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Colonic proteome signature in immunoproteasome-deficient stressed mice and its relevance for irritable bowel syndrome

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ABSTRACT

A role for immunoproteasome in the regulation of intestinal permeability has been previously suggested both in mice during water avoidance stress (WAS) and in patients with irritable bowel syndrome (IBS). Here, we provide evidence that the ubiquitin-proteasome system (UPS) contributes to the pathophysiology of IBS. Indeed, we report that colonic proteome is altered in WAS mice and that $\beta 2i$ subunit deficiency modifies the proteome response that is associated with a limitation of colonic hyperpermeability. Interestingly, we show specific alterations of proteins involved in UPS, mitochondrial and energy metabolism. We also report changes in the pattern of colonic ubiquitome in diarrhea-predominant IBS patients (IBS-D), and particularly a reduced expression of ubiquitinated proteins involved in the NF- κ B inflammatory signaling pathway. All these data suggest that immunoproteasome targeting may represent a new therapeutic strategy for the treatment of IBS patients with increased intestinal permeability.

Keywords: Colonic proteome; Immunoproteasome; Irritable bowel syndrome; Water avoidance stress.

1. INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder that affects 5-20% of the general population in industrialized countries.^{1,2} It is characterized by chronic abdominal pain associated with diarrhea and/or constipation, bloating and urgency.³ Recent studies have shown that IBS-associated symptoms, such as visceral hypersensitivity, low-grade inflammation or pain, may be linked to disturbances in intestinal barrier function.⁴⁻⁹ Alterations were observed in the expression and distribution of intestinal tight junctions (TJs), apical protein structures that control paracellular permeability.¹⁰⁻¹² Indeed, IBS patients with diarrhea-predominant symptoms (IBS-D) exhibited an increased permeability both in colon¹³ and jejunum¹², and a reduced expression of several TJ proteins such as occludin, zonula occludens-1 (ZO-1) and claudin-1.^{11,12} Emerging evidence supports the concept of altered colonic ubiquitin-proteasome system (UPS) in IBS patients that could be responsible of intestinal barrier dysfunction. Interestingly, occludin has been reported as a functional target of the E3 ubiquitin-protein ligase Itch¹⁴ and we have previously shown that the low expression of occludin in the colonic mucosa of IBS patients was related to its higher degradation by UPS.¹¹

UPS is a major pathway of proteolysis in eukaryotic cells and may contribute to the regulation of many intestinal biological processes, such as inflammatory response, cell proliferation and apoptosis.^{10,15,16} Targeted proteins by UPS are polyubiquitinated and then processed by the constitutive 20S proteasome, which comprises three catalytic subunits named β 1, β 2, and β 5. In mammals, the exposure of cells to inflammatory cytokines such as IFN- γ or TNF- α leads to the formation of an immunoproteasome that can be distinguished from its constitutive homologue through assembly of the catalytic immunosubunits named LMP2 (β 1i),

MECL-1 ($\beta 2i$) and LMP7 ($\beta 5i$).¹⁷ It has been reported an increased expression of immunoproteasome subunits in intestinal epithelial cell lines stimulated by IFN- γ .^{15,16} *In vivo*, the immunosubunits $\beta 1i$ and $\beta 2i$ were strongly upregulated in colon tissues of IBS¹⁰ and Crohn's disease (CD) patients,¹⁵ but not in patients with ulcerative colitis. Proteasome inhibitors such as bortezomib, MG132 and PR-957 have been developed and have achieved success in preventing experimental colitis in mice.¹⁸⁻²⁰ Similar effects have been observed with mice deficient in subunit $\beta 5i$.¹⁹ More recently, we showed a link between proteasome and intestinal permeability in mice. In this latter study, both pharmacological immunoproteasome inhibition and $\beta 2i$ subunit deficiency limited colonic hyperpermeability and improved occludin expression.²¹

To better understand the underlying mechanisms, we thus aimed (i) to evaluate the colonic proteome in *wild-type* and $\beta 2i$ immunoproteasome subunit knock-out ($\beta 2i^{-/-}$) mice during water avoidance stress (WAS) and (ii) to investigate the colonic expression of 49 ubiquitinated proteins in IBS-D patients.

2. MATERIAL AND METHODS

2.1. Animals

Male C57Bl/6 knock-out mice for MECL1 gene (or β 2i proteasome subunit) were a kind gift from Professors Marcus Groettrup and Mickael Basler of Konstanz University (Germany) with the permission of Professor John Monaco from Cincinnati University (Ohio). The protocol was approved by CENOMEXA, the Ethics Committee of Normandy for Animal Experimentation (acceptance number: N/04-11-12/27/11-15). In addition, all experiments were performed in accordance with relevant guidelines and regulations (Official Journal of the European Community L 358, 18/12/1986) and followed ARRIVE guidelines.²²

2.2. Water avoidance stress

The water avoidance stress (WAS) is a murine model of chronic stress that induces several symptoms associated to IBS. In particular, WAS increased visceral sensibility, colonic motility, intestinal permeability, pro-inflammatory cytokine production and mast cell count.^{21,23-25} After one week of acclimatization at 23°C (12-hour light-dark cycle; dark phase: 8:00 PM - 8:00 AM), WAS model was performed during 1 hour per day during 10 consecutive days as previously described.²¹ After the last WAS procedure, mice were euthanized. Colonic samples were either taken, washed with ice-cold PBS and immediately frozen until further analysis or immediately used to evaluate paracellular permeability.

2.3. Intestinal permeability

Colonic permeability was evaluated by measuring paracellular flux of FITC-dextran (4 kDa) through colonic samples mounted in Ussing chambers as previously described.²⁶ Briefly, FITC-dextran (5 mg/ml) was placed in the mucosal side. After 3 hours at 37°C, medium from the serosal side was removed and stored at -80°C. The fluorescence level of FITC-dextran was measured in 96-well black plate with spectrometer Chameleon V (excitation at 485 nm, emission at 535 nm; Hidex, Turku, Finland).

2.4. qRT-PCR

Mucosal total RNA was extracted from samples as previously described.²⁷ After reverse transcription of 1.5 µg total RNA into cDNA by using 200 units of SuperScript™ II Reverse Transcriptase (LifeTechnologies, Cergy-Pontoise, France), qPCR was performed by SYBR™ Green technology on BioRad CFX96 real time PCR system (BioRad Laboratories, Marnes la Coquette, France) in duplicate for each sample. GAPDH was used as the endogenous reference gene. Specific primers are displayed in Table S-1.

2.5. Proteasome activities

Proteasome activities were evaluated on colonic mucosa homogenized in ice-cold lysis buffer containing 30 mM Tris-HCl (pH 7.2), 1 mM dithiothreitol, 1% Triton X-100 by spectrofluorimetry on microtiter plate fluorometer Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany) as previously described.¹⁰

2.6. Protein extraction for comparative proteome and ubiquitome analysis

Colonic mucosa were homogenized, as previously described,^{28,29} in ice-cold lysis buffer containing 7 M urea, 2 M thiourea, 4% (wt:vol) CHAPS, 50 mM dithiothreitol, 25 mM spermine tetrahydrochloride, 1X protease inhibitor cocktail (P2714; Sigma Aldrich) and 0.5% (vol:vol) IPG buffer with pH 3-10 non-linear (GE Healthcare). The protein content was determined by using 2D Quant kit (GE Healthcare).

2.7. Comparative proteome analysis

Total protein extract (40 µg for silver staining or 500 µg for CBB G-250 staining) was used to rehydrate Immobiline DryStrip gels (pH 3-10 NL 18 cm; GE Healthcare). Proteins were resolved in the first dimension by isoelectric focusing for a total of 50 000 V-h by using the Ettan IPGphor 3 (GE Healthcare). After equilibration of the strips, the second dimension was performed on 8-16% polyacrylamide gradient gels (20 cm x 18 cm x 1 mm) and was run on an Ettan Daltsix vertical system (GE Healthcare). The 2D gels were silver-stained (PlusOne Silver Staining kit; GE Healthcare) for image analysis or dyed following the CBB G-250 staining method for mass spectrometric analysis.²⁸

(i) 2D image Analysis

Silver stained gels were scanned with an ImageScanner II (GE Healthcare) previously calibrated by a greyscale marker (Kodak) and 2D images were captured with Labscan 6.00 software (GE Healthcare). Differential analysis was realized by using ImageMaster 2D Platinum v7.0 software (GE Healthcare) for spot detection, background subtraction, spot volume normalisation and differences in spot expression among the groups and/or each experiment. Each tissue sample was subjected to 2DE three times to minimize run-to-run variation, and each set of

three gels was compared by using ImageMaster to confirm the nonappearance of statistically differential spots within the set of gels. The most representative silver-staining gels (gel migration, spot definition, and spot number) of each sample (n=5 per group) was used to identify differential colonic protein expression. The expression level was determined by the relative volume of each spot in the gel and expressed as %volume, calculated as spot volume/ Σ volumes of all spots resolved in the gel. Variations in abundance, corresponding to normalized spot volume, were calculated as the ratio of average values of %volume for each group. Only spots with a %volume variation >1.4 were included in analysis. Statistical differences between the groups were determined using ANOVA (significance level P values < 0.05).

(ii) In-gel trypsin digestion and protein identification by LC-ESI-MS/MS

Protein spots of interest were manually excised from colloidal Coomassie blue-stained 2D gels and digested. Briefly, gel fragments were washed three times for 20 min in destaining solution containing 50% (v/v) methanol and 50 mM ammonium bicarbonate. The excised spots were then air-dried for 1 h, which was followed by in-gel digestion in 30 μ L of a digestion buffer containing 50 mM ammonium bicarbonate and 6 ng/ μ L sequencing grade bovine trypsin (Roche Diagnostics, Meylan, France). After overnight digestion, the aliquots were treated with 50 % (v/v) acetonitrile and 5 % (v/v) formic acid. Speed-vas-dried peptide extracts were resuspended in 10 μ L of 5 % (v/v) acetonitrile and 0.1 % (v/v) formic acid and then analyzed with a nanoflow liquid chromatograph LC1200 system coupled to a Q-TOF 6550 mass spectrometer equipped with a nanospray source and an HPLC-chip cube interface (Agilent Technologies). A 20-min linear gradient (3–75% acetonitrile in 0.1% formic acid), at a flow rate of 350 nL/min, was used to separate peptides on a polaris-HR-Chip C18 column (150 mm long \times 75 μ m inner diameter).

Full autoMS1 scans from 290 to 1700 m/z and autoMS2 from 59 to 1700 m/z were recorded. In every cycle, a maximum of 5 precursors sort by charge state (2+ preferred and single-charged ions excluded) were isolated and fragmented in the collision cell that was automatically adjusted depending on the m/z . Active exclusion of these precursors was enabled after 1 spectra within 0.13 min, and the absolute threshold for precursor selection was set to 1000 counts (relative threshold 0.001%). For protein identification, MS/MS peak lists were extracted and compared with the protein databases using the MASCOT Daemon version 2.4.0 (Matrix Science, London, UK) search engine. The MASCOT searches were performed in *Mus musculus* database as previously described.³⁰ Protein hits were automatically validated if they satisfied one of the following criteria: identification with at least two top ranking peptides (bold and red) each with a MASCOT score of more than 41 ($P < 0.001$), or at least two top ranking peptides (bold and red) each with a MASCOT score of more than 31 ($P < 0.01$). To evaluate false-positive rates, all the initial database searches were performed using the “decoy” option of MASCOT. Results were considered relevant if the false-positive rate never exceeded 1%. The mass spectrometry data were deposited in the ProteomeXchange Consortium via the PRIDE partner repository (<https://www.ebi.ac.uk/pride/archive/>) with the data set identifier PXD011199 and 10.6019/PXD011199.³¹

2.8. Patients

Fourteen patients, who met the Rome III criteria for IBS with predominant diarrhea and seen in the Department of Gastroenterology of the Rouen University Hospital, were included between December 2012 and July 2016 (10 women/4 men, aged 41.2 ± 11.2 years). IBS patients with the following criteria were excluded: (i) patients with IBS predominant constipation or alternating

constipation and diarrhea, (ii) blood dyscrasia, (iii) personal history of auto-immune disease, (iv) personal history of inflammatory bowel disease, (v) personal history of digestive cancer, or (vi) macroscopic (except for diverticulosis, hyperplastic polyp, and angiodysplasia) and histological abnormality of the colonic mucosa. Fourteen control patients (8 women/6 men, aged 55 ± 17.4 years) were subjects admitted in the Department of Gastroenterology of the Rouen University Hospital for a colonoscopy indicated within the framework of screening (anemia, personal or family history of polyp, family history of colorectal cancer, rectal bleeding, screening campaign). To be selected, control subjects had to fulfill all of the following criteria: (i) macroscopically and histological normal colonic mucosa (diverticulosis, hyperplastic polyp, and angiodysplasia were tolerated), (ii) lack of any digestive symptom, and (iii) lack of any organic or functional bowel disease. A complete colonoscopy was performed under general anesthesia after large bowel cleansing with 4L of a macrogol solution. Biopsies were taken in the descending colon in all cases. Two biopsies for each patient were analyzed by a pathologist in order to exclude any histological abnormality. Written informed consent was obtained from each patient. The protocol was conducted in accordance with the Declaration of Helsinki and European GCP for biomedical research.

2.9. Ubiquitome analysis

Expression of 49 ubiquitinated proteins was assessed using the commercially Proteome Profiler™ Human Ubiquitin Array kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Briefly, 300 µg of colonic mucosal proteins were loaded on membranes previously coated with specific antibodies directed against ubiquitinated proteins. After incubation of these membranes with specific buffers and wash steps, ubiquitinated proteins

were detected using ECL reagents (GE Healthcare). Each spot was quantified by densitometry using ImageScanner III and ImageQuant TL software (GE Healthcare). Only spots with a %Vol variation ratio greater than 1.5 were considered relevant.

2.10. Gene ontology-based analysis

The PANTHER classification system (version 10.1, <http://www.pantherdb.org>) was used to categorize the identified proteins according to their molecular function and biological process. To identify significantly enriched biological themes and functional groups in the list of identified proteins, functional annotation clustering analysis was performed using the DAVID Gene Ontology (GO) tool version 6.7³² as previously described³⁰ with minor modifications: the reference dataset were *Mus musculus* or *Homo sapiens*, and $P < 0.05$ for Benjamini-Hochberg correction was considered significant.

2.11. Western blot analysis

20 µg of total protein extracts were separated by 4-20% SDS-PAGE gels and electrotransferred onto nitrocellulose membrane (GE Healthcare) as previously described.²⁸ Membrane was blocked with 3% (wt:vol) BSA in TBS-T solution [10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2% (vol:vol) Tween 20] and then incubated with corresponding primary antibodies overnight at 4°C: mouse monoclonal antibodies anti-UCH-L3 (sc-100340, 1:2500); goat polyclonal antibodies anti-GDH1 (sc-160382, 1:1000) or anti-HSP60 (sc-1052, 1:3000); rabbit monoclonal antibodies anti-Ub (sc-9133, 1:1000) from Santa Cruz Biotechnology. Membrane was washed three times for 10 min with TBS-T and then incubated with appropriate horseradish peroxidase-coupled secondary antibodies [HRP-goat anti-mouse IgG (sc-2005, 1:5000; Santa Cruz Biotechnology), HRP- donkey anti-goat IgG (sc-2020, 1:5000; Santa Cruz Biotechnology),

HRP-swine anti-rabbit IgG (P0399, 1:5000; Dako)] for 1 h at room temperature. After 2 washes with TBS-T, immunocomplexes were revealed by using ECL detection reagents (GE Healthcare). Protein bands were quantified by densitometry with ImageScanner III and ImageQuant TL software (GE Healthcare). The blot was stripped and reblotted with a mouse monoclonal antibody anti β actin (A5441, 1:5000; Sigma Aldrich) also used as a loading control.

2.12. Detection of carbonylated Proteins

Carbonylated proteins were detected and analyzed following derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), using the OxyBlot kit (Millipore, Billerica, MA). Briefly, colonic proteins were incubated with DNPH for 15 min at room temperature to convert carbonyl to hydrazone derivatives. The samples were neutralized and applied on SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was performed with a primary antibody directed against dinitrophenylhydrazone.

2.13. Statistical analysis

Results were compared in GraphPad Prism 5.0 (GraphPad software Inc, San Diego, CA, USA) by two-way ANOVA (stress x $\beta 2i^{-/-}$) with Bonferroni post-tests. To data from patients, t-test or Mann-Whitney test were used as appropriate. For all tests, $P < 0.05$ was considered significant.

3. RESULTS

3.1. Immunoproteasome subunit beta2i deficiency prevents hyperpermeability and pro-inflammatory cytokine production in the colonic tissue of stressed mice

The WAS model increased fecal pellet output from day 1 to day 10 both in *wild-type* (wt) and $\beta 2i^{-/-}$ mice (Figure S-1A). However, $\beta 2i^{-/-}$ WAS mice showed lower fecal pellet output than wt WAS mice. In $\beta 2i^{-/-}$ mice, trypsin-like activity was not affected by WAS (Figure S-1B). Interestingly, colonic mRNA levels for TNF- α and IL-1 β were not altered in $\beta 2i^{-/-}$ WAS mice (Figure S-1C). Gut barrier function was assessed by paracellular permeability of FITC-dextran. WAS induced enhanced passage of FITC-dextran across the colonic mucosa of wt mice (Figure S-1D). Interestingly, FITC-dextran flux was markedly reduced in $\beta 2i^{-/-}$ WAS mice compared to wt WAS mice (Figure S-1D). These data confirm that WAS did not affect colonic permeability in $\beta 2i^{-/-}$ mice, as previously described.²¹

3.2. WAS and immunoproteasome subunit beta2i deficiency alter colonic proteome in mice

To evaluate colonic proteome and then identify altered biological processes in wt and $\beta 2i^{-/-}$ mice both in control and WAS conditions, we used a proteomic approach combining 2DE mapping and mass spectrometry identification. Image analysis detected approximately 2100 protein spots per 2D gel, out of which 75% were successfully matched throughout all gels. Representative 2D gels from both conditions are shown in Figure 1A. Fifty protein spots were differentially and significantly expressed between the four mice groups (at least ± 1.4 fold change; two-way ANOVA, $p < 0.05$). These protein spots were analyzed by using liquid chromatography coupled

with electrospray ionization MS/MS, and 36 spots (72%) were identified (Table 1). Overall, 33 non-redundant proteins were obtained. Indeed, two proteins, named MICOS complex subunit Mic60 (spots 2 and 4, Figure 1A) and protein disulfide-isomerase precursor (spots 14, 16 and 17; Figure 1A), were detected at multiple spot positions that could be due to protein isoforms, i.e. difference in maturation state, degradation and/or post-translational modifications. Among the identified proteins, 22 proteins were downregulated in the colonic mucosa of wt WAS mice compared with wt mice, while only 6 proteins were affected by WAS in $\beta 2i^{-/-}$ mice. Interestingly, among these 6 proteins, only 2 were downregulated. In addition, we also observed that 7 proteins were differentially expressed in $\beta 2i^{-/-}$ WAS mice compared to wt WAS mice. In this latter case, proteasome subunit alpha type-3 (PSMA3), proteasome subunit alpha type-5 (PSAM5) and ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCH-L3) were up-regulated in the colonic mucosa of $\beta 2i^{-/-}$ WAS mice (range from 2.20 to 3.80-fold), whereas proteasome subunit alpha type-4 (PSMA4), alpha-enolase 1 (ENO1), 60 kDa heat shock protein (HSP60) and glutamate dehydrogenase 1 (GDH1) showed a reduced expression (with fold changes from 1.48 to 1.86).

To validate the data from proteomics experiments, three proteins were randomly selected to verify the expression level via Western blot analysis (Figure 2A). The colonic expression levels of GDH1 and HSP60 were downregulated in $\beta 2i^{-/-}$ WAS mice compared to wt WAS mice, whereas UCH-L3 expression was increased, which is consistent with the results given by 2-DE analysis, and thus confirmed the proteomic findings.

3.3. WAS and immunoproteasome subunit beta2i deficiency modify expression of protein involved in mitochondrial and energy metabolism in the colon

Panther Gene Ontology (GO) analysis allowed us to elucidate the different functions and processes in which the 33 proteins identified are putatively involved. According to the molecular function analysis (Figure 1B), most of the proteins were related with catalytic activity (53.1%) and binding (18.8%). Concerning biological processes, the identified proteins were mainly involved in metabolic process (45.5%, Figure 1B). To expand the GO analysis, we next analyzed the proteomic data with DAVID functional annotation tool. DAVID uses GO and other data sources to cluster proteins based on the shared annotations to similarity clusters. DAVID analysis identified 3 significant annotation clusters (Table 2), of which the highest ranked cluster had an enrichment score (ES) of 4.89 and was represented by 3 terms relating to mitochondrion.

The mitochondrial cluster accounted for 33% of the identified proteins and included: ATP synthase subunit beta (Atp5b), Elongation factor G1 (EF-G1mt), Elongation factor G2 (EF-G2mt), Electron transfer flavoprotein subunit alpha (ETFa), GDH1, HSP60, Heat shock protein 75 (HSP75), Isovaleryl-CoA dehydrogenase (IVD), MICOS complex subunit (Mic60), 3-mercaptopyruvate sulfurtransferase (MST), Vacuolar protein sorting-associated protein 4B (Vsp4b). The expression levels of Atp5b, EF-G1mt, EF-G2mt, HSP75, IVD, Mic60, MST and Vsp4b were downregulated in the colonic mucosa of wt mice during WAS (Figure 3). It was also observed a decreased expression of pyruvate kinase (PKM), adenylosuccinate synthetase isozyme (Adss2) and adenosine kinase (AK), which are involved in energy metabolism (Figure 3). These data suggest a significant decrease of mitochondrial activity and energy metabolism in the colonic mucosa during WAS. Invalidation of $\beta 2i$ expression alone induced similar modifications of colonic proteome except for Vsp4b, Atp5b and AK (Table 1). Interestingly, Vsp4b, Atp5b and AK remained unaffected in $\beta 2i^{-/-}$ WAS mice compared with $\beta 2i^{-/-}$ control mice. In addition, in $\beta 2i^{-/-}$ mice, WAS altered alpha-enolase-1 (ENO1), which was

downregulated, and Uridine phosphorylase 1 (UPase 1; Table 1). By comparing the response of wt and $\beta 2i^{-/-}$ mice to WAS, we observed a reduction of ENO1, HSP60 and GDH1 expression in $\beta 2i^{-/-}$ WAS mice (Table 1 and Figure 3).

3.4. WAS and immunoproteasome subunit beta2i deficiency alter ubiquitin-proteasome system in the colon.

We also observed that proteasome subunits were significantly enriched among identified proteins. The proteasome cluster (ES = 2.06, Table 2) was made up from three proteasome alpha subunits (PSMA3/4/5) and 26S protease regulatory subunit S10B (PSMC6). The expression levels of PSCM6 and two others UPS related-proteins, namely pre-mRNA-processing factor (Prpf19) and protein deglycase (DJ-1), were decreased in the colonic mucosa of wt mice during WAS (Figure 4). Interestingly, $\beta 2i^{-/-}$ WAS mice exhibited an increased expression of PSMA3 and PSMA5 as compared to $\beta 2i^{-/-}$ control mice, and of PSMA3, PSMA5 and UCH-L3 when compared to wt WAS mice (Table 1 and Figure 4). By contrast, PSMA4 were downregulated while Prpf19 and DJ-1 remained unaffected in $\beta 2i^{-/-}$ WAS mice. UCH-L3 is a deubiquitinating enzyme (DUB) that controls levels of cellular ubiquitin through processing of ubiquitinated proteins. We thus evaluated the level of ubiquitinated proteins in the colonic mucosa and we observed that both wt and $\beta 2i^{-/-}$ control mice exhibited a reduction of ubiquitinated protein levels in response to WAS (Figure 2B). Interestingly, $\beta 2i^{-/-}$ WAS mice exhibited lower ubiquitinated protein level than wt WAS mice. All these data suggest that (i) WAS affected UPS and (ii) that subunit $\beta 2i$ deficiency may regulate the degradation of specific proteins.

3.5. Ubiquitination of inflammation-related proteins is modulated in the colonic mucosa of IBS-D patients

Because stressed mice exhibited UPS changes in their colonic mucosa, we investigated in IBS-D patients the putative changes of ubiquitination of colonic proteins. For this purpose, we used macroarrays directed against 49 ubiquitinated proteins. Among these 49 proteins, the expression of 7 ubiquitinated proteins was significantly decreased in the colonic mucosa of IBS-D patients compared with control patients (Figure 5): TNF- α -induced protein 3 (A20), Prostaglandin G/H synthase 2 (COX-2), Heat shock 70 kDa protein (HSP70), Heat shock protein HSP 90 (HSP90), Interferon regulatory factor 3 (IRF3), Nuclear factor erythroid 2-related factor 2 (Nrf2), TNF receptor-associated factor 6 (TRAF-6). Nevertheless, the total expression of ubiquitinated proteins was not significantly different between control and IBS-D patients (Figure S-2A). Interestingly, enrichment analysis of KEGG pathways in DAVID showed that A20, COX-2, IRF3 and TRAF6 were involved in the NF- κ B signaling pathway (39.7-fold enrichment, $p=0.031$). All these data suggest that ubiquitination of these proteins may participate in the pathophysiology of IBS-D, which is commonly described as a low grade inflammatory state, by regulating apoptosis and disrupting UPS mechanism.

4. DISCUSSION

In the present study, we provide evidence that the ubiquitin-proteasome system contributes to the pathophysiology of IBS. Indeed, we report that colonic proteome is altered in stressed mice and that $\beta 2i$ subunit deficiency modifies the proteome response that is associated with a limitation of colonic hyperpermeability. Interestingly, we show specific alterations of proteins involved in UPS, mitochondrial and energy metabolism. We also report changes in the pattern of colonic ubiquitome in IBS-D patients, and particularly a reduced expression of ubiquitinated proteins involved in the NF- κ B signaling pathway.

Previous studies clearly demonstrated that UPS may be involved in the regulation of inflammatory response³³ or gut barrier function¹⁰. We and others have reported the formation of an alternative proteasome called immunoproteasome in the colonic mucosa of IBS¹⁰ and CD^{15,34} patients. It was also shown that proteasome composition and activity were different between CD and IBS patients.¹⁰ Indeed, trypsin-like activity was increased in the colonic mucosa of IBS patients³⁵ but not in CD patients. By contrast, chymotrypsin-like activity remained unchanged in IBS patients whereas it was upregulated in CD patients. In the present study, we used a stress model (the water avoidance stress “WAS” model) able to reproduce certain aspects of IBS such as visceral hypersensibility or intestinal hyperpermeability. Indeed, stress contributes to the development and aggravation of IBS symptoms.³⁶ WAS mice exhibited an increase colonic permeability, trypsin-like activity and $\beta 2i/\beta 2$ subunit ratio,²¹ that was previously observed in IBS-D patients.¹⁰ In our study, we showed that $\beta 2i$ subunit deficiency increased expression of proteasome subunits (PSMA3/5) in stressed mice, whereas PSMA4 and ubiquitinated proteins was downregulated. The shift from constitutive to immunoproteasome could be responsible for

the decrease of ubiquitinated proteins after WAS since Seifert *et al.* showed that immunoproteasome activity is higher than constitutive proteasome activity.³⁷ Nevertheless, Nathan *et al.* have reported that immunoproteasomes and constitutive proteasomes degraded ubiquitinated proteins at similar rates following IFN γ -treatment.³⁸ Other processes like ubiquitination and deubiquitination may also be involved. UCH-L3, which was increased in $\beta 2i^{-/-}$ mice in the present study, is a deubiquitinating enzyme performing hydrolysis of the bond at the C terminus of the ubiquitin and trimming down the polyubiquitin chain into monomers. UCH-L3 knock-out mice led to marked accumulation of ubiquitinated proteins in the skeletal muscles.³⁹ However, the effect of UCH-L3 overexpression on ubiquitinated protein level remains unknown. Thus, the underlying mechanisms responsible of the ubiquitinated protein level changes need to be deeply explored. UCH-L3 also processed an ubiquitin-like substrate called NEDD8 and regulated smad1 ubiquitination in osteoblast differentiation.⁴⁰ Interestingly, Sano *et al.* have reported that UCH-L3 prevented mitochondrial oxidative stress-related apoptosis in photoreceptor cells.⁴¹ UCH-L3 may then affect mitochondrial structure and the levels of reactive oxygen species (ROS). By contrast, it was considered as negative regulators of apoptosis in cancer.⁴²⁻⁴⁴ More interestingly, Zhao *et al.* have recently underlined a relationship between UCH-L3 and inflammatory response. Indeed, overexpression of UCH-L3 inhibited the activity of cellular SCF $^{\beta-TrCP}$ ligase E3 through a limitation of cullin-1 neddylation.⁴⁵ SCF $^{\beta-TrCP}$ ligase E3 induces ubiquitination and degradation of phosphorylated substrates including I- κ B, the inhibitor of NF- κ B.⁴⁶

Previous papers reported that NF- κ B activity can also be limited by glutamine through the regulation of I- κ B ubiquitination^{29,47} and particularly by the regulation of cullin-1 neddylation.⁴⁸ Interestingly, in the present study, we showed that $\beta 2i$ subunit deficiency leads to

a reduced expression of GDH1 in the colonic mucosa of WAS mice. GDH1 is a metabolic enzyme mainly located in the mitochondria that catalyzes the reversible NAD (P)+-linked oxidative deamination of glutamate into α -ketoglutarate and ammonia. This reaction is the final step of glutaminolysis after the conversion of glutamine to glutamate by glutaminase. In inflammatory experimental conditions, glutamine supplementation improves gut barrier function⁴⁹⁻⁵², regulates UPS^{29,48,53} and restores colonic TJ protein expression.^{49,50,54} We have recently demonstrated that glutamine supplementation prevented colonic hyperpermeability and reduced proinflammatory cytokine production in WAS mice.²¹ Zhou *et al.* have recently reported that reduced expression of glutamine synthase in colon tissues of IBS-D patients may lead to decreased claudin-1 expression, and consequently increased intestinal permeability.⁵⁵ In the same way, we have shown *ex vivo* that glutamine supplementation increased claudin-1 expression in the colonic mucosa of IBS-D patients.⁵⁴ In a randomized controlled trial, Zhou *et al* recently showed the beneficial effect of glutamine supplementation on intestinal permeability and gastrointestinal symptoms in post-infectious IBS patients.⁵⁶ All these data are in accordance with previous studies showing that glutamine supplementation or deprivation is able to modify TJ protein expression and influence intestinal permeability.^{50,57} The mechanisms of action for glutamine remain not completely understood and have been previously discussed.^{58,59} In our model, we can thus speculate that immunoproteasome inhibition induces higher glutamine content and overexpression of UCH-L3 leading to limitation of inflammatory response and improved barrier function (Figure 6). The assessment of colonic mucosal glutamine content in $\beta 2i^{-/-}$ WAS mice should be thus further investigated.

To confirm the putative role of proteasome system in the pathophysiology of IBS, we have investigated in IBS-D patients the changes of ubiquitination rate of colonic proteins.

Interestingly, we observed alterations of ubiquitome, and particularly a reduced expression of 7 ubiquitinated proteins involved in the NF- κ B signaling pathway. Cleynen *et al.* previously reported that adherent-invasive *Escherichia coli* (AIEC) was able to regulate UPS leading to a decrease of NF- κ B negative regulator CYLD in intestinal epithelial cells.⁶⁰ In patients with IBS-D, Zhou *et al.* recently underlined that colonic hyperpermeability caused by TNF- α may be due to activation of NF- κ B expression and disassembly of claudin-1.⁵⁵ All of these data suggest potential relationship between UPS, glutamine content, inflammatory response and TJ protein expression in the pathophysiology of IBS. Further investigations should be done to deeply explore these mechanisms.

In conclusion, we provide evidence that (i) β 2i subunit deficiency modified colonic UPS function in stressed mice that was associated with an improvement of intestinal hyperpermeability, (ii) IBS-D patients exhibited altered colonic ubiquitome leading to altered NF- κ B pathway. Therefore, immunoproteasome targeting represents a new therapeutic strategy for the treatment of IBS-D patients with intestinal hyperpermeability.

5. ASSOCIATED CONTENT

Supporting Information

Figure S-1. Colonic permeability and cytokine production in stressed mice.

Figure S-2. Expression levels of ubiquitinated and carbonylated proteins in colonic mucosa of IBS-D patients.

Table S-1. Sequences of used primers for qPCR

6. ACKNOWLEDGMENTS

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7. AUTHOR CONTRIBUTIONS:

AG, IG, WB, PC performed experiments and analyses. AG and MC designed experiments. PDu performed colonoscopy and sampling. AG, DV, PDe, PDu and MC interpreted the data. AG and MC wrote the main manuscript text and prepared figures. All authors reviewed the manuscript.

8. DISCLOSURE STATEMENT:

The authors declare that they have no conflict of interest.

9. ABBREVIATIONS:

A20, TNF- α -induced protein 3; Adss2, Adenylosuccinate synthetase isozyme; AK, Adenosine kinase; Atp5b, ATP synthase subunit beta; CD, Crohn's disease; COX-2, Prostaglandin G/H synthase 2; EF-G1mt, Elongation factor G1; EF-G2mt, Elongation factor G2; ENOA, Alpha-enolase-1; ETFA, Electron transfer flavoprotein subunit alpha; GDH1, Glutamate dehydrogenase 1; GO, Panther Gene Ontology; HSP60, 60 kDa heat shock protein; HSP70, Heat shock 70 kDa protein; HSP75, Heat shock protein 75 kDa; HSP90, Heat shock protein 90 kDa; IBS, irritable bowel syndrome; IBS-D, diarrhea-predominant irritable bowel syndrome; IVD, Isovaleryl-CoA dehydrogenase; IRF3, Interferon regulatory factor 3; Mic60, MICOS complex subunit; MST, 3-mercaptopyruvate sulfurtransferase; Nrf2, Nuclear factor erythroid 2-related factor 2; NF- κ B, nuclear factor-kappa B; PKM, Pyruvate kinase; Prpf19, Pre-mRNA-processing factor; PSMA3/4/5, Proteasome alpha subunits; PSMC6, 26S Protease regulatory subunit S10B; TJs, tight junctions; TRAF-6, TNF receptor-associated factor 6; UCH-L3, Ubiquitin carboxyl-terminal hydrolase isozyme L3; UPase 1, Uridine phosphorylase 1; UPS, ubiquitine-proteasome system ; Vsp4b, Vacuolar protein sorting-associating protein 4B; WAS, water avoidance stress; wt, wild-type.

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Factors Modulating the Ubiquitin Proteasome System in Inflammatory Bowel Disease.

Gut **2014**, *63* (8), 1265–1274.

FIGURE LEGENDS:

Figure 1. Colonic proteome alterations in *wild-type* (wt) and $\beta 2i^{-/}$ mice during WAS

(A) Silver-stained 2-dimensional gel images representing colonic proteome from wt control (Ct), $\beta 2i^{-/}$ Ct, wt WAS and $\beta 2i^{-/}$ WAS mice. For isoelectric focusing, 40 μ g of total colonic proteins was separated onto pH 3-10 NL IPG strip (18 cm). SDS-PAGE was performed 4-16 % gel and proteins were revealed with silver staining. The molecular weight of each protein was determined by standard markers. Differentially expressed proteins (> 1.4 fold difference; circled spots with a number) were determined by statistical analysis (two-way ANOVA, $P < 0.05$). Protein spots identification is displayed in Table 1. (B) Pie chart depicting the functional classification of proteins differentially expressed between the four groups mice. Using the online resource PANTHER classification system, proteins of interest were categorized into the broad GO classes biological process and molecular function. For each category, the number of identified proteins and their percentage of the total number of proteins in the pie chart are indicated for each GO term.

Figure 2. Expression levels of GDH1, HSP60, UCH-L3 and ubiquitinated proteins in the colonic mucosa of $\beta 2i^{-/}$ WAS mice

Representative immunoblots and densitometric analysis of GDH-1 (A), HSP60 (A), UCH-L3 (A), ubiquitinated protein (B) and β -actin levels in the colonic mucosa of *wild-type* (wt) or $\beta 2i$ knock-out ($\beta 2i^{-/}$) mice subjected to WAS (1 h/day for 10 days) or not (Control; Ct). Values are means \pm sem (n = 5 per group). Open and closed bars represent control and WAS mice, respectively. Two-way ANOVA P values: (A. GDH1) $P(\text{WAS}) = 0.0363$, $P(\beta 2i^{-/}) = 0.0305$,

$p(\text{interaction}) = 0.1709$; (A. HSP60) $P(\text{WAS}) = 0.0013$, $P(\beta 2i^{-/-}) = 0.0388$, $P(\text{interaction}) = 0.0805$; (A. UCH-L3) $P(\text{WAS}) = 0.1797$, $P(\beta 2i^{-/-}) = 0.0019$, $P(\text{interaction}) = 0.9172$; (B) $P(\text{WAS}) < 0.0001$, $P(\beta 2i^{-/-}) = 0.0243$, $P(\text{interaction}) = 0.1393$. *, $P < 0.05$ vs. wt Ct; #, $P < 0.05$ vs. wt WAS; and \$, $P < 0.05$ vs. $\beta 2i^{-/-}$ Ct. GDH-1, Glutamate dehydrogenase 1; HSP60, 60 kDa heat shock protein; UCH-L3, Ubiquitin carboxyl-terminal hydrolase isozyme L3.

Figure 3. Schema of mitochondrial and energy metabolism alterations in the colonic mucosa WAS induces protein expression changes in the colon tissue of wt mice (in red) and $\beta 2i^{-/-}$ mice (in mauve). These changes are different from those induced by $\beta 2i$ subunit deficiency during WAS (in green). Proteins in red, mauve or green are downregulated in the colonic mucosa of mice, excepted for UPase 1: Adss2, Adenylosuccinate synthetase isozyme 2; AK, Adenosine kinase; Atp5b, ATP synthase subunit beta; EF-G1mt, Elongation factor G1; EF-G2mt, Elongation factor G2; ENO1, alpha-enolase 1; GDH1, Glutamate dehydrogenase 1 ; HSP60, 60 kDa heat shock protein; HSP75, Heat shock protein 75; IVD, isovaleryl-CoA dehydrogenase; Mic60, MICOS complex subunit; MST, 3-mercaptopyruvate sulfurtransferase; PKM, Pyruvate kinase; Vsp4b, Vacuolar protein sorting-associating protein 4B. These modifications suggest a significant decrease of mitochondrial activity and energy metabolism in the colonic mucosa of wt mice during WAS. Interestingly, these metabolic disturbances were minored in $\beta 2i^{-/-}$ WAS mice.

Figure 4. Schema of ubiquitin-proteasome system (UPS) alterations in the colonic mucosa WAS induces protein expression changes in the colon tissue of wt mice (in red) and $\beta 2i^{-/-}$ mice (in mauve). These changes are different from those induced by $\beta 2i$ subunit deficiency during

WAS (in green). The external and internal rings constitute the 20S proteasome. The lid and base constitute the 19S regulatory complex. In some cases, it can be replaced by the PA28 or 11S regulatory complex, constituted of a single ring of 7 subunits. Proteins in red, mauve or green are upregulated (plus sign, +) or downregulated (minus sign, -) in the colonic mucosa of mice: DJ-1, Protein deglycase; Prp19, Pre-mRNA-processing factor 19; PSMA3, Proteasome subunit alpha type-3; PSMA4, Proteasome subunit alpha type-4; PSMA5, Proteasome subunit alpha type-5; PSMC6, 26S protease regulatory subunit S10B; UCH-L3, Ubiquitin carboxyl-terminal hydrolase isozyme L3. These modifications suggest altered UPS function in the colonic mucosa of wt mice during WAS. It was also observed disturbances in UPS in $\beta 2i^{-/-}$ WAS mice. In this case, immunoproteasome subunit beta2i deficiency induces increased colonic expression of UCH-L3, PSMA3 and PSMA5 in stressed mice, while PSMA4 were downregulated. α , proteasome alpha subunit; β , proteasome beta subunit, DUBs, deubiquitinating enzymes; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; Ub, Ubiquitin.

Figure 5. Expression of ubiquitinated proteins in the colonic mucosa of IBS-D patients. The ubiquitinated forms (Ub) of A20, COX-2, HSP70, HSP90, IRF3, Nrf2 and TRAF6 are underexpressed in the colonic mucosa of IBS-D patients

Representative macroarray results (A) and densitometric analysis (B) of ubiquitinated protein expression in the colonic mucosa of IBS-D or control patients. 300 μ g of colonic mucosal proteins from IBS-D or control patients were incubated with a Human ubiquitin macroarray (n = 7-8 per group). A ratio of signal intensity "R" (IBS-D / control) was calculated, and log transformed (base2). Significant changes ($\text{Log}_2 R < -0.584$ or $\text{Log}_2 R > 0.584$; Mann-Whitney test, $P < 0.05$) are showed by black bars. A20, Tumor necrosis factor alpha-induced protein 3;

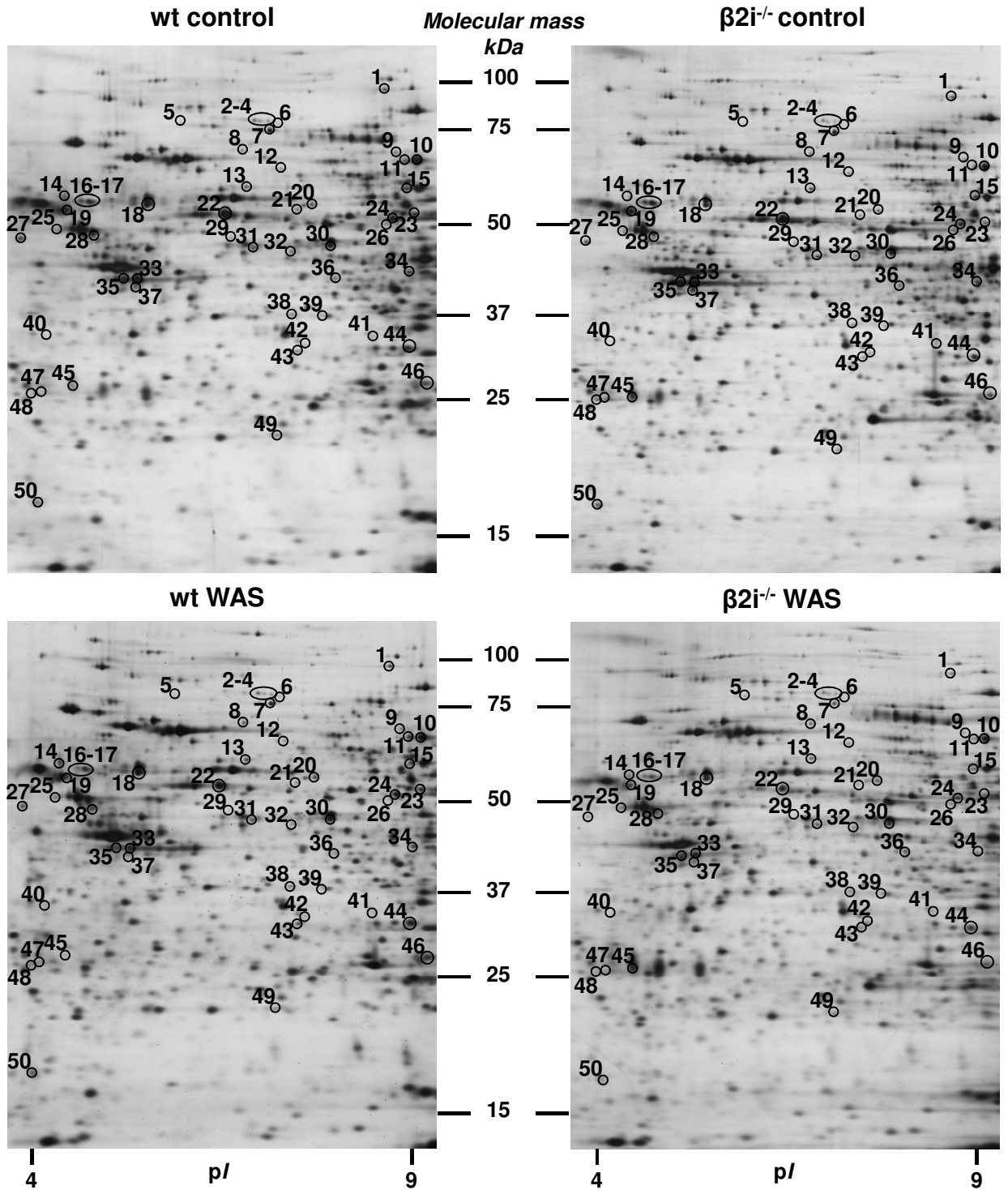
COX-2, Prostaglandin G/H synthase 2; HSP70, Heat shock 70 kDa protein; HSP90, Heat shock protein HSP 90; IRF3, Interferon regulatory factor 3; Nrf2, Nuclear factor erythroid 2-related factor 2; TRAF-6, TNF receptor-associated factor 6; Ub, Ubiquitin.

Figure 6. Speculative schema of interactions between UCH-L3, glutamine content and inflammatory response in the colonic mucosa of immunoproteasome-deficient stressed mice

In *wild-type* mice, stress induces the neddylation of cullin-1 that is necessary for activation of the ubiquitin ligase E3 complex, SCF- β -TrCP. Active SCF- β -TrCP induces ubiquitination of I- κ B, the inhibitor of NF- κ B, leading to the degradation of I- κ B, the translocation of NF- κ B in the nucleus and transcription of NF- κ B-dependent genes. In immunoproteasome deficient mice, the expression of deubiquitinase (UCH-L3) is increased, while the expression of glutamate dehydrogenase 1 (GDH1) is decreased. Underexpression of GDH1 may lead to higher glutamine content. Both UCH-L3 and glutamine are able to inhibit cullin-1 neddylation. Consequently, I- κ B is not degraded by proteasome and NF- κ B remains in the cytosol leading to the limitation of inflammatory response.

Figure 1.

A



B

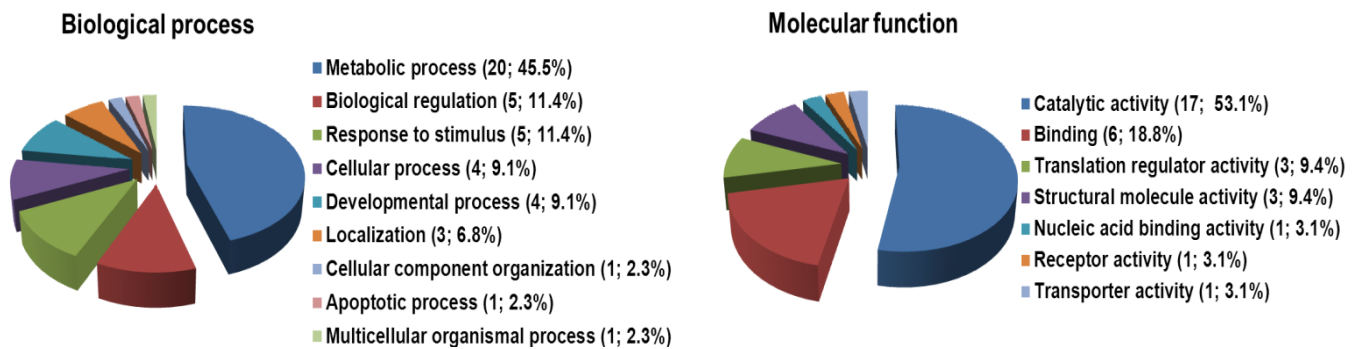
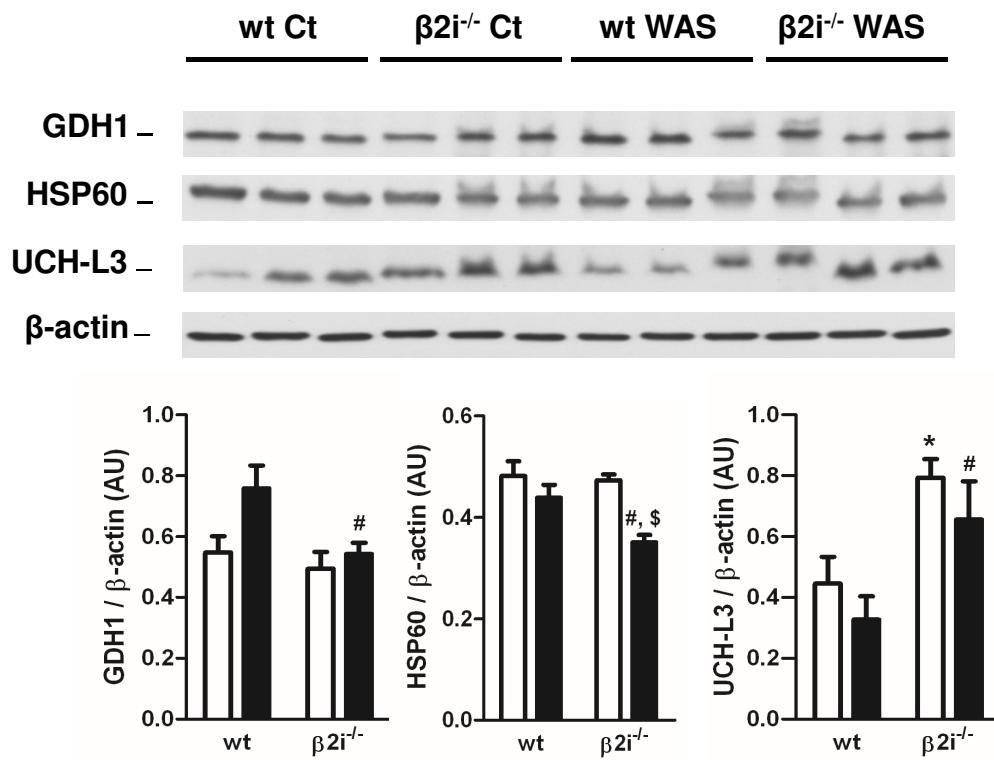


Figure 2.

A



B

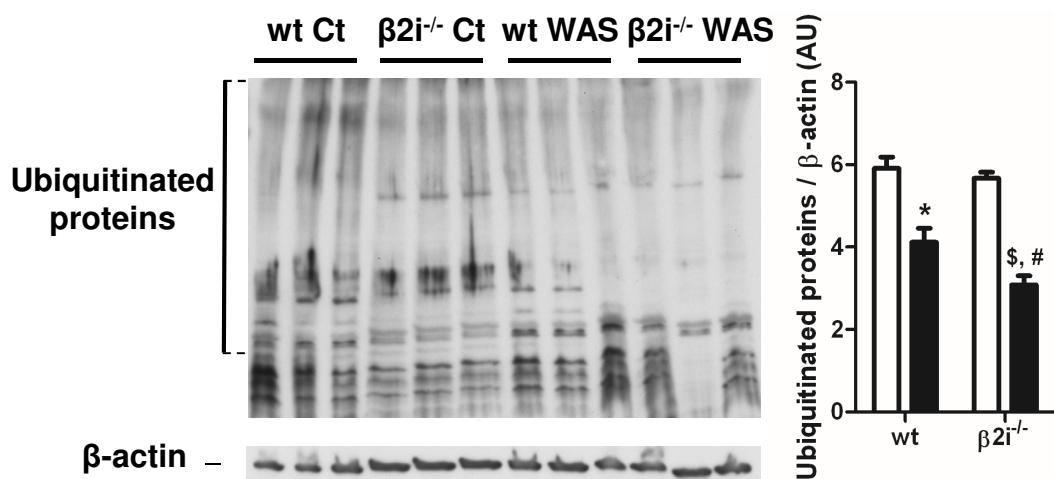


Figure 4.

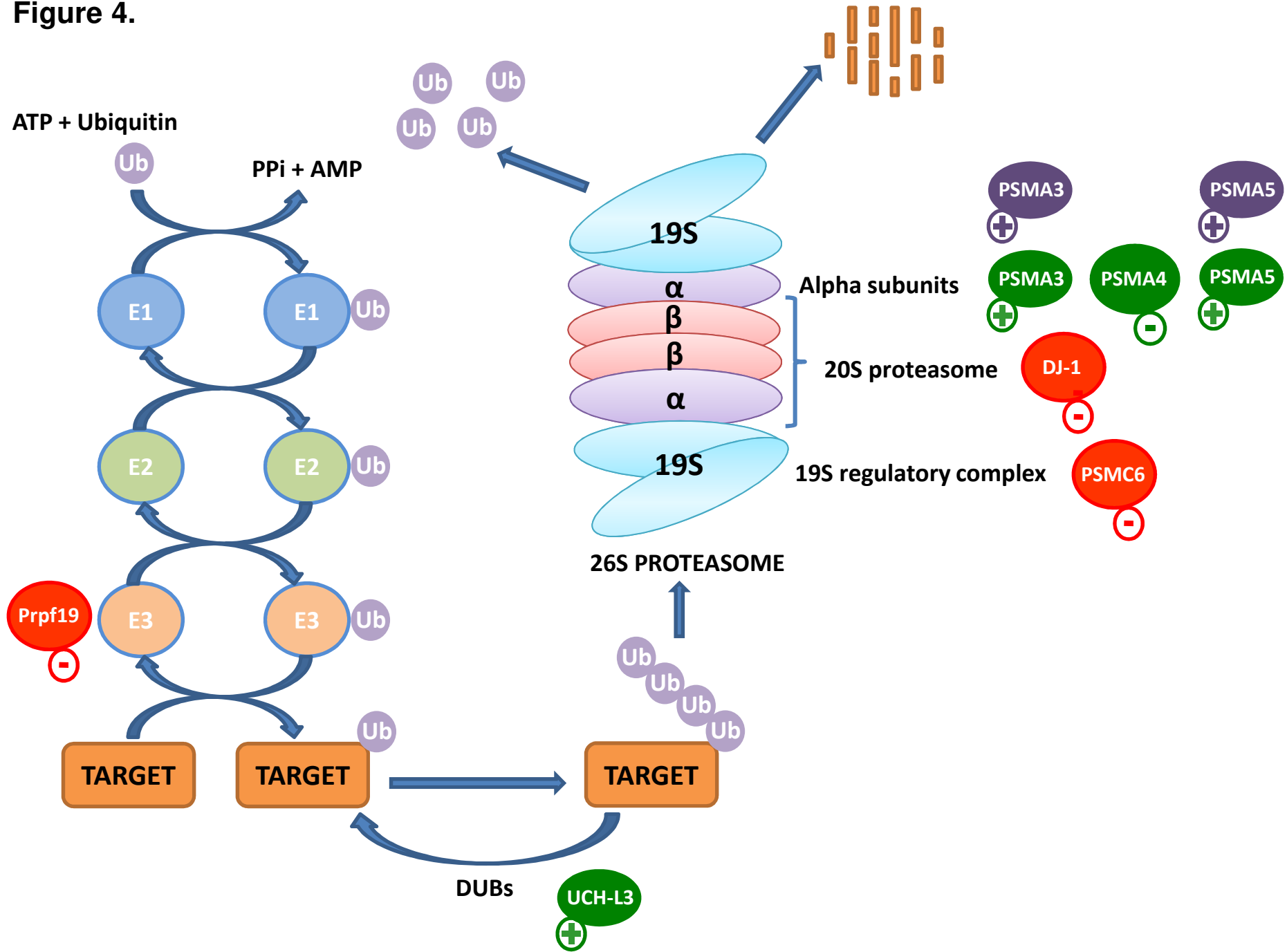
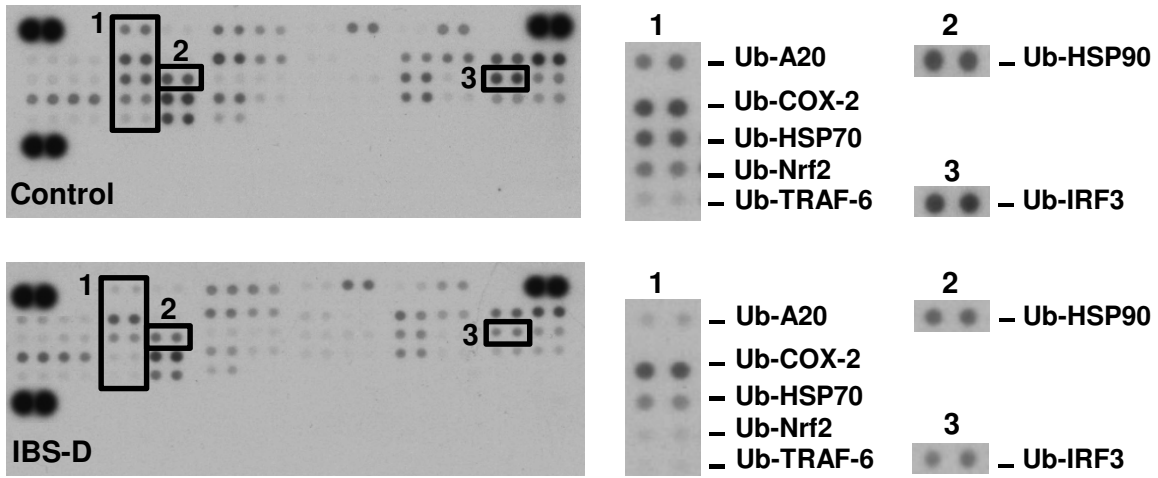


Figure 5.

A



B

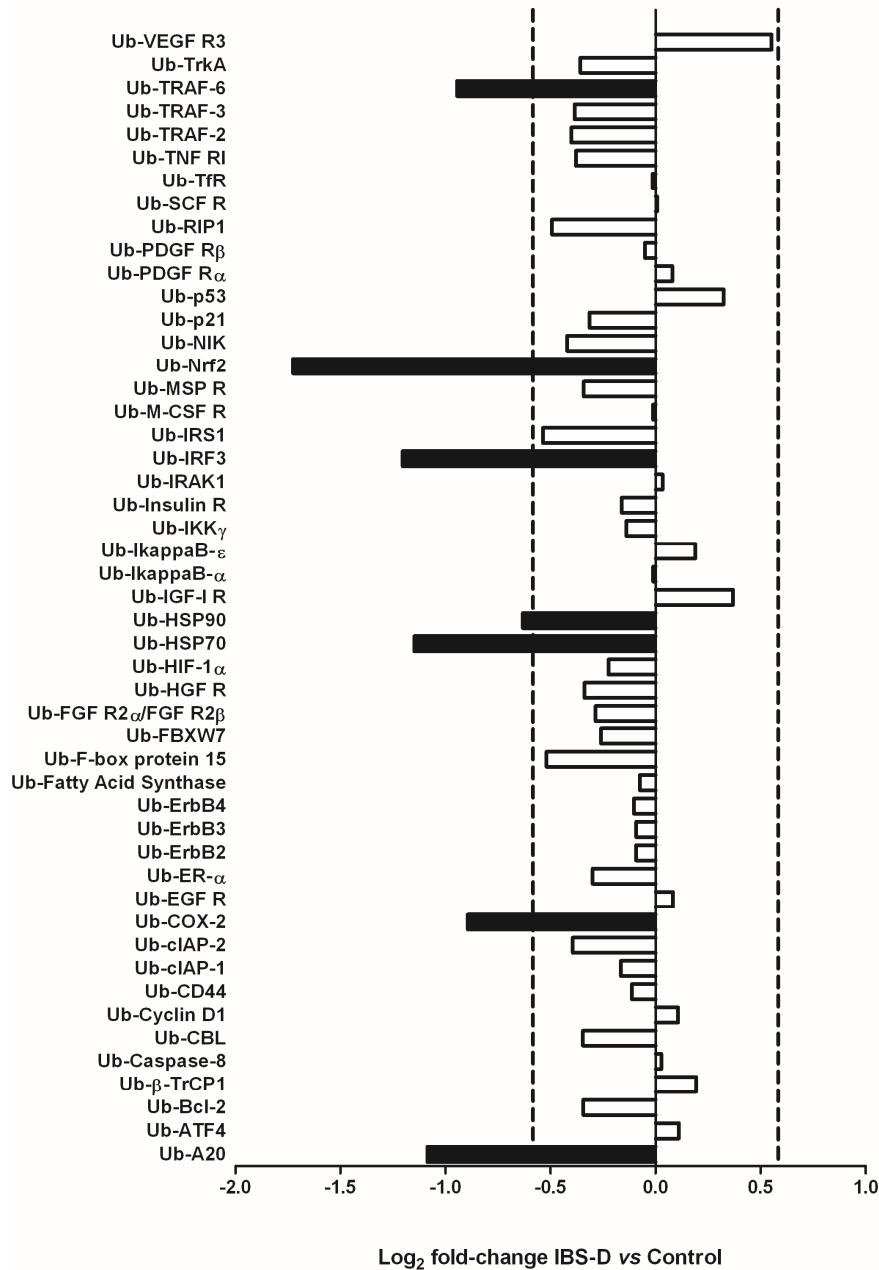


Figure 6.

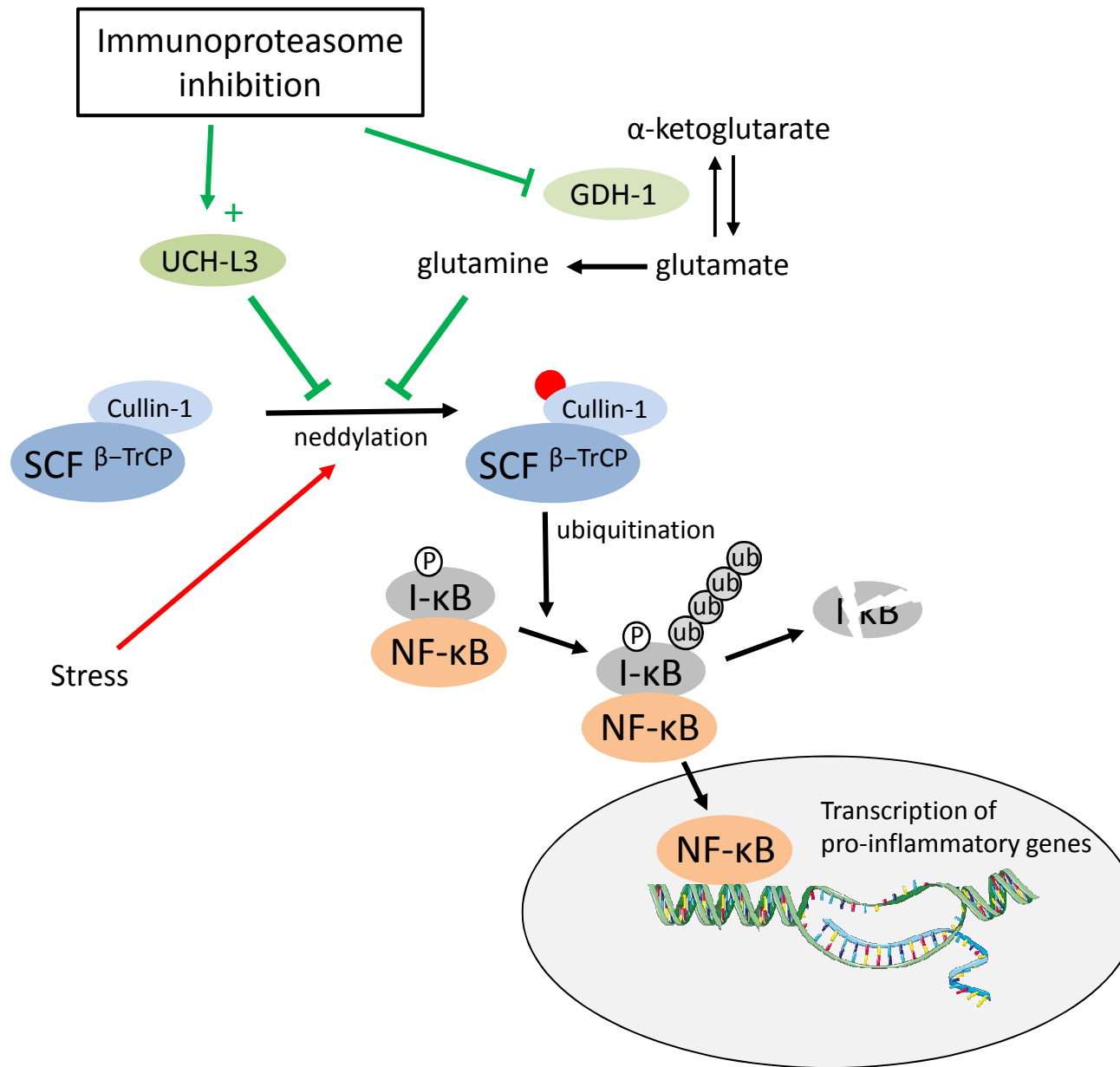


Table 1. Colonic protein expression changes in *wild-type* (wt) and $\beta 2i^{-/-}$ immunoproteasome subunit knock-out ($\beta 2i^{-/-}$) mice during water avoidance stress (WAS) or no stress (Ct)

Spot number	Swiss-Prot accession number	Protein name (short name)	Theoretical ^a		Mascot Score ^b , Sequence coverage (%), peptide hit ^c	Fold change ^d				Two-way ANOVA: <i>P</i> (WAS), <i>P</i> ($\beta 2i^{-/-}$), <i>P</i> (interaction)
			<i>pI</i>	<i>Mr</i> (kDa)		wt Ct/ wt WAS	wt Ct/ $\beta 2i^{-/-}$ Ct	$\beta 2i^{-/-}$ Ct / $\beta 2i^{-/-}$ WAS	wt WAS / $\beta 2i^{-/-}$ WAS	
1	P58252	Elongation factor 2 (EF-2)	6.41	95.3	94.0, 4%, 4	-3.23	-3.74	NS	NS	0.0553, 0.0345, 0.0786
2	Q8CAQ8	MICOS complex subunit Mic60 (IMMT)	6.18	83.8	166, 9%, 6	-1.41	-1.46	NS	NS	0.0498, 0.0134, 0.1482
3	Q8K0D5	Elongation factor G1 (EF-G1mt)	6.48	83.5	76.0, 6%, 4	-1.88	-2.17	NS	NS	0.0699, 0.0179, 0.0180
4	Q8CAQ8	MICOS complex subunit Mic60 (IMMT)	6.18	83.8	147, 8%, 4	-1.60	-1.52	NS	NS	0.0576, 0.1305, 0.0072
5	Q8R2Q4	Elongation factor G2 (EF-G2mt)	5.98	85.9	63.0, 3%, 2	-1.74	-1.98	NS	NS	0.2957, 0.0986, 0.0095
6	Q9WU78	Programmed cell death 6-interacting protein (Pcd6ip)	6.15	96.0	121, 7%, 5	-2.48	-2.14	NS	NS	0.0145, 0.0446, 0.0222
7	P26040	Ezrin (Ezr)	5.83	69.4	267, 9%, 6	-1.53	-1.93	NS	NS	0.0345, 0.0019, 0.2013
8	Q9CQN1	Heat shock protein 75 kDa (HSP75)	6.25	80.2	90.0, 8%, 4	-1.51	-1.63	NS	NS	0.2076, 0.0898, 0.0375
9	–	Undetermined	–	–	–	-2.23	-1.84	NS	NS	0.0002, 0.0047, 0.0028
10	–	Undetermined	–	–	–	-1.42	-1.59	NS	NS	0.0391, 0.0062, 0.1552
11	–	Undetermined	–	–	–	-1.58	-1.77	NS	NS	0.0244, 0.0056, 0.1831
12	–	Undetermined	–	–	–	-1.48	-1.71	NS	NS	0.2286, 0.0269, 0.0112
13	P11984	T-complex protein 1 subunit alpha A (TCP-1-A)	5.76	60.3	203, 11%, 5	-1.89	-1.79	NS	NS	0.0074, 0.0146, 0.0528
14	P09103	Protein disulfide-isomerase precursor (PDI)	4.79	57.1	151.0, 7%, 4	-2.36	-2.81	NS	NS	0.0056, 0.0010, 0.0018
15	P52480	Pyruvate kinase (PKM)	7.18	57.8	300, 24%, 11	-1.73	-1.77	NS	NS	0.0358, 0.0277, 0.0206
16	P09103	Protein disulfide-isomerase precursor (PDI)	4.79	57.1	201, 10%, 5	-2.89	-1.70	NS	NS	0.0097, 0.4592, 0.0099
17	P09103	Protein disulfide-isomerase precursor (PDI)	4.79	57.1	174, 14%, 6	-2.59	-1.89	NS	NS	0.0135, 0.2347, 0.0015
18	P63038	60 kDa heat shock protein (HSP60)	5.90	60.9	821, 28%, 13	NS	NS	NS	-1.86	0.0399, 0.0014, 0.4110
19	P99024	Tubulin beta-5 chain (Tubb5)	4.78	49.6	552, 24%, 9	-1.83	-1.85	NS	NS	0.0020, 0.0017, 0.0532
20	Q99KP6	Pre-mRNA-processing factor 19 (Prpf19)	6.14	55.2	169, 10%, 3	-1.48	1.72	NS	NS	0.0325, 0.0021, 0.0463
21	P46467	Vacuolar protein sorting-associating protein 4B (Vps4b)	6.65	49.4	79.0, 7%, 3	-1.68	NS	NS	NS	0.0276, 0.2884, 0.1122
22	–	Undetermined	–	–	–	-2.78	-2.85	NS	NS	0.0171, 0.0145, 0.0136
23	Q91ZJ5	UTP--glucose-1-phosphate uridylyltransferase (Ugp2)	7.18	57.0	216, 17%, 6	-1.60	-1.61	NS	NS	0.0059, 0.0055, 0.4087
24	P26443	Glutamate dehydrogenase 1 (GDH1)	8.05	61.3	393, 31%, 13	NS	NS	NS	-1.48	0.0201, 0.0041, 0.7822
25	P56480	ATP synthase subunit beta (Atp5b)	5.19	56.3	294, 21%, 7	-1.63	NS	NS	NS	0.0023, 0.9397, 0.4053

Table 1. (Continued)

Spot number	Swiss-Prot accession number	Protein name (short name)	Theoretical ^a		Mascot Score ^b Sequence coverage (%) peptide hit ^c	Fold change ^d				Two-way ANOVA: <i>P</i> (WAS), <i>P</i> ($\beta 2i^{-/-}$), <i>P</i> (interaction)
			pI	<i>M_r</i> (kDa)		wt Ct/ wt WAS	wt Ct/ $\beta 2i^{-/-}$ Ct	$\beta 2i^{-/-}$ Ct / $\beta 2i^{-/-}$ WAS	wt WAS / $\beta 2i^{-/-}$ WAS	
26	–	Undetermined	–	–	–	NS	NS	+1.59	+1.67	0.0277, 0.0127, 0.0312
27	–	Undetermined	–	–	–	-2.01	-1.70	NS	NS	0.0015, 0.0159, 0.0318
28	–	Undetermined	–	–	–	NS	-1.60	NS	NS	0.249, 0.0452, 0.03630
29	–	Undetermined	–	–	–	-1.65	-1.83	NS	NS	0.080, 0.0286, 0.11610
30	P17182	Alpha-enolase 1 (Eno1)	6.37	47.1	773, 26%, 9	NS	NS	-1.55	-1.65	0.030, 0.0078, 0.13510
31	P55264	Adenosine kinase (AK)	5.84	40.1	162, 16%, 5	-1.76	NS	NS	NS	0.0188, 0.1506, 0.2865
32	P46664	Adenylosuccinate synthetase isozyme 2 (Adss2)	5.98	50.0	111.0, 7%, 3	-1.45	-1.55	NS	NS	0.1908, 0.0202, 0.0465
33	–	Undetermined	–	–	–	-2.46	-2.31	NS	NS	0.0341, 0.0588, 0.0010
34	P62334	26S protease regulatory subunit S10B (Psmc6)	7.10	44.1	404, 30%, 8	-1.47	NS	NS	NS	0.0078, 0.0359, 0.1776
35	–	Undetermined	–	–	–	-2.57	-2.41	NS	NS	0.0096, 0.0187, 0.0002
36	Q9JHI5	isovaleryl-CoA dehydrogenase (IVD)	8.53	46.3	350, 20%, 7	-1.56	-1.73	NS	NS	0.118, 0.0354, 0.07550
37	Q9CQM9	Glutaredoxin-3 (Glr3)	5.42	37.8	93.0, 7%, 2	-1.64	-1.76	NS	NS	0.2368, 0.0992, 0.0010
38	Q9JMD3	PCTP-like protein (PCTP-L)	6.67	32.9	169.0, 8%, 2	NS	NS	NS	NS	0.0224, 0.0711, 0.6146
39	–	Undetermined	–	–	–	-1.67	NS	-1.84	NS	0.0002, 0.9421, 0.7163
40	–	Undetermined	–	–	–	NS	+2.35	NS	NS	0.1041, 0.0075, 0.1830
41	–	Undetermined	–	–	–	-1.97	-1.85	NS	NS	0.1486, 0.2342, 0.0151
42	P52624	Uridine phosphorylase 1 (UPase 1)	6.12	34.1	101.0, 6%, 2	NS	NS	+3.59	NS	0.0096, 0.2894, 0.2429
43	Q99J99	3-mercaptopyruvate sulfurtransferase (MST)	6.11	33.0	416, 36%, 9	-1.51	-1.44	NS	NS	0.0075, 0.0178, 0.2104
44	Q99LC5	Electron transfer flavoprotein subunit alpha (Alpha-ETF)	8.62	35.0	878, 49%, 13	NS	-1.50	NS	NS	0.0393, 0.0059, 0.4695
45	O70435	Proteasome subunit alpha type-3 (Psm3)	5.29	28.4	80.0, 10%, 2	NS	+5.06	+1.72	+3.80	0.0152, < 0.0001, 0.211
46	Q9R1P0	Proteasome subunit alpha type-4 (Psm4)	7.59	29.5	231, 19%, 4	NS	NS	NS	-1.70	0.204, 0.0167, 0.32380
47	Q9JKB1	Ub carboxyl-terminal hydrolase isozyme L3 (Uchl3)	4.96	26.1	142, 16%, 3	NS	NS	NS	+2.35	0.7986, 0.0105, 0.0445
48	Q9Z2U1	Proteasome subunit alpha type-5 (Psm5)	4.74	26.4	87.0, 7%, 2	NS	NS	+1.85	+2.22	0.2666, 0.0385, 0.0004
49	Q99LX0	Protein deglycase DJ-1 (DJ-1)	6.32	20.0	178, 12%, 3	-1.54	-1.70	NS	NS	0.0036, 0.0008, 0.4190
50	Q3THE2	Myosin regulatory light chain 12B (Myl12b)	4.71	19.8	105, 12%, 3	NS	NS	NS	NS	0.0519, 0.0117, 0.7918

^aTheoretical molecular mass (*M_r*) and isoelectric point (pI) calculated from the mature protein and amino acid sequence, respectively.

^bMASCOT, algorithm that uses mass spectrometric data to identify proteins from primary sequence databases.

^cNumber of peptides matched.

^dAverage spot volume ratio between two groups. +, upregulated; -, downregulated; NS, not significant.

Table 2. DAVID functional annotation clusters generated from the list of colonic proteins differentially expressed between the four mice groups

	Annotation category	Term	Enrichment Score ¹	Protein count	P value ²	Benjamini ³	Related genes ⁴
Annotation Cluster 1	SP_PIR_KEYWORDS	Mitochondrion	4.89	11	7.0E-6	2.2E-4	ATP5B, ETFA, GFM1, GFM2, GLUD1, HSPD1, IMMT, IVD, MPST, TRAP1, VSP4B
	SP_PIR_KEYWORDS	Transit peptide		8	1.3E-5	3.1E-4	ATP5B, ETFA, GFM1, GFM2, GLUD1, HSPD1, IVD, TRAP1
	UP_SEQ_FEATURE	Transit peptide: Mitochondrion		8	2.4E-5	2.5E-3	ATP5B, ETFA, GFM1, GFM2, GLUD1, HSPD1, IVD, TRAP1
Annotation Cluster 2	INTERPRO	Proteasome, alpha-subunit	2.06	3	8.2E-5	9.2E-3	PSMA3, PSMA4, PSMA5
	SP_PIR_KEYWORDS	Proteasome		4	1.2E-4	1.9E-3	PSMA3, PSMA4, PSMA5, PSMC6
	INTERPRO	Proteasome, subunit alpha/beta		3	5.5E-4	3.1E-2	PSMA3, PSMA4, PSMA5
	KEGG_PATHWAY	Proteasome		4	6.2E-4	1.9E-2	PSMA3, PSMA4, PSMA5, PSMC6
	SP_PIR_KEYWORDS	Threonine protease		3	6.4E-4	6.0E-3	PSMA3, PSMA4, PSMA5
Annotation Cluster 3	SP_PIR_KEYWORDS	gtp-binding	1.94	6	2.2E-4	2.3E-3	ADSS, EEF2, GFM1, GFM2, GLUD1, TUBB5
	PANTHER_MF_ALL	Translation elongation factor		3	5.3E-4	2.6E-2	EEF2, GFM1, GFM2

¹Fold enrichment of the cluster.

²P value from modified Fisher's exact test (EASE score).

³P value adjusted for multiple testing according to the Benjamini-Hochberg model.

⁴Genes encoding the differentially expressed proteins in *Mus Musculus*: ADSS - Adenylosuccinate Synthase; ATP5B - ATP Synthase, H⁺ Transporting, Mitochondrial F1 Complex, Beta Polypeptide; EEF2 - Eukaryotic Translation Elongation Factor 2; ETFA - Electron Transfer Flavoprotein Alpha Subunit; GFM1 - G Elongation Factor Mitochondrial 1; GFM2 - Elongation Factor Mitochondrial 2; TRAP1 - Heat shock protein 75 kDa, mitochondrial; GLUD1 - Glutamate Dehydrogenase 1; HSPD1 - Heat Shock Protein Family D (Hsp60) Member 1; IMMT - Inner Membrane Mitochondrial Protein; IVD - Isovaleryl-CoA Dehydrogenase; MPST - Mercaptopyruvate Sulfurtransferase; PSMA3 - Proteasome Subunit Alpha 3; PSMA4 - Proteasome Subunit Alpha 4; PSMA5 - Proteasome Subunit Alpha 5; PSMC6 - Proteasome 26S Subunit, ATPase 6; TUBB5 - Tubb5 tubulin, beta 5 class I; VSP4B - Vacuolar protein sorting-associated protein 4B.

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