These levels are comparable to what has been seen for hBD2. Interestingly, inhibitor treatment alone induced the pro-inflammatory cytokine IL8 (Figure 26b). In slight contrast to the study from Fischer et al (Fischer et al., 2016), HDAC inhibition in parallel with the stimulation had a synergistic effect on IL8 induction as well and also strongly increased the basal transcription level of this pro-inflammatory gene (Figure 26c-e) (Stebe-Frick et al., 2018). Interestingly, these findings are different from those observed in *ex vivo* biopsies within this study at hand, where HDACi did not further enhance IL8 expression in response to medium treatment alone or stimulation with EcN.

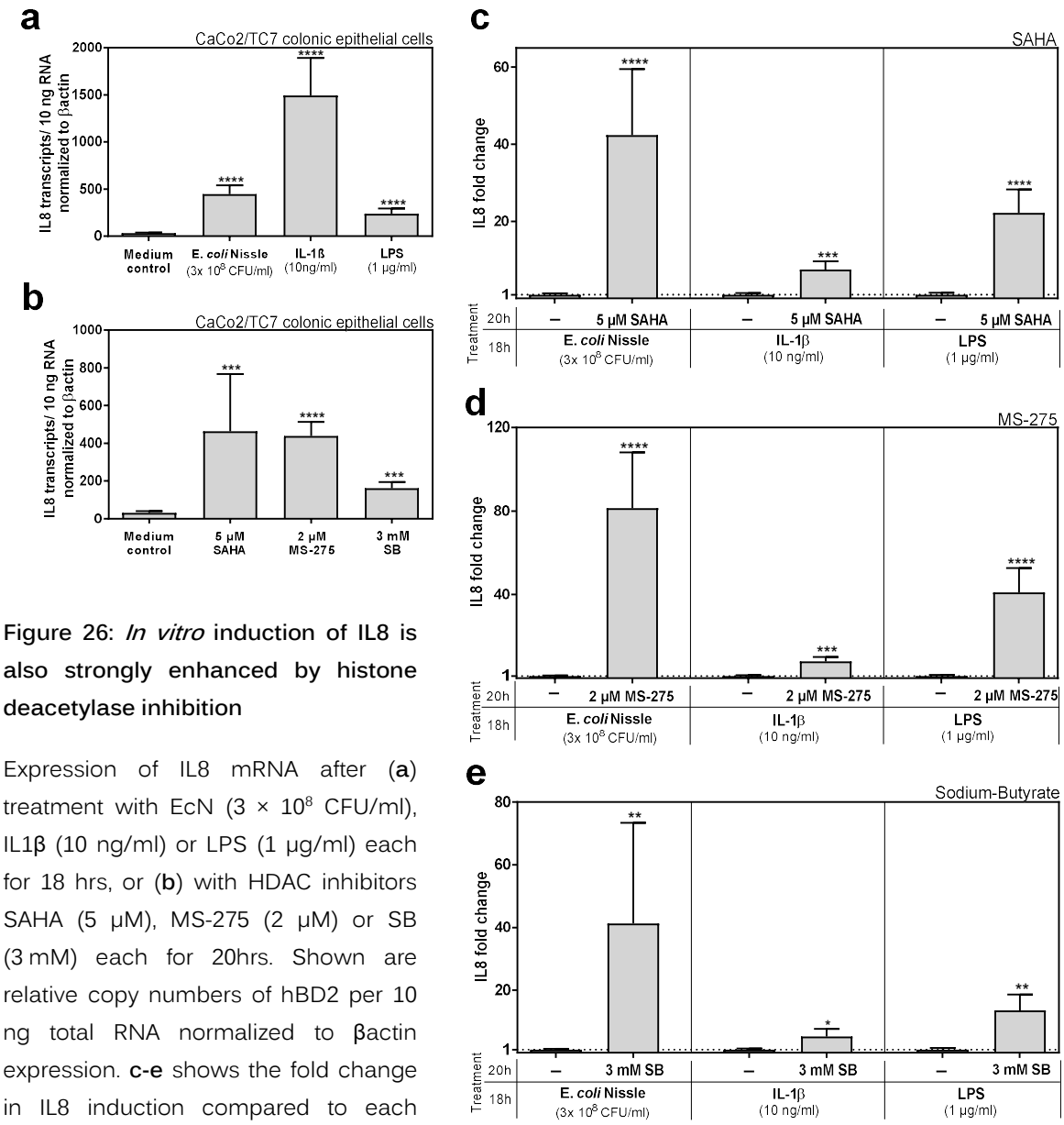

Figure 26: *In vitro* induction of IL8 is also strongly enhanced by histone deacetylase inhibition

Expression of IL8 mRNA after (**a**) treatment with EcN (3 × 10^8 CFU/ml), IL1β (10 ng/ml) or LPS (1 µg/ml) each for 18 hrs, or (**b**) with HDAC inhibitors SAHA (5 µM), MS-275 (2 µM) or SB (3 mM) each for 20hrs. Shown are relative copy numbers of hBD2 per 10 ng total RNA normalized to βactin expression. **c-e** shows the fold change in IL8 induction compared to each stimulation alone when co-treated with either SAHA (5 µM) (**c**), MS-275 (2 µM), (**d**) or SB (3 mM) (**e**). Inhibitor treatment started 2 hrs prior to the stimulations, which took then place in parallel to HDAC inhibition for another 18 hrs. Shown are results of at least three independent experiments carried out in biological triplicates. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p<0.0001 evaluated by Mann-Whitney u test. Figure from Stebe-Frick et al., 2018.

3.2.2.3. hBD1 expression under HDAC inhibition

Contrasting, human β-defensin 1 (hBD1), which is constitutively expressed in intestinal epithelial cells, was neither significantly affected by HDAC inhibition nor by the stimulants (Figure 27). However, a significant trend towards an enhanced hBD1 mRNA expression could be observed for MS-275 and SB (Figure 27b and c), especially when acting together with the stimulants (Stebe-Frick et al., 2018).

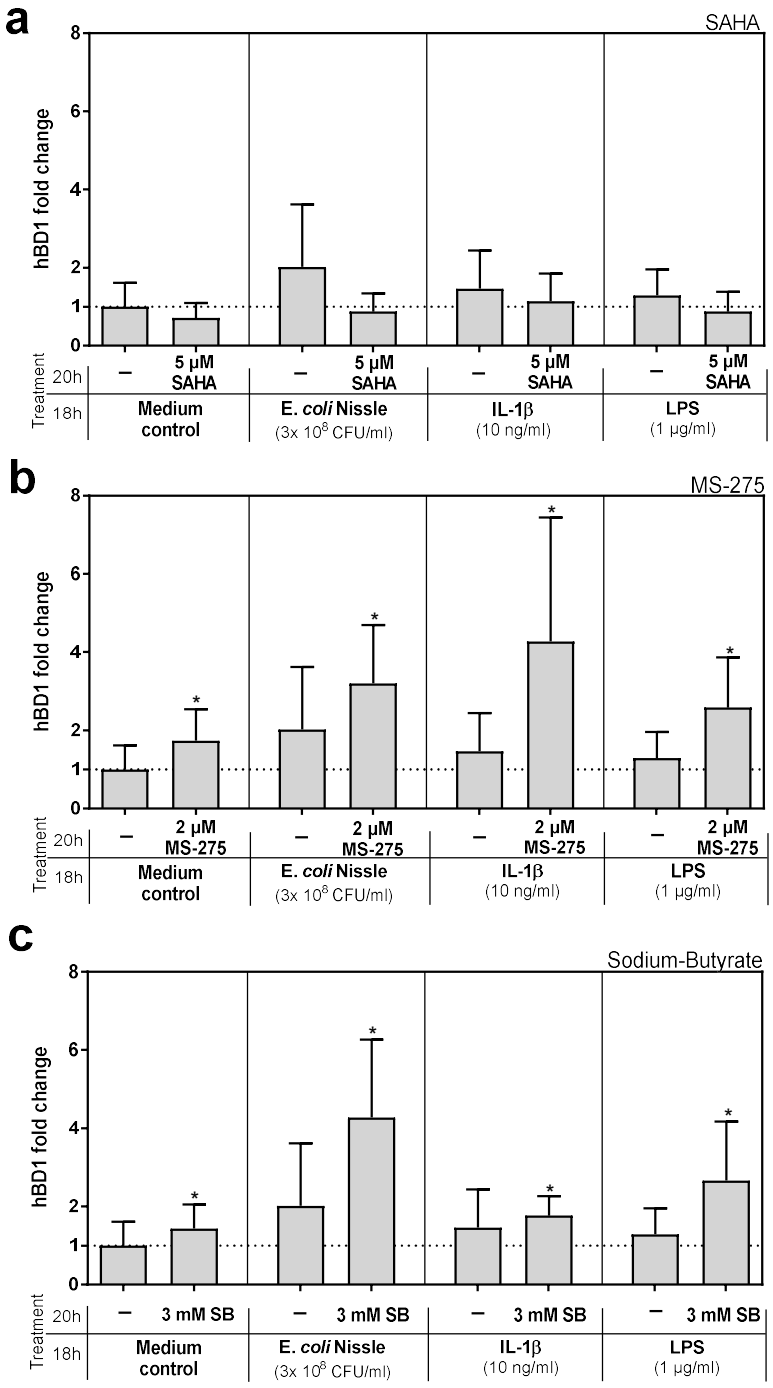

Figure 27: The effect of histone deacetylase (HDAC) inhibition on human β-defensin 1 (hBD1) expression *in vitro*

Fold changes in relative hBD1 mRNA expression after (**a**) treatment with HDAC inhibitor SAHA (5 µM), (**b**) MS-275 (2 µM) or (**c**) SB (3 mM) each for 20hrs together with or without the EcN (3 × 108 CFU/ml), IL1β (10 ng/ml) or LPS (1 µg/ml) each for 18 hrs. Expression of hBD1 according to 10 ng total RNA normalized to βactin expression. Inhibitor treatment started 2 hrs prior to the stimulations, which took then place in parallel to HDAC inhibition for another 18 hrs. Shown are results of at least three independent experiments carried out in biological triplicates. * $p<0.05$, ** $p<0.01$ evaluated by Mann-Whitney u test. Figure from Stebe-Frick et al., 2018.

3.2.2.4. Viability of CaCo2 cells after HDAC inhibition

Scrutinizing the viability of CaCo2 cells after the treatments, first the proliferation marker Ki67 (Scholzen and Gerdes, 2000) has been checked by measuring its mRNA expression (Figure 28). HDACi did not affect Ki67 expression. The stimulants EcN, IL1β, and LPS lead to a slight but not significant reduction of Ki67 indicating a decrease of the growth fraction of the regarded CaCo2 population (Scholzen and Gerdes, 2000).

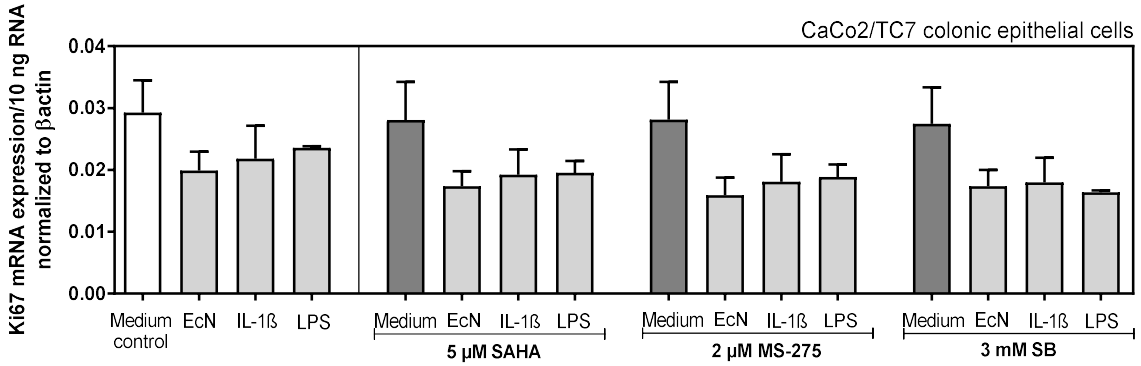

Figure 28: Expression of the proliferation marker Ki67 in treated CaCo2 cells
Shown are results of at least two independent experiments carried out in biological triplicates.

Furthermore, the metabolic activity of the cells has been assessed via MTT assays to infer on cell survival and proliferation (Figure 29). The left part of the figure shows that neither the stimulants alone, nor together with the HDACi had a severe effect on the cellular capability to reduce MTT. The right side of the graph shows the metabolic activity under the additional treatment with the NF-κB inhibitor Helenalin (relevant for the investigations described in the following chapter: 3.2.3 The role of NF-κB and AP1 transcription factors in the HDAC inhibition-dependent enhancement of hBD2 induction) which took place for 1h right at the beginning of the 20 hrs and was then removed to avoid cytotoxic effects. Helenalin reduced metabolic activity only by about 20% relative to untreated cells which is still in an acceptable range.

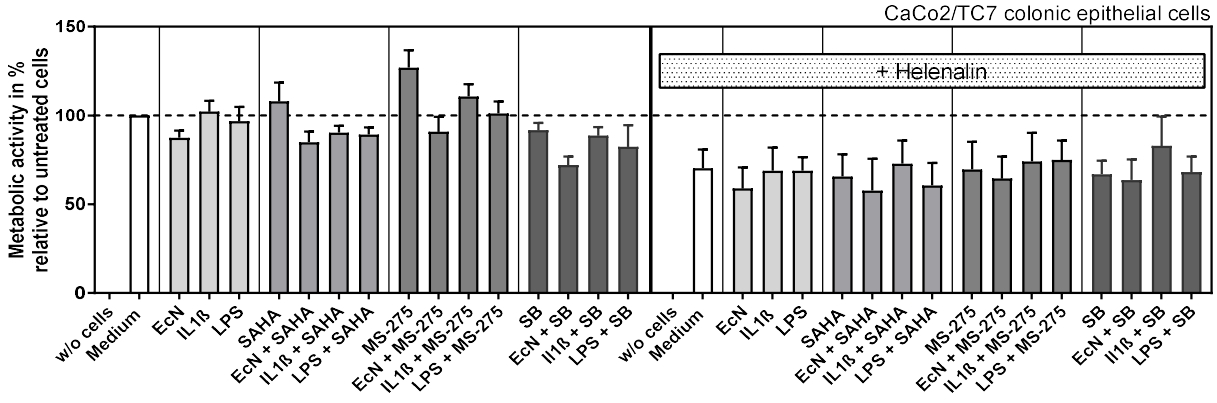

Figure 29: Metabolic activity of CaCo2 cells after treatment

CaCo2 metabolic activity as determined by the MTT reducing capacity of the differentially treated cells.

3.2.3. The role of NF-κB and AP1 transcription factors in the HDAC inhibition-dependent enhancement of hBD2 induction

In 2004, it has been shown that hBD2 induction via EcN is dependent on at least two NF-κB binding sites present in the promoter region of hBD2 (Wehkamp et al., 2004a). There are also NF-kB binding sites in the IL8 promoter (Hoffmann et al., 2002). The use of HDAC inhibition generally leads to a more relaxed chromatin structure by blocking their deacetylation function at histone proteins which then promotes transcription (Xu et al., 2007). But besides histones, HDACs have a large number of non-histone protein substrates such as transcription factors (Seto and Yoshida, 2014). Several reports implicated a role for HDACs in the epigenetic control of NF-κB signaling and its transactivation function. It has been shown, e.g. that HDAC1 and 2 interact with the p65 subunit exerting a corepressor function (Ashburner et al., 2001) and that HDAC3 deacetylates p65 and thereby facilitates the nuclear export of RelA which reduces NF-κB regulated gene expression (Chen et al., 2001; Huang et al., 2010). Inhibiting HDACs might therefore reduce the repressive effect they might have on NFkB. This could then lead to a fortified NF-kB-dependent gene expression (Stebe-Frick et al., 2018). So, based on these studies, it was aimed to test whether the pronouncing effects of HDACi on hBD2 induction might indeed depend on NF-κB function (Stebe-Frick et al., 2018).

3.2.3.1. Pharmacological inhibition of NF-κB

To verify the dependency on NF-κB, simultaneously to blocking HDAC function, NF-κB was inhibited by Helenalin (Figure 30). This compound hinders DNA binding of p65 via alkylation of the latter (Lyß et al., 1998). Therefore, Helenalin was expected to avert the enhancement of hBD2 expression under HDACi. Indeed, enhancement of hBD2 induction mediated by SAHA in response to all three tested stimulants was abolished (Figure 30a) (Stebe-Frick et al., 2018). In the case of the MS-275, simultaneous inhibition of NF-κB rather caused an attenuating effect when stimulation of hBD2 occurred via activation of TLRs (EcN, LPS) (Figure 30b, left and right panel) (Stebe-Frick et al., 2018). When IL1β was used to induce hBD2, the MS-275-mediated reinforcement of its expression was not blocked by NF-κB inhibition (Figure 30b, middle panel) (Stebe-Frick et al., 2018). For SB, the observed effect resembled the one with SAHA – blocking NF-κB in EcN- or LPS-stimulated cells caused an abolishment of the HDACi-mediated enhancement (Figure 30c, left and right panel) (Stebe-Frick et al., 2018). For IL1β, this trend demontrated to be weaker but still present (Figure 30c, middle panel) Overall, these findings show that the potentiation in hBD2 expression under pan-HDACi is mediated via NF-κB. This does not seem to be the case when the HDAC1 and 3 inhibitor MS-275 is used upon IL1β-stimulated cells. Here, signaling pathways and molecules other than NF-κB seem to be of importance (Stebe-Frick et al., 2018).

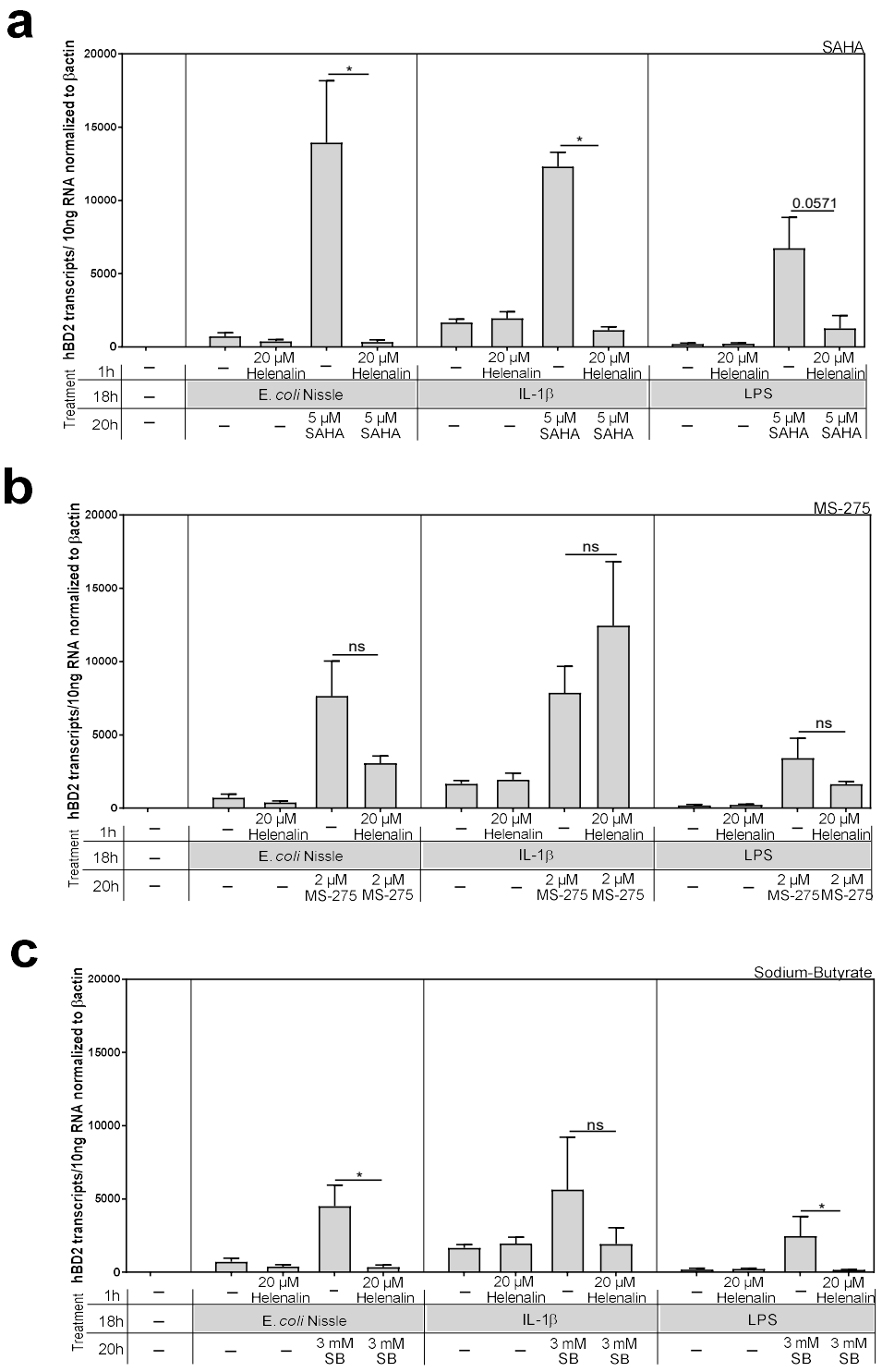

Figure 30: Pan-HDAC inhibition enhancement of hBD2 expression is dependent on NF-κB

a-c show the abolishment of the enhancing effect of HDAC inhibition through Helenalin in CaCo2/TC7 cells. HBD2 mRNA induction in response to EcN (3×10^8 CFU/ml), IL1β (10 ng/ml) or LPS (1 µg/ml) alone, together with SAHA (5µM) (**a**), MS-275 (2 µM) (**b**), or SB (3 mM) (**c**), and after pretreatment for 1 h with the NFkB inhibitor Helenalin (20 µM). Represented are the results of four independent experiments carried out in biological triplicates. Shown are relative copy numbers of hBD2 per 10 ng total RNA normalized to βactin expression. * $p<0.05$ evaluated by Mann-Whitney u test. Figure from Stebe-Frick et al., 2018.

3.2.3.2. Reportergene studies

To further elucidate the importance of NF-κB, CaCo2/TC7 cells were transfected with different hBD2 promoter constructs – with either a wildtype construct (hBD2-2338-luc) or with constructs that bear mutations in either the proximal (positions -205 to -186) or the distal NF-κB binding sites (positions -596 to -572), but the activator protein-1 (AP1) binding site (positions −127 to −121). All were either mutated alone or in different combinations (Figure 31). After treatment with either one of the stimulants EcN, IL1β, or LPS together with SAHA, hBD2 promoter activation was analyzed (Figure 31) (Stebe-Frick et al., 2018).

hBD2 promoter constructs

The wildtype construct (hBD2-2338-luc) displayed an about 13 fold activation of the hBD2 promoter when stimulated with EcN alone compared to untreated cells. Strikingly, together with HDACi this activation was enhanced up to approximately 43 fold (Figure 31 topmost row, EcN panel) (Stebe-Frick et al., 2018). This was similar for LPS but not for IL1β (Figure 31 topmost row, IL1β and LPS panel, respectively). Mutation of the proximal NF-κB binding site (NF-κB-mut1-luc) hindered EcN- as well as LPS-induced hBD2 reportergene activation, which confirmed a previous finding made by our group (Wehkamp et al., 2004a) and also dampened the EcN+SAHA-mediated enhancement, leaving over only an about ~4 fold activation (Figure 31 second row, EcN panel) (Stebe-Frick et al., 2018). Interestingly, the malfunction of the proximal binding site also abolished the IL1β induced promoter activation, indicating that the initial induction of the hBD2 gene via IL1β signaling is strongly dependent on this NF-κB binding site but in this case not the enhanced expression of hBD2 as has been observed for the intrinsic gene expression in CaCo2 cells (cf. Chapter 3.2.2.1 Induction of hBD2 by different stimuli – The effect of HDAC inhibition). So, the augmentation of IL1β-induced hBD2 is likely mediated via additional NF-κB binding sites that are missing from the constructs used here since blocking total NF-κB function with Helenalin had been capable of abolishing the SAHA-enhanced hBD2 expression. Mutation of the distal binding site (hBD2-mut2-luc) did, however, not show an effect on the inducibility of the promoter construct for neither one of the three stimulants (Figure 31 third row) (Stebe-Frick et al., 2018). The binding site for AP1 (AP1-mut-luc) seems to also play a role not only for the basal induction of the hBD2 promoter but also for the capacity of reinforcing the amplitude of its activation, leading to an elevation from ~5 fold to ~14 fold (Figure 31 fifth row, EcN panel) (Stebe-Frick et al., 2018). The observation was comparable for LPS as well as for IL1β where the lack of this AP1 site led to a diminished activation of the reportergene. Overall, the effect is minor compared to NF-κB but significant. Together, these findings substantiate the NF-κB involvement as well as demonstrating an involvement of AP1 in the HDACi-mediated augmentation of hBD2 induction on the promoter level (Stebe-Frick et al., 2018).

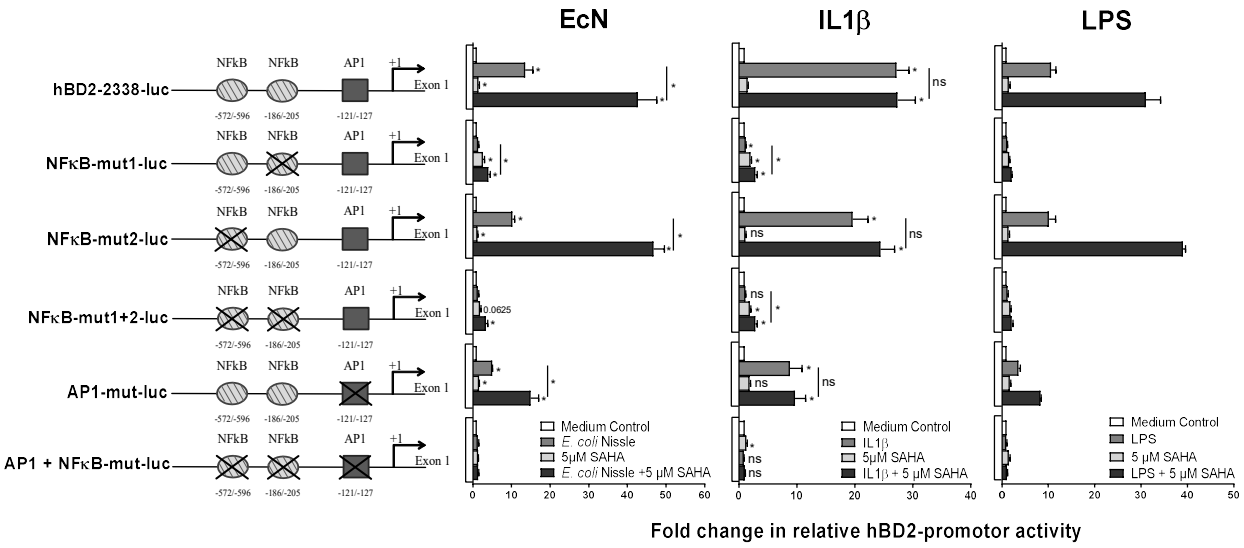

Figure 31: The proximal NF-κB binding site in the hBD2 promoter and an AP1 binding site are essential for the enhancing effect of HDACi on hBD2 expression

The left panel shows a diagram of the used hBD2 promoter constructs (bp -2338 to -1 linked to the luciferase gene). Two NF-κB binding sites and one AP1 binding site are marked relative to the hBD-2 transcription start. Constructs with mutated binding sites were used as indicated. Transfection of CaCo2/TC7 cells took place with either the wild-type (hBD2-2338-luc) or the mutated hBD2 promoter constructs together with a Renilla luciferase plasmid as internal standard. 24 hrs post transfection, cells were treated with either EcN (3×10^8 CFU/ml), IL1β (10 ng/ml), or LPS (1 µg/ml) for 18 hrs, with SAHA (5µM) for 20 hrs or a simultaneous combination of a stimulant and SAHA (18 and 20 hrs, respectively). Promoter activation was measured subsequently and is displayed as luciferase activity normalized to Renilla activity. Shown are the results of at least 3 independently conducted experiments carried out in biological triplicates. * $p<0.05$ evaluated by Wilcoxon signed rank test. Parts of this figure have been published before (Stebe-Frick et al., 2018).

NF-κB and AP1 constructs

In addition to the hBD2 promoter constructs, CaCo2/TC7 cells were transfected with two general luciferase reporter plasmids with either tandemly repeated NF-κB or AP1 binding sites to test their reaction to stimulation with EcN under pan-HDACi by SAHA (Figure 32). This has been done to answer the question on how isolated NF-κB or AP1 binding sites contribute to a promoter activation under the respective conditions and to allow drawing inferences about more general effects of pan-HDACi on these transcription factors. Of note, in contrast to the hBD2 promoter constructs, the reporter genes under the control of tandem NF-κB or AP1 binding sites showed an initial level of activation in CaCo2 cells indicating a certain "basal" level of activity by these transcription factors. This level has been set as one. Treatment with EcN alone slightly activated NF-κB but not AP1 binding sites. Both types of binding sites were activated by SAHA alone, whereas this was more distinctive for AP1. Most interestingly, EcN and SAHA acted synergistically on the NF-κB binding sites leading to an about doubled reportergene activation as compared to either EcN or SAHA treatment alone (Figure 32).

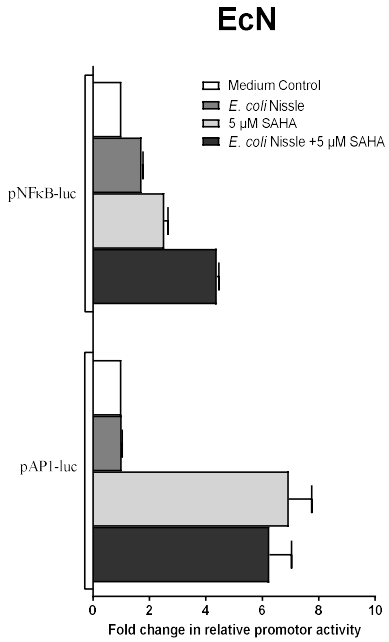

Figure 32: NF-κB binding sites are synergistically activated by EcN and HDACi

Tandemly repeated NF-κB-binding sites are slightly activated by EcN (3×10^8 CFU/ml) or SAHA (5µM) alone but together act synergistically (upper panel). AP1 binding sites are not activated by EcN treatment alone but strongly respond when HDACs are inhibited via SAHA (lower panel). Shown are fold changes in reportergene activation as results of 3 independently conducted experiments carried out in biological triplicates.

From this point of view, it can be concluded that in CaCo2/TC7 cells, EcN alone does not activate AP1 transcription factors or rather that AP1 binding sites alone are not sufficient to reach gene activation via EcN. However, administration of the pan-HDACi SAHA leads to a strong activation of AP1 sites and an enhanced expression of NF-κB-controlled genes supporting the hypothesis of hBD2 mRNA expression being indirectly repressively controlled by HDACs via NF-κB. Therefore, the pronouncing effects of HDACi on hBD2 induction might indeed depend on NF-κB function and activity.

3.2.4. Investigations on the discrepancy between the *ex vivo* and *in vitro* hBD2 transcriptional response to HDAC inhibition

As has been mentioned before, most studies on the effect of HDAC inhibition on hBD2 inducibility have been conducted in mono-layered *in vitro* cell culture approaches (Fischer et al., 2016; Lan et al., 2011; Schwab et al., 2008). Since HDACs are often overexpressed and deregulated in many cancers (Ishihama et al., 2007; Marks et al., 2001; Wilson et al., 2006; Yang et al., 2014), tumorous epithelial cell lines could, however, show differential responses as compared to non-cancerous complex tissue. A human colonic biopsy culture has been established as described in chapter 3.2.1 (Human colonic biopsy culture), which is able to survive in a culture medium almost identical to the one used for the CaCo2/TC7 assays as depicted in chapter 3.2.2 (Cell culture functional studies on hBD2 inducibility). This has been done to add the tissue context and potential crosstalks between different cell types to the picture. Stimulation with the probiotic EcN and treatment with the HDAC inhibitors SAHA, MS-275 and SB was conducted in the same way in both settings with only one exception (Stebe-Frick et al., 2018). The MS-275 concentration was 5 µM in the *ex vivo* experiments as compared to 2 µM *in vitro* which has been adopted from the literature (Tatamiya et al., 2004). This has been done since first results from the biopsies showed that MS-275 was not capable of hindering hBD2 expression after EcN stimulation whereas SAHA and SB could. Therefore, an initial check on whether maybe MS-275 was not able of diffusing as well into the tissue compound as it could diffuse into the mono-layered CaCo2/TC7 cells. The higher concentration also did not abolish hBD2 expression in *ex vivo* biopsies but due to the advanced status of the experiments with valuable patient samples, it has been decided to abide to the higher concentration for the ongoing *ex vivo* experiments.

As has been delineated in the previous chapters, the findings from the *ex vivo* and *in vitro* experiments showed that HDACi has an opposing effect on hBD2 induction levels- in cultured biopsies HDACi mostly prevents whilst in CaCo2/TC7 cells it strongly reinforces hBD2 mRNA expression (cf. Figure 19 versus Figure 23) (Stebe-Frick et al., 2018).

3.2.4.1. hBD2 response to IL1β in colonic epithelial HCT116 cells under HDAC inhibition

Since these differences from *ex vivo* and *in vitro* observations could be due to either the tumorous nature of the CaCo2 cells or due to the more complex tissue context of the biopsies, at first the effect of HDACi has been studied in an additional colorectal carcinoma cell line with an epithelial morphology. For this, HCT116 cells were treated with the same stimulants that have been used in the CaCo2 cells- EcN, IL1β or LPS (Figure 33). Likely due to a lack of detectable or at

least only a very low amount of TLR4 receptor expression in this cell line (Zhao et al., 2007; Doan et al., 2009), there was no induction of hBD2 in response to the TLR4 ligand LPS. However, LPS was able to induce the expression of a small amount of IL8 mRNA, possibly owed to a still present but very low level of TLR4 receptor expression (Doan et al., 2009). Interestingly, there was no TLR5-dependent induction of hBD2 in HCT116 cells following stimulation with EcN (Figure 33a) but IL8 was readily induced (Figure 33b). HCT116 cells seem to express TLR5 receptor at rather low levels (Zhao et al., 2007). Furthermore, the responsiveness to flagellin in cancer cells lines might be differentially modulated due to the presence different single nucleotide polymorphisms (SNPs) that have been found in the TLR5 receptor gene in colorectal cancers (Klimosch et al., 2013). This might the reason for an only very weak hBD2 response to EcN in these cells.

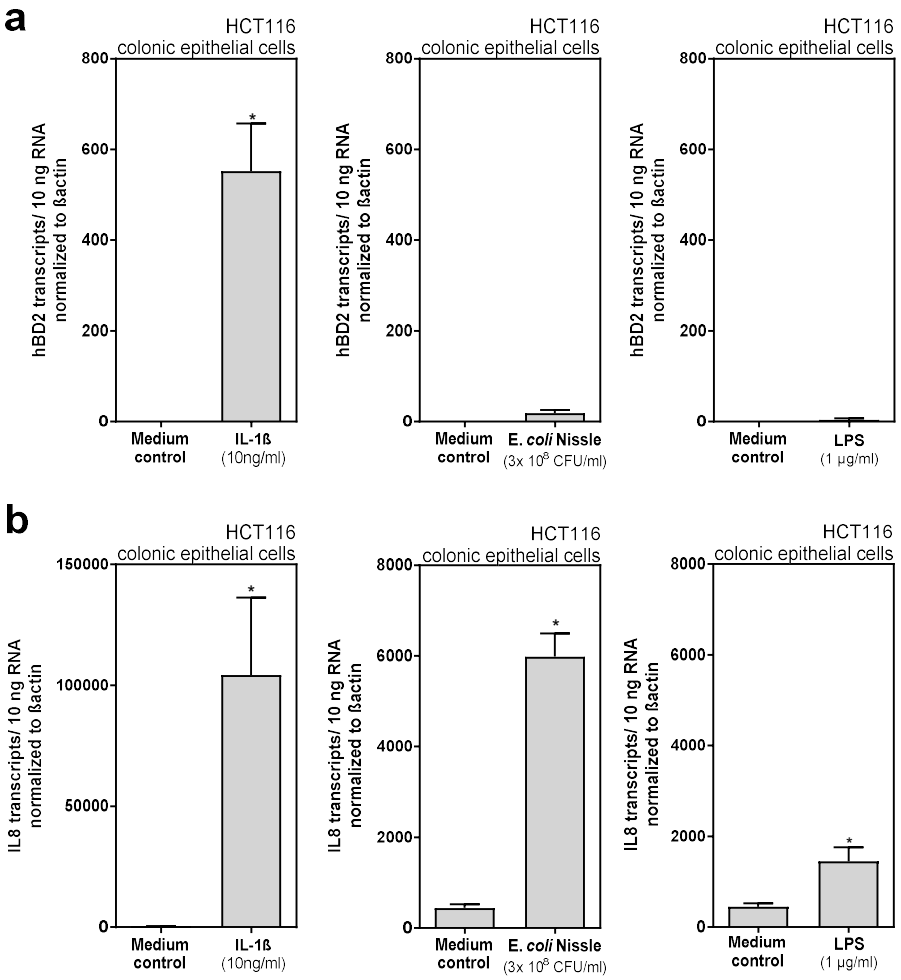

Figure 33: Induction of hBD2 and IL8 in HCT116 colonic epithelial cells following stimulation with EcN, IL1β, or LPS

Effect of HDACi on the induction of (**a**) human β-defensin 2 (hBD2) or (**b**) interleukin 8 (IL8) in HCT116 cells in response to the probiotic strain EcN (3×10^8 CFU/ml), IL1β (10 ng/ml), or LPS (1 µg/ml) for 18 hrs. * $p<0.05$ evaluated by Mann-Whitney u test. Parts of this figure have previously been published (Stebe-Frick et al., 2018).

However, HCTs strongly responded to IL1β stimulation with hBD2 induction (Figure 33). Since for IL1β-mediated hBD2 induction, this is the first report on an enhancement effect of HDACi in intestinal epithelial cells *in vitro*, the aim was to confirm the results for IL1β from CaCo2/TC7cells in HCT116 cells. HDACi alone did not affect hBD2 induction in HCT116 cells but had an elevating effect on basal IL8 expression (Appendix Figure 4). The enhancing effect on hBD2 expression by HDACi could also be observed (Figure 34a) (Stebe-Frick et al., 2018).

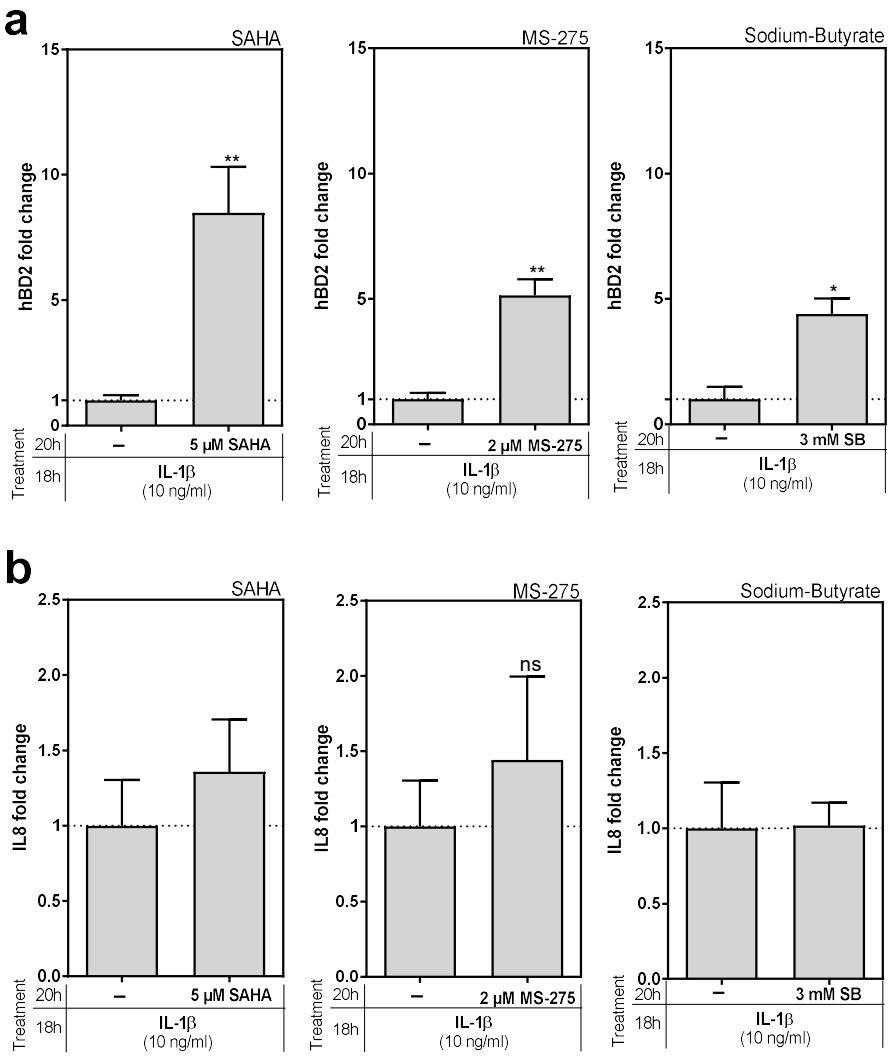

Figure 34: IL1β mediated hBD2 induction is enhanced by HDACi in colonic carcinoma HCT116 cells
a Induction level of hBD2 mRNA in HCT116 cells by IL1β (10 ng/ml) after 18 hrs of stimulation. Fold change in hBD2 (**a**) or IL8 (**b**) induction are shown for IL1β stimulation alone compared to when co-treated with either SAHA (5 µM), MS-275 (2 µM), or SB (3 mM). Inhibitor treatment and stimulation were done exactly as for CaCo2/TC7 cells. Depicted are results of at least three independent experiments carried out in biological triplicates. * $p<0.05$, ** $p<0.01$, evaluated by Mann-Whitney u test. Figure from Stebe-Frick et al., 2018.

In contrast to the CaCo2/TC7 cells, however, IL1β induced IL8 expression was not further enhanced when HDAC inhibitors were used (Figure 34b) indicating a possibly cell line specific

inflammatory response that differs in respect to IL8 between CaCo2/TC7 and HCT116 cells (Stebe-Frick et al., 2018).

3.2.4.2. hBD2 expression under HDAC inhibition in primary gingival epithelial cells

To gain insight into whether the observed enhancement effect of HDACi on hBD2 induction in the two cell lines CaCo2/TC7 and HCT116 was due to their cancerous nature or rather the mono-layered status of the *in vitro* system, in a first pilot experiment, the effect of HDACi was also tested in the non-tumorous human primary gingival epithelial cell line hgep. For this, hgep cells were cultivated until they nearly reached a mono-layered confluency. Thereafter, differentiation was induced to obtain mature gingival epithelial cells. Two timepoints were chosen where differentiation had taken place for either 48 or 72 hrs to see whether different timespans might play a role. Cells were then subjected to either stimulation with IL1β alone or together with SAHA or MS-275 at the same timepoints and in the same manner as in CaCo2/TC7 and HCT116 cells.

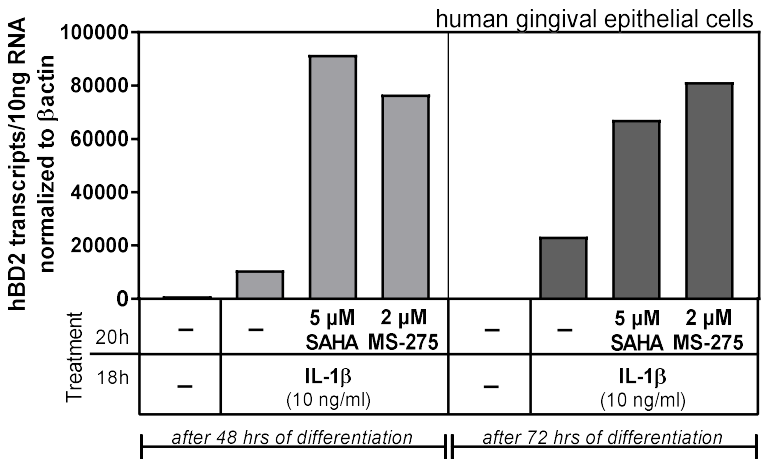

Figure 35: IL1β-mediated hBD2 induction is enhanced by HDACi in human primary gingival epithelial cells

Induction level of hBD2 mRNA in hgep cells by IL1β (10 ng/ml) after 18 hrs of stimulation. Relative transcript numbers (normalized to βactin) for hBD2 induction are shown for IL1β stimulation alone compared to when co-treated with either SAHA (5 µM) or MS-275 (2 µM) for a total of 20hrs starting. Inhibitor treatment and stimulation were done exactly as for CaCo2/TC7 or HCT116 cells. Depicted are preliminary results of a first experiment.

Interestingly, hgeps showed the same reaction patterns for hBD2 like CaCo2/TC7 or HCT116 cells. Inhibition of HDACs via SAHA or MS-275 also enhanced hBD2 mRNA expression,

irrespective of whether the duration of differentiation had been 48 or 72 hrs (Figure 35). This result, together with the findings from CaCo2/TC7 and HCT116 cells, points towards the conjecture, that rather the mono-layered and cell-typologically more homogeneous (as compared to the tissue compound of colonic mucosal biopsies) than the tumorous character of the *in vitro* cell lines is responsible for the mode of impact of the epigenetical intervention on hBD2 expression via HDACi.

3.2.4.3. Effect of HDAC inhibition on hBD2 induction in colonic tumor biopsies

To be able to shine further light on the hypothesis that the more complex, inhomogeneous nature of the colonic biopsies is the demarcating factor for the differing effect of HDACi on the level of hBD2 induction *ex vivo* versus *in vitro*, not only its mRNA expression of biopsies from non-cancerous healthy controls or IBD patients has been examined (see chapter 3.2.1 Human colonic biopsy culture), but also of biopsies obtained from colorectal tumors (n=4) (Figure 36).

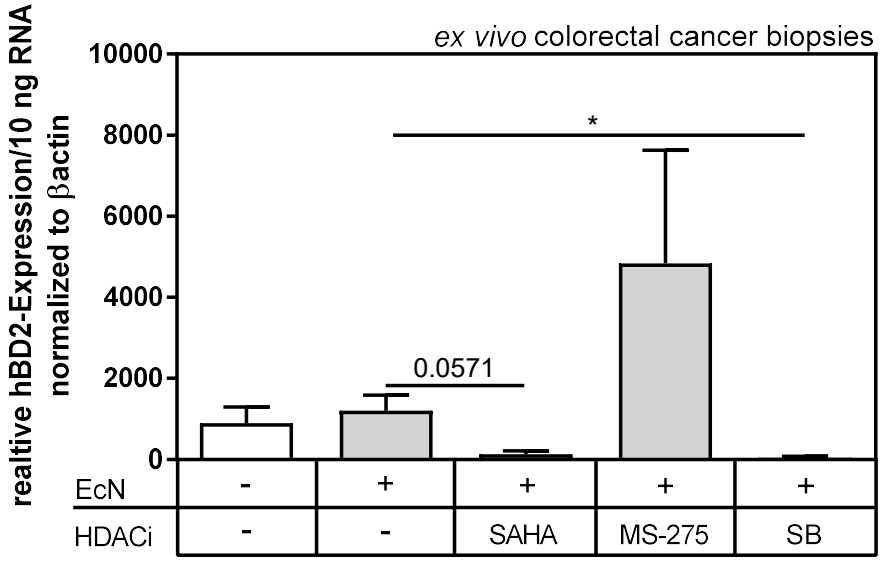

Figure 36: Inhibition of histone deacetylases HDACs hinders hBD2 induction in human colorectal tumor biopsies

HBD2 mRNA expression in cultured human colonic biopsies of four colorectal tumor specimens in response to EcN, alone or together with either SAHA (5 µM), MS-275 (2 µM), or SB (3 mM). Inhibitor treatment started 2 hrs prior to the stimulation with EcN, which took then place in parallel to HDAC inhibition for another 18 hrs. * p≤0.05 evaluated by Mann-Whitney u test. This figure has partly been published in Stebe-Frick et al., 2018.

Remarkably, in these tumorous biopsies HDACi via SAHA or SB averted hBD2 induction stimulated by EcN whereas blocking mainly HDAC1 and 3 through the inhibitor MS-275 did not suffice to do so (Figure 36), mirroring the situation in the non-tumorous tissue samples (cf. Figure 19). These results furthermore strengthen the hypothesis, that the more sophisticated

tissue compound of biopsies possibly creates an opposing epigenetic effect of HDACi on hBD2 inducibility as compared to the mono-layered, homogeneous epithelial cell lines.

4 Discussion

This study is an important contribution to the existing body of knowledge on the role of HDACs in the epigenetic control of AMP expression, focussed on that of hBD2. In this study, emphasis has been put on the therapeutically relevant probiotic *E. coli* Nissle 1917 (EcN) as hBD2-inducing factor in addition to the pro-inflammatory stimulant IL1β and the bacteria-derived factor LPS. It could be shown and confirmed that *in vitro* HDAC inhibition (HDACi) generates an NF-κB-dependent enhancement on EcN- and LPS-induced hBD2 expression, whereas for IL1β-induced hBD2 an augmentation of expression seems to be depending on additional transducing factors and conditions when only a subset of HDACs are inhibited. In the case of IL1β, the enhancing effect of HDACi could be evinced in a second intestinal epithelial cell line. In parallel, an *ex vivo* human colonic biopsy culture has been established as an effort to mimic the *in vivo* situation and allow a comparison to the *in vitro* results since it is of substantial interest to learn how a more complex, non-tumorous, human tissue compound reacts to HDACi. Strikingly, in this context, an opposing impact of HDACi on EcN-stimulated hBD2 expression was observed. Inhibiting HDAC function using pan-HDAC inhibitors hindered hBD2 expression instead of enhancing it. These contrary findings were further investigated using *ex vivo* treated human colorectal cancer tissue showing the same response as non-cancerous intestinal biopsies. Furthermore, first insights into the reactivity of primary gingival epithelial cells towards HDACi upon IL1β-stimulation were collected. Interestingly, this homogeneous, non-malignant cell line showed a comparable response towards IL1β as did the cancerous intestinal cell lines. Together, these investigations point towards a tissue context dependency of the effect of HDACi on hBD2 expression. In addition to these investigations, light has been shed on the expressional pattern of class I HDAC enzymes in respect to IBD via mRNA and prtoein analyses. This first systematic analysis is potentially opening new venues of targeted treatment.

This chapter first describes the possible means of HDAC-mediated epigenetic regulation of the antimicrobial hBD2. Furthermore, especially the involvement of NF-κB in the enhancement mechanism via HDACi is elucidated. The divergent effects between the *in vitro* and *ex vivo* approaches are discussed, and the limitiations of colonic tissue culture are addressed. Finally, the results of the HDAC expressional analysis in IBD patients are put into the literary context and discussed in the light of the use of HDAC inhibitors as possible therapeutics.

4.1. hBD2 expression is modulated by HDAC function

Within recent years, several studies have pointed towards the importance of HDAC function in the regulation of AMPs like the inducible hBD2 and thereby antimicrobial defense in different

tissues (Kallsen et al., 2012; Schwab et al., 2008; Yin and Chung, 2011). They could consequently be involved in the human β defensin-related defects observed in IBD. Complementing and extending previous investigations, this work also shows a TLR-dependent upregulation of hBD2 after HDACi *in vitro* (see Figure 23) (Fischer et al., 2016; Schlee et al., 2007, 2008; Stebe-Frick et al., 2018; Wehkamp et al., 2004a; Yin and Chung, 2011). Furthermore, the same effect has been observed using the pro-inflammatory cytokine IL1β as inducer of hBD2 where pan-HDACi as well as the more specific inhibitor MS-275, inhibiting only HDAC1 and 3, strongly enhanced hBD2 mRNA and intracellular peptide in CaCo-2 cells(see Figure 23 and Figure 25). Expression of hBD2 mRNA showed the same trends in HCT cells in response to IL1β and HDACi. These results, together with those of others, hint towards a consistency of the observed enhancing effect for several inducers tested in different *in vitro* systems, including several cell lines and even cultured human colonic organoids (Fischer et al., 2016; Stebe-Frick et al., 2018; Yin and Chung, 2011). This present work is, however, the first one to demonstrate that, under the given conditions, pan-HDACi has the opposite effect in *ex vivo* treated healthy control, IBD, and cancerous intestinal biopsies where it seems to hinder hBD2 induction (see Figure 19 and Figure 36) (Stebe-Frick et al., 2018). In an effort to illuminate the possible reasons for this disparity, the manifold ways in which HDACs are possibly impacting on hBD2 expression need to be taken into consideration.

4.1.1. NF-κB dependency of the *in vitro* enhancement effect of HDACi on hBD2 inducibility

Since the hBD2 promoter bears at least two binding sites for NF-κB transcription factors (Wehkamp et al., 2004a), the epigenetic control of NF-κB via HDAC-mediated mechanisms is likely a crucial working point in hBD2 transcriptional regulation.

Overall, remarkable progress has been made within the last two decades in understanding the complex regulation of NF-κB activation. Chromatin organization together with nucleosome remodelling has been shown to be a crucial regulatory layer in the highly complex orchestration of NF-κB-dependent inflammatory gene expression (Saccani et al., 2001; Natoli et al., 2005; Ramirez-Carrozzi et al., 2006; Natoli, 2009). Two distinct types of NF-κB-regulated genes have been described based upon the kinetics of their activation following a stimulus – the “fast” ones with constitutively and immediately accessible (CIA) promoters and the “slow” ones, that have promoters with a regulated and late accessibility (RLA) (Saccani et al., 2001). While both fast and slow genes bear high-affinity binding sites for NF-κB transcription factors, they are encompassed by different chromatin landscapes leading to a differential accessibility for NF-κB and the transcriptional machinery (Saccani et al., 2001). Chromatin surrounding readily accessible fast genes is, for example, characterized by histone marks that

correlate positively with transcription such as acetylated histones H3 and H4 or trimethylated lysine 4 on histone 3 (H3K4me3) (Henikoff and Shilatifard, 2011; Krogan et al., 2003; Natoli, 2009; Saccani et al., 2001; Yang and Seto, 2003). On the other hand, slow genes are rather shielded due to hypoacetylated and more condensed chromatin that is often also negative for H3K4me3 as long as cells rest in an unstimulated state. Upon stimulation, chromatin modifications undergo changes leading to easier accessible gene regulatory elements, e.g. NF-κB sites (Henikoff and Shilatifard, 2011; Natoli, 2009; Saccani et al., 2001). Furthermore, nucleosomes are being moved with the help of chromatin remodeling complexes such as the Swi/Snf or the Mi2/Nurd complex (Becker and Hörz, 2002; Ramirez-Carrozzi et al., 2006; Scarpa and Stylianou, 2012; Skiniotis et al., 2007), the latter's catalytic core comprises HDAC1 and HDAC2 (Yang and Seto, 2008). Together, the necessity of these events to take place leads to a delayed transcriptional start of slow genes (Saccani et al., 2001). Unfortunately, so far direct experimental evidence on the kinetics of NF-κB recruitment to hBD2 gene regulatory sequences is sparse. A hint that a change in histone modification at the hBD2 promoter is happening in CaCo2 cells under HDACi and upon *E.coli* K12 stimulation has been demonstrated recently with the detection of increased phosphorylation of serine 10 of H3 (Fischer et al., 2016) -a histone mark that is known to facilitate NF-κB access to DNA possibly by alleviating recruitment of chromatin-remodeling complexes at a subset of innate immune genes (Cheung et al., 2000; Saccani et al., 2002). At this point, it is worth mentioning that Saccani and colleagues could furthermore show that the IL6 promoter is activated at different velocities via NF-κB in mouse macrophages (rather slowly) in response to LPS as compared to in fibroblasts where NF-κB is recruited very fast (Natoli et al., 2005; Saccani et al., 2001, 2004). So the possibility exists that hBD2 transcriptional activation also does not exhibit the same kinetics in different tissues or cell types, e.g. in keratinocytes of the skin and intestinal epithelial cells. All in all, it would be worthwhile to investigate further, whether the NF-κB-dependent hBD2 promoter is indeed late accessible in the tissue or cells of interest to be able to further dissect or even better – predict what specific effect a certain HDACi might have there and at which rate.

By impacting on the overall cellular acetylation status, HDACs influence on this complex, dynamic and multilayered organization of chromatin (Heintzman et al., 2007; Seto and Yoshida, 2014; Yang and Seto, 2003), and furthermore on the posttranslational modification (PTM) of NF-κB itself, e.g. via class I HDAC activity on RelA. Many stimulus- and possibly cell-type specific PTMs have been shown to influence on the duration and strength of NF-κB nucelar activity (Huang et al., 2010). Chen et al demonstrated for example how acetylation of RelA at specific lysines differentially impacts on its DNA-binding capacity, the ability to assemble with inhibitory κB (IκB) molecules, delaying or facilitating its nuclear export, or affecting the completeness of its transcriptional activity (Chen et al., 2002). It has been shown that HDAC3, likely by deacetylating RelA at lysines 218 and 221, accelerates its nuclear export and abrogates NF-κB-dependent gene

expression (Chen et al., 2001; Huang et al., 2010). Interestingly, deacetylation of lysines 122 and 123 by HDAC3 unmasks the positive charge of those lysines which seem to be essential for the binding of RelA to a κB-enhancer (Kiernan et al., 2003). This is an example where deacetylation actually leads to increased rather than repressed transcription, adding another twist to the complexity of posttranslational regulation of NF-κB. Furthermore, HDAC1 together with HDAC2 have been shown to associate with the RelA subunit acting as corepressors (Ashburner et al., 2001). Recently, acetylated ReLA at lysine 310 has been reported to be increasingly recruited to the hBD2 promoter under HDACi (Fischer et al., 2016).

Effect of NF-κB-inhibition

Our results from the experiments with the NF-κB-inhibitor Helenalin in CaCo2 cells nicely reflect that prevention of NF-κB binding to DNA largely abolished SAHA- and SB-induced enhanced hBD2 expression, indicating that the "unleashed" activity of NF-κB transcription factors due to elevated acetylation under HDACi could be hindered via NF-κB-inhibition. Therefore, that "unleashed" activity is likely causative for the enhancing effect on hBD2 expression observed *in vitro*. However, not only the effect of HDACi on NF-κB modifications but also on histone acetylation possibly occurring at the hBD2 promotor after stimulation has to be taken into account. Considering all the above mentioned spots where HDACs influence on deacetylating histones in relevant nucleosomes and NF-κB itself, perturbing HDAC function with the use of inhibitors, especially with pan-inhibitors like SAHA or SB, can easily derange the order in inflammatory and innate immunity gene regulation by NF-κB on several levels. This also holds true for hBD2.

Interestingly, inhibiting only HDAC1 and HDAC3 by using MS-275 revealed that some level of hBD2-enhancement was still possible under simultaneous NF-κB inhibition (Figure 30b, left and right panel, EcN and LPS stimulation respectively), suggesting further HDACs to be substantially involved in a putative chromatin remodeling process at the hBD2 promoter which are blocked in the case of pan-HDACi (Stebe-Frick et al., 2018). The promoter might be left rendered accessible to further transcription factors, which are in turn possibly negatively regulated by other HDACs than HDAC1 or 3, accounting for the left-over enhanced hBD2 expression. Shedding some light on HDAC1 and 3 involvement at modifying histone acetylation signatures especially of nucleosomes surrounding the hBD2 promoter would be interesting.

For IL1β- induced hBD2, the MS-275-mediated enhancement has neither been sufficiently blocked nor attenuated by NF-κB inhibition (Figure 30b, middle panel) suggesting other signaling pathways and transcription factors to be involved in IL1β-mediated hBD2 induction (Stebe-Frick et al., 2018). Besides AP1 (Wehkamp et al., 2004a), further factors and signaling proteins like p38 mitogen-activated protein kinase (p38 MAPK), or c-Jun N-terminal kinase (JNK) could play a role (Jang et al., 2004). In addition, putative feedback loops involving

upregulated cytokines such as IL8 or the IL1β-responsive IL6 could be taking part in modulating signal transducers and activators of transcription (STAT) signaling-induced hBD2 transcription (Albanesi et al., 2007; Kanda and Watanabe, 2008; Vitkus et al., 1998). HDACs together with histone transferases (HATs) have been shown to regulate STAT signaling in multiple, sophisticated ways (Icardi et al., 2012a; Zhuang, 2013). A few years ago, Icardi et al reported that the SIN3 transcription regulator homolog A (Sin3a) co-repressor complex containing HDAC1 and 2, deacetylates STAT3 and thereby negatively regulates STAT3-dependent gene expression (Icardi et al., 2012b). But also possibly positive effects of HDAC activity on STAT function have been demonstrated, e.g. that in mouse macrophages binding of STAT1 to DNA is compromised when STAT1 is hyperacetylated (Guo et al., 2007), or that HDACi can lead to increased expression of suppressors of cytokine signaling (SOCS-)1 and 3 and thereby to downregulated STAT3 signaling in colorectal cancer cells (Xiong et al., 2012). Furthermore, NF-κB-STAT signaling crosstalk could be affected by the use of HDACi. Acetylated STAT1 has been shown to interact with RelA decreasing its DNA binding and nuclear localization (Krämer et al., 2006).

Those and many more findings on non-histone (de-)acetylation processes indicate how tightly, multilayered, and dynamic not only cytokine expression but also expression of the antimicrobial hBD2 are regulated and modulated. So, in general, there are very likely multiple signaling pathways that might be affected and modulated simultaneously by HDACi and thereby their interplay among each other. This possibly leads to a differential impact on hBD2 expressional control depending on the stimulatory context and furthermore the microenvironment of each cell especially in a tissue compound or organ. This microenvironment is probably additionally affected by the overall epigenetic state a certain individual is in. The development or discovery of HDAC isotype specific inhibitors is therefore very important, to lower possible undesirable side effects and to be able to interfere more specifically in certain disease aspects related to HDAC function or are at least mendable by altering it.

Of notice, comparing the enhancing effect of the specific inhibitor MS-275 on hBD2 to that of the pan-inhibitor SAHA when stimulating CaCo2 cells with EcN shows that the selective inhibition, at least in this setup, is similar. When hBD2 is stimulated via IL1β or LPS the enhancing capacities of the two inhibitors are significantly different. Here SAHA has a stronger effect. According to those results, HDACs 1 and 3 play a major role in negatively regulating TLR5-dependent hBD2 induction, whereas other HDACs seem to have greater importance when cells are stimulated via LPS or IL1β (cf. Figure 24) (Stebe-Frick et al., 2018).

Role of different transcription factor binding sites in the hBD2 promoter region

The role of NF-κB but also the AP1 binding site controlling hBD2 expression could be further confirmed inhibiting total HDAC function using SAHA on CaCo2 cells transfected with hBD2 promoter constructs (see Figure 31). The proximal NF-κB binding site in the hBD2 promotor region has proven to be the one essential for a great deal of the enhancing effect of SAHA on EcN- or LPS-induced reportergene activity. When the AP1 site was non-functional but the proximal NF-κB site still was, the enhancement of hBD2 was also clearly diminished. This suggests a cooperative function of those two regulatory elements where the proximal NF-κB site needs the AP1 site to be activatable for full enhancing effect on hBD2, while AP1 alone is not sufficient. Since those two transcription factor binding sites are in a similar manner necessary and responsible for the plain or basal inducibility of hBD2, as has been demonstrated before (Wehkamp et al., 2004a), it can be assumed that the HDACi effect in this experimental setting can be ascribed to changes in epigenetic regulation of signaling pathways and transcription factors. This assumption is furthermore strengthened by the finding that transiently transfected plasmids do not seem to be packed efficiently into nucleosomes (Smith and Hager, 1997), making the regulatory layer of chromatin modification unlikely to play a role in the above mentioned observation. Strikingly, mutation of the proximal binding site also hindered the IL1β-induced promoter activation, indicating that the initial induction of the hBD2 gene via IL1β signaling is strongly dependent on this NF-κB binding site, and AP1 also plays a similar part, but in this case not the augmentation of hBD2 as has been observed for the intrinsic gene expression in CaCo2 cells (cf. Chapter 3.2.2.1 Induction of hBD2 by different stimuli – The effect of HDAC inhibition). In the CaCo2 stimulation experiments, the observed enhancement of IL1β-induced hBD2 is likely mediated by additional NF-κB binding sites, which are not included in the constructs utilized here, since blocking total NF-κB function with Helenalin was capable of abolishing the SAHA-enhanced IL1β-induced hBD2 expression (see Figure 30).

More general inferences on the pan-HDACi effect on these transcription factors could be drawn from the use of plasmids with either tandemly repeated NF-κB or AP1 binding sites reacting to stimulation with EcN under SAHA application (see Figure 32). EcN alone did not activate AP1 binding sites but slightly elevated NF-κB controlled reportergene expression. SAHA alone could activate NF-κB sites, but even more so AP1 sites. AP1 has been shown to be as well negatively regulated by deacetylation processes (Yang et al., 2017). Seemingly, the sole use of HDACi might have relieved some sort of repression from AP1 transcription factors exerted by HDACs. Most interestingly, a synergistic effect of EcN and SAHA treatment could be observed on the reportergene activity mediated by NF-κB site tandem repeats, additionally supporting the hypothesis of hBD2 mRNA expression to be indirectly repressively regulated by HDACs via NF-κB transcription factors.

Summarizing, the means via which HDACs can influence on the transcriptional regulation of hBD2 are via a combination of chromatin accessibility together with the impact they can exert on transcription factors themselves, such as NF-κB, AP1, or STATs, and other signaling molecules.

4.1.2. Divergent effects in tissue versus monolayered cells

The data regarding hBD2 presented here show a striking divergence in the direction of the effect of HDACi on hBD2 inducibility when investigating different experimental setups, namely homogeneous monolayered cancerous cell lines as opposed to the more complex tissue compound of colonic mucosal biopsies (Stebe-Frick et al., 2018). This finding is unprecedented and highlights the importance of more *in vivo* like experimental approaches.

Enhanced hBD2 induction in vitro

While *in vitro* in CaCo-2 intestinal epithelial cells, hBD2 mRNA and peptide are enhanced when induced via bacterial or pro-inflammatory stimuli and HDACs are inhibited in parallel, the complete opposite effect has been observed in *ex vivo* biopsies where pan-HDACi with SAHA or SB abolished hBD2 mRNA expression. In quest of an explanation, a second intestinal epithelial cell line, HCT116 cells, has been examined. It displayed the same answer in hBD2 expression in response to IL1β and HDACi as did CaCo2 cells. Neither EcN nor LPS lead to a significant induction of hBD2 in HCT116 cells. Therefore and since this is the first report on an enhancing effect of HDACi on IL1β-induced hBD2 *in vitro*, HCT116 cells served as a second intestinal epithelial cell line to confirm the CaCo2 results concerning IL1β. The non-tumorous human primary gingival epithelial cell line hgep was tested in a pilot experiment and showed the same answer to HDACi+IL1β treatment as did the two cancerous cell lines suggesting that tumorous cell lines and primary cell culture seem to show the same reaction in this respect (cf. Figure 33-Figure 35) (Stebe-Frick et al., 2018). Inhibiting HDAC function in rather homogeneous cell line *in vitro* systems seems to release a potentially repressive effect of HDACs on hBD2 expression, leading to an enhancement. So, this appears consistent for multiple inducers. A very similar effect has also been observed in cultured human organoids derived from colonic epithelial cells (Fischer et al., 2016). The means via which a repression by HDACs could possibly be established, have been discussed in the previous chapters.

Of note, HDACi alone did not induce hBD2 in neither CaCo2 nor HCT116 cells but had an elevating effect on basal IL8 expression, a finding that needs to be kept in mind when talking about the use of HDACi as anti-inflammatory therapeutic. Furthermore in this context, in contrast to a previous report (Fischer et al., 2016), the results presented herein clearly show an upregulation of the pro-inflammatory IL8 in response to stimulation under HDACi (Stebe-Frick et al., 2018) (also see Chapter 4.3 HDACi-mediated enhancement of hBD2 as therapeutic option in IBD). The study at hand does therefore not support the proposition of HDACi as a pharmacological mean to reinforce antimicrobial defense without risking an increase in the inflammatory response in parallel. This discrepancy in respect to IL8 could, however, be due to the different study designs used. Fischer et al pretreated with HDACi for 16 hrs before

stimulating with E.coli K12 for only one or two hours, while in this study, HDACi started 2 hrs prior to the addition of the different stimulants for another simultaneous treatment for 18 hrs. Consequently, it would be of interest to further investigate the pharmacokinetics of HDACi in respect to antimicrobial and cytokine regulation in epithelial cells. The concentrations and timepoints used in this study are drawn from the literature stating reasonable durations for and concentrations of HDACi (Boffa et al., 1978; Butler et al., 2002; Leoni et al., 2002; Oger et al., 2010; Tatamiya et al., 2004; Wilson et al., 2006), and fitted to known kinetics of hBD2 (Möndel et al., 2009; Schlee et al., 2008; Wehkamp et al., 2004a).

In contrast to the observation in CaCo2 cells, where stimulus-induced IL8 transcription was further increased by the use of HDACi, IL1β-induced IL8 expression was not enhanced in HCT116 cells (Figure 34b) indicating a potential cell line specific inflammatory response- additionally potentially under a different epigenetic control. This assumption is underlined by the observed primary inducibility of IL8 by IL1β stimulation which lead to a much higher IL8 level (about 100 000 transcripts/10ng RNA normalized to βactin) in HCT cells than in CaCo2 cells (about 1300 transcripts/10ng RNA normalized to βactin). The differing epigenetic statuses, owed to the cancerous nature of the cell lines (Ahmed et al., 2013), could be accountable for both the difference in the initial IL8 response towards IL1β and also for the absent enhancing capacity of the applied HDACi.

At this point, it is worth noting that in HCT116, the response to EcN with hBD2 was very low but a substantial induction of IL8 was possible. So, TLR5-dependent induction of hBD2 in HCT116 cells following stimulation with EcN (see Figure 33a) seems to be impaired. HCT116 cells have been shown to express TLR5 receptor at rather low levels (Zhao et al., 2007). Furthermore, the responsiveness to flagellin in cancer cells lines might be differentially modulated due to the presence of different SNPs that have been found in the TLR5 receptor gene in colorectal cancers (Klimosch et al., 2013). This might explain why only a very weak hBD2 response could be observed downstream to EcN stimulation in these cells while IL8 could be induced to some extent. Likely due to a lack of detectable or at least only a very low amount of TLR4 receptor expression in this cell line (Zhao et al., 2007; Doan et al., 2009), there was no induction of hBD2 in response to the TLR4 ligand LPS. However, LPS was able to induce the expression of a small amount of IL8 mRNA, possibly owed to a still present but very low level of TLR4 receptor expression which possibly was sufficient to activate the IL8 promotor (Doan et al., 2009).

Repressed expression of hBD2 ex vivo

Analyzing the role of HDACs in hBD2 regulation in human intestinal tissue, however, unraveled a completely opposite effect, at least when all HDACs were inhibited collectively. Under the use of SAHA or SB on *ex vivo* cultured colonic biopsies, the upregulation of hBD2 mRNA by the

probiotic EcN was completely prevented and expression levels returned to. The more selective inhibitor MS-275, that only blocks HDACs1 and 3 also did not cause an enhancement, as has been observed in CaCo2 cells *in vitro*, but still allowed for hBD2 induction by EcN baseline (Stebe-Frick et al., 2018). At this stage, it can be concluded, that SAHA and SB both had a clear effect on the tissue response, whereas in the case of MS-275 a possible insufficient functionality could not be completely excluded as the reason for the lacking or differing effect. Technical issues such as a limited uptake of the compound by and/or reduced diffusibility within the tissue have been tried to be ruled out. Firstly, by an initial check to clarify whether MS-275 was potentially not able of diffusing as well into the tissue as it could diffuse into the mono-layered CaCo2/TC7 cells. Therefore the MS-275 concentration used in the *ex vivo* experiments was increased to 5 µM as compared to 2 µM *in vitro* which has been adopted from the literature (Tatamiya et al., 2004). The higher concentration also was not able to abolish hBD2 expression in *ex vivo* biopsies. Due to the advanced progress of the experiments with the valuable patient samples, it has been decided to abide to the higher concentration for the ongoing *ex vivo* experiments. And secondly, by conducting a western blot analysis to attest that HDAC proteins have been inhibited efficiently, in one feasibility test, the acetylation status of histone 3 has been checked after the 20 h treatment with EcN and SAHA, MS-275 and SB in the biopsies of one patient. A marked increase in histone 3 acetylation could be observed in response to all HDACi used, indicating that at least enough of each of the inhibitors has diffused into the cells to cause an effect on chromatin modification (see Appendix Figure 3). However, no direct inferences on the actual effect at the transcription factor level can be drawn from these results, as to which extend the applied concentrations and durations of HDACi have an impact on e.g. the acetylation status of NF-κB, AP1, or STATs. Those and others are known targets of HDAC1 and 3 and could thereby still affect hBD2 regulation at the deployed concentration of MS-275(Ashburner et al., 2001; Chen et al., 2001; Huang et al., 2010). In this regard, a limited mode of action of MS-275 within the tissue cannot be ruled out. Furthermore, due to the limited amount of the specimens, it was unfortunately not possible to conduct these additional experiments for each patient included in the study. This data still allows to hypothesize that the specific inhibition of HDACs 1 and 3 cannot hinder hBD2 in *ex vivo* stimulated human intestinal epithelial tissue from neither healthy individuals, nor ones with IBD, nor ones colorectal cancer (which are further discussed below).

At this point, it cannot be excluded that, further *ex vivo* testing of time- and dose-responses towards HDACi might deliver a different picture of the effect on the transcriptional profile, including that of hBD2, of the *ex vivo* cultured biopsies. Maybe even one resembling the effects observed *in vitro*. Unfortunately, investigating pharmacokinetics in *ex vivo* tissue culture has not been feasible due to the limited amount of patient specimens. Nevertheless, the described profound effect clearly demonstrates that pan-HDACi of all class I isoforms might

potentially hinder hBD2 expression under certain circumstances and thereby impair antimicrobial defense. This needs to be taken into serious consideration when evaluating HDAC inhibitors, also more selective ones, as a therapeutic (also see Chapter 4.3 HDACi-mediated enhancement of hBD2 as therapeutic option in IBD), since the abolishment of hBD2 has equally been observed in IBD patient samples (cf. Figure 19).

In the quest of exploring the underlying mechanisms of the divergent findings from the *in vitro* and *ex vivo* approaches, it has been important to further consider the possibility, that the reason for this could be lying in the cancerous nature of the intestinal cell lines (as mentioned in the previous chapter) as opposed to the non-malignant tissue samples. Therefore, biopsies from colorectal tumors have been examined under the same *ex vivo* conditions. Strikingly, here pan-HDACi also seemed to disable hBD2 induction, whereas MS-275 again still allows for hBD2 transcription to take place (see Figure 36). This result supports the assumption that the discrepancy between *in vitro* and *ex vivo* effects are unlikely to be caused by the altered HDAC function in cancers (Ahmed et al., 2013; Ropero and Esteller, 2007). The findings from the primary cell culture experiments with differentiated gingival epithelial cells complement this assumption and strengthen the hypothesis, that the more sophisticated tissue compound of biopsies possibly creates an opposing epigenetic effect of HDACi on hBD2 inducibility as compared to the mono-layered, homogeneous epithelial cell lines. This could be due to possibly still feasible paracrine crosstalks within the tissue, for example, with lamina propria cells. Consequently, further investigation is needed, e.g. in dissecting the effect of HDACi on hBD2 in single cells from *ex vivo* treated biopsies via single cell sequencing as well as in co-cultures of intestinal epithelial and stromal cells such as fibroblasts (Viney et al., 2009). Such co-cultures, also with immune cells like intestinal intraepithelial lymphocytes (IELs) (Beagley et al., 1995; Nozaki et al., 2016), bear the potential of investigating the exact molecular mechanisms of the HDAC-dependent gene regulation in cells embedded in a more diverse cellular microenvironment. As for now, future epigenetic considerations of cell culture systems need to be carefully evaluated in the light of these divergent findings from tissue culture.

Last but not least, the expression of IL8 in *ex vivo* treated intestinal biopsies appeared to not be significantly affected by any of the HDAC inhibitors in neither of the patient groups tested. As opposed to the results from the CaCo2 cells, HDACi did not further enhance IL8 expression in response to medium treatment alone or stimulation with EcN. Strikingly however, HDACi also did not abolish IL8 expression as compared with hBD2, pointing towards more distinctive epigenetic regulatory mechanisms for IL8 and hBD2 on the level of HDAC function (Stebe-Frick et al., 2018).

Taken together, the remarkable divergence from the *ex vivo* culture and the cell culture experiments presented in this study and the findings from others prompt the speculation that the role of HDACs in hBD2 transcriptional regulation likely is more complex and context-

dependent than so far anticipated, not only in terms of the microenvironment of the cells but also in terms of stimulus type and duration. Fischer and colleagues reported a very similar result in cultured organoids derived from human colonic epithelial cells (Fischer et al., 2016) as has been observed in classical cell culture. This furthermore suggests the involvement of other non-epithelial mucosal cells, potentially infiltrated immune cells as well as IELs that could modulate and possibly reverse the HDACi effect on the epithelium. Up until now however, the exact mechanisms underlying the gap between cell-/organoid- and *ex vivo* tissue culture remain elusive(Stebe-Frick et al., 2018).

Brief evaluation of the in vitro and ex vivo approach used in this study

This study at hand is the first to show that HDACi seems to have a different potential in affecting hBD2 and also IL8 expression depending on the experimental system used. Therefore, it highlights the importance of more *in vivo* like experimental approaches employing human patient material. At this point it is important to be said, that using mice for the study of the role of HDAC function in the regulation of antimicrobials is a necessary endorsement that allows proof of concept conclusions and to receive *in vivo* insights. However, mice are not humans, physiologically differing in respect to defensins (Cunliffe and Mahida, 2004) or the immune system in general (Mestas and Hughes, 2004). This has to be kept in mind when working with mouse systems, even though HDACs are generally well conserved among mammals (Gregoretti et al., 2004).

In vitro investigations such as those that have been undertaken here, are indispensable for studying the mechanistics of the observed effects, since high throughput experiments as would for example be necessary for pharmacokinetic studies are mostly unfeasible with patient tissue material. It has become obvious though, that the establishment of more sophisticated *in vitro* culture systems such as co-cultures of different cellular components of the intestinal epithelium like epithelial cells, fibroblasts and even lymphocytes, would possibly reveal much needed insights whilst incorporating more aspects than homogeneous cell culture. The *ex vivo* culture of epithelial biopsy tissue employed in this study definitely offers the advantage of evaluating the impact of HDACi treatment on the probiotic response in tissue in the context of the surrounding multicellular microenvironment.

Tissue culture limitations

However, like each experimental system, tissue culture as well bears some limitations. Given the procedure of obtaining colonic biopsies and the fact, that they are samples that have been torn out of a greater context, it is important to keep in mind, that by this, they are likely primed towards a pro-inflammatory status. This is reflected by the substantial expression of IL8 in medium-treated samples (cf. Appendix Figure 2). Therefore, in this setup, additional auto- and paracrine effects of pro-inflammatory mediators on the inducibility of hBD2 cannot be ruled out

and have to be taken into consideration when evaluating the microenvironment of the cells in *ex vivo* tissue. Those effects would however already be present in the medium-control samples. Therefore, those samples have been used as references focusing on fold changes compared to the already basally hBD2-induced medium control samples.

Besides this, biopsies from different patients also are not as homogeneous in terms of their background concerning genetics, lifestyle of the individual patient, and also the epigenetic status. Therefore, quite a large number of samples was needed to receive a representative population profile. A positive aspect of *ex vivo* culture is, that biopsies also contribute primary defects present in IBD patients (Courth et al., 2015; Stebe-Frick et al., 2018).

Furthermore, histological stainings of biopsies treated for 20 hrs using hematoxylin and eosin (cf. Figure 18 and Figure 20) revealed the tissue to be partially affected to the extend that disintegrated laminae propriae and epithelial linings could be observed equally throughout all the culturing conditions. This is due to the short viability of the tissue *ex vivo*. However, overall the tissue was still relatively intact after 20 hrs of incubation. In general, cellular viability seemed to still be in quite a good condition since most of the determined LDH values of the biopsies were in low-end to intermediate levels (data not shown) which allows to reckon on reasonable testing conditions. Furthermore, e.g. Aldhous and colleagues also have successfully *ex vivo*-treated and investigated colonic biopsies over a period of even 24 hrs which exceeds the duration employed herein by another 4 hrs (Aldhous et al., 2009).

As has been elaborated above, unfortunately only one time point and concentration per HDACi could be tested as well as one stimulant (EcN) due to the limited number of samples per patient. Up till now, this leaves blanks as to whether the stimulation and inhibition might be able to elicit a different effect at earlier timepoints, but still gave an important and unexpected insight which ultimately underlines the importance of reconciliation of the use of HDACi as therapeutic treatment especially for IBD, if it possibly has this dampening effect on the expression of at least one AMP shown to be important for the barrier function of the intestinal lining (Ostaff et al., 2013; Wehkamp et al., 2002, 2005a).

Reflections on the inducibility of hBD2 in ex vivo cultured biopsies

Generally speaking, the study of hBD2 has been of particular interest in the context of IBD, especially UC and colonic CD (Jäger et al., 2013). Those two forms of IBD display different potentials in upregulating hBD2, which seems to be readily inducible in actively inflamed UC, however in CD patients hBD2 induction attenuated which might renders the epithelium more prone to be infected by resident microbiota (Nuding et al., 2007; Wehkamp et al., 2003). This variation has been found in several IBD cohorts investigated by different groups (Fahlgren et al., 2003; Wehkamp et al., 2002; Zilbauer et al., 2010) even though the mechanisms remain unclear. Gene copy number variations of the beta defensin cluster bearing reduced gene copies of hBD2

have been suggested as the underlying cause in German and US American patients (Fellermann et al., 2006). However, this association could not be confirmed in another cohort (Aldhous et al., 2009). Our herein presented data on hBD2 induction in *ex vivo* cultured biopsies could indeed help in further elucidating those differences in the two IBD entities. This work on biopsies from IBD patients is the first to show that the probiotic strain EcN alone is capable of inducing hBD2 in *ex vivo* colonic mucosa and that there are clear differences in the inducibility between healthy individuals and L1 CD patients, but also in comparison to L3 (Stebe-Frick et al., 2018). This nicely adds to previous work from our group were it has been shown, that oral intake of EcN leads to an increase in hBD2 levels in stool samples of healthy individuals (Möndel et al., 2009).

In line with the above mentioned investigations describing distinct hBD2 expression levels in CD and UC, it could also be demonstrated that on the level of fold changed induction, Crohn´s disease (L1 and L3) patient mucosa indeed seems compromised in inducing hBD2 using *ex vivo* EcN-treated biopsies. This is especially marked in the group of L1 patient samples. However, this finding only became apparent, when comparing the fold change in induction of hBD2 under EcN stimulation to the respective unstimulated, only medium-treated control biopsies. It is of note, that when looking at the relative expression levels of L3 patient biopsies (as depicted in Appendix Figure 1) it became apparent that those samples show a higher median starting point than the other groups tested and then end up at the same level as the UC samples. The reason for the higher median basal induction in the L3 medium-treated group is so far unclear, but can be credited to the samples of 5 out of the 14 patients tested. Four out of those 5 L3 “high starters” also show the highest end levels in hBD2 expression under EcN stimulation (labelled with red shapes in Appendix Figure 1). There were however no explicit reasons to exclude those particular L3 patients from the cohort. Doing so, would have leveled the L3 median relative hBD2 expression under both treatment conditions into the range of the L1 group. Of note, an exclusion of the indicated UC “high starters” with high end levels after EcN stimulation from the UC group in parallel to hypothetically excluding the L3 “high starters” would not change the picture (Appendix Figure 1, sample values marked in blue). Apriori, it is however rather necessary to include further L3 colonic samples to decipher whether these few “high inducers” truly are exceptions and do separate completely from the rest of the L3 group. Furthermore, it is a stand-alone interesting finding, that L1 disease-unaffected colonic samples also seem to be compromised in inducing hBD2 on the relative expression level in an *ex vivo* setup with probiotic bacteria as the hBD2 stimulant.

4.2. Changes in class I HDAC expression levels in patients with IBD

To this date, not much is known about the expression status of HDACs in IBD patients which have been shown to be involved in chronic intestinal inflammation (Glauben et al., 2006; Turgeon et al., 2014). Especially class I HDACs seem to play a role. Therefore, expression of class I HDACs has been strategically analyzed in ileal and colonic mucosa from active as well as inactive IBD.

4.2.1. Diverse findings from mRNA and protein analyses

The results of these analyses provide first insights into the overall deacetylation capacity of the intestinal tissue in IBD based on the inflammatory status, as well as in comparison to and within overall healthy tissue. Data from the RT PCR analysis of mRNA expression levels showed that overall if there were changes in the expression levels of IBD patients as compared to healthy controls, they were always diminished (cf. Figure 6). No increased levels could be detected. In ileal tissue only HDAC2 was reduced in both L1 and L3 CD, independent from the inflammatory status (only ileal or ileal and colonic affliction respectively). In the colonic samples of CD (L2 and L3 patients examined, L2 being only affected in the colon by the disease) reductions have been observed in HDAC1 and HDAC2 levels. In UC samples of the colon, however, all 4 class I HDACs seemed to be affected- HDAC8 independently of the inflammation status, HDACs 1,2, and 3 were even further reduced during inflammation. At this point, it is worth mentioning that Tsaprouni et al could demonstrate that histone 4 acetylation is slightly increased in the uninflamed ileal tissue of CD patients, but was markedly elevated in inflamed specimens. This was also shown in the inflamed mucosa of a murine model of colitis (Tsaprouni et al., 2011). These results hint towards a reduced HDAC function in CD ileal tissue, especially during inflammation.

Interestingly however, the findings from the western blot experiments display a diverting picture - at least in some aspects (cf. Figure 7 and Figure 8). For instance, HDAC2 protein seems to not be reduced in L3 specimens but instead rather markedly increased compared to healthy controls. A tendencial increase in protein levels has also been found for HDAC1 in the ileum of L3 but not so for the mRNA level. Colonic specimens, for example showed a trend towards reduced HDAC1 protein levels in UC, which is in line with the corresponding mRNA data, but HDAC2 protein in UC seemed tendentially increased not decreased as was HDAC2 mRNA. HDAC3 protein on the other hand again rather corresponded to its mRNA. L3 tissue from the colon showed no changes in HDAC1 or 2 protein, thereby did not correspond to their mRNA. Before going into discussing possible underlying causes, it needs to be remarked that different, meaning new biopsies not identical to those used for RT PCR, had to be used for

western blotting. Therefore, a direct deducibility for conclusions regarding mRNA and protein correlations is not given. Although small, considering the IBD samples investigated via western blot as an at least to some extend comparable patient cohort still allows to speculate about potential coherences. Unfortunately, overall not much is known so far about the expressional regulation of neither HDAC mRNA nor its translation into protein. Several reports point towards feedback mechanisms involving HDACs themselves. For HDAC1, for example, it has been shown in hepatocellular carcinoma cells that HDACi by Trichostatin A leads to HDAC1 upregulation (Gray and Ekström, 1998). Schuettengruber and colleagues could thereafter demonstrate that transcriptional regulation of the mouse HDAC1 is autoregulated and involves specificity protein 1 (SP1) and nuclear transcription factor Y (NF-Y) transcription factors, which can recruit HDAC1 to its own promotor leading to HDAC1-mediated HDAC1 transcriptional repression (Schuettengruber et al., 2003). HDAC1 has furthermore been shown to also negatively regulate the expression of HDAC2 and 3 in mouse embryonic stem cells (Lagger et al., 2002). At the level of alternative splicing, HDAC3 mRNA has been shown to be regulated by diverse stimuli and pathways, even more so in differing ways between murine and human cells (Gray et al., 2003). HDAC2 seems to furthermore be repressively regulated by the adenomatosis polyposis coli (APC) tumor suppressor in a Wnt pathway-dependent manner in colorectal cancer cells (Zhu et al., 2004). A large number of microRNAs (miRNAs) has been predicted via in silico analyses to interact and thereby potentially post-transcriptionally regulate HDAC mRNA (Felice et al., 2014). A link to IBD could be found in miRNA expression profiles of IBD biopsies where for example miR-192, predicted to target HDAC2, has been reported to be reduced in active UC (Wu et al., 2008). All the above-mentioned findings display a complex multilayered and potentially species- and/or cell-type-specific regulation of HDAC transcription where distinct IBD regardings still remain due. It becomes clear, that there is unlikely an easy answer to the question whether the downregulated class I HDAC mRNA levels in intestinal IBD tissue presented herein are cause of changes in corresponding protein levels and/or whether the manifold ways of post-translationally modulating HDAC function and stability (Segré and Chiocca, 2011; Sengupta and Seto, 2004) are rather feeding back on mRNA expression rates. Furthermore, a possible inflammation dependency of HDAC expression but also activity needs to be considered since HDAC1 mRNA as well as protein have been found to be increased in response to tumor necrosis factor α (TNFα) in rheumatoid arthritis synovial fibroblasts (Kawabata et al., 2010). To be able to draw more precise conclusions, not only mRNA and protein from the very same biopsy need to be examined, but furthermore, a larger cohort than could be used for western blotting here, would be needed. This is also true since there are partly substantial interindividual variations that have been found in protein levels, especially for ileal HDAC1 in L3 and HDAC2 in controls and L3 patients. This finding is in line with the growing knowledge on seemingly substantial inter- and even intraindividual variations in epigenotypes (Flanagan et al., 2006; Fraga et al.,

2005; Petronis et al., 2003). Nevertheless, the findings presented here concerning mRNA and protein levels in IBD patients as well as healthy individuals deliver some important first systematic insights and definitely show changes in mRNA expression of members of the class I HDAC family in this inflammatory condition. Those are valuable findings for the discussion on HDACi as a therapeutic intervention in IBD. The herein presented data might suggest that, while mRNA expression differences exist, a possible inflammation mediated change in HDAC protein levels could be minor due to for example possible compensatory mechanisms in stabilizing protein.

4.2.2. Insights into tissue distribution of HDACs in IBD

In general, immunohistochemical stainings are well recognized for the investigation of the cellular distribution of the protein of interest, less for quantitative analyses (Cregger et al., 2006). Staining intensity can vary due to technical reasons (de Matos et al 2010), therefore, in this study, IHC was mainly used to describe the spatial distribution of the investigated HDAC proteins. Only the different amount in HDAC1 positive cells in ileal tissue of CD patients could be semi quantitatively assessed, not considering staining intensity, and has been evaluated in percentage terms by four independent individuals (cf. Figure 10). This particular finding is interesting, since it shows a trend towards an increased number of HDAC1 positive cells in the mucosa, and more specifically, in the villus epithelial linings of CD patients as compared to healthy controls. This indicates, that HDAC1 protein levels are higher in villus epithelial cells of ileal CD, not only delivering important spatial information of HDAC1 expression, but also underpinning the finding from the western blot depicted in Figure 7a. Since, of course, those are only a snapshots in time, it remains elusive, whether this elevation of HDAC1 protein is sustained over a longer period of time, whether it is causative for or rather an effect of the disease, or to which extend and at which specific point of disease progression it is relevant. It seems however likely to be of importance, as HDAC1 and also 2 have been shown to impact on the inflammatory response in rat intestinal epithelial cells (Gonneaud et al., 2014) and the general intestinal homeostasis in mice (Turgeon et al., 2013, 2014). The negative feedback autoregulatory mechanism described for HDAC1 (Schuettengruber et al., 2003) could potentially be underlying to an oscillation in the amount of epithelial HDAC1 protein level and thereby be involved in the interindividual differences seen in the western blot (see Figure 7 and Figure 8). Furthermore, an increase in HDAC1 expression could possibly cause a decrease in HDAC2 in affected cells as well as the other way around - potentially transiently auto-downregulated HDAC1 could have the effect that HDAC2 is upregulated. Such an interdependent expression of HDAC1 and 2 has been demonstrated in several mouse cell types and knockdown or knockout studies (Jurkin et al., 2011; Lagger et al., 2002, 2002; Senese et al., 2007). Future experiments

could comprise attempts to knockdown HDAC1, but also other HDACs, via small interfering RNA (siRNA) molecules or using gene-editing tools such as the CRISPR/Cas9 system in human intestinal organoids (Driehuis and Clevers, 2017) to get additional mechanistic information about the complex expressional regulation of those epigenetic players in the human gut.

The cellular distributions of class I HDACs found in this study of intestinal tissue confirm the described mainly nuclear localization of those proteins (Karagiannis and Ververis, 2012; Khochbin et al., 2001; The Human Protein Atlas, 2018; Thul et al., 2017; Uhlen et al., 2010); with the exception of HDAC3, which was herein also found to display cytoplasmatic occurrence in both ileal and colonic mucosa, irrespective of disease status. Cytoplasmatic staining was especially seen in the epithelial cells (see Figure 14 and Figure 15). For HDAC8, slight differences between IBD patients and healthy individuals could be observed. Patients with CD and UC displayed weaker HDAC8 staining in the mucosa, but no differences in spatial staining patterns could be seen. The differences in HDAC8 staining intensity were, however, unquantifiable and to this point remain solely an observation. Furthermore, the general staining patterns of the single HDACs appeared to be quite alike in ileal and colonic tissue.

4.3. HDACi-mediated enhancement of hBD2 as therapeutic option in IBD

In this study, class I HDAC mRNA expression levels have been shown to be reduced in IBD intestinal specimens. Furthermore, distinctions in protein levels could also be made out in a small patient cohort. The chicken-and-egg problem concerning HDAC levels remains unresolved at this stage, however, this is so far the first systematic approach on differential HDAC expression in human intestinal tissue samples of IBD patients and therefore a valuable contribution in the evaluation of HDACi as a therapeutic option in IBD. Furthermore, the *in vitro* and *ex vivo* results on the effect of HDACi on hBD2 expression obtained herein deliver relevant new aspects demanding consideration in the quest of strengthening the intestinal antimicrobial barrier while at the same time suppressing an overshooting inflammatory response via the use of HDACi.

A link between intestinal inflammation, gut homeostasis and HDAC function has been established and HDACi are widely discussed as potential anti-inflammatory agents (Edwards and Pender, 2011; Felice et al., 2014; Glauben and Siegmund, 2011; Scarpa and Stylianou, 2012; Ventham et al., 2013). *In vivo* animal studies have demonstrated the efficacy of HDACi in murine colitis models (Glauben et al., 2006; Turgeon et al., 2014) and have helped in deciphering HDAC roles in intestinal tissue homeostasis via epigenetic control of gene expression (Alenghat et al., 2013; Gonneaud et al., 2016; Turgeon et al., 2013). Several *in vitro* investigations have shown general anti-inflammatory effects of HDACi (Chen et al., 2012; Glauben et al., 2006; Leoni et al., 2002; Segain, 2000). HDACi led to a dose-dependent suppression of cytokine production and induction of apoptosis *in vitro*, where lower doses were needed to affect cytokines. *In vivo*, HDACi reduced the severity of dextran sulfate sodium (DSS)-induced colitis and the expression of colonic pro-inflammatory cytokines. Interestingly, HDACi-induced local hyperacetylation only occurred in parallel with DSS treatment and only at the site of inflammation, namely in the colon, suggesting a need of cell activation in the presence of an HDACi to result in an increase in histone acetylation (Glauben et al., 2006). Turgeon and colleagues could demonstrate recently, that an intestinal epithelial cell-specific double knockout of HDAC1 and HDAC2 increased DSS colitis severity and led to higher inflammatory gene expression. However, HDAC2 depletion alone seemed to protect against DSS colitis, did not lead to chronic intestinal inflammation per se and resulted in an upregulation of colonic antimicrobials (Turgeon et al., 2013). A loss of HDAC1 only on the other side disturbed intestinal architecture and increased susceptibility to DSS (Gonneaud et al., 2016). Additionally, it has been found that butyrate administration on colonic biopsies, lamina propria mononuclear cells (LPMC), and human peripheral blood mononuclear cells (PBMC) of CD patients led to reduced TNFα expression under LPS stimulation (Segain, 2000). Furthermore, PBMCs secrete less TNFα, IL-1-beta, IL-12, and IFNγ after

stimulation with LPS upon simultaneous inhibition with SAHA, but levels of IL8 were not affected (Leoni et al., 2002). HDAC3 has also been identified as an important governor of intestinal homeostasis and the barrier function (Alenghat et al., 2013; Navabi et al., 2017). Alenghat and her coworkers investigated the effects of an intestinal epithelial cell-specific deletion of HDAC3 in mice and found it to be crucial for the integration of commensal-bacteria-derived signals by the epithelium, modulating host cellular responses for the establishment of a homeostatic coexistence of the epithelium and its inhabitant microbiota. Absence of HDAC3 in conventionally housed mice caused Paneth cell loss, spontaneous inflammation and increased susceptibility to intestinal damage. This phenotype was widely lost and barrier function, dysregulated gene expression and homeostasis were largely restored in HDAC3 deficient mice re-derived into germ-free conditions (Alenghat et al., 2013). Another study attributes further protective features to HDAC3, where it has been demonstrated that a lack of HDAC3 in mice intestinal epithelial cells were rendered more susceptible to an infection by *Citrobacter rodentium* due to a defective communication between intestinal epithelial cells and resident lymphocytes via IL18 and IFNγ. This suggests HDAC3 as an epigenetic effector important in regulating host defense (Navabi et al., 2017). On the contrary, HDAC3 has also been marked essential for the activation of about half of the inflammatory gene expression program in LPS-stimulated mouse macrophages indicating (Chen et al., 2012).

Therefore, when looking at the available literature it becomes obvious that there is no one-size-fits-all answer to the question on whether and how to employ HDACi as an effective anti-inflammatory therapeutic. The current body of knowledge highlights that HDACs seem to hold anti- as well as pro-inflammatory properties. This became especially apparent in a study, where HDAC1 was found to repress but also activate different subsets of inflammatory genes in response to IL1β in rat intestinal epithelial cells (Gonneaud et al., 2014).

The findings in this study at hand are of note for this discussion, since HDACi use alone already had an elevating effect on basal pro-inflammatory IL8 expression in colonic epithelial cells, whereas the antimicrobial defense molecule hBD2 was elevated only after primary stimulation of the cells with hBD2 inducing molecules or bacteria. In addition to this, stimulus-induced IL8 expression in CaCo2 cells was even further elevated after treatment with HDACi (Stebe-Frick et al., 2018). Therefore, this contrasting finding to a previous report from Fischer and colleagues (Fischer et al., 2016) does not support an unconditional proposition of HDACi as a way to bolster antimicrobial defense without risking an increase in the inflammatory response in parallel (Stebe-Frick et al., 2018). Not only *in vitro*, but also the results of the *ex vivo* investigations conducted in this study here advise caution, since increased IL8 levels could not be reduced nor abolished by HDACi use. Whether this is a technical artefact of the tissue culture remains to be clarified. At this point, it could however be physiologically relevant and therefore be important in an inflamed state of the mucosa of an IBD patient potentially treated with an

HDACi. In parallel, hBD2 could not only *not* be enhanced *ex vivo* but was even abolished by pan-HDACi.

Hence, further detailed investigations are needed to sort out the different targets and mechanisms of action of HDACs, deciphering their manifold cell-, tissue- and organ-specific functions, factoring in the stimulus-dependent activation of inflammatory programs that have been shown to influence the downstream response modulation by HDACs (Huang et al., 2010; Natoli, 2009). Considering the fact that HDACs regulate the acetylation status not only of histones but of thousands of different proteins explains their involvement in diverse cellular processes (Choudhary et al., 2009). Furthermore, impacting on transcriptional programs via HDACi likely leads to secondary effects that might even overwrite the direct effects caused by the inhibition (Chen et al., 2012). One of these secondary transcriptional effects might be exerted by hBD2 itself if it should prove possible to elevate its expression by combining HDACi with the therapeutic EcN *in vivo*. As do other antimicrobials, hBD2 also possesses immunomodulatory properties (Lai and Gallo, 2009; Niyonsaba et al., 2005, 2007). Overly abundant hBD2 expression levels are thought to be a driver in psoriasis as it has been demonstrated that hBD2 triggers the activation of dendritic cells by breaking self-DNA tolerance (Lande et al., 2015). On the contrary, in cells originating from a different organ, the lung, hBD2 displayed anti-inflammatory properties by reducing pro-inflammatory cytokines under LPS-stimulation (Donnarumma et al., 2007).

Nonetheless, as discussed above, HDACi bear intriguing promises to be effective as anti-inflammatory agents or adjuvants in IBD therapy. It is therefore worth to continue studies on the specific effects of single HDAC isoforms and combinations thereof acting as HDAC complexes on cytokine and antimicrobial peptide expression. With more precisely targeted HDACi and a better understanding of their pharmacokinetics within the different cell and tissue types undesired side effects could potentially be significantly reduced.

4.4. Arising conclusion and outlook

The data obtained and presented in this study deliver new knowledge about the expression status of class I HDACs in a large cohort of IBD patients, revealing reductions on the mRNA levels of several HDACs together with a more diverse expression pattern on the protein level. Furthermore, this study strengthens the link between HDAC-mediated epigenetic control of the transactivating function of the transcription factor NF-κB, a controller of hBD2.

Induction of the antimicrobial hBD2 in response to the therapeutically relevant probiotic EcN, the pro-inflammatory stimulant IL1β, and the bacteria-derived factor LPS has been shown to be strongly augmentable in a NF-κB-dependent manner by the use of HDACi in colonic

epithelial cells *in vitro*. The effect could also be found for the likewise NF-κB-dependent pro-inflammatory cytokine IL8. The establishment of an *ex vivo* human colonic biopsy culture as an effort to closer mimic an *in vivo* situation and to test the effect of HDACi in a complex, non-tumorous, human tissue compound, however, revealed contrasting results – pan-HDACi seemed to impede hBD2 expression while IL8 was not downregulated. Respective observations from a second transformed colonic epithelial cell line, colorectal tumor biopsies, but also from a non-transformed primary epithelial cell line, suggest not the cancerous nature of the *in vitro* cell lines to be the underlying cause of these opposing effects between the *in vitro* and *ex vivo* approaches. Rather the complex, multicellular composition of the biopsies might be leading to a tissue-specific transcriptional repression by HDACs creating a different outcome of inhibiting HDACs within the tissue. Hence, these results argue for a cellular context-dependent modulation of the epigenetic regulation of hBD2 expression by HDACs.

To expediently evaluate the controversially discussed issue of the use for HDACi as a safe and effective therapeutic for inflammatory conditions, such as IBD, and/or infectious diseases, further research is required. With future studies, a more detailed understanding of the potential functional consequences of more selective HDACi in specific inflammatory disease contexts and in specific organs must be achieved. Potential experiments could utilize parallel treatment with HDACi and different isolated innate immune stimulants but also with e.g. supernatants from peripheral lymphocytes from patients. Stronger focus could be laid on *ex vivo* cultures of additional tissues other than colonic epithelia, co-cultures of intestinal epithelial with stromal cells such as fibroblasts together with intestinal intraepithelial lymphocytes, as well as animal studies. Understanding the complex and dynamic mechanisms by which HDACs synergize or interfere with different stimulants, immune mediators and signaling pathways to control antimicrobial and cytokine expression, will be decisive in translating the knowledge on these epigenetic factors into potential therapy development.

Overall, this work at hand corroborates the growing understanding of epigenetics as the conjoining integrative mechanism between genome and environment, bridging the way to answering many yet elusive questions in the pathogenesis of IBD.

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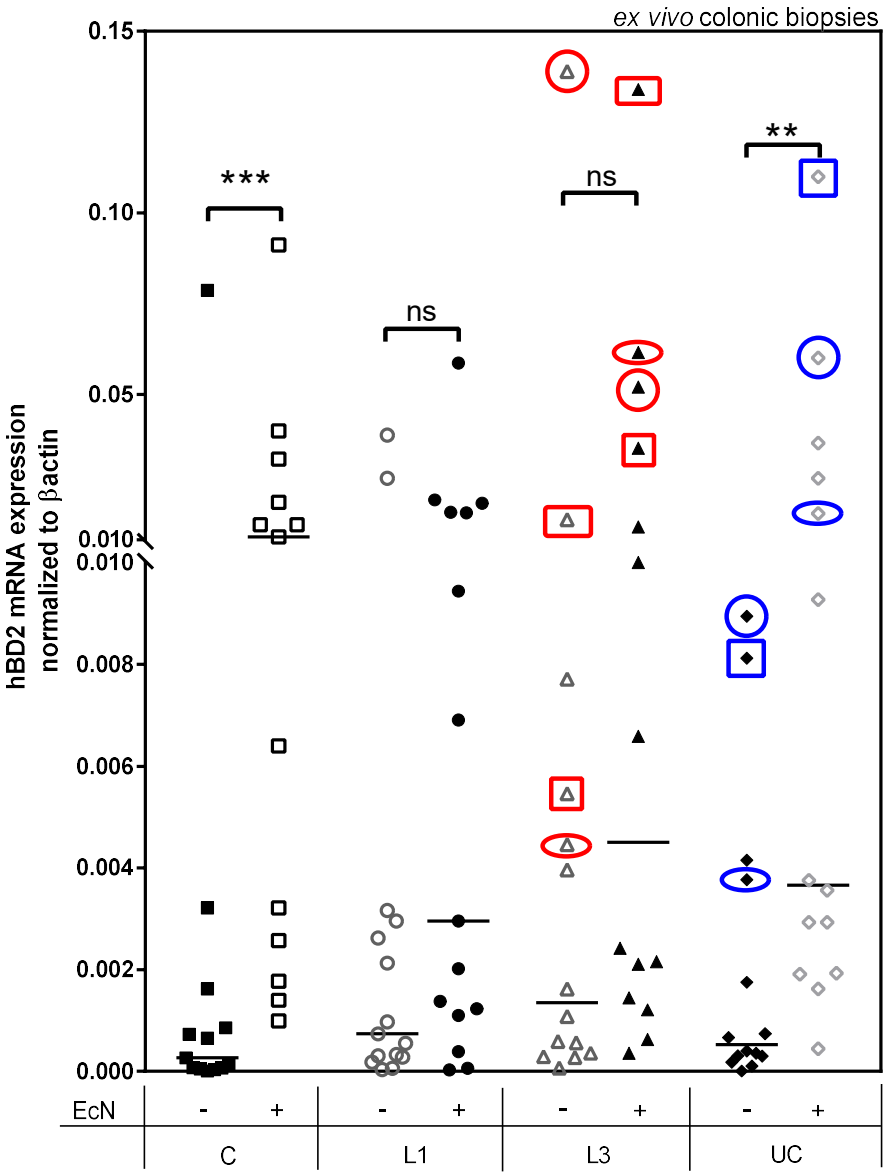
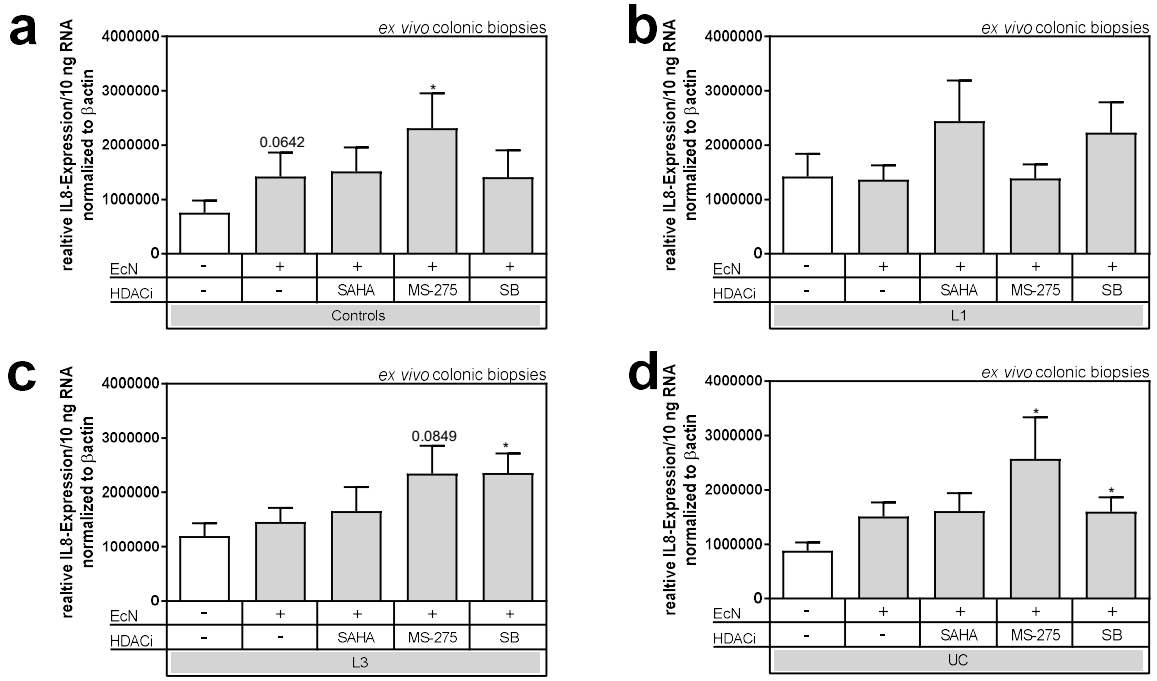
Appendix



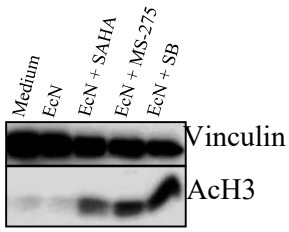
Appendix Figure 1: *E. coli* Nissle stimulates hBD2 expression in ex vivo cultured human colonic biopsies

HBD2 mRNA in cultured human colonic biopsies of controls (C, n=13), L1 (n=15) or uninflamed L3 (n=14) CD patients, or uninflamed UC patients (n=14) in response to 18 hrs of EcN stimulation alone or together with either SAHA (5 μM), MS-275 (5 μM), or SB (3 mM). Inhibitor treatment started 2 hrs prior to the stimulation with EcN, which took then place in parallel to the HDAC inhibition for another 18 hrs. Shown is relative hBD2 mRNA expression according to 10 ng total RNA normalized to βactin expression. Horizontal bars denote median values. Red and blue shapes mark each one value of two separate biopsies taken from the same patient but treated either with or without EcN. ** p≤0.01, *** p≤0.001, ns not significant, evaluated by Mann-Whitney u test.



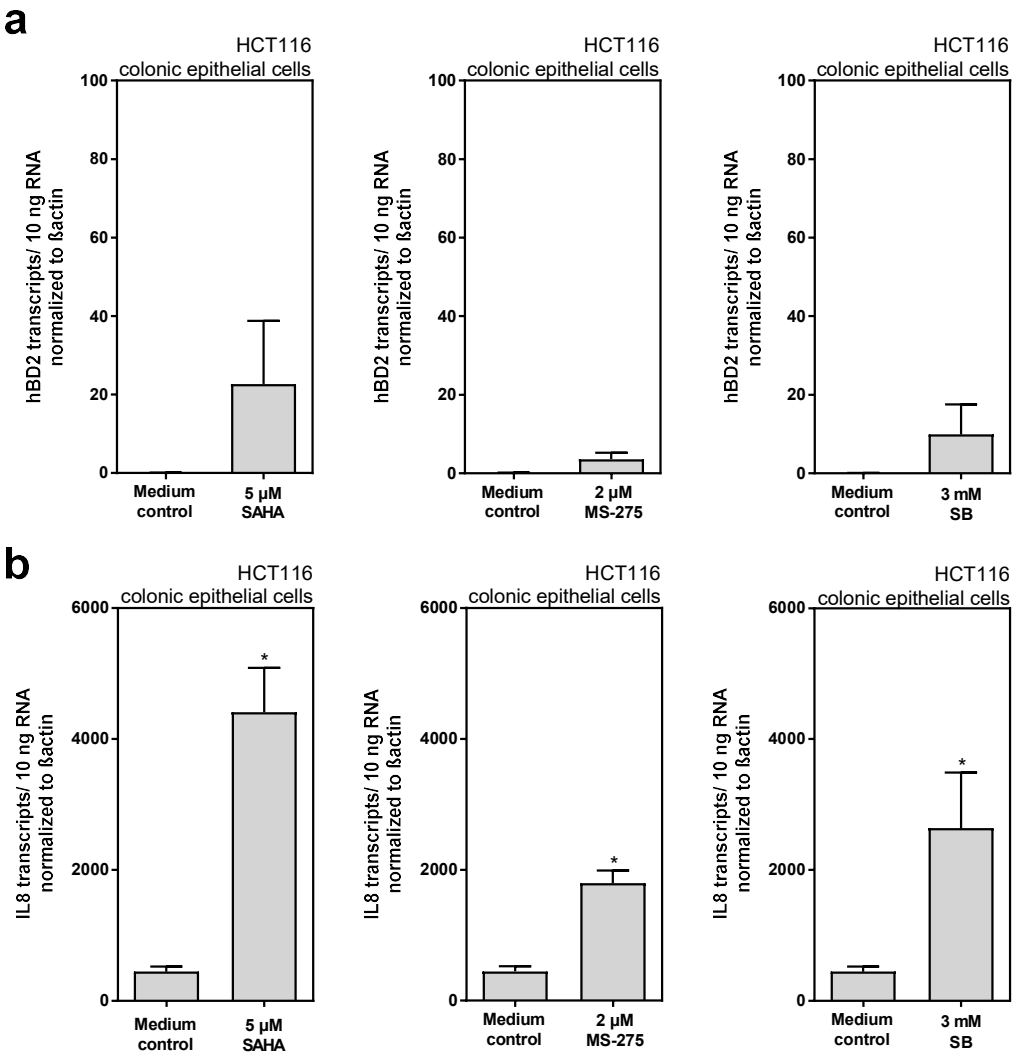
Appendix Figure 2: IL8 expression levels in *ex vivo* cultured intestinal biopsies

IL8 mRNA in cultured human colonic biopsies of (**a**) controls (n=13), (**b**) L1 (n=15) or (**c**) uninflamed L3 (n=14) CD patients, or (**d**) uninflamed UC patients (n=14) in response to medium treatment only, 18 hrs of EcN stimulation with or without HDACi with SAHA (5 μM), MS-275 (5 μM), or SB (3 mM) shown in the right panels of each graph respectively. Shown are relative expression levels according to 10 ng total RNA normalized to βactin expression. * p≤0.05 evaluated by Mann-Whitney u test.



Appendix Figure 3: Histone H3 acetylation status in *ex vivo* biopsies of one patient tested after treatment with HDACi

Western blot analysis of colonic tissue homogenate from a healthy control patient. Each lane on the western blot represents one biopsy of the same patient stimulated with EcN (3 × 108 CFU/ml) with or without HDACi with SAHA (5 μM), MS-275 (5 μM), or SB (3 mM). Inhibitor treatment started 2 hrs prior to the stimulation with EcN, which took then place in parallel to HDAC inhibition for another 18 hrs.



Appendix Figure 4: Expression of hBD2 and IL8 in HCT116 colonic epithelial cells following HDACi

Effect of HDACi on the induction of (**a**) human β-defensin 2 (hBD2) or (**b**) interleukin 8 (IL8) in HCT116 cells in response to either SAHA (5 µM), MS-275 (2 µM), or SB (3 mM) for 20 hrs. * $p<0.05$ evaluated by Mann-Whitney u test.