Loss of Mucosal p32/gC1qR/HABP1 Triggers Energy Deficiency and Impairs Goblet Cell Differentiation in Ulcerative Colitis

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SUMMARY

Oxidative phosphorylation promoting p32 critically maintains mitochondria function essential for terminal goblet cell differentiation. Reduced colonic p32 expression in ulcerative colitis patients possibly explains a great range of disease-specific histopathological hallmarks.

BACKGROUND & AIMS: Cell differentiation in the colonic crypt is driven by a metabolic switch from glycolysis to mitochondrial oxidation. Mitochondrial and goblet cell dysfunction have been attributed to the pathology of ulcerative colitis (UC). We hypothesized that p32/gC1qR/HABP1, which critically maintains oxidative phosphorylation, is involved in goblet cell differentiation and hence in the pathogenesis of UC.

METHODS: Ex vivo, goblet cell differentiation in relation to p32 expression and mitochondrial function was studied in tissue biopsies from UC patients versus controls. Functional studies were performed in goblet cell-like HT29-MTX cells in vitro. Mitochondrial respiratory chain complex V-deficient, ATP8 mutant mice were utilized as a confirmatory model. Nutritional intervention studies were performed in C57BL/6 mice.

RESULTS: In UC patients in remission, colonic goblet cell differentiation was significantly decreased compared to controls in a p32-dependent manner. Plasma/serum L-lactate and colonic pAMPK level were increased, pointing at high glycolytic activity and energy deficiency. Consistently, p32 silencing in mucus-secreting HT29-MTX cells abolished butyrate-induced differentiation and induced a shift towards glycolysis. In ATP8 mutant mice, colonic p32 expression correlated with loss of differentiated goblet cells, resulting in a thinner mucus layer. Conversely, feeding mice an isocaloric glucose-free, high-protein diet increased mucosal energy supply that promoted colonic p32 level, goblet cell differentiation and mucus production.

CONCLUSION: We here describe a new molecular mechanism linking mucosal energy deficiency in UC to impaired, p32-
dependent goblet cell differentiation that may be therapeutically prevented by nutritional intervention. *(Cell Mol Gastroenterol Hepatol 2021;12:229-250; https://doi.org/10.1016/j.jcmgh.2021.01.017)*

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**U**lcerative colitis (UC), as one main phenotype of inflammatory bowel disease (IBD), is a chronic, relapsing-remitting immune mediated disorder of the human gastrointestinal tract, in which inflammation is localized to the large intestine and restricted to the mucosa. While the exact pathophysiology is still not fully understood, genetic, environmental, and immune-mediated factors contribute to disease onset and recurrence in UC. Loss of intestinal epithelial and mucus barrier integrity leading to bacterial translocation is commonly accepted as a major cause of inflammation. Correct cellular differentiation, which is pivotal for the cryptic architecture and thus barrier integrity, is a highly energy demanding process, strongly suggesting that mitochondrial dysfunction plays a key role in both the onset and recurrence of the disease. Of main interest, mitochondrial dysfunction in epithelial cells, defective goblet cell differentiation, and mucus depletion in UC have been independently reported in several studies. Nevertheless, mechanistic evidence linking cellular energy metabolism to goblet cell differentiation and UC pathogenesis is still missing.

Cells of the colonic mucosa utilize different mechanisms to maintain their energy homeostasis. Energy generation in cells of the lower third of the crypt (eg, intestinal stem cells) mainly depend on glycolysis, while short-chain fatty acids (SCFAs) inhibit stem and progenitor cell proliferation. In contrast, differentiated postmitotic cells of the upper third of the crypt (eg, goblet cells) maintain their energy level through mitochondrial OXPHOS function, induces a metabolic shift from mitochondrial OXPHOS to cytosolic aerobic glycolysis. This metabolic shift led to an enhancement of cell proliferation and a decrease in cell differentiation of cancer cells and is potentially involved in the transition of transient amplifying cells into postmitotic cells.

In the intestinal crypt, differentiation of goblet cells occurs along the metabolic trajectory of shifting energy source. Secretory precursor cells in the transit-amplifying zone are characterized by high expression of atonal basic helix-loop-helix transcription factor 1 (ATOH1) (also referred to as HATH1 in humans and Math1 in mice) and further by high level of SAM pointed domain-containing Ets transcription factor 1 (SPDEF). Kruppel-like factor 4 (KLF4) expressing, terminally differentiated goblet cells are particularly specialized in the production and secretion of highly glycosylated proteins, so called mucins, with mucin 2 (MUC2) being the most abundant in the colon and small intestine. Notably, klf4-deficient mice display defective goblet cell differentiation with a decrease of about 90% of colonic goblet cells. Reduced numbers of goblet cells in line with colonic mucus depletion have been suggested as histological hallmarks of UC. Gersemann et al showed that induction of goblet cell differentiation during inflammation is impaired in UC but not in Crohn’s disease (CD). Furthermore, differentiation defects of intestinal stem cells have been found to be accompanied by a barrier dysfunction, leading to intestinal inflammation or cancer development.

The OXPHOS system has been found to be highly active in cells of the upper part of the intestinal crypt. Therefore, differentiated goblet cells are expected to be highly affected by reduced OXPHOS activity. Supporting this hypothesis, we recently published that loss of OXPHOS-stabilizing p32 by inflammasome-driven cleavage reduces goblet cell differentiation state in vitro.

In 1980, Roediger hypothesized that pathogenesis of UC is linked to energy deficiency. More specifically, Roediger found reduced butyrate oxidation rates in isolated colonicocytes from UC patients compared with healthy control subjects. Furthermore, 2 independent studies reported reduced mitochondrial respiratory chain complex activity accompanied by mucosal ATP depletion in UC patients. Interestingly, alterations in all 3 studies were already present in noninflamed tissue, implicating mitochondrial dysfunction as a pathophysiological cause, rather than a consequence, in UC.

The ubiquitous nuclear encoded multifunctional protein p32 critically maintains OXPHOS and was independently identified as a subunit of the human pre-messenger RNA (mRNA) splicing factor SF2, as C1qbp (complement component 1q binding protein) (gC1qR; globular C1q receptor), and as HABP1 (hyaluronic acid binding protein). p32 expression is integral to mitochondrial energy maintenance, with energy generation via OXPHOS being nearly absent in p32 knockout cells and p32-deficient mice being embryonic lethal.

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Abbreviations used in this paper: ATOH1, atonal basic helix-loop-helix transcription factor 1; CD, Crohn’s disease; DMEM, Dulbecco’s modified Eagle medium; ECAR, extracellular acidification rate; ELISA, enzyme-linked immunosorbent assay; GFHP, glucose-free high-protein; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; IgA, immunoglobulin A; KLF4, Kruppel-like factor 4; mRNA, messenger RNA; MUC2, mucin 2; MUC5AC, mucin 5AC; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; SDS, sodium dodecyl sulfate; siRNA, silencing RNA; SPDEF, SAM pointed domain-containing Ets transcription factor; UC, ulcerative colitis; WT, wild-type.

*Most current article*
Taken together, we hypothesized p32 to be involved in the maintenance of the metabolic trajectory within the intestinal crypt, thereby enabling the metabolic switch from glycolysis to mitochondrial OXPHOS, which is necessary for terminal differentiation of intestinal stem cells toward goblet cells. Because mitochondrial dysfunction and defects in goblet cell differentiation have been attributed to UC pathogenesis, we aimed at investigating colonic expression of p32 in UC patients, as well as studying mechanistic backgrounds and possible modulation of OXPHOS-driven goblet cell differentiation.

Results

UC Patients in Remission Display Decreased Colonic p32 Expression, Increased Glycolysis, and Cellular Energy Deficiency

Stem cells in the lower part of the colonic crypt are mainly dependent on glycolysis, while there is a gradient toward an increase in energy generation via OXPHOS toward the differentiated cells at the tip of the crypt. Cellular differentiation occurs alongside this gradient of shifting energy source (Figure 1A), and we postulated p32, as a main driver of mitochondrial OXPHOS, to be involved in its maintenance. Indeed, p32 is highly expressed in the upper part of the colonic crypt, together with mitochondrial marker TOMM22 and goblet cell differentiation marker KLF4 (Figure 1B and C). When p32 mRNA expression was analyzed in colonic biopsies obtained from UC patients in remission and non-IBD control subjects, we found significantly reduced p32 level in UC (Table 1, Figure 1D). At least 2 isoforms of p32 have been described, 1 encoding and 1 lacking the mitochondrial leader sequence in exon 1. Hence, we investigated exon expression in a subset of intestinal biopsies from 10 non-IBD control subjects and 9 UC patients in remission. Expression of all exons, and therefore most likely all isoforms, of the p32 transcript was reduced in the intestine of UC patients compared with non-IBD control subjects (Figure 1E). Detection rates of the p32 exon 1 and exon 1–2 transcripts were more than 100-fold lower than for all other p32 exons when quantified by the dCt method. This was at least partially due to low binding capacity of corresponding anti-p32 probes as revealed by binding assays of these probes against a human full-length p32 plasmid (Figure 1F).

Owing to the fact that mitochondrial function is highly affected by aging and various therapeutic regiments, we related p32 mRNA expression to patients’ age and tested for potential influences of commonly prescribed therapeutics within our cohort such as prednisolone, mesalazine, and azathioprine. p32 mRNA expression did not correlate with age in either non-IBD control subjects or UC patients (Figure 1G). In line with previous studies, which showed azathioprine to impair cell proliferation, azathioprine treatment was associated with higher p32 mRNA level in UC patients, an effect observed under neither mesalazine nor prednisolone therapy (Figure 1H and I). Therefore, biopsies from patients receiving azathioprine treatment were excluded from data presented in Figures 1D, E, and J and 2.

To investigate whether differences in p32 mRNA level are also reflected on protein level, a set of 10 colonic biopsies collected from non-IBD patients was compared with 9 colonic biopsies collected from UC patients in remission via immunohistochemistry staining of the p32 protein (clone EPR8871). p32 staining was densitometrically quantified in the upper third of the crypt and revealed a significantly lower p32 positive area in UC patients compared with non-IBD control subjects (Table 1, Figure 1J). Further, UC patients displayed increased L-lactate level in plasma and serum samples compared with non-IBD control subjects as well as high phosphorylation of AMPK (adenosine monophosphate kinase) in colonic biopsies, pointing to increased glycolysis activity and mucosal energy deficiency in UC patients (Table 2, Figure 2A–C).

Colonic Goblet Cell Differentiation Is Impaired in UC Patients in Remission and Goblet Cell Number Decreases With Increasing Degree of Inflammation

High glycolytic activity characterizes cell metabolism in proliferating precursor cells, rather than in nondividing differentiated cells. Because goblet cell function has been previously proposed to be impaired in UC, we focused on analyzing differentiation status of this cell entity. Interestingly, expression of terminal goblet cell differentiation marker KLF4 was significantly down-regulated in colonic biopsies (hepatic flexure to sigmoid colon) from UC patients in remission compared with non-IBD control subjects (Figure 2D). Additionally, colonic KLF4 mRNA expression significantly correlated with p32 mRNA expression (Figure 2E), supporting the hypothesis that impaired terminal goblet cell differentiation in UC is a result of defective energy generation via p32-driven OXPHOS. Meanwhile, transcript levels of goblet cell precursor markers ATOH1 and SPDEF1 were not statistically altered (Figure 2D).

In the next set of experiments, we analyzed p32 expression and goblet cell appearance in noninflamed and inflamed tissue sections of UC patients in remission or active disease. Inflammasomes, as part of the innate immune system, are responsible for the initiation of inflammatory responses, mediated by the activation of caspase-1 among others. We have recently published that active caspase-1 cleaves p32 at 2 distinct sites (exon 1–2 junction and in exon 5), thereby preventing mitochondrial import of p32. This mechanism results in a shift in energy generation of tumor cells from OXPHOS towards aerobic glycolysis (Figure 3A) and abrogation of differentiation of goblet cell–like HT29-MTX cells, in vitro. p32 levels were not altered in UC tissue biopsies from inflamed versus noninflamed regions, when analyzing mRNA expression in paired biopsies or p32 protein expression utilizing an anti-p32 antibody detecting total p32 (binding to epitopes encoded by exon 5 before the C-terminal caspase-1 cleavage side (Table 1, Figure 3B and C)). In line with data published by Gersemann et al in 2009, we did not observe induction of KLF4 expression in biopsies obtained from inflamed regions.
### Table 1. Patient Characteristics of Native and Paraffin-Embedded Biopsies

| mRNA Expression | IHC Analysis |
|-----------------|--------------|
| **Total number of patients included** | **24** |
| Non-IBD | 39 | 11 (28.2) | 9 (37.5) | 10 (41.7) |
| UC remission NI | 18 (46.2) | 9 (50.0)/5 (50.0)/— |
| UC I | 10 (25.6) | 5 (50.0)/5 (50.0)/— |
| **Female/male/unknown** | **5 (50.0)/5 (50.0)/—** |
| Non-IBD | 4 (36.4)/6 (54.5)/1 (9.1) | 5 (50.0)/5 (50.0)/— |
| UC remission NI | 9 (50.0)/9 (50.0)/— | 7 (77.8)/2 (22.2)/— |
| UC I | 5 (50.0)/5 (50.0)/— | 3 (60.0)/2 (40.0)/— |
| **Age (unknown), y** | **53.4 ± 11.3** |
| Non-IBD | 62.2 ± 19.0 (1) | 53.4 ± 11.3 |
| UC remission NI | 52.4 ± 16.2 (1) | 60.0 ± 13.5 |
| UC I | 38.51 ± 13.4 | 51.6 ± 7.7 |

| Origin of biopsies | Non-IBD | UC NI | UC I | Non-IBD | UC NI | UC I |
|-------------------|---------|------|------|---------|------|------|
| Cecum | — | 4 (22.2) | — | — | — | — |
| Colon ascendens | 2 (18.2) | 2 (11.1) | — | — | — | — |
| Flexura hepatica | 1 (9.1) | — | — | — | — | — |
| Colon transversum | 1 (9.1) | 1 (5.6) | — | 1 (10.0) | — | — |
| Colon descendens | — | 1 (5.6) | 1 (10) | 3 (30.0) | 3 (33.3) | 1 (20.0) |
| Colon sigmoideum | 6 (54.5) | 9 (50.0) | 9 (90) | 6 (60.0) | 6 (66.7) | 4 (80.0) |
| Rectum | 1 (9.1) | 1 (5.6) | — | — | — | — |
| Colon unclassified | — | — | — | 1 (10.0) | 1 (11.1) | — |

| Medication | Non-IBD | UC NI | UC I | Non-IBD | UC NI | UC I |
|------------|---------|------|------|---------|------|------|
| Mesalazine/mesalazine klysmen | — | 12 (66.7) | 5 (50) | — | 5 (55.6) | 2 (40.0) |
| Prednisone | — | 6 (33.3) | 7 (70) | 1 (10.0) | — | 2 (40.0) |
| Azathioprine | — | 5 (27.7) | 1 (10) | — | — | — |
| Sulfasalazine | — | 2 (11.1) | 1 (10) | — | 1 (11.1) | — |
| Tacrolimus | — | 2 (11.1) | — | — | — | 1 (20.0) |
| Budesonide klysmen | — | — | — | — | 2 (40.0) |
| Metronidazole | — | — | 3 (30) | 1 (10) | — | 1 (20.0) |
| Sirolimus | — | 1 (5.6) | — | — | — | — |
| Hydrocortisone rectal foam | — | 1 (5.6) | — | — | — | — |
| Olsalazine | — | 1 (5.6) | — | — | — | — |
| Ciprofloxacin | — | — | — | 3 (30.0) | 1 (11.1) | 1 (20.0) |

**NOTE.** Values are mean ± SD or n (%), unless otherwise indicated. I, inflamed; IBD, inflammatory bowel disease; IHC, immunohistochemistry; mRNA, messenger RNA; NI, noninflamed; UC, ulcerative colitis.

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**Figure 1.** (See previous page). UC patients in remission not receiving azathioprine display reduced colonic p32 level. (A) A model for energy generation and goblet cell differentiation in the colonic crypt and (B) schematic subcellular localization of proteins of interest were generated by modifying images from Servier Medical Art. (C) Representative immunohistochemistry staining of p32 (clone EPR8871), Tomm22, and KLF4 in paraffin-embedded human colonic biopsies. Scale bar = 50 μM. (D) p32 mRNA expression was measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in colonic biopsies from non-IBD and UC patients in remission. (E) p32 exon expression was analyzed by TaqMan assay. Non-IBD: n = 10; UC: n = 7 and 6 for exon 1 and exon 1–2, respectively, and n = 8–9 for all other exon junctions. (F) Binding of TaqMan probes to the p32 plasmid was analyzed by qRT-PCR. (G) Intestinal p32 transcript expression was correlated against age. (H) p32 mRNA level in colonic biopsies from UC patients in remission treated with or without mesalazine, prednisolone, or azathioprine was measured by qRT-PCR. (I) Exemplary measurement of patients receiving azathioprine or control treatment applying TaqMan probes for every exon-exon junction. UC w/o azathioprine n = 5 for exon 1–2 and n = 8–9 for all other exons; UC with azathioprine n = 3–4. (J) Representative immunohistochemistry staining and corresponding quantification of p32 (clone EPR8871) expression in the upper part of the colonic crypt in paraffin-embedded biopsies from non-IBD control subjects and UC patients in remission. Scale bar = 10 μM. (D, E) Unpaired t test with Welch’s correction; (G) Spearman’s rank correlation coefficient; (H–J) unpaired t test; results are shown as (D, J) mean ± 95% CI, (E, I) box-and-whisker plot min to max, or (H) mean ± SD. *P ≤ .05, **P ≤ .01.

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of patients with active UC compared with noninflamed biopsies from UC patients in remission (Table 1, Figure 3D). Of note, protein expression of pro-caspase-1 was reduced in inflamed but not in noninflamed tissue areas of UC patients indicating inflammasome activation in respective regions. Consistent with reported caspase-1-induced p32 cleavage, binding of an antibody against p32 exon 6 was reduced in UC inflamed tissue sections compared with non-IBD control subjects in a disease activity-dependent manner. Furthermore, blinded evaluation of PAS-Alcian blue staining revealed reduced staining intensity of goblet cell granules in UC noninflamed tissue compared with non-IBD control subjects under basal conditions. The amount of mucus-filled goblet cells was reduced under low-grade inflammation and further decreased with increasing degree of mucosal inflammation (Table 1, Figures 3E and 4). Overall, these findings support our previous observation that caspase-1 cleavage of p32 leads to abrogation of goblet cell differentiation, thereby further reducing mitochondria-localized and functional p32 and differentiated goblet cells in UC.

**Goblet Cell Differentiation Is Dependent on OXPHOS and p32 In Vitro**

To test our hypothesis that OXPHOS-driven goblet cell differentiation in the intestinal crypt is dependent on p32, we next screened a range of human colorectal carcinoma cell lines for expression of goblet cell differentiation markers, MUC2, Mucin5AC (MUC5AC), and p32. HT29-MTX cells depicted high basal mRNA level of SPDEF1, indicating a goblet cell precursor phenotype as well as MUC5AC but not MUC2. While DiFi cells displayed high levels of both ATOH1 and KLF4, the analyses of T84 cells indicated terminal differentiation reflected by high expression of KLF4 and MUC2. All these 3 goblet cell–like cell lines similarly expressed p32 mRNA (Figure 5A). To find an inducible cell line model to study dependency of goblet cell differentiation on mitochondrial activity in vitro, β-oxidation and hence OXPHOS in HT29-MTX, T84, and DiFi cells was boosted through stimulation with the SCFA butyrate in the presence or absence of the proinflammatory stimulus lipopolysaccharide, frequently present in the intestine (Figure 5B and C).

| Total number of patients included | Serum/Plasma | Western Blot Analysis |
|----------------------------------|-------------|----------------------|
| Non-IBD                          | 17 (51.5)   | 5 (55.6)             |
| UC remission                     | 16 (48.5)   | 4 (44.4)             |

| Female/male                      |              |                      |
|----------------------------------|-------------|----------------------|
| Non-IBD                          | 9 (52.9)/8 (47.1) | 3 (60.0)/2 (40) |
| UC remission                     | 7 (43.8)/9 (56.3) | 1 (25)/3 (75)       |

| Age (unknown), y                  |              |                      |
|----------------------------------|-------------|----------------------|
| Non-IBD                          | 30.3 ± 9.7  | 49.8 ± 24.2          |
| UC remission                     | 43.8 ± 7.7 (10) | 60.15 ± 17.04 |

**Table 2. Patient Characteristics Serum Samples and Western Blot Biopsies**

NOTE. Values are n (%) or mean ± SD, unless otherwise indicated. IBD, inflammatory bowel disease; TNF-α, tumor necrosis factor α; UC, ulcerative colitis.
Butyrate stimulation induced terminal goblet cell differentiation of HT29-MTX but not of T84 or DiFi cells, reflected by induction of KLF4 expression, which was abrogated in the presence of lipopolysaccharide (Figure 5C). Butyrate-triggered terminal goblet cell differentiation of HT29-MTX cells was accompanied by an increase in oxygen consumption rate (OCR) but not in extracellular acidification rate (ECAR) (Figure 5D), underlining the importance of a metabolic switch toward OXPHOS in goblet cell differentiation. Furthermore, differentiated HT29-MTX cells displayed increased mucin granule formation, decreased cell proliferation, and enhancement of secreted Muc5AC (Figure 5E–H). Of note, p32 mRNA expression was not altered upon butyrate stimulation (Figure 5I). To test whether goblet cell differentiation is indeed dependent on p32, we performed silencing RNA (siRNA)-induced silencing experiments in HT29-MTX cells. Of main interest, induction of goblet cell differentiation via butyrate was abrogated in p32-silenced HT29-MTX cells accompanied by increased lactate level, indicating a switch in energy metabolism toward aerobic glycolysis (Figure 5J and K). OXPHOS is a lot more efficient in the production of ATP compared with aerobic glycolysis. Therefore, we proposed a pivotal role for cellular energy supplied by the mitochondrial OXPHOS system not only for goblet cell differentiation, but also for mucus secretion. To test this hypothesis, HT29-MTX cells were first terminally differentiated by postconfluent growth, followed by stimulation with OXPHOS complex V blocker oligomycin or the uncoupling agent DNP (2,4-dinitrophenol) (Figure 6A and B). As expected, blocking of OXPHOS function by oligomycin resulted in a shift of cellular energy metabolism from OXPHOS to glycolysis (Figure 6C). Moreover, mucus secretion was impaired by oligomycin as well as by DNP, reflected by a dose-dependent downregulation of secreted but not intracellular Muc5AC (Figure 6D–G), supporting the idea that mucus secretion is a highly energy demanding process enabled by efficient OXPHOS activity.

ATP8 mutant Mice Display Low Colonic p32 Expression in Concert With Loss of OXPHOS and Goblet Cells

To investigate the observed UC phenotype of low colonic p32 level, energy deficiency, and defective goblet cell differentiation in a mouse model, we applied conplastic respiratory chain complex V mutant mice. These mice carry a mutation in the mitochondrial encoded ATP8-synthase resulting in diminished respiratory capacity and ATP production with parallel induction of energy generation via nonmitochondrial glycolysis in various cell entities. (Figure 7A). ATP8 mutant mice did not
exhibit signs of colonic inflammation under basal conditions, as defined by an inconspicuous colonoscopy and similar mRNA expression of cxc11/keratinocyte chemoattractant in colonic biopsies compared with wild-type (WT) B6 mice (Figure 7B and C). Furthermore, intestinal barrier integrity was still intact, with comparable level of immunoglobulin A (IgA) in fecal samples of FVB mutant compared with B6 WT mice and similar amounts of mucosa attached bacteria in colonic biopsies (Figure 7D and E). Specifically, we found that ATP8 mutant mice displayed reduced p32 mRNA expression and diminished p32 protein level especially in differentiated intestinal epithelial cells in the upper part of colonic crypts (Figure 7F and G), while serum L-lactate levels were similar between strains (Figure 7H). In line with the phenotype observed in UC patients, loss of p32 in ATP8 mutant mice was associated with altered colonic goblet cell differentiation represented by decreased klf4 mRNA expression, diminished mucus filling of goblet cells and a reduced thickness of the colonic mucous layer. As observed in the human colon, klf4 mRNA expression significantly correlated with p32 mRNA expression in colonic samples from B6 WT and ATP8 mutant mice (Figure 7I–K). Expression of atoh1 and spdef1 was not altered, which was comparable to

Figure 4. UC patients in remission display reduced PAS-Alcian staining intensity. Follow-up of representative immunohistochemistry staining against pro-caspase-1 and p32 exon 6 as well as PAS-Alcian staining in paraffin-embedded tissue biopsies from the descending colon or sigmoid colon; #5 and #6: non-IBD noninflamed; #7 and #8: UC noninflamed.

Figure 3. (See previous page). Goblet cell loss correlates with inflammasome activation and decrease of full-length p32 level in active UC. (A) Schematic visualization of p32 cleavage by active Caspase-1. (B) p32 mRNA expression in paired biopsies from noninflamed and inflamed intestinal tissue sections were quantified by qRT-PCR. (C) Representative immunohistochemistry staining and corresponding quantification of p32 protein expression (clone EPR8871, anti-p32 exon 5) in the upper part of the colonic crypt in paraffin-embedded biopsies from noninflamed and inflamed colonic tissue sections from UC patients. Scale bar = 100 μM. (D) KLF4 mRNA expression was quantified by qRT-PCR in noninflamed colonic biopsies from UC patients in remission and in inflamed colonic biopsies from UC patients with active disease. (E) Representative immunohistochemistry staining against pro-caspase-1 and p32 exon 6 as well as PAS-Alcian staining in paraffin-embedded tissue biopsies from the descending colon or sigma; #1: non-IBD noninflamed; #2.1: UC noninflamed; #3: UC low-grade inflammation; #2.2: UC medium-grade inflammation; #4: UC high-grade inflammation. Representative images from 8 biopsies each categorized as non-IBD control subjects or UC noninflamed and 5 UC inflamed samples are displayed. Scale bar = 50 μM. Results are shown as (C) mean ± 95% CI or (D) median ± interquartile range. i., inflamed; n.i., noninflamed.
observations in UC patients (Figure 7I). Furthermore, expression of the proliferation marker ki67 and the stem cell marker lgr5 were not different in colonic biopsies from ATP8 mutant mice (Figure 7L). Taken together, we here present a mouse model with low intestinal p32 level and diminished energy generation via OXPHOS to depict reduced numbers of terminally differentiated goblet cells in the colon, strengthening the notion that especially goblet cell differentiation is highly sensitive to mitochondrial dysfunction.

A Glucose-Free Nutritional Intervention Promotes Colonic p32 Expression and Goblet Cell Differentiation in Mice

Finally, we aimed to study regulation of goblet cell differentiation via the enhancement of intestinal p32 expression in a glucose-free, high-protein nutritional intervention in mice. Because availability of nutrients critically affects cellular metabolism,33 we hypothesized that withdrawal of glucose from the diet and isocaloric replacement of glucose by the protein casein may result in a metabolic shift toward

Figure 5. (See previous page). Goblet cell differentiation is dependent on p32 expression. (A) Transcripts of goblet cell differentiation factors, mucins, and p32 were measured in colorectal cancer cell lines by qRT-PCR. (B) Graphical setup of cell culture experiments. (C) Western blot experiments were performed from whole protein extracts with respective antibodies in cells stimulated with 1.25-mM butyrate and/or 1-μg/mL lipopolysaccharide (LPS) (p32 clone EPR8871). (D) Basal OCR and ECAR were measured by Seahorse assay.13 (E) Representative image of HT29-MTX cell growth characteristics. (F) Cell counts are presented as fold change for each individual experiment. (G) Muc5AC levels in the supernatant were measured by ELISA, were normalized to cell count, and are displayed as fold change for each individual experiment. (H) KLF4 and MUC5AC immunohistochemistry staining was performed in paraffin-embedded butyrate-stimulated or control HT29-MTX cells. Scale bar = 10 μM. (I) p32 mRNA expression in butyrate-treated or untreated HT29-MTX cells was measured by qRT-PCR. (J, K) For siRNA knockdown, HT29-MTX cells were stimulated with 50-nM p32 siRNA or respective control for 96 hours and butyrate for 72 hours. (J) Representative Western blot and quantification from whole protein extracts (p32 clone 60.11) and (K) L-lactate level from corresponding cell culture supernatants. (D) Paired t test; (F, G) unpaired t test; (J, K) uncorrected Fisher’s test; data are shown as mean ± SD with the exception of (A) mean ± SEM. *P ≤ .05, **P ≤ .01.
mitochondrial oxidation (Figure 8A). Adult C57BL/6 mice were fed a glucose-free high-protein (GFHP) diet or an isocaloric control diet for an average of 70 days before organ sampling and molecular analysis. Food consumption, body weight, serum glucose level, and lactate level were similar between diets (Figure 8B–E). Of main interest, GFHP diet–fed mice exhibited increased p32 protein level in the upper part of the colonic crypt, which was not due to elevated p32 mRNA level (Figure 8F–H). Simultaneously, GFHP diet–fed mice displayed high colonic energy level, reflected by low phosphorylation of AMPK (Figure 8H). Eventually, we tested whether enhanced p32 expression would also result in enhanced goblet cell differentiation. In comparison with control mice, increased KLF4 mRNA and protein expression as well as a thicker colonic mucus layer were potent indicators for induction of terminal differentiation of goblet cells under GFHP diet (Figure 8I and J). Further, spdef1 as a marker for secretory progenitor cells tended to be reduced in GFHP diet mice (without statistical significance, $P \leq .06$), supporting the idea that p32 expression is pivotal for the transition from secretory precursors toward terminal differentiated goblet cells (Figure 8I). Expression of intestinal stem cell marker lg5 and proliferation marker ki56 were unaltered upon GFHP diet (Figure 8K). Taken together, nutritional intervention by glucose restriction in the presence of high protein intake appears as a promising tool to enhance colonic p32, thereby improving cellular energy supply and finally promoting goblet cell differentiation.

Discussion

Within the colonic crypt, mitochondria maintain the energy gradient, which is necessary for efficient cell differentiation and proliferation and thereby critical in determination of epithelial cell fate.\textsuperscript{2,10,12} Mitochondrial disturbance and dysfunction of goblet cells are hallmarks of UC pathology,\textsuperscript{4,6,34} which presents as a multifactorial disease, in which inflammation is caused by a disruption of the colonic epithelial and mucus barrier. Terminally differentiated goblet cells have a pivotal role in the maintenance of intestinal barrier integrity, and their differentiation is presumably regulated by a metabolic switch from glycolysis to mitochondrial OXPHOS.\textsuperscript{5,16} In order to understand the molecular basis and disease origin of UC, it is necessary to find the underlying cause of mitochondrial dysfunction and to unravel a potential link to impaired goblet cell function.

Both IBD subtypes, UC and CD, are disorders of the gastrointestinal tract, which display dysfunctional mitochondria. Nevertheless, while mitochondrial disturbance results in aberrant development of Paneth cells in CD,\textsuperscript{35} we here present abrogation of goblet cell differentiation through insufficient mitochondrial respiration as a potential cause for disease development in UC. Impaired induction of goblet cell differentiation in inflamed UC but not CD has been previously reported.\textsuperscript{15} Our data indicate that defective terminal differentiation of goblet cells is already present in noninflamed colonic tissue of UC patients in remission, defined by diminished mucus filling of goblet cells and reduced expression of terminal goblet cell differentiation marker KLF4 compared with non-IBD control subjects. In line with this tenet, reduced numbers of goblet cells and a defective colonic mucus layer enabling bacterial invasion were already published for UC patients in remission.\textsuperscript{6,7,36} Very recently differentiation of intestinal stem cells into the secretory lineage has been proposed to depend on mitochondria by a mechanism involving FoxO (forkhead box O) transcription factors and Notch signaling–regulated mitochondrial fission.\textsuperscript{37} Data presented here suggest that loss of p32, which is postulated to be the main driver of OXPHOS, is the underlying cause of metabolic dysfunction and secondarily of defective goblet cell function in UC.

In addition to the observation that low p32 expression is accompanied by mitochondrial dysfunction and defective goblet cell maturation in UC, we present experimental evidence that induction of goblet cell differentiation is dependent on p32-regulated mitochondrial function in vitro. Stimulation of a mucus producing goblet cell–like cell line with the SCFA butyrate resulted in induction of OXPHOS and terminal differentiation. Of main interest, differentiation was abolished by p32 silencing, and mucus secretion was impaired after treatment with OXPHOS inhibitors. In line with these observations, polymorphisms in nuclear-encoded mitochondrial genes involved in ATP generation, namely uncoupling protein 2 (UCP2) and SLC22A5, encoding the OCNT2 (organic carnitine transporter 2), have been described as risk factors for UC.\textsuperscript{38,39} In addition, inhibition of intestinal fatty acid β-oxidation as well as genetic ablation.
Low p32 Level Drive Goblet Cell Loss in UC

Table 3. Chow Composition as Provided by the Manufacturer

| Diet composition | Isoenergetic control diet | Glucose-free high-protein diet |
|------------------|---------------------------|-------------------------------|
| Casein, %        | 20                        | 60                            |
| Brewer’s yeast, %| —                         | 2                             |
| Corn starch, %   | 28                        | —                             |
| Maltodextrin, %  | 14.5                      | —                             |
| Sucrose, %       | 10                        | —                             |
| Cellulose powder, % | 15                      | —                             |
| L-cystine, %     | 0.3                       | —                             |
| Vitamin premixture, % | 1                     | 1                             |
| Minerals and trace elements, % | 6              | 6                             |
| Choline chloride, % | 0.2                       | 0.2                           |
| Soybean oil, %   | 5                         | —                             |
| Crude protein, % | 17.7                      | 53.4                          |
| Crude fat, %     | 5.1                       | 11.4                          |
| Crude fiber, %   | 15.8                      | 20.3                          |
| Crude ash, %     | 5.4                       | 5.9                           |
| Starch, %        | 26.9                      | 0.1                           |
| Sugar, %         | 9.9                       | —                             |
| NFE, %           | 51.7                      | 1.9                           |
| Physiological fuel value, MJ/kg | 13.6               | 13.6                          |
| Protein, kcal%   | 22                        | 66                            |
| Fat, kcal%       | 14                        | 32                            |
| Carbohydrates, kcal% | 64                    | 2                             |

of UCP2 or OCNT2 in mice resulted in experimental colitis. Conversely, conplastic mice with high mucosal OXPHOS and ATP levels have been already demonstrated by our group to be protected from experimental colitis.

Apart from its role as a regulator of mitochondrial function, p32 has been described to interact with various proteins localized on the cell surface, the nucleus, the cytoplasm, or the extracellular space. Binding of p32 to the globular heads of C1q reportedly inhibits classical pathway complement activation. Furthermore, interaction of p32 with serum proteins involved in blood clotting and fibrin polymerization as well as binding to various bacterial or viral antigens might play a role in the prevention of intestinal inflammation. Whether low levels of p32 observed in UC might lead to impairment in any of the mentioned pathways will be a topic of further investigations.

Here, we describe high level of KLF4-expressing terminally differentiated goblet cells as a healthy state and as necessary for mucus barrier integrity. In ATP8 mutant mice, carrying a mutation in complex V of the respiratory chain, we observed low colonic expression of p32 accompanied by reduced klf4 mRNA expression, a diminished number of goblet cells and a thinned mucous layer. The transcription factor KLF4 specifically controls goblet cell fate, since in mice with intestinal deletion of Klf4 both colonocytes and enteroendocrine cells appear to undergo normal maturation. Additionally, cell proliferation and cell death rates appear unchanged in Klf4-deficient mice, while goblet cell numbers are reduced by 90%. In general, goblet cells are recognized to be a major line of defense in the intestinal mucosa. The 2-layered colonic mucus system separates bacteria from the host epithelium and the continuous self-renewal pushes bacteria out into the lumen, while animals with a penetrable mucus layer develop spontaneous colitis. Notably, high KLF4 levels suppress development and progression of intestinal neoplasia and colitis-associated colorectal cancer upon azoxymethane or dextran sulfate sodium treatment in mice.

Proliferation, rather than differentiation, of intestinal epithelial cells is highly important for tissue repair during

**Figure 8.** (See previous page). GFHP diet increased mucosal energy supply, induced colonic p32 protein expression, and promoted goblet cell differentiation. (A) Hypothesized metabolic switch upon GFHP dietary intervention in mice. Weekly (B) food consumption and (C) mice body weight was determined (n = 6 from 2 independent experiments). (D) Glucose and (E) L-lactate level were measured in serum samples from control and GFHP diet mice. Expression of transcripts was measured (F) via TagMan probes for p32 exon 3–4 or (I, K) by SYBR qRT-PCR in colonic biopsies from control and GFHP diet mice. (G, J) Representative p32 (clone EPR8871) and KLF4-Alcian blue immunohistochemistry staining of parafomaldehyde-fixed colonic tissue samples as well as MUC2 fluorescent staining of Carnoy’s fixed tissue are presented with corresponding quantifications. Scale bar = 100 μM (10×), 50 μM (20×), 10 μM (63×). Arrow indicates inner mucus layer. (H) Western blot experiment of whole protein extracts from colonic samples from control and GFHP diet mice (p32 clone EPR8871). (G, J) (thickness of inner mucus layer) unpaired t test; (I, K) (KLF4 staining) unpaired t test with Welch’s correction; results are shown as mean ± SD. *P ≤ .05.
active UC. Additionally, mitochondrial dysfunction in the colonic epithelium of patients with active UC has been reported to be accompanied by a reduction in fatty acid oxidation.34 We have recently published that caspase-1 mediated cleavage of p32 results in a metabolic switch from mitochondrial oxidation to glycolysis, thereby shifting cell fate toward proliferation.13 In line, mice deficient for caspase-1 display defects in mucosal tissue repair, being detrimental under dextran sulfate sodium–induced colitis, while derepression of the inflammasome complex results in enhanced repair and resistance to acute colitis.46 Here, we demonstrate caspase-1 to be indeed activated in inflamed colonic tissue sections of UC patients, accompanied by a reduction of antibody binding against p32 exon 6 and a decrease of differentiated goblet cells. Taken together, loss of p32 in noninflamed colonic tissue appears highly problematic, owing to a decrease in differentiated goblet cells and thereby impaired mucus barrier function. Meanwhile, during colitis, p32 cleavage might be a physiologic mechanism necessary for induction of rapid cell proliferation for tissue repair.

We here propose nutritional intervention as a potential strategy to improve colonic p32 expression. A Westernized diet, rich in glucose, is a major environmental factor contributing to UC17 and was found to continuously activate the NLRP3 inflammasome.48,49 Having shown that caspase-1 mediated cleavage of p32 boosts cell proliferation,13 we vice versa proposed an isocaloric GFHP diet to result in increased p32-mediated goblet cell differentiation. Indeed, mice receiving a GFHP diet exhibited induction of p32 protein expression in the colon, which was not due to elevated p32 mRNA level compared with control subjects. In line with human and in vitro data, GFHP mice depicted high mucosal energy level, an increased number of KLF4-positive terminally differentiated goblet cells, and a thickening of the colonic mucus layer compared with control subjects. Considering this, dietary intervention appears as a promising tool to modulate p32 expression, mitochondrial function, and goblet cell differentiation in the intestine.

In conclusion, we identified a new pathway linking low colonic expression of OXPHOS-regulating p32 to mitochondrial dysfunction, defective goblet cell differentiation, and impaired mucus barrier formation, frequently observed in UC. Furthermore, we present a diet low in glucose as an option to induce colonic expression of p32, opening new pathways in the preventive treatment and therapy of UC.

### Table 4. Information on Applied Primer Pairs and TaqMan Probes

| Target gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|-------------|------------------------|------------------------|
| Human       |                        |                        |
| ATOH1       | CCAGCTCGGCAATGTTATCC   | TGCTGTTTTCCCTCTGCAC    |
| β-ACTIN     | ACATCCGCAAAGACCTGCTAC | TTGCTGATCCACATCTGCTG   |
| KLF4        | CCACTCCTTCAAGCTGCTG   | ATCGGATAGGTAAGCTGCA    |
| Muc2        | GACGGAGCTGAACTGTTAG   | GGACAGGATAGGTTAGGAT    |
| MUC5AC      | CTGGTGCAAGTGTGCGTCC   | TTGATCACCACCGTCTG      |
| p32         | CTGGACCCAGGAGAGAAG    | CATATAAGGCCAGTCCAG     |
| SPDEF1      | GATTCACCTAGTGCGTCCAG  | ATGCTGAGCCTCCAGGAT     |
| Mouse       |                        |                        |
| atoh1       | GTGGGGTTGATGAGGAGAG   | GGTTGCCTCGGACATTG     |
| β-actin     | GATGCCTCCGCGGCTGTATT  | GGTTGATCCAGGGTGAT     |
| cxcl1/kc    | CTCGGGATACCTAAAGAG   | CTGGGACACCTTTAGATC    |
| ki67        | CTCGAGGGACCTTCAAAAT   | TTGCTCACAGCATGAGG     |
| klf4        | GATAGGGTGTCAGGCTTGG  | GATAGGGTGTCAGGCTTGG   |
| lgr5        | GCACACACGACGATGGAGCTCT | GACACGTCAGGAGCGAGC  |
| muc2        | GCTGGAGATGAGGAGTCTGAG | CCAGGTCCTCGTGTCGCA    |
| p32         | CGCCCTACCTTGTGCAGAAG  | CGCCCTACCTTGTGCAGAAG  |
| spdef1      | GGAGAAGGCGACCATACAGGA | GGAGAAGGCGACCATACAGGA |

| Target genes | Assay ID (Thermo Fisher Scientific) |
|--------------|-----------------------------------|
| Human β-ACTIN| Exon-exon junction  Hs99999903_m1 |
| Human p32    | Exon 1                             Hs05053979_g1 |
| Human p32    | Exon 1-2                           Hs00214254_m1 |
| Human p32    | Exon 2-3                           Hs00911223_g1 |
| Human p32    | Exon 3-4                           Hs00911220_g1 |
| Human p32    | Exon 4-5                           Hs00911221_g1 |
| Human p32    | Exon 5-6                           Hs00911222_g1 |
| Murine β-actin| Exon 2-3                          Mm01205647_g1 |
| Murine p32   | Exon 3-4                           Mm01245770_m1 |
Materials and Methods

Study Cohort

Tissue biopsies from the terminal ileum and colon were obtained during endoscopy as part of regular patient management in the Medical Department 1, University Hospital Schleswig-Holstein, Campus Lübeck, Germany. Blood samples were collected at the University Hospital Schleswig-Holstein, Campus Lübeck; at the University Hospital Münster, North Rhine-Westphalia, Germany; and at the University Hospital Rostock, Mecklenburg Western Pomerania, Germany. Characteristics of histologically confirmed UC patients and non-IBD control subjects at time of endoscopy or sample collection are listed in Tables 1 and 2, respectively. The control group included patients who presented for a regular check-up or underwent endoscopy due to non–IBD-related reasons and presented without macroscopically and histologically evidenced of mucosal inflammation. Diagnosis of UC and classification of patients into remission and disease flare was based on clinical, endoscopic and histopathologic findings. Categorization into inflamed and noninflamed tissue was based on histopathologic presentation and activation of inflammasome components caspase-1. Groups were age and sex matched. Non-IBD control subjects or UC patients with reported colon cancer for mycoplasma contamination every 3 months and when any water. Procedures involving animals and their care were conducted in accordance with national and international laws and regulations. Glucose-free high-protein (GFHP) and isocaloric control diet were purchased from Ssniff (Soest, Germany). Compositions of corresponding diets are specified in Table 3. C57BL/6 mice were ordered at an age of 7–8 weeks from Charles River Laboratories (Wilmington, MA), were left to acclimatize on a standard chow diet until an age of 20 weeks and were than randomly distributed into GFHP diet– and isocaloric control diet–receiving groups. Mice were kept on the corresponding diet on an average of 70 days before sampling. Food consumption and body weight were measured once a week. Dietary intervention was performed in 2 independent experimental rounds.

Cell Culture

The human colorectal carcinoma cell lines HT29-MTX-E12 (Sigma-Aldrich, St Louis, MO) and DiFi were kept in Dulbecco’s modified Eagle medium (DMEM) medium supplemented with or without 1% nonessential amino acids, respectively. The human colorectal carcinoma cell line T84 was kindly provided by Markus Huber-Lang, University Hospital Ulm, Baden-Wuerttemberg, Germany, and grown in DMEM/F12 1:1 medium containing 1.5% HEPES. All cell culture media were supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100-U/mL penicillin, and 100-
mg/mL streptomycin. Cells were incubated at 37°C and 5% CO2 in a humidified incubator. Cells were cultivated up to a maximum of 20 passages and confirmed to be negative for mycoplasma contamination every 3 months and when freshly thawed.

For terminal differentiation, HT29-MTX cells were either grown post confluent for 9 days as described previously or stimulated with 1.25-mM butyrate (Merck Millipore, Burlington, MA) for 72 hours in the presence or absence of 1 μg/mL LPS-EB ultrapure (InvivoGen, San Diego, CA). DNP (Santa Cruz, Dallas, TX) or oligomycin (Agilent, Santa Clara, CA) were applied at indicated concentrations for 24 hours to inhibit mitochondrial respiration. Further, HT29-MTX cells were transiently transfected with 50-μM siRNA specific for human p32 (exon 3; s2138; Thermo Fisher Scientific) or control siRNA (Thermo Fisher Scientific) by reverse lipofection using lipofectamine 3000 reagent (Thermo Fisher Scientific) for 96 hours or were left untreated. After 24 hours, cells were stimulated with 1.25 mM butyrate for 72 hours or were left untreated.

RNA Extraction, Complementary DNA Synthesis, and Quantitative Reverse-Transcription Polymerase Chain Reaction

Isolation of total RNA from tissue biopsies or cell pellets was performed using the innuPREP RNA Mini Kit (Analytic Jena AG, Jena, Germany) according to manufacturer’s guidelines. Additional DNA digestion was performed 2 times after binding of RNA to RNA column with 4 units DNase (Sigma-Aldrich) in according reaction buffer for 20 minutes at RT. For complementary DNA synthesis, 1 μg of endoscopy (Hopkins Optik 64019BA; Aida Vet; Karl Storz, Tuttingen, Germany).
isolated RNA was transcribed with 100 pmol Oligo(dt)18 (Metabion, Steinischnagen, Germany), 20 U RiboLock RNase inhibitor (Thermo Fisher Scientific), dNTP Mix (0.2 mM for each dNTP), and 200 U RevertAid H Minus reverse transcriptase (Thermo Fisher Scientific) in corresponding reaction buffer at 42°C for 60 minutes. Target amplification was performed by quantitative reverse-transcription polymerase chain reaction on the StepOne real-time system (Thermo Fisher Scientific) applying Perfecta SYBR Green Supermix (Thermo Fisher Scientific) and 0.5-µM forward and reverse primer. Following cycling conditions were applied: initial denaturation at 95°C for 5 minutes; 40 cycles of denaturation at 95°C for 45 seconds, annealing at appropriate temperature (55°C) for 30 seconds and elongation for at 72°C for 30 seconds. Melting curve profiles were produced and data were analyzed following the 2^−ΔΔCt algorithm by normalized to β-actin. Primer sequences are listed in Table 4.

p32 exon expression was additionally analyzed by TaqMan probes (Thermo Fisher Scientific) (Table 4) according to manufacturer’s instructions using the StepOnePlus Real-Time polymerase chain reaction system. The following cycling conditions were applied: preincubation at 50°C for 2 minutes and 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing and elongation at 60°C for 1 minute. Ct-Values of targets were acquired via the StepOne system software and normalized to β-actin that served as an internal housekeeping transcript via the 2^−ΔΔCt algorithm.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Immunoblotting**

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and immunoblotting was performed according to standard protocols. In short, whole-protein extracts from homogenized tissue samples or cells were prepared by cell lysis in denaturing lysis buffer containing 1% SDS, 10-mM Tris (pH 7.4), phosphatase II, phosphatase III, and protease inhibitor (Sigma-Aldrich). Protein extracts were separated by denaturing SDS polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) under reducing conditions and transferred onto polyvinylidene difluoride

| Primary antibody | Species | Company | Working concentration |
|------------------|---------|---------|-----------------------|
| Anti-AMPKα (#2532) | rabbit | Cell Signaling Technology, Danvers, Massachusetts | 1 µg/mL (WB) |
| Anti-α-TUBULIN (#2125) | rabbit | Cell Signaling Technology, Danvers, Massachusetts | 1 µg/mL (WB) |
| Anti-β-ACTIN (#4967) | rabbit | Cell Signaling Technology, Danvers, Massachusetts | 1 µg/mL (WB) |
| Anti-CASPA3-1 (#2225) | rabbit | Cell Signaling Technology, Danvers, Massachusetts | 1:100 (IHC); 1:1000 (WB)* |
| Anti-mouse IgA α-chain (PAB9360) | rabbit | Abnova, Taipei, Taiwan | 0.5 µg/mL (ELISA) |
| Anti-mouse Ig light chain k (407201) | rat | BioLegend, San Diego, CA | 1 µg/mL (ELISA) |
| Anti-mouse Ig light chain λ (407302) | rat | BioLegend, San Diego, CA | 1 µg/mL (ELISA) |
| Anti-human KLF4 (AF3640) | goat | R&D Systems, Minneapolis, Minnesota | 4 µg/mL (IHC); 1 µg/mL (WB) |
| Anti-murin KLF4 (AF3158) | goat | R&D Systems, Minneapolis, Minnesota | 3.3 µg/mL (IHC) |
| Anti-MUC2 (clone H-300) | rabbit | Santa Cruz Biotechnologies, Dallas, Texas | 2 µg/mL (IF) |
| Anti-Muc5AC (clone 45M1) | mouse | Bioss, Woburn, Massachusetts | 2 µg/mL (IHC); 1 µg/mL (ELISA) |
| Anti-p32 (clone EPR8871) | rabbit | Abcam, Cambridge, United Kingdom | 2 µg/mL (IHC); 0.4 µg/mL (WB) |
| Anti-p32 (clone 60.11) | mouse | Abcam, Cambridge, United Kingdom | 1 µg/mL (WB) |
| Anti-p32 exon 6 | rabbit | kindly provided by Berhane Ghebrehiwet | 5 µg/mL (IHC) |
| Anti-TOMM22 (#WH0056993M1) | rabbit | Sigma-Aldrich, St Louis, Missouri | 2.5 µg/mL (IHC) |

| Secondary antibodies/labeled polymers | Company | Working concentration |
|--------------------------------------|---------|-----------------------|
| Alexa Fluor 594 nm goat anti rabbit | Thermo Fisher Scientific, Waltham, Massachusetts | 8 µg/mL (IF) |
| Anti-mouse IgG HRP | Cell Signaling Technology, Danvers, Massachusetts | 1:1000 (ELISA); 1:4000 (WB)* |
| Anti-rabbit IgG HRP | Cell Signaling Technology, Danvers, Massachusetts | 1:2000 (ELISA); 1:4000 (WB)* |
| Anti-goat IgG HRP | Agilent, Santa Clara, California | 1 µg/mL (IHC); 0.5 µg/mL (WB) |
| EnVision anti-rabbit HRP system | Agilent, Santa Clara, California | Not applicable |
| EnVision anti-mouse HRP system | Agilent, Santa Clara, California | Not applicable |

ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; WB, Western blot. *If stock concentration was not provided by the manufacturer, dilution from stock solution was listed.
membranes. After blocking, membranes were probed with specific primary antibodies followed by respective horse-radish peroxidase (HRP)–conjugated secondary antibodies. To determine similar transfer and equal loading, membranes were stripped and reprobed with an appropriate housekeeper. Proteins of interest were visualized on a ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories). Applied antibodies are listed in Table 5.

**Histology and Microscopy Analyses**

Immunohistochemical staining in paraformaldehyde-fixed and paraffin-embedded tissue biopsies was performed according to standard protocols. After deparaffinization, rehydration, endogenous peroxidase blockage, and antigen retrieval, tissue slides were probed with specific primary antibodies or isotype control antibodies, followed by respective HRP-conjugated secondary antibodies or HRP-labeled polymers (both listed in Table 5). Tissue slides were incubated with DAB-substrate (Dako, Jena, Germany) and counterstained with Mayer’s hemalum solution or Alcian blue. Images were obtained and analyzed on an Axio Scope.A1 microscope (Zeiss, Oberkochen, Germany) utilizing the ZEN imaging software (Zeiss). If appropriate, stained areas were quantified via the color deconvolution plugin for the software ImageJ version 1.53e (National Institutes of Health, Bethesda, MD).51

For Muc2 immunofluorescent staining and quantification of mucus layer thickness, colonic biopsies were fixed in Carnoy’s solution before paraffin-embedding. Slides were probed with specific antibodies for murine Muc2 or an appropriate isotype control, followed by incubation with respective fluorochrome-labeled IgG secondary antibody and counterstaining using DAPI (Sigma-Aldrich). Applied antibodies are listed in Table 5. Mucus layer thickness was measured at least at 4 different representative positions per slide per animal using the AxioCam software (Zeiss).

**Enzyme-Linked Immunosorbent Assay**

For detection of extracellular Muc5AC by enzyme-linked immunosorbent assay (ELISA), pure supernatants from cells was coated at 4°C overnight. Intracellular protein was detected in native protein isolates, coated with 50% coating buffer, containing 0.3% (w/v) Na2CO3 × 10 H2O and 0.6% (w/v) NaHCO3, pH 9.6. After blocking, Muc5AC was detected using a Muc5AC-specific primary antibody in combination with a respective HRP-conjugated secondary antibody listed in Table 5. Optical density was measured at 450 nm against a reference wavelength of 540 nm on a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA).

In order to quantify IgA in mouse fecal pellets, a sandwich ELISA was performed according to standard procedures. Applied antibodies are listed in Table 5. Fecal protein was extracted by homogenization in 10× volume extraction buffer (20-mM Na3PO4, 650-mL NaCl, 1-mM EDTA, 1-mM PMSP, pH 7.4) per gram of feces. Coating of capture antibody against anti-mouse Ig light chain κ and λ was performed at 4°C overnight. Fecal protein extracts and IgA RSG ELISA standard (Affymetrix, Santa Clara, CA) were diluted in 0.5% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20 in PBS and applied for 2 hours at room temperature. After applying an anti-mouse IgA α-chain specific detection antibody followed by a respective HRP-conjugated secondary antibody, optical density was measured as described previously.

**Seahorse Assay**

For determination of OCR and ECAR via Seahorse assay, 5 × 10³ HT29-MTX cells were seeded in a Seahorse XF24 cell culture plate in DMEM medium containing 5-mM glucose, 1% nonessential amino acids, 10% (v/v) heat-inactivated fetal calf serum, 100-U/mL penicillin, and 100-mg/mL streptomycin. Cells were stimulated with 1.25-mM butyrate for 72 hours or were left untreated. OCR and ECAR was determined in standard Seahorse medium on day 3 after seeding before and after injection of 2-μM oligomycin on a XF24 analyzer (Agilent) according to manufacturer’s instructions.

**Lactate Assay**

L-lactate levels were measured in serum or plasma samples (1:5 diluted in PBS) and in cell culture supernatants (1:10 diluted in PBS) according to manufacturer’s instructions (Megazyme, Wicklow, Ireland). Lactate level in cell culture supernatant were normalized to cell count.

**Quantification of Mucosa Attached Bacteria**

Extraction and quantification of mucosa-attached bacteria from mouse colonic tissue was performed as previously described.52 In short, mucus was removed by incubation of biopsies in 500-μL PBS containing 0.016% DTT at 800 g for 1 minute. After wash, hypotonic lysis of eukaryotic cells was achieved by vortexing tissue in 500 μL ddH2O at 800 g for 30 minutes. Bacteria and tissue debris were pelleted by centrifugation at 12,000 g for 3 minutes, taken up in 16% glycerol, and stored at −80°C. To quantify bacteria via flow cytometry, mucosa-attached bacteria were thawed on ice, washed with 1% (w/v) bovine serum albumin and 0.1% Na2HPO4 in PBS and incubated with 10-μM Syto BG green fluorescent nucleic acid stain (Thermo Fisher Scientific) for 20 minutes on ice. Flow cytometry was performed on an Attune NxT cytometer (Thermo Fisher Scientific) with a forward scatter threshold set to 10,000 to exclude cell debris from measurement.

**Author Approval**

All authors had access to the study data, reviewed and approved the final manuscript.

**Statistics**

Statistical analysis was performed using Prism version 6 (GraphPad, San Diego, CA). Outliers were identified by
Grubbs’ test (significant level $\alpha = 0.05$). The $F$ test was used to compare variances and D’Agostino–Pearson test was applied to test for normal distribution. Statistical differences between 2 groups were analyzed by unpaired t-test or paired t-test (normally distributed data), unpaired $t$ test with Welch’s correction (significant different variances) or Mann-Whitney $U$ test (not-normally distributed data). For comparison of more than 2 groups, 1-way analysis of variance with Bonferroni posttest was applied. Uncorrected Fisher’s least significant difference test was employed for datasets with 2 variables. Correlation analysis was performed by obtaining the Spearman’s rank correlation coefficient. $P$ values were calculated and null hypotheses were rejected when $P \leq 0.05$. Data are shown as mean ± 95% confidence interval, as mean ± SD for small datasets, or as median with interquartile range for datasets with large variances.

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