Loss of Sirt1 Function Improves Intestinal Anti-Bacterial Defense and Protects from Colitis-Induced Colorectal Cancer

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Abstract

Dysfunction of Paneth and goblet cells in the intestine contributes to inflammatory bowel disease (IBD) and colitis-associated colorectal cancer (CAC). Here, we report a role for the NAD+−dependent histone deacetylase SIRT1 in the control of anti-bacterial defense. Mice with an intestinal specific Sirt1 deficiency (Sirt1int−/−) have more Paneth and goblet cells with a consequent rearrangement of the gut microbiota. From a mechanistic point of view, the effects on mouse intestinal cell maturation are mediated by SIRT1-dependent changes in the acetylation status of SPDEF, a master regulator of Paneth and goblet cells. Our results suggest that targeting SIRT1 may be of interest in the management of IBD and CAC.

Introduction

Paneth and goblet cells are highly specialized small intestinal epithelial cells that synthesize and secrete anti-microbial peptides and mucus. These factors represent the first line of defense against pathogens and are essential to maintain the subtle balance among different bacterial species that colonize the mammalian gut [1]. Dysbiotic microbiota impact on the health of the host and contribute to the pathogenesis of several intestinal diseases, such as inflammatory bowel disease (IBD) and colitis-associated colorectal cancer (CAC) [2].

The differentiation and maturation of Paneth and goblet cells is controlled by the Wnt and Notch signaling cascades that cooperate to promote the specification of the different cell lineages [3]. Moreover, the SAM pointed domain containing ETS transcription factor (SPDEF), a downstream effector of both Wnt and Notch pathways, is known to enhance the differentiation of both Paneth and goblet cells from their common progenitor [4]. SPDEF was initially identified as a regulator of the prostate-specific antigen [5], but later also associated to breast, lung, and intestine epithelium, with possible involvement in cancer progression in those tissues [6,7].

Sirtuin 1 (SIRT1), a NAD+-dependent deacetylase [8], is involved in a wide range of cellular processes including metabolism, cell proliferation and apoptosis, and immune response [9]. The role of SIRT1 in the regulation of intestinal homeostasis is only beginning to be elucidated. Recently an involvement of intestinal SIRT1 in systemic bile acid and cholesterol metabolism has been proposed [10]. Furthermore, studies focusing on the role of SIRT1 in colorectal cancer development using Apcmin/+ mice as a model, showed conflicting results supporting both tumor promoting [11] and tumor suppressing [12,13] functions.

Using mice with an intestinal-specific Sirt1 deletion (Sirt1int−/−), we show here that SIRT1 regulates Paneth and goblet cell maturation and production of anti-bacterial proteins. These effects depend on SIRT1-mediated changes in the acetylation status of SPDEF. Moreover, intestinal Sirt1 deletion has a major impact on the gut microbiome and protects mice from IBD and CAC. Notably, the effects of Sirt1 deficiency on the production of anti-bacterial proteins are evolutionary conserved in C. elegans highlighting the ancient nature of this function of SIRT1. Taken together our results suggest that targeting SIRT1 may be of interest for the management of IBD and CAC.
Materials and Methods

Generation of Sirt1int−/− and Sirt1int−/−-LGR5EGFP-ires-CRE-ent2 mice

For the generation of Sirt1 floxed (Sirt1lox/lox) mice, genomic DNA covering the Sirt1 locus was amplified from the 129Sv strain by high-fidelity PCR. The resulting DNA fragments were assembled into the targeting vector of the Institut Clinique de la Sœur (Strasbourg, France). The construct in which exons 5, 6 and 7 were flanked by LoxP sites was then electroporated into 129Sv embryonic stem (ES) cells (Figure S1B). G418-resistant colonies were selected and analyzed for homologous recombination by PCR and positive clones were verified by Southern blot hybridization. Correctly targeted ES cell clones were injected into blastocysts and transferred into pseudopenregnant females, resulting in chimeric offspring that were mated to female C57BL/6J mice. Bacterial feeding RNAi experiments were carried out as described [18]. The mutated allele and that lost the Flp transgene (Sirt1int−/−) were then electroporated into 129Sv blastocysts and transferred into pseudopregnant females, resulting in chimeric offspring that were mated to female C57BL/6J mice for ten generations. For the gut microbiota analysis Sirt1int−/− and Sirt1lox/lox were co-housed under specific pathogen free conditions within the same room. Mice treated with AOM/DSS were singly housed after weaning to avoid differences in the DSS intake. However, Sirt1int−/− and Sirt1lox/lox were treated under specific pathogen free conditions within the same room. Notably, Sirt1int−/− and Sirt1lox/lox mice come from the same parents. All animal experiments were done in accordance with institutional and Swiss guidelines and approved by the Cantonal authorities of the Canton of Vaud. Moreover, all animal experiments were performed to the Swiss Animal Welfare legislation and reviewed by the State Ethical Board of the Canton de Vaud (Animal Welfare Act 2005; Project License No. 2463-2463.1-2605 license to Prof. Johan Auwerx).

Luciferase assay

PC3 cells (ATCC) in 96/wells plates were co-transfected with a reporter containing the SPDEF response element of the human E-cadherin promoter, pGL2Basic-EdaK (plasmid 10963) [16] and pGL2Basic-EdaK (plasmid 19290) [17] were purchased by Addgene. pGEX-GST-SPDEFK294Q and pCruzHA-SIRT1G261A expression vectors (jetPEI transfection; Polyplus). Media was removed after 24 hr, cells washed with cold PBS, and Luciferase Substrate (Bright-Glo Luciferase Assay System, Promega) was added before Luciferase measurement by the Victor x3. β-galactosidase was used for normalization.

Plasmids

Mammalian expression vector pUSE-SIRT1 was purchased from Upstate. The SPDEF coding sequence belongs to the Mouse Transcription Factor Resource [15]. It was amplified and ligated to the pCDA3-FLAG or pGEX-GST. pCruzHA SIRT1G261A (plasmid 10963) [16] and pGL2Basic-EdaK (plasmid 19290) [17] were purchased by Addgene. pGEX-GST-SPDEF-294Q was generated using site-directed mutagenesis.

C. elegans assays

C. elegans strains were grown at 20°C on nematode growth media agar plates (NGM) seeded with E. coli strain OP50 unless stated otherwise. Strains used were wild-type Bristol N2, VC199 sir-2.1(ad434) IV, SAL129 [pha-1(e2123)[III];jpy-1(GFP+pha-1[+)], and SAL105 [pha-1(e2123)[III];jfy-2(GFP+pha-1[+])]. Strains were provided by the Caenorhabditis Genetics Center (University of Minnesota). Bacterial feeding RNAi experiments were carried out as described [18]. The sir-2.1(R11A8)4 clone (University of California) was purchased from GeneService and sequenced. Quantification of GFP expression was carried out according to described protocols [19]. For picture acquisition of jpy-2::GFP expression, animals were mounted on 2% agarose pads in 10 mM tetramisole (Sigma) and examined using a Zeiss Axioplan-2 microscope (Carl Zeiss).

mRNA extraction and RT-qPCR analysis

RNA was isolated from tissues using the TriPure reagent (Roche) according with manufacturer’s instructions. cDNA was generated from 1 μg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was carried out using LightCycler 480 SYBR Green I Master Mix (Roche) and analyzed through ΔΔCT calculation. Values were normalized to Cycloplicin expression. Primers are listed in Table S1 in File S1.

In vitro acetylation and deacetylation assays

In vitro deacetylation assays were performed as originally described [20]. Briefly, 1 μg of recombinant SPDEF protein, obtained from the BL21 strain, were incubated with 500 ng of recombinant p300 in acetylation buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 10% glycerol, 1 mM PMFS, 1 mM DTT, 1 μg/ml bpsatin, 1 μg/ml leptin, 1 μg/ml pepstatin, 1 mM sodium butyrate and 150 μM acetyl-CoA) for 1 hour at 30°C. After incubation, samples were resolved on SDS-PAGE and analyzed by western blot or used for in vitro deacetylation assays. For deacetylation assays, 1 μg of acetylated SPDEF was incubated with 500 ng recombinant SIRT1 protein in deacetylation buffer (50 mM Tris-HCl, pH 9, 4 mM MgCl2, 0.2 mM DTT, 1 μg/ml bpsatin, 1 μg/ml leptin, 1 μg/ml pepstatin, and 1 mM NAD+) for 30 minutes with constant agitation. The incubated samples were resolved on SDS-PAGE and analyzed by western blot or used to map an acetylated residue with nano-LC-MS/MS.

Cell culture, transfection, and antibodies

HEK293 and PC3 cells were cultured in Dulbecco’s modified Eagle’s medium including 4.5 g/l glucose, 10% fetal calf serum, 0.1 mM NEAA, and 50 μg/ml gentamicin at 37°C under a 5% CO2 atmosphere. HEK293T and PC3 cells were transfected using JetPEI reagent (Polyplus Transfections, Illkirch, France) according to the manufacture’s instructions. Antibodies: Lysozyme (Abcam,
Ab36362), ChRα (Santa Cruz, sc-13090), Acetylated lysine (Cell signaling, 9441L), Sirt1 (Abcam, ab12193), β-catenin (Sigma, A5441), L-FABP (Santa Cruz, sc-50300), PCNA (Santa Cruz, sc-56), HSP90 (BD Transduction Laboratories, 610415), anti-FLAG (Sigma, F1804), Tubulin (Santa Cruz, sc-52860), SPDEF for western blot (Sigma, AV32533), SPDEF for IHC was kindly provided by Prof. J. Whitsett.

Subcellular fractionation

Cytoplasm and nucleus fractionation were obtained from Sirt1lox/lox and Sirt1ΔΔ/ΔΔ isolated crypts. Crypts were isolated following a published protocol [21]. Single crypt-derived cells were finally washed with ice-cold PBS, and incubated in buffer A (10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 10 mM, 10 HEPES-KOH, pH 7.4) including a protease inhibitor cocktail (Roche) for 5 min. After 50 strokes in a dounce homogenizer, cytoplasm fraction was collected by centrifugation (1.4 k × g for 5 min, 4°C). Pellets were washed two times with buffer A. Nuclei were incubated in buffer B (150 mM NaCl, 0.1% NP-40, 50 mM Tris-HCl, pH 7.4) containing a protease inhibitor cocktail for 30 min on ice. Nucleoplasm was collected by centrifugation (2 k × g for 5 min, 4°C). Proteins were quantified using the Lowry method and processed for western blot assay.

Fractionation of cells along the crypt–villus axis

The sequential isolation of mouse small intestinal epithelial cells along the crypt–villus axis was performed as described previously [23] with few modifications. Briefly, the entire small intestine (duodenum to terminal ileum) was removed, flushed, and cut into small pieces (2–5 mm) and incubated at 37°C for 15 min in 15 ml of buffer A (96 mM NaCl, 1.5 mM KCl, 27 mM Na-citrate, 8 mM KH2PO4, and 5.6 mM Na2HPO4, pH 7.3). Then it was incubated for 15 min in 15 ml of buffer B (PBS plus 1.5 mM EDTA, 0.5 mM dithiothreitol, and 1 mg/ml bovine serum albumin) in a shaking 37°C incubator. At the completion of the 15-minute incubation, detached enterocytes were collected (Fraction 1) and 15 ml of fresh buffer B added to the tissue. This procedure was repeated four more times, the steps lasting 25, 25, 25 and 30 min, respectively (Fractions 2, 3, 4 and 5), for a total of 120 min of incubation time. At the completion of the final incubation period, cells collected from each of the 5 fractions were harvested by centrifugation at 1500 rpm at 4°C for 5 min. Cell pellets were washed twice and lysed in RIPA buffer. Alkaline phosphatase activity was assayed by Alkaline Phosphatase Assay kit (BioVision).

Mass spectrometry analysis

Gel lanes were cut into pieces and subjected to in-gel digestion with endoproteinase Glu-C or trypsin. Peptide digests were resuspended and analyzed by nano-LC-MS/MS using an Orbitrap Elite mass Spectrometer (Thermo Fischer Scientific) coupled to an ultraperformance LC (UPLC) system (Thermo Fischer Scientific Ultimate 3000 RSLC). Data analysis was performed with Proteome Discoverer (v. 1.3) and searches were performed with Mascot and Sequest against a mouse database (UniProt release 2013_01). Data were further processed, inspected and visualized using Scaffold 3 software.

In situ hybridization

The in situ probes used in this study correspond to expressed sequence tags or fully sequenced cDNAs obtained from Open Biosystems. The accession numbers for these probes are as follows: mouse Olfm4 BC141127 (9055739), mouse Defa4 BC134360 (40134597). To ensure the specificity of the probes, we generated both sense and antisense probes by in vitro transcription using DIG RNA labeling mix (Roche) according to the manufacturer’s instructions and to published methods [24]. ISH was done using the fully automated instrument Ventana XT (Roche). Chemicals were from Roche Diagnostics. Briefly, formalin fixed paraffin-embedded sections were de-paraffinized and rehydrated and pretreated by enzymatic digestion (protease1, 4 min at 37°C). Hybridization was performed adding to each slide 50 or 100 ng of the probe diluted in Ribohybe at 65°C for 6 hours.

Bactericidal assay

Bactericidal assays were carried out as described [25] with few modifications. Briefly, the small intestine of adult mice was rinsed with ice-cold PBS and segments incubated in 30 mM EDTA to elute epithelial cells. Total proteins from the epithelial cells were extracted using NP40 lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2.5 mM KCl, 5 mM EDTA, 5% vol/vol glycerol, 1% [vol/vol] NP40) supplemented with complete protease inhibitor. 100 μg of proteins were incubated for 60 minutes at 37°C in 10 mM iPipes buffer containing exponentially growing Escherichia coli K12 (ATCC, 106 CFU/ml). 20 μL of sample was diluted and plated in LB-agar solid medium. Surviving bacteria were quantified as CFU on plates after overnight incubation at 37°C.

Colitis and Colitis-associated colorectal cancer models

DSS-induced colitis was induced as previously described [26]. Daily changes in body weight were assessed. Rectal bleeding was scored on a scale from 0 to 5, indicating no (0) or highly severe (5) rectal bleeding. Ileal and colonic tissue was snap-frozen or fixed with 4% formaldehyde solution or 4% Formaldehyde (SeraLab). Colitis-induced colorectal cancer (AOM/DSS model) was induced as previously described [28]. After sacrifice, mouse colon was opened longitudinally and after 2 hours fixation in 4% Formaldehyde solution, briefly stained with Coomassie Blue to visualize tumors. Tumors were counted in a blinded fashion by a pathologist. Tumor size was assayed by gauge. Small pieces of terminal ileum and proximal colon were fixed and stained with H&E. Bacteria incubated in iPipes buffer without added proteins were considered as controls.

Statistical analysis

Data were checked with the Shapiro–Wilk test for normality in R prior to performing significance tests. Variables with a W value ≥0.80 were considered as approximately normally distributed. Two variable comparisons were calculated using Welch’s two-tailed t-test. For multiple comparisons, Bartlett’s test was performed to check for equal variance (p>0.10) and then one-way ANOVA was performed with the Bonferroni post-hoc test. Variables with a Shapiro-Wilk W value <0.80 were considered as non-normal and comparisons were calculated using the Wilcoxon signed-rank test. The Kaplan-Meier method was used for the survival analysis in worms. Data are expressed as mean ± SEM, and for all significance comparisons, p values smaller than 0.05 were considered as statistically significant. **p<0.01; ***p<0.001. This study had an exploratory nature and there was...
no pre-specified effect size. The sample size was chosen based on the study feasibility and potential statistical power. Sample sizes are consistent with those reported in similar studies. Statistical analysis for the gut microbiota analysis is reported in the Supplemental Information.

Results

Sirt1 deletion increases anti-microbial response in mammals and nematodes

We first determined the localization of SIRT1 protein along the villus/crypt axis. Since SIRT1 appears highly enriched in the small intestine crypts (Figure S1A), we bred Villin-Cre transgenic mice with mice in which exons 5–7 of the Sirt1 gene were flanked by LoxP sites (Sirt1<sup>fl/fl</sup>) to generate intestinal-specific Sirt1 knockout mice (Sirt1<sup>int</sup>−/−) (Figure S1B–C). Unlike a previously reported deletion of Sirt1 exon 4 [29], no truncated and potentially active SIRT1 fragments were detected in Sirt1<sup>int</sup>−/− mice (Figure S1C). Sirt1<sup>int</sup>−/− intestines showed a significant increase in the number of Paneth (lysozyme<sup>e</sup> and Defa4<sup>+</sup>) and goblet (PAS<sup>+</sup>), but not of enteroendocrine (ChR<sub>α</sub>) cells (Figure 1A, Figure S1D). Accordingly, mRNA levels of Paneth (Ly6, Crypt1, Crypt4, Defa-Ro) and goblet (Klf4, Muc2) cell markers, as well as lysozyme protein abundance and Defa mRNA detected through in situ hybridization, were induced in the proximal and distal intestine of Sirt1<sup>int</sup>−/− mice (Figures 1B–C, Figure S1D–E).

We next evaluated whether Sirt1 is correlated to the genes involved in anti-bacterial defense using the BDX mouse genetic reference population (www.genenetwork.org) [30]. As no intestinal gene expression data are available, we analyzed hematopoietic cells, which are part of the arsenal of cells that protect against pathogens. In line with our observations, Sirt1 expression correlates negatively with the expression of several Defensin-related genes (Figure S1F).

To test whether the link between Sirt1 and the anti-bacterial defense was evolutionary conserved, we used C. elegans. Remarkably, the expression of a wide range of genes involved in anti-microbial defense (lyz-1, lyz-7, lyz-6) [31], as well as that of genes induced by specific pathogens (F01D5.5, F56D6.2, C17H12.8, C29P3.7, K06D8.5) [32] was enhanced in sirt-2.1 [33] mutant worms (Figure 1D). Furthermore, in lyz-1::GFP and lyz-7::GFP reporter strains [31], sirt-2.1 siRNA significantly induced both lyz-1- and lyz-7-dependent GFP expression (Figure 1E). These data hence indicate that the effect of Sirt1 in the anti-microbial response is evolutionary conserved from mammals to worms.

Intestinal Sirt1 deletion impacts on gut microbiota

Considering the fundamental role that Paneth and goblet cells play in protecting the mammalian gut from aberrant bacterial colonization, we next analyzed the impact of a Sirt1 deletion on gut microbiota. Sirt1<sup>int</sup>−/− intestines did not only have a larger cecum (Figure 2A), indicative of a modified microbiome [34], they also had a higher bactericidal capacity (Figure 2B). Using DNA extracted from either the intraluminal cecum content or colon tissue, we amplified the V3 region of the bacterial 16S rRNA gene. After removal of low quality reads and all singleton Operational Taxonomic Units (OTUs) (Table S2 in File S1), 98% of the reads fell into the Core Measurable Microbiome (CMM—see methods). The microbial community was dominated by 4 phyla, Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia (Figure 2C) [35]. To map the microbial community composition and structure across the Sirt1 mutation we used an OTU-based method. In network-based analyses, the cecum microbial communities of Sirt1<sup>L2/L2</sup> and Sirt1<sup>int</sup>−/− mice highlighted community differences between genotypes (Figure 2D), an observation further supported by Principal Coordinates Analysis (PCA; Figure 2E). Indicator microbial species that contribute towards community differences between Sirt1<sup>L2/L2</sup> and Sirt1<sup>int</sup>−/− mice were identified and OTUs displayed in Figure 2F and Table S3 in File S1. Interestingly, a majority of enriched OTUs in Sirt1<sup>int</sup>−/− mice were of the genus Bacteroides, Bacteroides, and Prevotella (phylum Bacteroidetes), found in the normal human and mouse gut, which are notably decreased in IBD [36,37]. In contrast, the genus Clostridium (phylum Firmicutes) was expanded in Sirt1<sup>L2/L2</sup> mice (Figure 2F and Table S3 in File S1). These results show that intestinal Sirt1 deletion and the consequent changes in Paneth and goblet cells modify the gut microbiome.

Intestinal Sirt1 deletion protects from colitis and colitis-induced colorectal cancer

Given these last observations, we studied the impact of Sirt1 on the pathogenesis of both colitis and CAC. Sirt1<sup>int</sup>−/− mice, exposed to dextran sulfate sodium (DSS), showed a milder colitis characterized by lower body weight loss, bleeding score, a trend towards reduced intestinal permeability, and a decreased expression of inflammatory genes (Figure S2A–D). These results incited us to study how Sirt1<sup>int</sup>−/− mice react to CAC. Mice were hence injected with the AOM (azoxymethane) carcinogen before a subsequent exposure to three cycles of DSS (Figure 3A). Remarkably, the number and size of tumors in Sirt1<sup>int</sup>−/− intestines were smaller (Figures 3A–B). The better health status of Sirt1<sup>int</sup>−/− mice was further underlined by the longer colon length and faster body weight recovery after the last DSS cycle (Figures 3C–D).

Analysis of the microbial community after DSS/AOM exposure revealed significant changes in Sirt1<sup>int</sup>−/− mice (Figure 3E), while the change was smaller in Sirt1<sup>L2/L2</sup> mice (Figure 3F). Indicator microbial species, before and after DSS/AOM, were identified (Figures 3G–H and Table S4 in File S1). Of note, OTUs belonging to genus Helicobacter and Desulfovibrio were only increased after DSS/AOM in Sirt1<sup>L2/L2</sup> mice (Figure 3G and Table S4 in File S1). Interestingly, although the role of Helicobacter in colorectal cancer remains controversial [38], Desulfovibrio is considered to take part in the pathogenesis of ulcerative colitis and Crohn’s disease [39]. Microbial communities in Sirt1<sup>L2/L2</sup> and Sirt1<sup>int</sup>−/− mice after DSS/AOM treatment were not significantly different (Figure 3I). Altogether, we hypothesize that changes in the general microbial community under normal conditions (Figure 2), as well as in specific genera after DSS/AOM, could determine the different susceptibility of the two genotypes to colitis and CAC.

Hyper-acetylated SPDEF drives Paneth and goblet cell maturation upon intestinal-specific Sirt1 deletion

To understand the molecular changes underlying these prominent phenotypes, we explored different possible pathways that contribute to Paneth and goblet cell development and maturation. Since the intestinal cell types differentiate from intestinal stem cells (ISC) [3], we first evaluated whether Sirt1 deletion might impact the ISC population. In situ hybridization using RNA probes for Olf4, an ISC marker, failed to show differences in number and localization of ISC between Sirt1<sup>int</sup>−/− and Sirt1<sup>L2/L2</sup> mice (Figure S3A). By crossing Lgr5<sup>EGFP-RES-CreERT2</sup> [40] with Sirt1<sup>int</sup>−/− or Sirt1<sup>L2/L2</sup> mice, we generated a mouse line with an intestinal-specific Sirt1 deletion, which concomitantly expresses GFP in ISCs. FACS analysis of GFP-positive cells (Figure S3B), as well as confocal imaging of crypts (Figure S3C), and gene expression profiling (Figure S3D–E), revealed no differences in ISC number.
We then analyzed the molecular pathways involved in intestinal cell commitment. The Wnt/β-catenin signaling pathway is one of the main driving forces of intestinal cell differentiation. Of note, the relationship between β-catenin and SIRT1 has been extensively studied in both mouse and cell lines, producing conflicting results [11,12]. Neither the number nor the localization of proliferative cells upon BrdU incorporation was different between Sirt1int2/2 and Sirt1L2/L2 mice (Figure S4A). Accordingly, villi and crypt lengths were indistinguishable (Figure S4B). Furthermore, no SIRT1-dependent modulation of β-catenin levels or localization could be observed by β-catenin immunostaining and protein fractionation from crypts (Figure S4C–D). Thus, in our model nothing heralds a possible involvement of β-catenin.

Paneth and goblet cells share also other transcription factors downstream from the Notch and Wnt signaling cascades, such as GIF1, SPDEF, and SOX9, which are required for their differentiation [4,41,42]. Thus, we compared the expression of their target genes in Sirt1int−/− and control small intestines. Of note, only SPDEF target genes, Slug, Mmp7, and Crypt4, Defa-Rs, and gastrointestinal (GIF1, Muc2) markers are increased in the proximal and distal small intestine of Sirt1int−/− mice (N = 6–8). For RTqPCR analysis, rps12 was used as reference gene. β-Actin was used as loading control in the western blot. D). Increased expression of genes involved in pathogen defense in the sir-2.1(ok434) C. elegans mutant (N = 6; each N means a single population of ~500 worms). Act1 and Y45 were used as reference. E, sir-2.1 siRNA induces lys-1 and lys-7 driven GFP expression in lys-1::GFP and lys-7::GFP reporter worms. In the same figure a representative image of lys-7::GFP before and after sir-2.1 siRNA is shown (DIC, differential interference contrast). Results are expressed as mean±SEM. *P<0.05; **P<0.01; ***P<0.001.

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Figure 1. SIRT1 deletion increases Paneth and goblet cell number and up-regulates anti-bacterial gene expression in mice and worms. A, Representative images of Paneth (Lysozyme+ cells), goblet (Periodic acid–Shiff+ cells), and enteroendocrine (Chromogranin A+ cells) cell staining. (N = 3 mice; 5–10 field per mouse, 50–100 crypt/villi per field). Bar = 50 μm. B–C, mRNA and protein levels of Paneth (Lys, Mmp7, Crypt1, Crypt4, Defa-Rs) and goblet (Kif4, Muc2) markers are increased in the proximal and distal small intestine of Sirt1int−/− mice (N = 6–8). For RTqPCR analysis, rps12 was used as reference gene. β-Actin was used as loading control in the western blot. D). Increased expression of genes involved in pathogen defense in the sir-2.1(ok434) C. elegans mutant (N = 6; each N means a single population of ~500 worms). Act1 and Y45 were used as reference. E, sir-2.1 siRNA induces lys-1 and lys-7 driven GFP expression in lys-1::GFP and lys-7::GFP reporter worms. In the same figure a representative image of lys-7::GFP before and after sir-2.1 siRNA is shown (DIC, differential interference contrast). Results are expressed as mean±SEM. *P<0.05; **P<0.01; ***P<0.001.

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HEK293T cells co-transfected with SIRT1 or SIRT1G261A [16], a catalytically inactive SIRT1 mutant. We also used a mutant of SPDEF protein, in which the K294 lysine residue was replaced by a glutamine (SPDEFK294Q), mirroring, at least in the charge, the hyperacetylated status of SPDEF (Figure 4D). While SIRT1 was able to fully deacetylate the wild type SPDEF, SIRT1G261A only minimally affected SPDEF acetylation. Furthermore, the SPDEFK294Q mutant was not acetylated, confirming that the K294 is the key acetylation site (Figure 4D).

To understand how the acetylation status could affect SPDEF activity, we assayed both wild type and SPDEFK294Q in protein stability assays. Remarkably, in presence of cycloheximide, wild type SPDEF deacetylation by SIRT1 significantly reduced its stability (Figure 4E), while that of the SPDEFK294Q mutant was not affected (Figure 4F). This change in the SPDEF protein stability also influenced SPDEF transcriptional activity. Hence, the presence of SIRT1 reduces the transcriptional activity of wild type SPDEF on a cognate target reporter [17], but not that of the SPDEFK294Q, which seems constitutively active (Figure 4G). For technical reasons it was impossible to immune-precipitate SPDEF and determine its acetylation status in vivo from mouse intestines, but total protein quantification by western blot showed an increase in the amount of total SPDEF in Sirt1^{−/−} mice (Figure 4H). SIRT1-dependent deacetylation of SPDEF hence reduces both its stability and transcriptional activity. We can therefore assume that SIRT1 deletion and the consequent SPDEF hyperacetylation, stabilizes the SPDEF protein and increases its transcriptional potential, ultimately favoring Paneth and goblet cells maturation.

**Discussion**

To bypass the pleiotropic effects of SIRT1 and focus on its intestine-specific role, we generated a new intestine-specific Sirt1 mutant mouse model. Intestinal Sirt1 knockout mice have been previously generated [10,11] through conditional deletion of Sirt1 exon 4, which encodes the catalytically active domain of the protein. This strategy generates a truncated SIRT1 protein [29], which has lost its enzymatic activities but still interacts with other proteins, potentially confounding the interpretation of results. Our model, in contrast, results in the full absence of SIRT1 in the intestine, eliminating these possible confounders. The characterization of this new Sirt1^{−/−} mouse line reveals an evolutionary conserved process in which Sirt1 (or Sir-2.1) inactivation protects the intestine from pathogens. This effect is, at least partially, mediated by the hyperacetylation of SPDEF, which we identify as a new SIRT1 deacetylation target. Acetylation stabilizes the SPDEF and increases its capacity to enhance Paneth and goblet cells differentiation and maturation, ultimately enhancing the intestinal anti-bacterial potential and remodeling the gut microbiota.

Recently, an SPDEF-dependent increase of intestinal goblet cells together with the inhibition of progenitor cells proliferation,
and a reduction in Paneth and enteroendocrine compartment has been reported in a doxycycline-dependent intestinal-specific SPDEF over-expressing mouse model [7,43]. These data, however, are in apparent contrast to the original characterization of the SPDEF knockout mouse model [4], in which SPDEF deficiency was responsible for both Paneth and goblet cells depletion, without affecting epithelial cell proliferation. Moreover, doxycycline treatment is known to heavily affect gut microbiota [44], hence potentially affecting Paneth and goblet cells function and maturation. Thus, the SPDEF-dependent increases of Paneth and goblet cells observed in our model (Sirt1int$^{+/+}$ mice) seems to fit better with the original discovered role of SPDEF [4].

Furthermore, together with the Paneth and goblet cell number, key antimicrobial peptides, such as lysozyme and the cryptidines, are increased in the intestine of Sirt1int$^{+/+}$ mice. The induction of these genes and proteins enhances the bactericidal capacity of Sirt1int$^{+/+}$ crypt content, and most likely underlies the rearrangement of the gut microbiome that we observed. SIRT1 in the intestine was recently suggested to control ileal bile acid absorption and as such impacts on systemic bile acid homeostasis [10]. In our study, the expression of genes involved in bile acid transport and sensing was, however, not altered, making changes in bile acids an unlikely contributor to the altered gut flora (Figure S1E).

Importantly, our Sirt1int$^{+/+}$ mice develop fewer tumors and show milder inflammation after AOM/DSS. Previous studies on the role of Sirt1 in colorectal cancer have used mice, in which the exon 4 of Sirt1 was deleted [11] or in which Sirt1 was overexpressed in the intestine [12]. The outcome of these studies showed both tumor promoting and inhibiting effects related to Sirt1 [11,12]. Besides the fact that we used a different Sirt1int$^{+/+}$ mouse model, another notable difference between our work and these studies is that they use Apcmin/+ mice. Apcmin/+ mice are representative of hereditary and rare forms of colorectal cancer, based on mutations in the APC gene. The sporadic form of colorectal cancer, i.e. CAC, commonly found in the general population, however, is better represented by the AOM/DSS.
mouse model. These previous studies using the APC\(^{min/+}\) mouse model furthermore showed conflicting results regarding the involvement of the Wnt/\(\beta\)-catenin pathway. The reduction of polyp number in a Sirt1 over-expressing APC\(^{min/+}\) mouse model was in one study attributed to the inhibition of \(\beta\)-catenin, a downstream target of the Wnt signaling [12], whereas another study suggested that SIRT1 promotes the Wnt signaling both \textit{in vitro} and \textit{in vivo} [11]. Our mouse model did not show differences in intestinal cell proliferation, \(\beta\)-catenin activation/inhibition, and intestinal stem cells involvement (Figure S3 and S4). However, a growing body of evidence indicates that the possible role of SIRT1 in the Wnt signaling is complex and tightly related to possible indirect mechanisms which involve other proteins, as well as differences in cellular context [45,46,47,48].

Our results hence may be relevant for a large number of subjects suffering from various forms of IBD and CAC and may open a potential perspective to treat these conditions in which the gut microbiota is involved by targeting SIRT1.

**Supporting Information**

**Figure S1** Generation and characterization of Sirt1\(^{int-/-}\) mice. A, Villus/crypt fractionation shows a decrease in the alkaline phosphatase (ALP) activity from the top of the villus (F1) to the bottom (crypt, F5). SIRT1 shows a gradient increasing from the top to the bottom of the villus/crypt unit. PCNA and LFABP, respectively a proliferative and a differentiation marker, showed opposite protein distribution, confirming the validity of the fractionation protocol. Tubulin is the loading control. B, In vitro acetylation/deacetylation assays demonstrate that p300 acetylates SPDEF and SIRT1, but not SIRT6 and SIRT7, deacetylates SPDEF. C, Nano-LC-MS/MS shows SIRT1-dependent in vitro deacetylation of Ack294 (left panel). Sequence alignment showing the evolutionary conserved K294 residue (right panel). D, SIRT1, but not SIRT1G261A, deacetylates SPDEF in HEK293 immunoprecipitates. The SPDEFK294Q mutant is not acetylated. Tubulin was used as loading control. E-F, SPDEF, SPDEFK294Q, and SIRT1 were transfected in the HEK293 cell line and visualized by immunoblotting before (0 min) and after cycloheximide (CHX) treatment. HSP90 is used as loading control. The relative stability of SPDEF or SPDEFK294Q was calculated by ImageJ (right panels in E and F). G, Immunoblotting of crypt enriched fractions from Sirt1\(^{int-/-}\) and Sirt1\(^{+/+}\) mice show increased SPDEF protein levels in Sirt1\(^{int-/-}\) intestines. \(\beta\)-Actin was used as loading control. Results are expressed as mean± SEM. *\(P<0.05; \)**\(P<0.01; \)**\(P<0.001.
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Schematic graph of the gene targeting strategy of exons 5–7 of the Sirt1 gene. C Western blot analysis of SIRT1 expression in intestine and liver of Sirt1+/−/− and control mice showing the tissue-specific deletion of SIRT1. *Non-specific band. β-Actin is the loading control. D, In situ hybridization using Defact DNA probe for Sirt1 in Paneth cells in Sirt1+/−/− and Sirt1+/+/+ mice. E, RTqPCR analysis of bile acids transport and sensing mRNAs in proximal and distal small intestine of Sirt1+/−/− and Sirt1+/+/+ mice (N = 6–8 mice). For RTqPCR analysis, tps12 is used as reference. F, inverse correlation between expression of Sirt1 (y-axis) and mRNAs of each indicated defensin-related gene (x-axis) in hematopoietic cells of B6D2F1 mice (N = 22). Results are expressed as mean ± SEM.

**Figure S2** Intestinal Sirt1 deletion impacts on the development of colitis. A, Percentage of body weight loss observed during 7 days of 2% DSS treatment. B, Rectal bleeding score. Sirt1+/−/− mice show significantly less weight loss and a reduced bleeding score compared with wild type mice. Scoring details are in Supplemental Materials & Methods. ANOVA statistical analysis with Bonferroni post-hoc test was performed for each time point. C, In vivo intestinal permeability measurement. Sirt1+/−/− mice show a reduced FTC-derived fluorescence in the blood suggesting less permeability, consequence of less inflammation. D, RT-qPCR analysis of lfs, Crypt1, Tgfa, and Mmp1 mRNAs in the distal ileum of Sirt1+/−/− and Sirt1+/+/+ after 2% DSS. Cyclophilin is used as reference. For the colitis experiment, 8 mice per genotype were used. Results are expressed as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001. (TIF)

**Figure S3** Intestinal Sirt1 deletion does not impact on intestinal stem cells. A, Representative images of In situ hybridization using RNA probes for Olfm4, B, Crypt from Sirt1+/−/−/Lgr5EGFP-IRES-CreERT2 and Sirt1+/+/+/Lgr5+/− mice were isolated and GFP positive cells were detected by FACS analysis. No changes are observed in the percentage of GFP positive cells between the two groups (N = 6). C, Representative confocal images from Sirt1+/−/−/Lgr5EGFP-IRES-CreERT2 and Sirt1+/+/+−/− Lg5+/−/−/−/−/−/− mice showing GFP+ ISC. Bar = 50 μm. D, Gene expression from the small intestine of Sirt1+/−/−/−/−/−/−/−/− and Sirt1+/−/−/−/−/−/−/−/− mice shows no difference in mRNA levels of ISC genes (Lgr5, Olfm4, Ascl2). E, RTqPCR analysis of Lgr5, Olfm4, and Pom1 mRNAs is proximal and distal small intestine of wild type and Sirt1−/− mice. For RTqPCR analysis tps12 is used as reference. Results are expressed as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001. (TIF)

**Figure S4** Proliferation assay and β-catenin detection in Sirt1+/−/− and control mice. A, BrDu+ cell staining and counts in Sirt1−/− mice and control mice 2, 24, and 48 hours after injection. No differences are detected, highlighting the absence of a change in proliferation (N = 3 mice per time point; 5–10 fields per mouse/slide, 20–50 crypt/villi per field). Bar = 50 μm. B, Villi and crypts length in Sirt1+/−/− and Sirt1+/+/+ (N = 9 mice. 5–10 fields per mouse, 20–50 crypt/villi per field). C, Immunostaining of β-catenin in the ileum and colon of Sirt1+/−/− and control mice. DAPI is used for nuclei staining. No differences are observed. D, Proteins from isolated crypts of Sirt1+/−/− and Sirt1+/+/+ were fractionated in order to detect β-catenin localization. No changes between the two genotypes are observed. Protein quantification was carried out through Imagel software [lower graph]. Hsp90 is used as loading and fractionation control (Cyto = Cytoplasm; Nucl = nuclei). (TIF)

**File S1** Supplemental Materials and Methods and four tables.

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