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Oit1/Fam3D, a gut-secreted protein displaying nutritional status-dependent regulation☆

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Abstract

Oncoprotein-induced transcript 1 (Oit1) was previously identified as a dietary fat-induced gene in the small intestine of C57Bl/6J mice. In this study, we further characterized Oit1 and its human ortholog family with sequence similarity 3, member D (Fam3D), on the messenger RNA as well as the protein level. Oit1 and Fam3D were found to be predominantly expressed in the gastrointestinal tract of mice and humans, respectively. Dietary fat induced a clear and acute up-regulation of Oit1, especially in the jejunum, whereas fasting led to a reduced gene expression in the small intestine. Regarding protein expression, we found a remarkable pattern of Oit1 along the longitudinal axis of the intestine, a predominant villus-restricted expression in the proximal small intestine and a more pronounced crypt expression in the distal parts of the intestine. Using transfection experiments, we confirmed secretion of the Oit1 protein, as was predicted by a signal peptide sequence. Detection of Oit1 and Fam3D in plasma samples indicated that both proteins are secreted to the basolateral site of enterocytes. Moreover, in human plasma samples, we also found an effect of nutritional status on Fam3D levels, with a postprandial elevation and a reduction after fasting. In conclusion, Oit1 and Fam3D are gut-derived proteins that are expressed and secreted in a nutritional status-dependent manner.

Keywords: Oit1; Fam3D; Intestine; Gut; Nutritional status; Secretion

1. Introduction

The gut is an endocrine organ, secreting cytokines, hormones and other signaling molecules that can have local but also systemic effects. Gut hormones, especially, are known to play an essential role in many metabolism-related biological processes such as digestion and absorption of nutrients (e.g., secretin, somatostatin) [1], satiety (e.g., cholecystokinin, peptide YY) [2] and glucose homeostasis (e.g., incretins, gastrin inhibitory polypeptide and glucagon-like peptide 1) [3]. In a previous study, we performed secretome analysis on mucosal scrapings of C57BL/6J mice fed a low-fat (LF) and high-fat (HF) diet, to identify novel gut-secreted proteins that might play a role in metabolic homeostasis [4]. We identified the potentially secreted protein oncoprotein-induced transcript 1 (Oit1), which showed a dietary fat-induced up-regulation in the small intestine. The mouse Oit1 transcript (Gene ID 18300, http://www.ncbi.nlm.nih.gov/gene/) encodes for a protein of 223 amino acids (predicted molecular weight of approximately 25 kDa). Family with sequence similarity 3, member D (Fam3D) is the human ortholog of Oit1 (Gene ID 131177). The homology between the mouse and human transcript and protein is 77% and 72%, respectively. Fam3D was previously identified as a member of a cytokine-like family with a four-helix-bundle structure similarity [5]. The family exists of four genes: Fam3A, Fam3B, Fam3C and Fam3D. Fam3D shows 53%, 28% and 50% homology to Fam3A, Fam3B and Fam3C, respectively. Fam3B, also called Pander (pancreatic-derived factor), is the most extensively studied. Fam3B is specifically expressed in the islets of Langerhans of the endocrine pancreas. Fam3B has delayed effects on β-cell function, inhibiting basal insulin secretion in a dose-dependent manner by affecting cell viability [6]. Also, Fam3D has been linked to the pancreas, as Souza et al. [7] reported that Fam3D is associated with diabetes mellitus in pancreatic adenocarcinoma patients. However, the function of Fam3D in pancreatic cells or even in general is still unknown.

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Moreover, Zhu et al. [5] did not find expression of Fam3D in pancreas but showed messenger RNA (mRNA) expression of Fam3D only in placenta and small intestine.

In this study, we now further characterized Oit1/Fam3D on the mRNA as well as the protein level. We studied its tissue distribution in mice and human in more detail. We analyzed the protein expression in the small intestine and verified the potential secretion of Oit1. Furthermore, we examined the effects of nutritional status on Oit1/Fam3D expression and secretion in mice as well as in humans.

2. Materials and methods

2.1. Mouse intervention studies

The mouse intervention studies that we used to determine intestinal Oit1 expression were previously described [4,8,9]. In short, in the LF vs. HF diet intervention study, C57BL/6J mice were fed a purified 10 energy (En)% fat diet and 45 En% fat diet, respectively, for 2 weeks (six mice per diet group) [4]. Mice were killed in the postprandial state, and mucosal scrapings of the small intestine and colon were collected. Prior to scraping, the small intestine was divided into three equal parts (proximal, middle, distal). The mucosal scrapings were stored at −80°C until RNA isolation. In a similar parallel LF vs. HF diet intervention study (also six mice per diet group), the proximal, middle and distal small intestine and colon were embedded in paraffin for immunohistochemistry. In the acute study, wild-type (WT) SV129 mice and peroxisome proliferator-activated receptor α (Pparα) knockout (KO) mice with a similar genetic background received an oral gavage with 400 μl of the synthetic triglycerides triolein (18:1), trilinolein (18:2), trilinolenin (18:3), trieicosapentaenoin (20:5) or tridocosahexaenoin (22:6) or the control vehicle 0.5% carboxymethylcellulose (five mice per treatment group) [8]. Prior to the gavage, all mice were fed a modified AIN76A diet containing 5% wt/wt corn oil (~10 En%) for 2 weeks. Six hours after the gavage, the mice were killed. The small intestines were excised, flushed with ice-cold phosphate-buffered saline (PBS) and stored at −80°C until RNA isolation. The small intestines in total were analyzed for gene expression. In the fasting study, WT SV129 mice and Pparα KO mice with a similar genetic background were fasted for 24 h (three mice per treatment group) [9]. Before and after this fasting period, the mice were killed. Small intestines were excised, flushed with ice-cold PBS and stored at −80°C until RNA isolation. The small intestines in total were analyzed for gene expression. For all experiments, the institutional and national guidelines for the care and use of animals were followed, and all experiments were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University.

2.2. Human intervention studies

Ten healthy nonsmoking men—without a history of any gastrointestinal disorders or complaints—aged between 18 and 65 years were included in the HF vs. LF diet intervention study. The study had a randomized crossover design. The subjects were randomly divided into two groups. During the first 3-week period, one group consumed an HF diet and the other group consumed an LF diet. After a washout period, during which the participants consumed their habitual diet, subjects crossed over to the other diet for another period of 3 weeks. The HF diet was aimed to contain 40 En% fat, 45 En% carbohydrates, 15 En% proteins and the LF diet 20 En% fat, 65% carbohydrates and 15% proteins. The diets were instructed by a dietician, and the participants had to buy the diets themselves as normal but changed some products to provide more or less fat than in their usual diets. To help the subjects, they had to score their fat and total energy intake daily by means of a scoring system in diaries. After 1 week of each intervention period, the diet was evaluated with the dietician by means of their daily food diaries, and if necessary, instructions to correct their intake were given. At the end of the study, the genuine fat intake was calculated based on the diaries of the participants; the HF diet contained 38 En% fat and the LF diet 21 En% fat. Although the study diets were aimed to be isocaloric, the mean energy intake was a little higher during the HF diet intervention period (~2400 vs. 2000 kcal/day). Following the 3-week HF or LF diet intervention period, the volunteers ingested an isocaloric HF (39 En% fat) or LF (20 En% fat) milkshake, respectively, for breakfast, after an overnight fast. Blood was sampled before consumption and 2 and 4 h after consumption of this milkshake. Blood was drawn in EDTA tubes (BD Vacutainer Systems; Becton Dickinson BV, Breda, the Netherlands), which were immediately (within 1 h) centrifuged at 2000×*g* for 30 min at 4°C. Plasma was then aliquoted, snap frozen and stored at −80°C. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. The Medical Ethical Committee of Maastricht University approved the study, and all subjects gave written informed consent.

For studying the effect of fasting on Fam3D plasma levels, we used blood samples that were obtained from a human study that was previously described by Hammer et al. [10]. In short, blood was sampled from 10 healthy nonsmoking men at baseline, while subjects followed a normal diet but abstained from alcohol for 3 days (mean intake 2065 kcal/day) and after a 3-day period of complete starvation (0 kcal/day; only water was allowed). Baseline blood samples were preceded by 4 h of fasting. Similar to the human study described above, blood was drawn in EDTA tubes (BD Vacutainer Systems; Becton Dickinson BV) and centrifuged at 2000×*g* for 30 min at 4°C, and plasma was aliquoted, snap-frozen and stored at −80°C.

2.3. RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. The isolated RNA was further column purified using the SV total RNA isolation system (Promega, Leiden, the Netherlands). RNA concentration was measured on a NanoDrop ND-1000 UV–Vis spectrophotometer (Isogen, Maarssen, the Netherlands) and analyzed on a bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips according to the manufacturer's instructions.

2.4. Complementary DNA synthesis and real-time quantitative PCR

Single-stranded complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the reverse transcription system (Promega) following the supplier's protocol. cDNA was PCR amplified with Platinum Taq DNA polymerase (all reagents were from Invitrogen). Primer sequences used for real-time quantitative PCR (qPCR) of Oit1 are 5′-GCATAACCCCCAAGGACATCC-3′ and 5′- GGCAGCTCCACTGCTGATTT -3′ and, for Fam3D, 5′-CTGCCCAGCCAACTACTTTG-3′ and 5′-CTCCCGTGGTTCCATTCAC-3′. qPCRs were performed using SYBR green and a MyIQ thermal cycler (Bio-Rad laboratories BV, Veenendaal, the Netherlands). Mouse (5′-TGTCTTTGGAACTTTGTCTG-CAA-3′ and 5′-CAGACGCCACTGTCGCTTT-3′) and human (5′-TCTTTGGGACCTTGTCTG-CAA-'3 and 5′-CCCACCGTGTTCTTCGACAT-3′) cyclophilin A and mouse hypoxanthine–guanine phosphoribosyltransferase (5′-GTTAAGCAGTACAGCCCCAAA-3′ and 5′-AGGG-CATATCCAACAACAAACTT-3′) were used as housekeeping genes for normalization. The following thermal cycling conditions were used: 8 min at 94°C, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min. PCR reactions were all performed in duplicate. To analyze tissue distribution of mouse Oit1, a healthy female FVB mouse was killed in the postprandial state and RNA was isolated from distinct tissues. To determine tissue distribution of human Fam3D, the FirstChoice Human Total RNA Survey Panel (Applied Biosystems/Ambion, Austin, TX, USA) was used. This panel contains pools of total RNA (10 μg) of different human tissues. Each pool comprises of RNA from at least three tissue donors.

2.5. Microarray analysis

RNA was hybridized to mouse genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Detailed methods for the labeling and subsequent hybridizations to the arrays are described in the eukaryotic section in the GeneChip Expression Analysis Technical Manual Rev. 3 from Affymetrix, which is available upon request. Arrays were scanned on an Affymetrix GeneChip Scanner 3000. Data analysis was performed as described previously [8].

2.6. Cloning

mOit1-F-Kpn (5′-GGGGTACCCCGCCACCATGAGAGTGGCAGGT-3′) and mOit1-R-BamH I primers (5′- CGGGATCCCGTACATTACTTTCCGTGG-3′) were used to clone the coding sequence of Oit1 in frame into the pEGFP-N2 vector (BD BioSciences, Franklin Lakes, NJ, USA). For amplification of the Oit1 coding sequence, the Expand high-fidelity PCR system kit (Roche Diagnostics Nederland BV, Almere, the Netherlands) and the PCR T3Thermocycler (Biometra) were used. Oit1 PCR product was cloned into pEGFP-N2 with an optimal 1:2 ratio between vector and insert (total amount of DNA added was 100 ng). Next to the DNA, the ligation mix consisted of 1 μl T4 DNA ligase and 2 μl 10× ligation buffer (New England Biolabs, Ipswich, MA, USA). This mixture was incubated for 30 min at room temperature. Transformation was performed in competent *Escherichia coli* JM 109 cells by a 1-min heat shock at 42°C. Cells were plated on LB agar (1:1000 kanamycin) and incubated overnight at 37°C. The sequence of pOit1-EGFP-N2 plasmid was verified using the ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). After DNA amplification, plasmids were purified by using the miniprep DNA purification system (Promega), according to the manufacturer's protocol.

2.7. Transient transfection

HEK-293 cells were seeded into 6-well plates, and after 24 h, when cells reached 80% confluency, the cells were transiently transfected with 5 μg of plasmid DNA (pEGFP-N2 or pOit1-EGFP-N2) together with 200 μl $CaCl_2$ and 200 μl of 2× HEPES-buffered saline buffer. In addition, 1.5 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) was added to the cells. Oit1 expression was analyzed at 24 and 48 h after transfection. To determine the expression after 24 h, 15 h after transfection, the medium was removed, the cells were washed twice with PBS, and DMEM without FBS was added to the cells. To determine the expression after 48 h, the medium was changed 24 h after transfection. At the time point of harvesting, medium was removed and stored at −20°C. PBS was then added to the cells, the cells were scraped and centrifuged at 100×*g* for 5 min, and the cell pellet was stored also at −20°C.

For transfection experiment with brefeldin A (Sigma-Aldrich Corp., St. Louis, MO, USA), inhibiting the classical secretory pathway, 0.7 μl brefeldin A (10 μg/ml, dissolved in ethanol) was added to 700 μl of DMEM medium without FBS, 24 h after transfection. Control cells received 0.7 μl ethanol in 700 μl DMEM medium without FBS. After a 24-h incubation with this mixture, the medium and cells were collected and stored as mentioned above.

2.8. SDS–PAGE and Western blotting

Prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the cells were lysed with cell lysis buffer (50 mM Tris–HCl, pH 7.4, 0.1 M NaCl, 5 mM EDTA, 0.1% wt/vol SDS, 1% vol/vol NP40), and SDS–PAGE sample buffer (2 ml 100% glycerol, 1.2 ml 1M Tris–HCl, pH 6.8, 0.4 g SDS, 2 mg bromophenol blue, 0.25 ml β-mercaptoethanol, 5 ml MilliQ) was added. Recombinant mouse Oit1 (Podiceps BV., Houten, the Netherlands) was taken along as a positive control for antibody specificity. In addition, for analysis of Oit1 secretion, the medium of transfected cells was first incubated for 2 h with trichloroacetic acid (final concentration, 20%) and centrifuged at 16,000×*g* at 4°C to precipitate proteins. The precipitated proteins were then dissolved in the sample buffer. Mouse plasma samples were albumin depleted prior to SDS–PAGE, as previously described by Colantonio et al. [11]. Protein concentrations in tissue homogenates and cell lysates were determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific Inc., Rockland, IL, USA). Proteins were separated using 12% SDS–PAGE gels, and the PageRule prestained protein ladder (Fermentas GmbH, St. Leon-Rot, Germany) was used as a marker.

After separation by SDS–PAGE, proteins were electrotransferred onto a polyvinylidene fluoride (PVFD) membrane (Immobilon-P; Millipore Corp., Billerica, MA, USA). The membranes were then blocked using Tris-buffered saline (pH 7.5) with 0.1% Tween-20 and 5% dry milk (ELK) for 1 h at room temperature, and the membrane was incubated with monoclonal antibody against green fluorescent protein (GFP; Sigma-Aldrich Corp.) (1:3000 dilution) or polyclonal antibody against Oit1 (R&D Systems, Minneapolis, MN, USA) (1:2000 dilution), overnight at 4°C. This was followed by incubation with a horseradish peroxidase (HRP)-linked rabbit antimouse (Sigma-Aldrich Corp.) or donkey antigoat secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (1:2000 dilution) at room temperature for 2 h. The signals were detected with the enhanced chemiluminescence detection system (Amersham ECL, GE Healthcare, Little Chalfont, UK). To additionally check loading of equal amounts of protein, membranes were incubated with a polyclonal antibody against actin (Sigma-Aldrich Corp.) (1:1000 dilution). Goat antirabbit HRP (Sigma-Aldrich Corp.) was used as a secondary antibody. Oit1 protein expression was quantified using GNU Image Manipulation software (GIMP 2.6.11).

2.9. Protein deglycosylation

Forty micrograms of recombinant mouse Oit1 (Podiceps BV.) was subjected to deglycosylation using the Protein Deglycosylation Mix (P6039S) of New England Biolabs Inc. (Ipswich, MA, USA). Deglycosylation under denaturing reaction conditions was performed according to the manufacturer's protocol. Untreated and deglycosylated recombinant mouse Oit1 samples were then loaded on a 12% SDS–PAGE gel, which was stained with ORIOLE Fluorescent Gel Stain (Bio-Rad Laboratories, Veenendaal, the Netherlands) and visualized with UV light.

2.10. Fam3D analysis in human plasma samples

Fam3D concentrations were measured in human EDTA plasma samples by using the Ab-Match Assembly Human FAM3D kit in combination with the Ab-Match Universal Kit (MBL International, Woburn, MA, USA), according to the manufacturer's protocol.

2.11. Immunohistochemistry

Four-micrometer sections of paraffin-embedded parts of the intestine were mounted on Superfrost microscope slides. These sections were dewaxed in xylene and rehydrated in a series of graded alcohols. To block endogenous peroxidase activity, slides were incubated with 3% H_2O_2 for 20 min. Antigen retrieval was performed by placing the slides in citrate buffer (pH 6.0) and heat them in a microwave oven for 5 min at 700 W (without lid) and four times for 5 min at 500 W (with lid). After cooling down to room temperature, the sections were briefly washed with PBS. The sections were stained in a three-step procedure utilizing the following incubations: overnight incubation at 4°C with goat polyclonal antibodies against Oit1 (R&D Systems), diluted 1:2000 in PBS. Thereafter, the sections were incubated with a biotinylated donkey antigoat antibody for 30 min, followed by 45 min incubation with peroxidase-labeled avidin–biotin complex (Vector Laboratories). Between all incubations, sections were washed three times in PBS. 3-Amino-9-ethylcarbazole (Vector Laboratories) was used as substrate to visualize the bound antibodies. After counterstaining with Meyer's hematoxylin, sections were mounted with Imsol Mount (Klinipath, Zevenaar, the Netherlands).

2.12. Statistical analysis

Physiological data and qPCR results are reported as the mean ± the standard error of the mean (S.E.M.). The differences between the mean values were tested for statistical significance by a two-tailed Student's *t* test or a one-way analysis of variance (ANOVA) with a Bonferroni post hoc test. Human Fam3D plasma data were tested for statistical significance by general linear model-repeated measures (PASW Statistics 17.0 software; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Tissue distribution of Oit1 and Fam3D

To determine the tissue distribution of Oit1 in mouse, we analyzed its mRNA expression in various normal tissues of a female FVB mouse under normal feeding conditions (chow) (Fig. 1A). We found that Oit1 is mainly expressed in the gastrointestinal tract, with a descending gradient from the stomach to the jejunum and expression going up again toward the colon, where the highest expression is detected. A similar intestinal Oit1 expression pattern was found in male mice of other mouse strains, such as SV129 (data not shown) and C57BL/6J mice fed a purified LF diet (Fig. 2). This indicates that the expression of Oit1 along the longitudinal axis of the intestine is independent of gender and mouse stain. Also, in normal human tissues, we analyzed the tissue-restricted expression

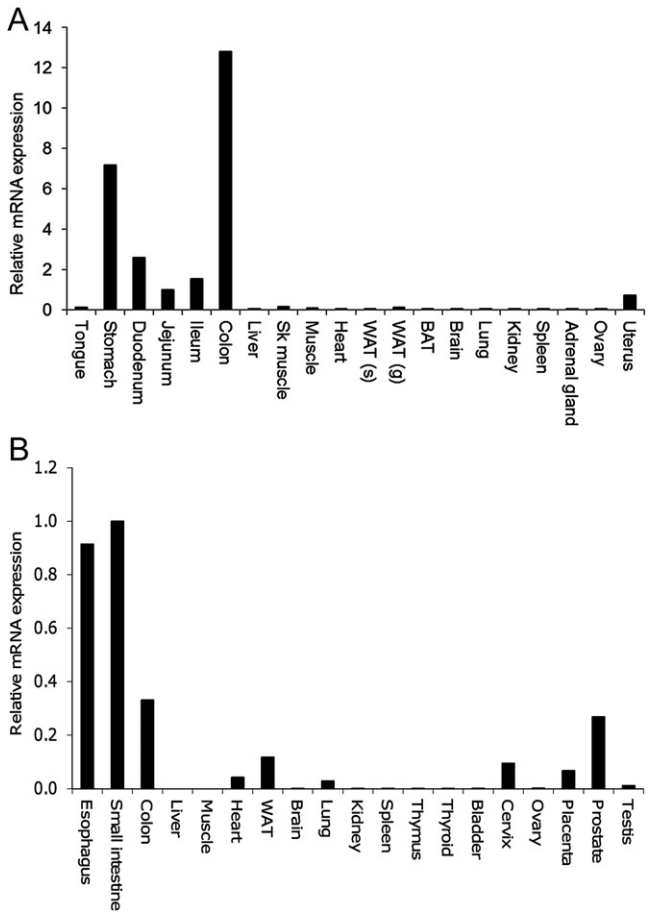

Fig. 1. Tissue distribution of mouse Oit1 and human Fam3D. Tissue distribution of mouse Oit1 (A) and human Fam3D (B) was analyzed by qPCR. mRNA expression of Oit1 and Fam3D in the various mouse and human tissues is visualized relative to jejunal Oit1 expression and intestinal Fam3D expression (set to 1), respectively. For normalization, cyclophilin A was used as a housekeeping gene in mouse as well as human tissue samples.

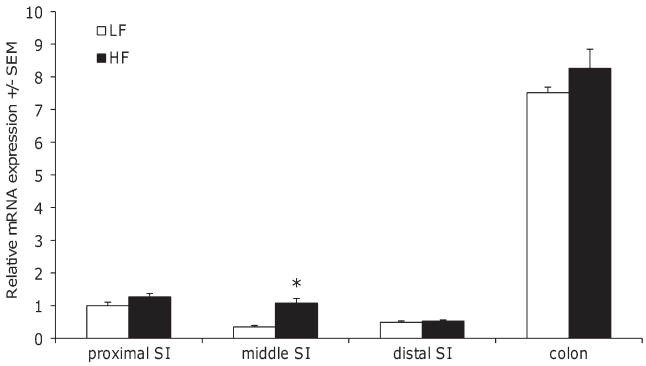

Fig. 2. Distribution of Oit1 along longitudinal axis of the intestine on LF and HF diets. After 2 weeks of LF and HF diet intervention, Oit1 mRNA expression was determined in the proximal, middle and distal parts of the small intestine and colon of C57BL/6J mice. qPCR data are visualized as the mean±S.E.M. expression of individual mice (n=6), relative to LF proximal SI, which was set to 1. Oit1 expression was normalized for housekeeping gene HGPRT. *Significant differential gene expression between the LF and HF diets (P<.05, two-tailed Student's t test).

of Fam3D. Fig. 1B also shows strong expression of Fam3D in the gastrointestinal tract, with the highest expression in the small intestine. In both mouse and human organs belonging to the reproductive system, we also found a low but substantial expression. These tissue-restricted expression patterns of Oit1 and Fam3D highly resemble expression data that are available in public databases such as BioGPS (http://biogps.gnf.org) and the Gene Expression Omnibus (GEO) database (GDS868, GDS3142).

3.2. Nutritional modulation of Oit1 mRNA expression

In a 2-week HF vs. LF diet intervention study [4], we determined the effect of dietary fat (mainly palm oil) on Oit1 expression along the longitudinal axis of the intestine of C57BL/6J mice (Fig. 2). We found a significant dietary fat-induced up-regulation of Oit1 in the middle part of the small intestine. Also, in the proximal small intestine and colon, there seemed to be an induction by dietary fat, but this difference was not significant. Next, we analyzed Oit1 expression in an acute study [8], in which the intestines of WT SV129 mice were isolated 6 h after an oral gavage with synthetic triglycerides (triolein, trilinolein, trilinolenin, trieicosapentaenoin or tridocosahexaenoin) (Fig. 3A). Unfortunately, saturated fats could not be included in this study due to their insolubility at room temperature. We found that all synthetic triglycerides already stimulated the expression of Oit1 after a 6-h exposure. These data indicate that the dietary fat-induced up-regulation of Oit1 is an acute effect, and there seems to be no differential effect among the different types of fat. In addition to postprandial effects, we also determined fasting-induced effects on intestinal Oit1 expression. Therefore, we analyzed the changes in Oit1 expression in a previously performed study reporting the effects of 24 h of fasting on gene expression in the small intestine of WT SV129 mice (GEO; accession number GSE6864) [9]. Fasting induced a 1.8-fold down-regulation of Oit1 (Fig. 3B).

Besides WT SV129 mice, also Pparα KO mice were included in the previously mentioned acute triglyceride study and the fasting study. In both mouse studies, we found similar effects on Oit1 expression in the intestines of WT and Pparα KO mice, indicating an up-regulated expression after a 6-h exposure of the intestine to synthetic triglycerides and a down-regulated Oit1 expression after 24 h of fasting (Fig. 3A and B). This indicates that the nutritional status-dependent regulation of Oit1 expression is not Pparα dependent.

3.3. Oit1 protein expression in the intestine

Based on its amino acid sequence, the size of the Oit1 protein is predicted to be ~25 kDa. We determined the protein expression of Oit1 in mouse small intestine and colon samples using anti-Oit1-specific antibodies (Fig. 4A). As a control, we also analyzed liver samples, as mRNA expression data (Fig. 1A) indicated that liver samples should have a very low Oit1 expression. Western blotting showed at least two bands of ~25 and ~33 kDa in all intestinal samples, of which the highest band was clearly most pronounced. Recombinant mouse Oit1 showed similar bands of ~25 and ~33 kDa, validating the specificity of the Oit1 antibody and the presence of at least two forms of Oit1 protein. The 33-kDa protein is probably the glycosylated form of Oit1, whereas the 25-kDa product is likely to be the native, nonglycosylated Oit1 protein. This was validated by performing a deglycosylation experiment in which the most common *N*- and *O*-linked glycans are removed from the Oit1 protein. Fig. 4C shows that deglycosylation of recombinant mouse Oit1 resulted in attenuation or even vanishing of the upper glycosylated Oit1 band(s) of ~30–33 kDa, whereas the lowest band of ~25 kDa, the native protein, became more apparent. The 25-kDa Oit1 protein could not be detected in the liver (Fig. 4A), but there seems to be low expression of the glycosylated Oit1 protein (~33 kDa). The 40-kDa band that is only visible in the liver probably represents cross-reactivity of the antibody with a non-Oit1-specific protein that is highly expressed

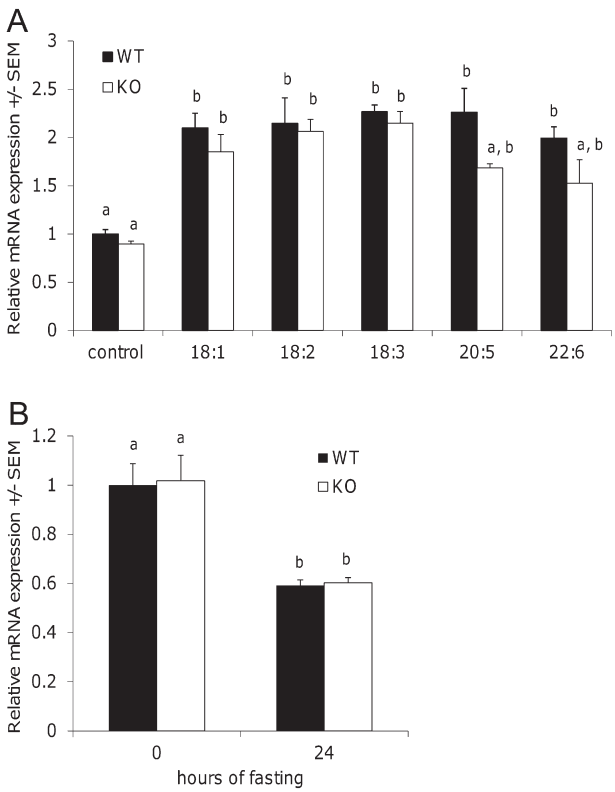

Fig. 3. Effects of nutritional status on intestinal Oit1 expression. Intestinal Oit1 expression in WT SV129 mice and Pparα null mice (KO). (A) Oit1 mRNA expression 6 h after an oral gavage with different synthetic triglycerides (triolein (18:1), trilinolein (18:2), trilinolenin (18:3), trieicosapentaenoin (20:5) and tridocosahexaenoin (22:6); control mice were gavaged with only vehicle (0.5% carboxylmethyl cellulose; n=5 per treatment group). (B) Oit1 mRNA expression before and after 24 h of fasting (n=3 per treatment group). Microarray results are visualized as the mean± S.E.M. expression of individual mice, relative to the WT control, which was set to 1. a and b indicate that bars with different letters are significantly different from each other (P<.05) (one-way ANOVA with Bonferroni correction).

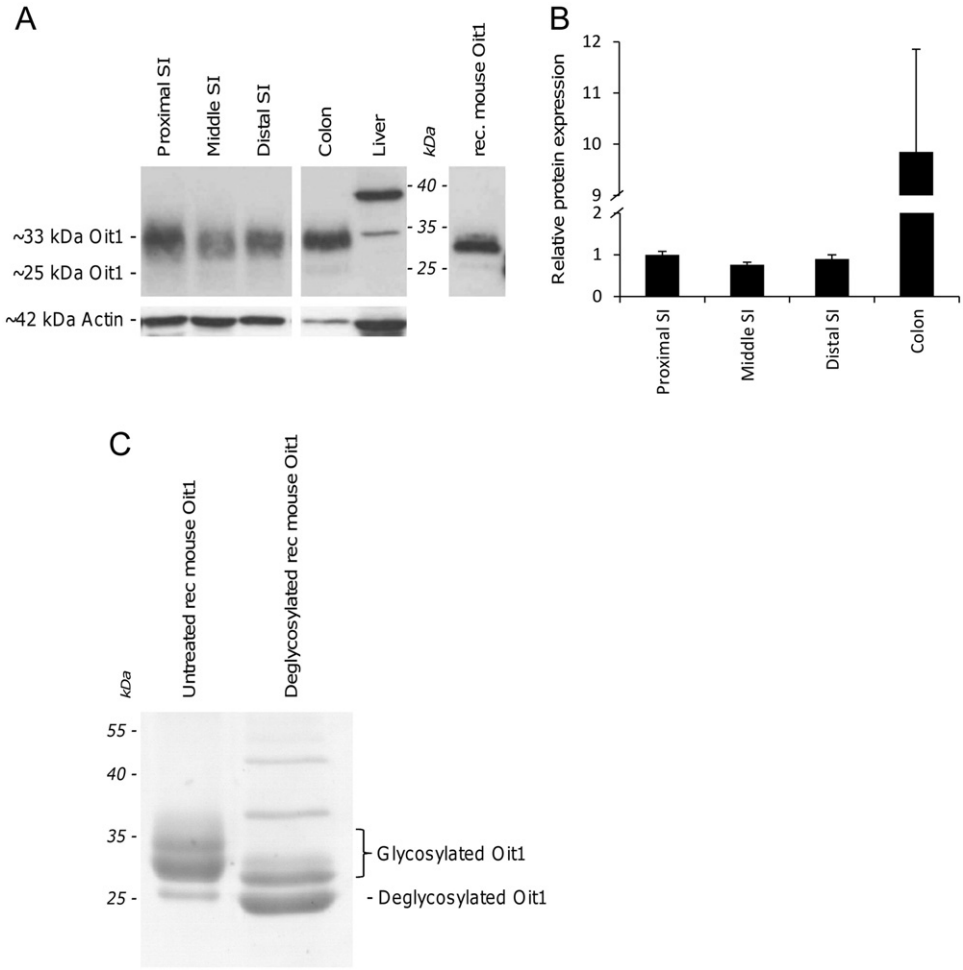

Fig. 4. Oit1 protein expression in the small intestine (SI), colon and liver. (A) Western blot analysis of Oit1 protein expression in the SI (divided in three parts of equal length), colon and liver of a C57Bl/6J mouse fed an LF diet for 2 weeks. Recombinant mouse Oit1 (50 ng) was taken along as a positive control for the anti-Oit1 antibody specificity. The protein input of SI samples and the liver was 20 μg, and for the colon, it was 1 μg. Actin was included as a loading control. (B) Oit1 protein expression was quantified for each part of the intestine in five mice fed an LF diet for 2 weeks. The results are visualized as the mean± S.E.M. Oit1 protein expression of individual mice, relative to the expression in the proximal SI, which was set to 1. (C) Deglycosylation of the Oit1 protein. Four micrograms of untreated and deglycosylated recombinant mouse Oit1 was loaded on a SDS–PAGE gel (ORIOLE staining). The protein bands in the deglycosylated sample of ~35 and ~40 kDa represent the deglycosylation enzymes used in this protocol.

in the liver but not in the intestine. To determine whether the Oit1 protein expression follows the Oit1 mRNA expression pattern that we found along the longitudinal axis of the intestine (Fig. 2), we quantified the total Oit1 protein expression (native+glycosylated forms) for each part of the intestine in five mice fed an LF diet (Fig. 4B). The results clearly showed that Oit1 mRNA and protein expression exhibit a highly similar pattern in the intestine, indicating a descending expression from proximal to middle small intestine with an increasing expression toward colon, where the highest expression was found. Furthermore, we explored differential protein expression in intestinal samples of mice fed an LF or HF diet. However, Western blot analysis was not sensitive enough to detect differences in Oit1 protein levels between these diet groups.

To localize protein expression of Oit1 in the intestine in more detail, we performed immunohistochemistry on the proximal, middle and distal parts of the small intestine and colon (Fig. 5A–D). In the proximal small intestine, we found that Oit1 mainly localized in the enterocytes of the villi, whereas in the middle and distal parts of the small intestine, Oit1 displays a higher expression in the crypt cells. The colonic expression of Oit1 seems to be equally distributed throughout the surface and crypt-epithelium. Interestingly, cellular Oit1 protein expression seems to be restricted to the Golgi apparatus in all parts of the intestine. As mentioned before, transcriptome analysis showed that an HF diet increases Oit1 mRNA expression, especially in the middle part of the small intestine. Staining of Oit1 protein now showed that this dietary fat-induced stimulation of Oit1 expression is mainly present in the villi of the middle small intestine (Fig. 5E–F).

3.4. Secretion of Oit1/Fam3D

Prediction programs, such as SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), indicated that Oit1 and Fam3D contain a signal peptide sequence, which suggests extracellular secretion. To validate secretion, we transiently transfected HEK293 cells with Oit1 linked to GFP (Oit1–GFP) at the C-terminal site. In addition, we transfected cells with the GFP protein alone, to control for the effect of GFP on cellular localization. The HEK293 cells transfected with Oit1–GFP showed a specific cellular localization of the fusion protein, which seems to be restricted to the Golgi apparatus. Cells transfected with GFP alone showed an equal distribution of fluorescent protein all over the cell (Fig. 6). Next, the medium and cell lysates of the transfected HEK293 cells were analyzed for GFP and Oit1–GFP expression. Fig. 7A shows that Oit1–GFP levels

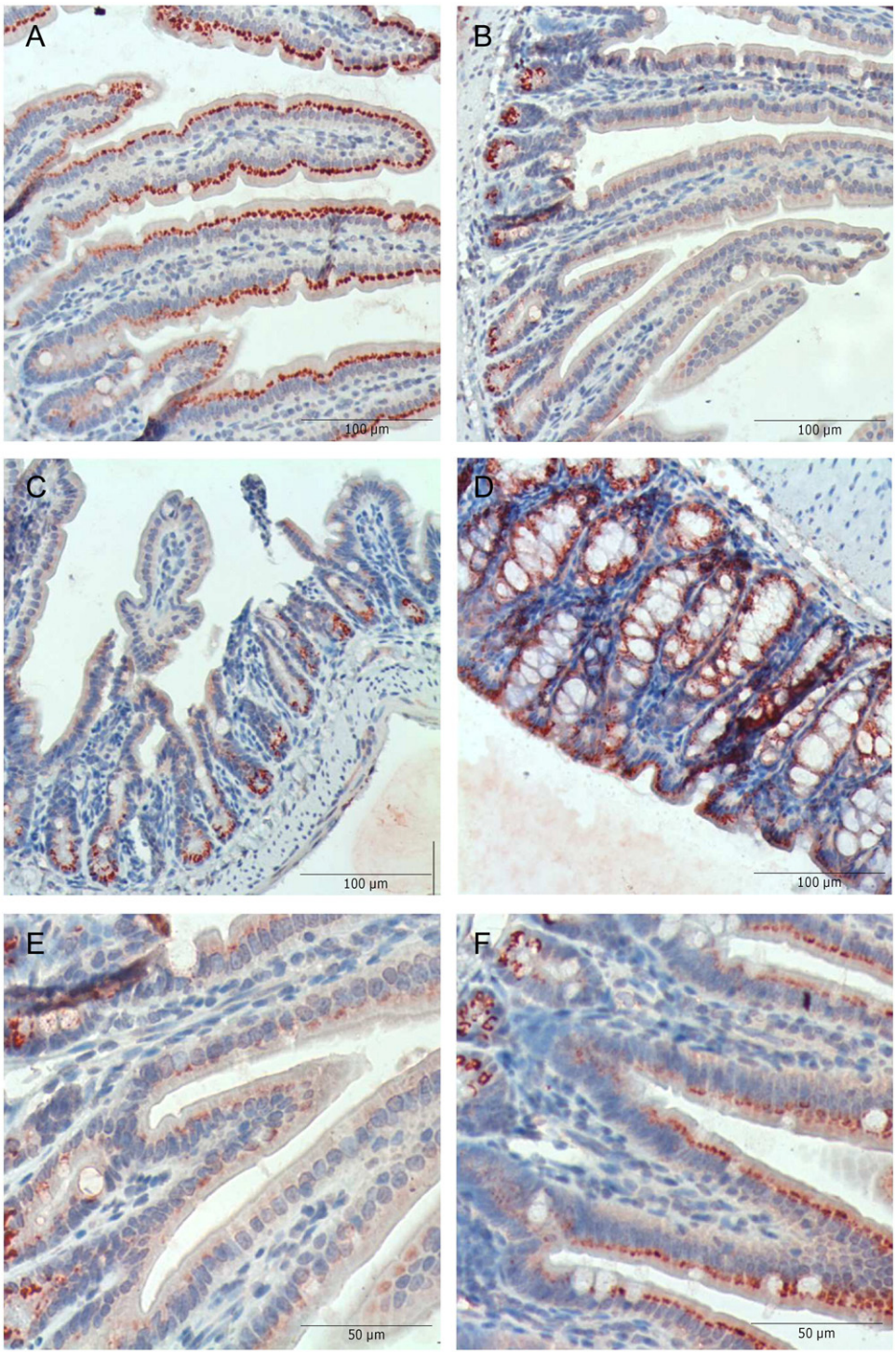

Fig. 5. Intestinal localization of Oit1 protein. Immunohistochemistry was performed on paraffin slides (5 µm) using anti-Oit1-specific antibodies. Red staining shows Oit1 protein expression under basal conditions (LF diet) in the proximal small intestine (A), middle small intestine (B), distal small intestine (C) and colon (D) (×200 magnification). (E–F) Oit1 protein expression in the middle part of the small intestine, after 2 weeks of LF diet (E) and HF diet (F) intervention (×400 magnification).

are much higher in the medium than in the cell lysates. For GFP alone, expression could be detected in the cells as well as in the medium. These secretion data for Oit1–GFP and GFP alone were verified by results that were obtained in stable transfected HEK293 cells (data not shown). To verify that the secretion of Oit1–GFP is following the signal peptide-dependent classical route via the Golgi apparatus, brefeldin A was added to transiently transfected HEK293 cells. Brefeldin A inhibits transport of proteins from the endoplasmatic reticulum to the Golgi apparatus, thereby blocking Golgi-dependent classical secretion. Fig. 7B shows that brefeldin A indeed blocked the secretion of Oit1–GFP, whereas GFP alone could still be detected in the medium. This indicates that Oit1–GFP is secreted via the signal peptide-dependent classical route, whereas GFP alone is "leaking" out of the cells.

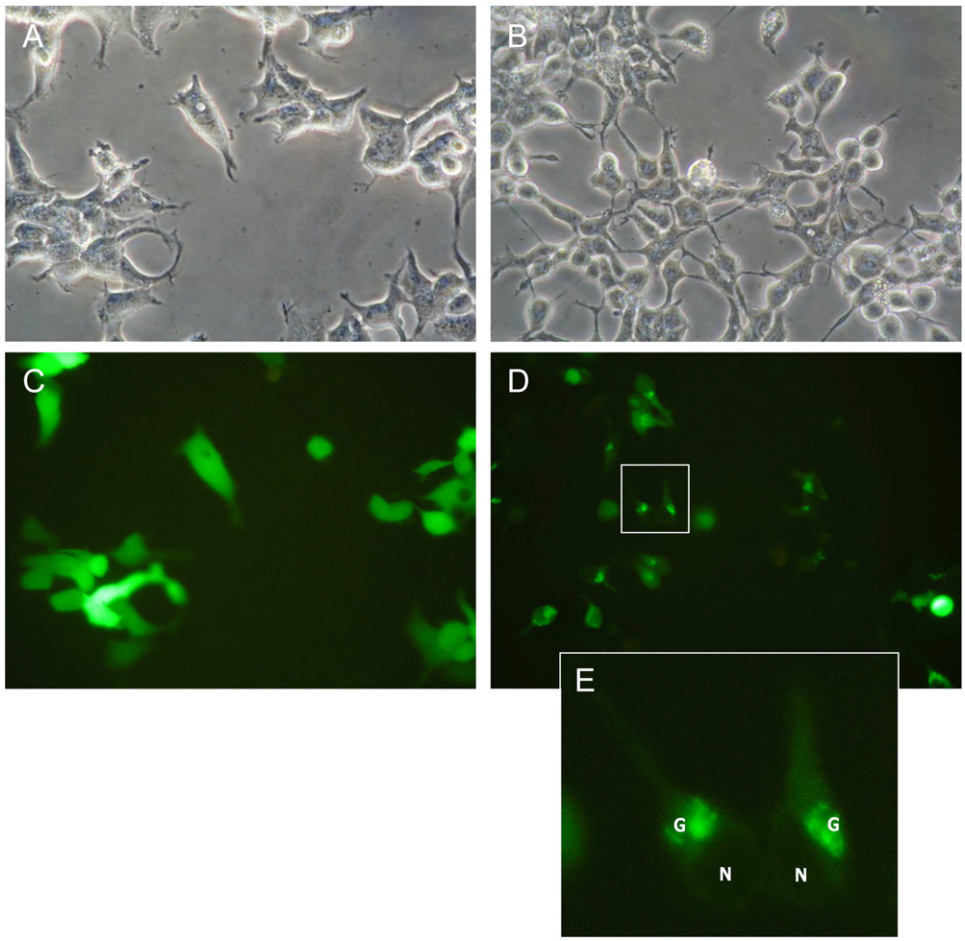

Fig. 6. Cellular localization of GFP and Oit1–GFP after transient transfection of HEK293 cells. Bright field microscopy (A, B) and fluorescence microscopy images (C–E) of HEK293 cells transiently transfected with the empty GFP vector (A, C) or the GFP vector containing Oit1 (B, D). Forty-eight hours after transient transfection, the expression of GFP alone (C) or Oit1–GFP (D) was determined (×400 magnification). (E) A higher digital magnification of the marked area in panel D is showing two cells with Oit1–GFP localized in the Golgi apparatus of HEK293 cells. N indicates nucleus; G, Golgi apparatus.

The transfection studies clearly showed that Oit1 is a secreted protein. However, in the intestine, proteins can be secreted to the luminal and/or basolateral site (into the blood stream). Johansson et al. [12] previously detected Oit1 in the intestinal mucus layer, indicating luminal secretion. In our study, Western blot analysis revealed the presence of the 33-kDa Oit1 protein in mouse plasma (data not shown). Unfortunately, we were not able to accurately quantify Oit1 content in mouse plasma, as it could only be detected after albumin depletion. In humans, we found basal Fam3D plasma levels after an overnight fast ranging from 0.2 to 18.0 ng/ml, with an average of 3.84

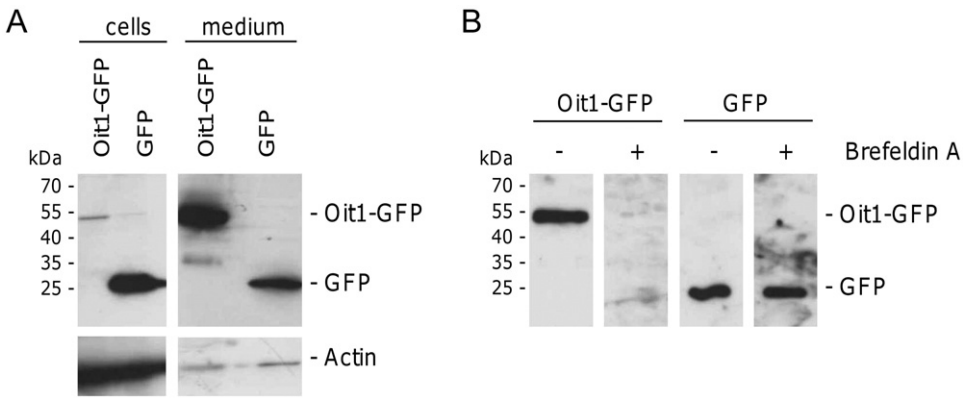

Fig. 7. Extracellular secretion of Oit1 in transient transfected HEK293 cells. Oit1–GFP and GFP protein levels were determined by Western blot analysis in HEK293 cells and medium, 48 h after transient transfection (A). Transient transfected HEK293 cells were additionally treated with brefeldin A, to study secretion of Oit1–GFP and GFP protein to the medium via the classical secretion route (B). Anti-GFP-specific antibodies were used to visualize the overexpressed proteins.

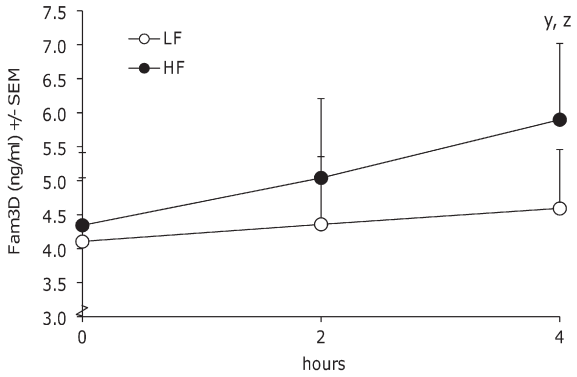

Fig. 8. Postprandial human Fam3D plasma levels after consumption of an HF or an LF diet/shake. Human Fam3D plasma levels were determined 0, 2 and 4 h after consumption of an HF or an LF shake, following a 3-week intervention on an HF or an LF diet, respectively. Plasma Fam3D levels are visualized as the mean± S.E.M. of individual subjects (n=10). y=time effect (P=.04), z=diet effect (P<.01) (GLM-repeated measures).

ng/ml (n=42). The presence in plasma indicates that Oit1 and Fam3D can be secreted via the basolateral site of enterocytes.

3.5. Effect of nutritional status on human Fam3D plasma levels

In our animal studies, we found that diet, especially dietary fat, up-regulated Oit1 expression, whereas fasting reduced mRNA expression in the small intestine. To determine whether changes in nutritional status could also affect human Fam3D levels, we analyzed plasma samples in postprandial conditions after HF and LF diets and after fasting. In a human diet intervention study, subjects consumed an HF (38.0 En% fat) or an LF (21 En% fat) diet for 3 weeks, followed by consumption of an HF or an LF shake, respectively. Fam3D levels were measured at baseline and 4 h after ingestion of the shake. We detected a postprandial elevation of Fam3D plasma levels, which was more pronounced after consumption of an HF diet/shake compared with an LF diet/shake (Fig. 8). Statistical analysis revealed a significant time (P=.04) as well as diet effect (P<.01). Three days of fasting led to a clear reduction of Fam3D levels from 3.76 (±0.9) to 1.77 (±0.4) ng/ml (P<.01). These data indicate that plasma levels of Fam3D are affected by nutritional status.

4. Discussion

In this study, we identified mouse Oit1 as a secreted protein with a predominant expression in the gastrointestinal tract. Also for human Fam3D, a profound expression in the gastrointestinal tract was found, although the distribution along the tract seems to be a little different from the mouse data (Fig. 1A and B). Especially, the expression ratio between the small intestine and the colon seemed to be different. Additional *in silico* analyses in mouse and human samples, using the GEO database (DataSet Record GDS182 and GDS3113) and BioGPS (http://biogps.gnf.org), showed, in general, a higher expression of Oit1 and Fam3D in colon compared with the small intestine. The discrepancy with our human tissue distribution analysis might be caused by the usage of different donors for each tissue, which makes it hard to quantitatively compare Fam3D expression levels between the different tissues.

Staining of intestinal sections for Oit1 revealed a striking villus/crypt pattern, as strong expression in the enterocytes of villi was found in the proximal small intestine and a more pronounced expression in the crypt cells in the middle and distal intestine. To our knowledge, such an expression pattern in the intestine has never been described for other proteins. Next, we determined the presence of Oit1 and Fam3D in plasma samples. This indicated basolateral secretion, whereas in a previous study, Oit1 was detected in the mucus layer of the colon [12]. Luminal secretion of Fam3D is also suggested by its previously described presence in saliva samples [13]. The differential secretion of Oit1 to the luminal or basolateral site of intestinal cells might be related to the differences in intestinal localization along the longitudinal axis. A potential underlying mechanism for basolateral or luminal secretion of Oit1/Fam3D might be differential glycosylation in the different regions/cells of the intestine. It is reported that plasma proteins and mucosal-secreted proteins can have a different glycosylation pattern (e.g., mucins are mainly *O*-glycosylated) [14]. In accordance with this hypothesis, we also found specific gene expression patterns along the longitudinal axis of the intestine of enzymes involved in *O*-glycosylation, such as UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (Galnts). For instance, Galnt4, Galnt7 and Galnt12 showed a higher expression in colon, whereas Galnt1, Galnt2 and Galnt6 were mainly expressed in the proximal small intestine (expression data can be found in GEO; accession number GSE8582). We speculate that region-specific expression patterns of glycosylation enzymes might be associated with region and/or cell type-specific glycosylation of Oit1/Fam3D and, thus, region and/or cell type-specific secretion. The characteristic localization of Oit1 along the longitudinal axis of the intestine and the differential secretion might indicate that Oit1 is a multifunctional protein with local as well as systemic effects. This will be investigated in future studies.

On the mRNA as well as the protein level, we found that Oit1/Fam3D expression is influenced by nutritional status. For gene expression, we found a postprandial up-regulation after intake of dietary fat and a down-regulation after fasting. Similar nutrition status-related results were found for human plasma Fam3D levels. It is very intriguing that dietary fat seems to induce an up-regulation of Oit1 protein expression in the villi, especially in the middle part of the small intestine (Fig. 5E–F). These data suggest that expression of Oit1 localized in villus cells is most likely related to postprandial changes in plasma levels.

The suggested dietary fat-dependent regulation of Oit1 expression that we found in our study might indicate that Oit1 is a Pparα target gene, as Pparα is known to be a transcription factor that is activated by fatty acids. However, analysis of dietary fat-induced and fasting-induced gene expression regulation in Pparα KO mice (Fig. 3) did not show a Pparα-dependent effect on Oit1 expression. Moreover, most Pparα target genes are regulated in the same direction by fasting as well as dietary fatty acids [8,9], which is not found for Oit1. Together, these data clearly indicate that Pparα is not a common regulator of Oit1 mRNA expression. Pparβ/δ and Pparγ are also expressed in the small intestine [4] and can also be activated by fatty acids. However, little is known about their diet- and/or fasting-related activation in the small intestine. These transcription factors might be candidate regulators of Oit1/Fam3D expression, although we found no potential binding sites (DR1) in the Oit1 and Fam3D promoter region by performing a NHR (nuclear hormone receptor) scan (www.cisreg.ca/NHR-scan/). Other nuclear receptors that can be activated on an HF diet and are thus potential regulators of Oit1 expression are liver X receptor (Lxr), farnesoid X receptor (Fxr) and pregnane X receptor (Pxr). Lxr, which is mainly activated by oxysterols, can be excluded as a regulator of Oit1 expression, as we found a similar dietary fat-induced up-regulation of Oit1 in WT and Lxr null mice (data not shown). Fxr and Pxr are known to be activated by bile acids. However, Fxr is not very likely to regulate Oit1 expression, as it is mainly present and active in ileum and not so much in the more proximal parts of the small intestine [15]. So far, the most plausible candidate for Oit1 transcription regulation is Pxr, especially because we found potential DNA binding sites (DR3/4 and/or ER6) for this nuclear

receptor in the promoter of both Oit1 and Fam3D by performing an NHR scan. This receptor is expressed throughout the intestine and is predominantly involved in detoxification mechanisms. In addition, based on the tissue- and intestinal region-specific gene expression of Oit1/Fam3D, it is likely that transcription factors with a more tissue-restricted expression pattern, such as Pdx1 and Cdx [16], are also involved in transcription regulation of Oit1/Fam3D.

In summary, mouse Oit1 and its human ortholog Fam3D are gastrointestinal-derived proteins that are secreted by enterocytes to the basolateral site. Moreover, Oit1/Fam3D shows a strong nutritional status-dependent expression on the mRNA as well as the protein level.

References

[1] Nicholl CG, Polak JM, Bloom SR. The hormonal regulation of food intake, digestion, and absorption. Annu Rev Nutr 1985;5:213–39.

[2] D'Alessio D. Intestinal hormones and regulation of satiety: the case for CCK, GLP-1, PYY, and Apo A-IV. J Parenter Enteral Nutr 2008;32:567–8.

[3] Drucker DJ. The role of gut hormones in glucose homeostasis. J Clin Invest 2007;117:24–32.

[4] de Wit N, Bosch-Vermeulen H, de Groot P, Hooiveld G, Grootte Bromhaar M, Jansen J, et al. The role of the small intestine in the development of dietary fat-induced obesity and insulin resistance in C57BL/6J mice. BMC Med Genomics 2008;1:14.

[5] Zhu Y, Xu G, Patel A, McLaughlin MM, Silverman C, Knecht K, Sweitzer S, Li X, McDonnell P, et al. Cloning, expression, and initial characterization of a novel cytokine-like gene family. Genomics 2002;80:144–50.

[6] Cao X, Yang J, Burkhardt BR, Gao Z, Wong RK, Greene SR, et al. Effects of overexpression of pancreatic derived factor (FAM3B) in isolated mouse islets and insulin-secreting betaTC3 cells. Am J Physiol Endocrinol Metab 2005;289: E543–50.

[7] Souza JJS, Machado MCC, Fortes MAHZ, Giorgi RR, Cunha JEM, Jukemura J, Nery M, Wajchenberg BL, Siqueira SAC, et al. Cytokine-like Fam3d gene is associated to diabetes mellitus in pancreatic adenocarcinoma. Pancreas 2006;33:498.

[8] de Vogel-van den Bosch H, Bunger M, de Groot P, Bosch-Vermeulen H, Hooiveld G, Muller M. PPARalpha-mediated effects of dietary lipids on intestinal barrier gene expression. BMC Genomics 2008;9:231.

[9] van den Bosch H, Bunger M, de Groot P, van der Meijde J, Hooiveld G, Muller M. Gene expression of transporters and phase I/II metabolic enzymes in murine small intestine during fasting. BMC Genomics 2007;8:267.

[10] Hammer S, van der Meer RW, Lamb HJ, Schar M, de Roos A, Smit JWA, et al. Progressive caloric restriction induces dose-dependent changes in myocardial triglyceride content and diastolic function in healthy men. J Clin Endocrinol Metab 2008;93:497–503.

[11] Colantonio DA, Dunkinson C, Bovenkamp DE, Van Eyk JE. Effective removal of albumin from serum. Proteomics 2005;5:3831–5.

[12] Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci USA 2008;105:15064–9.

[13] Ramachandran P, Boontheung P, Xie Y, Sondej M, Wong DT, Loo JA. Identification of N-linked glycoproteins in human saliva by glycoprotein capture and mass spectrometry. J Proteome Res 2006;5:1493–503.

[14] Spiro RG. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology 2002;12:43R–56R.

[15] Houten SM, Volle DH, Cummins CL, Mangelsdorf DJ, Auwerx J. In vivo imaging of farnesoid x receptor activity reveals the ileum as the primary bile acid signaling tissue. Mol Endocrinol 2007;21:1312–23.

[16] Walters JR, Howard A, Rumble HE, Prathalingam SR, Shaw-Smith CJ, Legon S. Differences in expression of homeobox transcription factors in proximal and distal human small intestine. Gastroenterology 1997;113:472–7.