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Title: Recognition of purified beta 1,3/1,6 glucan and molecular signalling in the intestine of Atlantic salmon

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Abstract

Atlantic salmon was orally intubated with a highly purified β-glucan product (MacroGard®) to study the recognition of the molecule by the receptor genes, the regulation of the downstream signalling genes and global proteins, and the micromorphological changes in the intestine.

The β-glucan receptor genes of Atlantic salmon, *sclra*, *sclrb*, *sclrc* and *cr3*, seem to recognize the molecule, and initiate the downstream ITAM-motif signalling, as evident from the significantly high mRNA levels of *ksyk*, *mapkin2*, *il1b* and *mip2a* levels. Among the altered proteins, the Apoa4 (involved in carbohydrate and lipid metabolism); Tagln, Actb (uptake of β-glucan); Psma2 (associated with substrate recognition); and Ckt (energy metabolism-related) were the overexpressed ones. The underexpressed proteins included the Uk114, Rpl9, Ctsb and Lgal that are connected to proliferation, LPS-stimulation, Il1b and lactose recognition, respectively. Furthermore, the mRNA levels of *igt* and the number of immune cells in the distal intestine were found to increase upon β-glucan uptake by the fish. This study provides some clues on the mechanisms by which the β-glucan evokes response in Atlantic salmon, particularly at the intestinal level.

Keywords: Atlantic salmon; beta-1,3/1,6 glucan; MacroGard®; C-type lectin receptor genes; Tagln, Actb, Psma2

1. Introduction

Immunomodulatory feed additives are relied on to enhance the performance and health of farmed animals, including fish. The purified β-glucan derived from yeast is considered as an additive that supports the immune system and improves the health of the host (Mantovani et al., 2008; Volman et al., 2008). These molecules are not digested and absorbed in the gut of animals, but are recognized by the surface receptors of leukocytes; mainly by Dectin-1 and the Toll-like receptors (TLRs), and to a certain extent by others including the complement receptor 3 (CR3) (Chan et al., 2009; Kim et al., 2011). The receptors are known to act singly or in combination with ligands. Dectin-1, a C-type lectin belonging to group V has a calcium (Ca)-independent carbohydrate recognition domain (CDR), an extracellular stalk region, a transmembrane region, a short cytoplasmic tail and an immunoreceptor tyrosine-based activation (ITAM)-like motif (Carter, 2013; Goodridge et al., 2009; Huysamen and Brown, 2009). Once the pattern recognition receptor of a host identifies a fungal pattern, Src kinases phosphorylates tyrosine in the ITAM-like motif to cause the transduction of the downstream signalling (Brown, 2006). Additionally, two phosphotyrosines bind to the spleen tyrosine kinase (SYK) and induce cellular responses (Brown, 2006).

Group V C-type lectins, which are the main fungal pattern recognition receptors (C-type lectin receptor, CLR) in mammals have not been identified in bony fish. Instead, in teleosts, group II members have been characterized, e.g. salmon C type lectin receptors a, b, c - Sclra, Sclrb and Sclrc in Atlantic salmon (Soanes et al., 2004). While CLRs and TLRs can recognize fungal patterns directly, CR3 identifies pathogen recognition receptor (PRR)-coated fungal particles (Brown, 2006). Collaborative action of Dectin-1 and TLRs induces inflammatory responses (Brown, 2006), and β-glucans are capable of initiating the production of inflammatory mediators such as TNFα and MIP-2 (Abel and Czop, 1992). Furthermore, the Dectin-1–dependent pathway initiated by β-glucans activates the transcription of the

proinflammatory cytokine IL-1β (Kankkunen et al., 2010). The TLR pathway starts with the recognition of the yeast pattern by TLR 2 or TLR 6, after which the association of the key signalling cytosolic domain of TLR, Toll/IL-1R domain (TIR) with the adaptor protein, Myd88 is initiated, leading to the activation of mitogen-activated protein kinases, MAPKs (O'Neill and Bowie, 2007). Furthermore, as mentioned before, Src family kinase-induced phosphorylation of tyrosine causes, among others, MAP kinase signalling (Goodridge et al., 2009; Huysamen and Brown, 2009). Additionally, teleost IgT is associated with gut mucosal surfaces and has immunoprotective roles (Zhang et al., 2011), and in mammals immunomodulins induce TGF-β, APRIL and BAFF to simulate lymphocytes to produce IgA (Preidis and Versalovic, 2009).

Although it is accepted that dietary β-glucan exerts immunomodulatory effects in fish, their mechanism of action has not been uncovered. When included in feeds containing multiple ingredients, it would be difficult to single out the mode of action of β-glucan. Therefore, an oral intubation study with a purified beta 1,3/1,6 glucan product was performed on Atlantic salmon to precisely examine the ensuing intestinal stimulation. The recognition of the molecule by the receptor genes (*sclra*, *sclrb*, *sclrc*, *cr3*) and the downstream signalling based on gene transcriptional changes (of *srckin*, *ksyk*, *myd88*, *mapkin2*, *il1b*, *mip2a*, *igt*) were studied. The changes in the proteome and the micromorphology of the intestine were also considered to obtain a better understanding of the physiological processes at the molecular level.

2. Materials and Methods

2.1 Fish and rearing conditions

Hatchery produced Atlantic salmon (*Salmo salar*, AquaGen strain), procured as smolts (from Cermaq, Bodø, Norway) and maintained on commercial feeds in the indoor rearing

facilities of the Research Station, University of Nordland (UiN), Bodø, Norway were used for the study. Zero-year class of healthy fish (av. wt. 275 g) were transferred to 500 L experimental tanks and allowed to acclimatize for 2 weeks. Two replicate tanks, each with 20 fish, were set up for the two treatments. The water temperature of the flow-through seawater system was 7°C and the oxygen saturation was above 90%. The experiments were conducted with the approval of the National Animal Research Authority (Forsøksdyrutvalget, FDU; ID - 5595) in Norway. The fish were handled by authorized personnel and the procedures were in accordance with the guidelines of FDU.

2.2 Preparation of the β-glucan suspension

The commercial product MacroGard® containing highly purified beta 1,3/1,6 glucans from *Saccharomyces cerevisiae* (Biorigin, Lençóis Paulista, Brazil) was employed in the study. An appropriate amount of the product was suspended in 5 ml of sterile phosphate-buffered saline (PBS), and sonicated (Vibra-Cell VC 750, Sonics and Materials Inc., Newtown, USA) for 3 min at a pulse rate of 20 s. The resulting suspension was employed for intubating the fish.

2.3 Oral intubation of fish

The oral intubation study was conducted on 2 groups of fish, which were starved for 2 days ahead of the procedure. The beta 1,3/1,6 glucan-intubated fish (at the rate of 15 mg/kg fish) constituted the treatment group (NL), while the PBS-intubated group served as the control group (CO). To perform the intubation, individual fish were netted out from each tank and sedated using MS-222 (Tricaine methane sulphonate; Argent Chemical Laboratories, Redmond, USA; 80 mg/l), approximately 4 min prior to initiating the intubation process. After ensuring that the fish were sedated, each fish was intubated with 500 µl of either the beta 1,3/1,6 glucan suspension or the saline using a Buster Cat Catheter 1.3 x 130 mm (Jorgen Kruuse A/S Denmark) connected to 1 ml syringe. Following the intubation, the fish were

allowed to recover from sedation. Then, they were transferred to the original holding tanks for the rest of the experimental period (7 days).

2.4 Intestinal tissue collection

At 1 and 7 days post intubation (dpi), 10 fish each from the study groups CO and NL were sampled to isolate the entire distal intestine. Immediately after the dissection, the distal intestinal region was divided into anterior, mid and posterior parts. The anterior and mid segments were snap-frozen in liquid nitrogen and stored at -80°C prior to RNA/protein extractions, respectively. The posterior portion was used for the histological studies (see section 2.7).

2.5 Assaying the expression of the target genes

The genes of the β-glucan receptors (salmon C type lectin receptors A, B, C - *sclra, sclrb, sclrc*, complement receptor 3, *cr3*); the genes involved in the downstream signalling pathway (Src kinase, *srckin*; spleen tyrosine kinase, *ksyk*); and other relevant immune genes (myeloid differentiation primary response gene 88, *myd88*; mitogen-activated protein kinase, *mapkin2*; interleukin 1b, *il1b*; macrophage inflammatory protein-2-alpha, *mip2a*; immunoglobulin T, *igt*) were studied.

All the qPCR reactions were performed in duplicate and the attributes of the gene specific primers used are presented in Table 1. The primers were designed flanking the intro-exon border to confirm the primer specificity. The total RNA was extracted from the distal intestine following the TRI-reagent method (Sigma, St. Louis, MO, USA), as described earlier (Lokesh et al., 2012). The RNA quality was assessed on 1% (W/V) agarose gels and subsequently quantified using Qubit® 2.0 Fluorometer and Quant-iT RNA assay kit (Life Technologies, Carlsbad, CA, USA). Total RNA (1000 ng) was reverse transcribed to complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The resulting cDNA was then diluted 50 times to perform

quantitative real time PCR (qPCR) on StepOnePlus™ Real-Time PCR system (Applied Biosystems, Carlsbard, CA, USA). The reaction mixture for qPCR (10 µl) contained 4 µl of diluted cDNA, 5 µl of the Fast SYBR® Green PCR Master mix (Applied Biosystems) and 1 µl of gene specific primer mix (5 pM each of forward and reverse). Conditions set for the qPCR reaction were: initial holding at 95°C for 20 s followed by 40 cycles of denaturation at 95°C for 3 s and isothermal annealing and extension at 60°C for 30 s. A melt curve analysis was performed to confirm the amplification specificity of the PCR products from each primer pair. Further, the amplicons generated by each of the gene specific primers were sequenced to confirm the specificity of the primers. Two negative controls, namely, water (control for cDNA template) and minus reverse transcriptase (i.e., pooled RNA treated with DNase) were also included. Additionally, 3-fold dilutions (1:1-1:243) of cDNA template (pooled) was used to prepare standard curves included in every qPCR reaction plate to evaluate the amplification efficiency (E) of each gene specific primer using the formula: $E = (10^{-1/\text{slope}}\text{-}1) .\cdot 100$.

Four reference genes - elongation factor 1 AB (*ef1ab*), hypoxanthine phosphoribosyltransferase 1 (*hprti*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and ubiquitin (*ubi*) - were run on all the samples. Quantification cycle values (Cq) obtained for every sample within a particular gene were converted to relative quantities. Finally, the geNORM (Vandesompele et al., 2002) was used to identify the most stable reference gene pair and subsequently to calculate the normalization factor. *ubi* and *gapdh* were found to be the most stable pair, with an M-value below 0.5.

2.6 Identifying the differentially expressed proteins

On the basis of the observations in the gene expression study, the comparisons of the intestinal protein spots were performed on the samples procured at 7 dpi. The protein extracts from the distal intestine of the CO and NL groups (n = 6 from each group) were used to perform 2-dimensional gel electrophoresis (2-DE). The proteins were extracted following a

slightly modified version of the procedure described earlier (Vasanth et al., 2015). Exactly 100 µg of the extracted protein was used to rehydrate 17 cm isoelectric (pI) strips pH 3-10 (Bio-Rad), as per the manufacturer's instructions. The isoelectric focusing (IEF) was performed on the pI strips using the Protean IEF cell (Bio-rad), as described by Vasanth et al. (2015). The electro-focused pI strips were first reduced and then alkylated for 15 min in equilibration buffer (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol) containing 0.2% DTT and 0.3% iodoacetamide (Bio-Rad), respectively. The second dimension gel electrophoresis was performed on a 12.5% polyacrylamide gel in the PROTEAN II xi system (Bio-Rad). The obtained gels were stained with the Sypro®Ruby protein gel stain (Life Technologies), and the gel images were captured using the ChemiDoc™ XRS imaging system (Bio-Rad). The images were analysed using the PDQuest Advanced software (Bio-Rad). The differentially expressed protein spots (those with 1.5-fold change in expression and $p < 0.1$) in the NL group compared to those in the CO group were identified.

The differentially expressed protein spots were selected for the liquid chromatography and tandem mass spectrometry (LC-MS/MS). A preparative gel employing 300 µg protein was used to excise the target spots. The LC-MS/MS analyses (ESI Quad TOF; Micromass/Water, MA USA) were performed at the University of Tromsø, Norway. The peak list (PKL) files generated with Protein Lynx Global server software (version 2.1, Micromass/Waters, MA, USA) was used for protein inference at UiN, Bodø. The Mascot search engine (version 2.5.00) was used to remove non-fish contaminants and perform a search in the vertebrate EST database, as described by Vasanth et al. (2015). Based on a prediction using Poisson distribution, protein inference was performed based on two unique peptides.

2.7 Examining the micromorphologic changes

The portion of the distal intestine for histology was fixed in 4% neutral phosphate buffered formalin and kept for 24 h at 4˚C. Employing a Citadel 2000 Tissue Processor (Thermo Fisher

Scientific, Waltham, MA, USA), the samples were dehydrated using graded alcohol series, equilibrated in xylene and embedded in paraffin. Sectioning was done using microtome (Microm HM355S, MICROM International GmbH, Walldorf, Germany). Five-micrometer thick cross sections were cut and mounted on glass slides (Superfrost1, Mentzel, Braunschweig, Germany). A staining robot (Microm HMS 760X, MICROM International GmbH) was used to dewax, rehydrate and stain the slides.

Alcian Blue (pH2.5) /Periodic Acid-Schiff's (AB/PAS) method [described by Suvarna et al. (2013)] was used to stain the acid and neutral mucins. The stained slides were mounted using Pertex medium (Histolab Products AB, Göteborg, Sweden). Photomicrographs were prepared using light microscopy employing the Olympus BX61/Camera Color View IIIu (Olympus Europa GmbH, Hamburg, Germany) and the photoprogram Cell P (Soft Imaging System GmbH, Munster, Germany).

A modified version of the immunohistochemistry protocol of Romarheim et al. (2011) (employing mouse monoclonal IgG2α-k, horse secondary Ab and Avidin/biotin staining) was adopted for studying the proliferating cell nuclear antigens (PCNAs). The modifications included the use of 1:500 dilution of the primary antibody and 3,3'-Diaminobenzidine tetrahydrochloride (DAB, D5905, Sigma) for the peroxidase reaction. After the reaction, the sections were counterstained with haematoxylin for 15 s, dehydrated, cleared and mounted with Pertex medium. The photomicrographs of the slides were obtained as mentioned above.

2.8 Statistical analysis

GraphPad Prism V6.03 was used to analyse the qPCR data. The Two-way ANOVA revealed the interaction between the factors, time and treatment. The Tukey's multiple comparisons test was employed to understand the differences between two groups for a particular factor. All the assumptions of the ANOVA were checked prior to the analyses, and transformations were employed wherever necessary. The non-parametric data were analysed

using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test. The significance level for the hypotheses testing was set to $p<0.05$.

3. Results

3.1 Intestinal genes affected by the β-glucan

The mRNA levels of the three CLRs in the distal intestine of salmon that were orally intubated with the β-glucan product were analysed. Interaction between the two factors (treatment X time) was detected ($p<0.05$) only in the case of *sclrb*. At 7 dpi, *sclra*, *sclrb* and *sclrc* were significantly ($p<0.05$) higher in NL group, compared to the values in CO (Fig.1). Furthermore, *sclrc* was higher ($p<0.05$) in NL group even at 1 dpi. *sclra* and *sclrb* levels in CO were lower ($p<0.05$) at 7 dpi compared to the respective values at 1 dpi.

In the case of *cr3*, an interaction of treatment and time was not evident. At 1 dpi, the mRNA level of *cr3* was significantly ($p<0.05$) higher in NL compared to that in CO. Furthermore, *cr3* in the two groups were higher ($p<0.05$) at 7 dpi compared to the respective values at 1 dpi.

A significant interaction ($p<0.05$) between the treatment and time was not detected for *ksyk*, and *srckin* (Fig. 2). At 7 dpi, the levels of *ksyk* was significantly ($p<0.05$) higher in NL compared to the level in CO. The values in CO and NL were significantly ($p<0.05$) higher at 7 dpi compared to the respective values at 1 dpi.

Significant differences were not detected for *myd88* ($p>0.05$) (Fig. 3). Interaction ($p<0.05$) was evident for *mapkin2*, and the level of the gene in NL was significantly ($p<0.05$) higher than that in CO at 7 dpi. Interaction between the factors was evident ($p<0.05$) in the case of *il1b*. At 7 dpi, *il1b* and *mip2a* were significantly ($p<0.05$) higher in NL compared to the levels in CO (Fig. 3). Furthermore, the level of *il1b* in CO at 7 dpi was significantly ($p<0.05$) lower

than the value at 1 dpi. The mRNA levels of *igt* was significantly ($p<0.05$) upregulated in the NL group compared to the CO group, at 7 dpi (Fig. 4).

3.2 Intestinal proteins affected by the β-glucan

The analyses of the global intestinal protein expression of the intubated fish groups revealed 10 differently expressed protein spots in the NL group compared to the CO group (Fig. 5). They were identified as Apolipoprotein A-IV precursor (Apoa4), Ribonuclease UK114 (Uk114), 60S ribosomal protein L9 (Rpl9), Cathepsin B precursor (Ctsb), Transgelin (Tagln), Actin, cytoplasmic 1 (2 spots of Actb), Galectin (Lgal), Proteasome subunit alpha type 2 (Psma2), Creatine kinase, testis isozyme (Ckt). Of these proteins, 6 were overexpressed and 4 were underexpressed in the NL group (Tables 2, 3).

3.3 Changes in intestinal micromorphology caused by the β-glucan

The normal structure of the distal intestine was evident from the intestinal photomicrographs. There were more number of goblet cells and other immune cells in the NL group compared to the control fish (Fig. 6a, b and Supplementary fig. 3a,c), and the goblet cells were distributed throughout the villi of the distal intestine. PCNA staining in the villi of the NL group was not different from that in the CO group (Fig. 7a, b). Furthermore, PCNA staining observed on crypt-like structures (yellow arrow heads in Supplementary fig. 4a) were also not different in both the groups.

4. Discussion

The known benefits of β-glucan (or its derivatives) on mammals include immunomodulation, enhancement of wound healing, reduction of inflammation, and improvement of the skin health and lipid profile (Di Franco et al., 2013; Kim et al., 2007; Ravo et al., 2011). β-glucans that have high molecular weight directly activate leukocytes and modulate the production of proinflammatory cytokines and chemokines, while those with low molecular weight activates the leukocytes via the stimulation of nuclear transcription factors

(Brown and Gordon, 2003). It has been shown that the uptake of the β-glucan particles (derived from *Saccharomyces cerevisiae*) by macrophages is actin-dependent and follows Dectin-1 linked recognition (McCann et al., 2005).The wound healing (Przybylska-Diaz et al., 2013) and immunomodulatory properties (Bonaldo et al., 2007; Falco et al., 2012; Marel et al., 2012; Pietretti et al., 2013) of β-glucan have been reported in different studies on fish. Although the response of immune cells following the uptake of β-glucan is reasonably well-known, evidences on the regulation of β-glucan receptor genes, and the alteration of genes and proteins involved in the signalling pathway in teleost intestinal immune system has not been reported.

4.1 Recognition and uptake of the beta 1,3/1,6 glucan

In vitro studies employing murine macrophages have revealed that Dectin-1, rather than TLR2, is involved in the binding and internalization of purified β-glucan particles (McCann et al., 2005). The results from the present study on Atlantic salmon indicate the participation of the three C-type lectins and cr3 in the recognition of β-glucan patterns of the beta 1,3/1,6 glucan. The higher levels of the genes at 7 dpi in NL compared to the levels in CO could be indicative of the ability of the C-type lectin receptor genes in recognizing the patterns of the purified beta 1,3/1,6 glucan. Additionally, the higher levels of *sclrc* in NL compared to the levels in CO at both the time points provide added evidence of the involvement of the C-type lectins in responding to the β-glucan. The mRNA levels of *sclra* and *sclrb* were lower in the CO group at 7 dpi compared to the respective values at 1 dpi. A similar decreasing pattern was observed for the transcript of a C-type lectin (*MjHeCL*) in the hemocytes of the control (PBS-injected) kuruma shrimp, *Marsupenaeus japonicus* (Wang et al., 2014). The higher level of *cr3* at 1dpi in NL compared to the level in CO indicate the additional recognition of the β-glucan at the early time point as CR3 is a distinct opsonic receptor (Brown, 2006). Furthermore, soluble beta-glucan polysaccharide primes CR3 of phagocyte/NK cells to cause

cytotoxicity of only the iC3b targeted tissues (Vetvicka et al., 1996). CR3 on NK cells/cytotoxic T cells resembles those on phagocytes, and cellular activation promotes the cytoplasm-derived expression of CR3 on cell surfaces (Muto et al., 1993). The protein, Beta-galactoside-binding lectin (LGAL) that shows affinity towards beta-galactosides like lactose is a calcium-independent type, unlike the group II C-type lectins reported in this study (Arason, 1996). The underexpression of Lgal in the present study points to the non-involvement of the protein in the β-glucan recognition.

Following the recognition of β-glucan, the Src family of kinases phosphorylate tyrosines of ITAM-like motif of CLRs, leading to the induction of the intracellular signalling cascade (Brown, 2006). Furthermore, Dectin-1 interacts with Syk and induces cellular responses, including, among others, MAPK and NFκB pathways (Goodridge et al., 2009; Huysamen and Brown, 2009). The significantly higher level of *ksyk* in NL compared to the value in CO at 7 dpi could be indicating the initiation of the immune signalling after the stimulation of *sclra*, *sclrb* and *sclrc*. The presence of tyrosine phosphorylation sites in SCLRA and SCLRC and the functional similarity between SCLRB and SCLRA suggests their involvement in immune responses (Soanes et al., 2004).

TLR2 and 6 are also known to recognize yeast patterns, and the association of the key signalling cytosolic domain of TLR, Toll/IL-1R domain (TIR) with the adaptor protein, Myd88 initiates a number of TLR-specific signals, including MAP kinase signalling (O'Neill and Bowie, 2007). These signalling cascades cause the activation of NFκB and the production of pro-inflammatory cytokines and chemokines (Brown, 2006). Although a significant upregulation of *myd88* was not evident, the higher levels of *mapkin2*, *il1b* and *mip2a* in NL compared to the values in CO could be indicating the initiation of the TLR pathway after the recognition of the β-glucan by the PRRs (TLR2 and TLR6 not yet described in salmon) in the distal intestine of Atlantic salmon. β-glucans are capable of initiating the production of the

inflammatory mediators such as TNFα and MIP-2 (Abel and Czop, 1992). In human macrophages, Dectin-1-dependent pathway initiated by β-glucans activates the transcription of the proinflammatory cytokine IL-1β (Kankkunen et al., 2010), although the process is dependent on trypsin-sensitive receptors (Abel and Czop, 1992). Furthermore, particulate β-glucan was found to increase *il1b, il6* and *il11* in carp (*Cyprinus carpio*) macrophages (Pietretti et al., 2013). Although inflammatory responses were evident, the characteristic features of intestinal inflammation (Vasanth et al., 2015) were not evident in the photomicrographs. The protein, Cathepsin B (CTSB) that has been linked to cell death and inflammation (Broker et al., 2004; Lenarcic et al., 1988) was underexpressed in the distal intestine of Atlantic salmon. The underexpression of Ctsb precursor in the NL group did not coincide with the mRNA levels of *il1b* at 7 dpi.

The protein Proteasome subunit alpha type-2 (PSMA2) - that takes part in substrate recognition and influences the specificity of the proteasome (Jung and Grune, 2012) - was overexpressed in the distal intestine of Atlantic salmon. Psma2 was present in the MHCIIβ-positive exosomes of CpG-stimulated head kidney leukocytes of Atlantic salmon (Iliev et al., 2010). In one of our recent studies that examined the ability of another microbial product to maintain intestinal epithelial homeostasis, Psma5 (protein of the α-ring of the proteasome complex) was overexpressed (Vasanth et al., 2015). Thus, the application of immunomodulatory substances such as β-glucan seems to favour the expression of Proteasome complex alpha ring proteins, implying that Psma components are very important in pattern recognition.

The delivery of antigens via goblet cells has been reported in mammals. Low molecular weight soluble antigens from the small intestinal lumen is transported to the underlying $CD103^{+}$ lamina propria and dendritic cells via goblet cells, and thus epithelial cells of this lineage help in intestinal immune homeostasis (McDole et al., 2012). There were more

number of goblet cells in the NL group compared to the control fish (Fig. 6a, b), and they were distributed throughout the villi of the distal intestine.

The mechanisms of the actin-dependent uptake of microbial particles, including those of yeast, by PRRs are not well described. *Edwardsiella ictaluri*, an enteric pathogen of catfish uses actin polymerization as one of the mechanisms of uptake, as demonstrated in rat intestinal epithelial cell line (IEC-6) (Li et al., 2012). In the present study, the distal intestine of Atlantic salmon treated with the beta 1,3/1,6 glucan, two actin-related proteins (3 protein spots) were overexpressed. One is Transgelin (TAGLN; also known as Actin 22α) – it is reported that this protein is expressed in B-1 cells, and is specific to smooth muscles, myoepithelium and mesenchymal cells (Frances et al., 2006). The other protein is Actin, cytoplasmic 1 (ACTB) - in its dynamic state this protein helps in the formation of transitory filaments that are needed for cell motility and active phagocytosis, and the protein is present in the permanent microfilaments of the intestinal microvilli (Nowak et al., 2005). The overexpression of the actin-related proteins (two significantly different spots of Actb and one spot of Tagln) may be indicating the actin-dependent β-glucan uptake (McCann et al., 2005).

4.2 Additional responses in the distal intestine

Gut mucosal surfaces of teleosts are associated with IgT, which has immunoprotective roles (Zhang et al., 2011). The higher levels of *igt* in the NL group could be indicative of the immunomodulatory property of the beta 1,3/1,6 glucan since immunomodulins are known to stimulate lymphocytes to secrete IgA in mammals (Preidis and Versalovic, 2009). The abundance of the immune cells (Supplementary fig. 3a, c) in the NL group could also be indicating the immunomodulatory property of the glucan product. Furthermore, in human dendritic cells, activation by LPS caused the downregulation of polysome-bound mRNA of (60S ribosomal protein L9, RPL9) *RPL9* (Ceppi et al., 2009). Similarly, in Atlantic salmon of

372 the NL group, the glucan molecules might have caused the underexpression of the protein,
373 Rpl9.

374 The immunomodulant induced the expression of the protein, Apolipoprotein A-IV (Apoa4)
375 that is associated with the carbohydrate and lipid metabolic processes. APOA4, a major
376 component of chylomicrons, HDL, and to a small extent VLDL, is synthesized by intestinal
377 enterocytes, and secreted into systemic circulation as a consequence of long-chain fatty acid
378 absorption (Weinberg et al., 2000). *apoa4* as well as *apoa1* were higher in rainbow trout,
379 *Oncorhynchus mykiss* fed on a carbohydrate-rich vegetable oil diet (Kamalam et al., 2013).
380 Additionally, the beta 1,3/1,6 glucan appears to be associated with a high energy demand.
381 Creatine kinase isozymes including testis isozymes (CKT), are involved in ATP binding and
382 catering to the energy needs of excited cells. The high levels of creatine kinase in blood is a
383 biomarker of muscle damage, and in Atlantic salmon the protein has been associated to heart
384 and skeletal muscle inflammation and cardiomyopathy syndrome (Yousaf and Powell, 2012).
385 However, our observations on intestinal overexpression of Ckt may be indicating a higher
386 energy demand rather than an intestinal damage because the histological observations did not
387 reveal any intestinal damage.

388 The overexpression of perchloric acid-soluble protein (which has high homology to
389 endoribonuclease UK114) has been linked to suppression of cell proliferation (Kanouchi et
390 al., 2001). However, our histological observations (PCNA staining) does not suggest a link
391 between Uk114 and cell proliferation.

392

393 5. Conclusions

394 In summary, the evidences point to the recognition and uptake of the purified β-glucan
395 molecules by the distal intestinal cells of Atlantic salmon to initiate immune signals. The
396 genes of *sclra*, *sclrb*, *sclrc*, *cr3*, *ksyk*, *mapkin2*, *il1b* and *mip2a* were upregulated in the NL

group. The overexpression of the proteins, Tagln and Actb, and the abundance of goblet cells in the NL group could be indicating the uptake of the beta 1,3/1,6 glucan particles. The high Psma2 may imply the involvement of the Psma components in pattern recognition. The upregulation of *igt*, the overexpression of Apoa4, Rpl9, Ckt and the abundance of the immune cells may be indicating the impact of the glucan molecule on immune and metabolic responses. This study provides some clues on the mechanisms by which the β-glucan evokes response in the fish, at the intestinal level.

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Figure legends

Figure 1. Relative mRNA levels of the β-glucan receptors in the distal intestine of Atlantic salmon. Expression of *sclra, sclrb, sclrc* and *cr3* in the distal intestine of Atlantic salmon after oral intubation with buffer saline (CO) or beta 1,3/1,6 glucan at 15 mg/kg fish (NL). Different letters above the bars indicate significant differences between the study groups at a particular time point. Solid line connectors indicate significant difference between the levels at two time points of a particular study group.

Figure 2. Relative mRNA levels of the genes involved in the downstream pathway following the recognition of β-glucan receptors. Expression of *srckin* and *ksyk* in the distal intestine of Atlantic salmon orally intubated with buffer saline (CO) or beta 1,3/1,6 glucan at 15 mg/kg fish (NL). Different letters above the bars indicate significant differences between the study groups at a particular time point. Solid line connectors indicate significant difference between the levels at two time points of a particular study group.

Figure 3. Relative mRNA levels of selected immune relevant genes in the distal intestine of Atlantic salmon. Expression of *myd88*, *mapkin2*, *il1b* and *mip2a* in the distal intestine of Atlantic salmon orally intubated with buffer saline (CO) or beta 1,3/1,6 glucan at 15 mg/kg fish (NL). Different letters above the bars indicate significant differences between the study groups at a particular time point. Solid line connectors indicate significant difference between the levels at two time points of a particular study group.

Figure 4. Relative mRNA level of immunoglobulin T in the distal intestine of Atlantic salmon. Expression of *igt* in Atlantic salmon orally intubated with buffer saline (CO) or beta 1,3/1,6 glucan at 15 mg/kg fish (NL). Different letters above the bars indicate significant differences between the study groups at a particular time point.

Figure 5. Representative 2-DE gels generated using the protein samples from the distal intestine of Atlantic salmon. The gels were generated to focus the proteins from the distal

intestine of Atlantic salmon orally intubated with buffer saline (CO) or beta 1,3/1,6 glucan at 15 mg/kg fish (NL). The two gels were prepared employing the samples procured at 7 dpi. Intestinal proteins from the fish were isoelectrically focused on 17 cm IPG strips (pI 3-10) and were subjected to 12.5% SDS-PAGE. The 2-DE gels were stained with Sypro®Ruby protein gel stain and the spots were annotated using the data from LC-MSMS. The spot numbers in the gels correspond to the protein identities mentioned in Table 3.

Figure 6. Photomicrographs of the distal intestine of Atlantic salmon. The images show PAS positive acid and neutral regions in the distal intestine of Atlantic salmon orally intubated with buffer saline (CO) and or beta 1,3/1,6 glucan at 15 mg/kg fish (NL). Yellow arrows point to the goblet cells and blue arrows indicate the intraepithelial lymphocytes. Scale: 100 μm (a), 20 μm (b).

Figure 7. Photomicrographs of the distal intestine of Atlantic salmon. The images show PCNA immunopositive regions of the distal intestine of Atlantic salmon orally intubated with buffer saline (CO) and or beta 1,3/1,6 glucan at 15 mg/kg fish (NL). Intense nuclear staining are considered positive for PCNA. Scale: 100 μm (a), 20 μm (b).

Table 1
List of primers used in the present study

Gene name	Sequence (5'-3')	Amplicon size (bp)	R^2	Reference
sclra	F- GACAACACAACACTGACAAACAAG R- GTGATCCTCCTGACTGATGATT	75	0.998	This study, GenBank : AY572832.1
sclrb	F- TGGACAACACAACGCTCACA R-AGATGCGGCGGTAGGTAAAG	159	0.994	This study, GenBank : AY572833.1
sclrc	F- ATGGAGAAAGAAGACCTTGTG R- AGTGGAGATGGGAGTAATGG	100	0.995	This study, GenBank : AY572834.1
cr3(itb2)	F- ATGACATGGACTACCCATCTGTT R-TCTGACAATACTCCCACCTCA	151	0.998	This study, GenBank : BT058776.1
scrkin	F- CCAGAGGCAATCAACTACGG R- TTCGTCATCCCTGGATATGGT	112	0.997	This study, GenBank : AF321110.1
ksyk	F- GTTCTTATCCAGAGCGACTTACA R-CCACCCACCACAATAGCTTT	145	0.998	This study, GenBank : NM001173673.1
myd88	F- GACAAAGTTTGCCCTCAGTCTCT R- CCGTCAGGAACCTCAGGATACT	87	0.996	GeneBank: EF672332.1
mapkin2	F- TCACAGAGACATCAAGCCAG R-CCCAGAGACCACATATCACAG	201	0.999	This study, GenBank : BT045910.1
igt	F- CAACACTGACTGGAACAACAAGGT R- CGTCAGCGGTTCTGTTTTGGA	97	0.996	(Tadiso et al., 2011) GenBank: GQ907004
il1b	F- GCTGGAGAGTGCTGTGGAAGA R- TGCTTCCCTCCTGCTCGTAG	73	0.997	GenBank: AY617117
mip2a	F- GACACTGAGATCATTGCCACT R- GCATCTTCTCAATGACCCTCTT	93	0.980	This study, GenBank: NM001141422.2
Reference genes				
ef1ab	F- TGCCCCTCCAGGATGTCTAC R- CACGGCCCACAGGTACTG	59	0.999	GenBank: BG933853
hprt1	F- CCGCCTCAAGAGCTACTGTAAT R- GTCTGGAACCTCAAACCCTATG	255	0.998	GenBank: BT043501
gapdh	F-AAGTGAAGCAGGAGGGTGGAA	96	0.999	GenBank: BT050045

	R-CAGCCTCACCCCATTTGATG			
ubi	F- AGCTGGCCCAGAAGTACAACTGTG R- CCACAAAAAGCACCAAGCCAAC	162	0.998	This study, GenBank: AB036060.1

Table 2
Information of the peptides identified using Mascot search engine

Spot no.	Protein details	Acc. No	Apparent pI/MW (kDa)	Protein score	ST[a]	Mp / Up[b]	SU[c]	Peptide sequence[d]
GM1	Clone ssal-plnb-013-037 Apolipoprotein A-IV precursor putative	Ssa.41274	4.0/29.9	703	55	8/7	629	TADDTVQMIR **ATQTADDTVQMIK** **AQLTALYQAFTNTN** **VAPLAENLQSELTTR** **EMQSQLGPYTDELK** **SVAPLAENLQSQLTTR** **QDLAPYAESLDSEALR** **AQMVQQSLAPYAEDLKDK**
GM2	TSA: *Salmo salar* isotig13060.Sasaskin mRNA sequence	Ssa.1898	4.8/13.6	204	56	2/2	204	**TFFSSSFPAR** **APAAIGPYSQAVVVDR**
GM3	rpl9 Ribosomal protein L9	Ssa.919	4.6/24.7	234	55	3/3	234	**EFNHINLELSLLGK** **TILSNQTVDIPDGVEVR** **SVYAHFPINVVMQESGALVEIR**
GM4	Transcribed locus, strongly similar to NP_001117776.1 procathepsin B precursor [*Oncorhynchus mykiss*]	Ssa.7877	5.1/27.8	435	55	5/5	435	**EQQIMSELYK** **GKDECGIESEIVAGIPR** **TGVYQHVTGQMLGGHAIK** **NGPVEAAFSVYEDFLLYK** **DGPVEAAFSVYEDFLLYK**
GM5	TSA: Salmo salar isotig04712.Sasaskin mRNA sequence	Ssa.7863	5.5/15.7	262	56	3/3	262	**IASSSMAFK** **TLMSLGSVAVTK** **QMEQISQFLTAAESFGVIK**
GM6	LOC100136352 Beta actin	Ssa.7935	5.6/50.0	424	55	4/4	424	**SYELPDGQVITIGNER** **VAPEEHPVLLTEAPLNPK** **DLYANTVLSGGTTMYPGIADR** **LCYVALDFEQEMGTAASSSSLEK**
GM7	Beta actin	Ssa.7935	5.9/45.6	355	55	4/2	161	SYELPDGQVITIGNER **DLYANTVLSGGTTMYPGIADR** LCYVALDFEQEMGTAASSSSLEK **MTQIMFETFNTPAMYVAIQAVLSLYASGR**

GM8	leg Beta-galactoside-binding lectin	Ssa.31246	5.9/13.5	243	55	3/3	243	**EGGFPFNQGEEFK** **EQFLVSLPDGSEIHFPNR** **LGQTLTITGIPNSEATHFVINVGNSEDDIALH** **MNPR**
GM9	TSA: Salmo salar isotig06760.Sasaskin mRNA sequence	Ssa.5609	6.5/31.5	608	55	7/5	445	**ASNGVVLATEK SILYDETSVHK** **GYSFSLTTFSPSGK** **LVQIEYALSAVAAGAPSVGIK** **YNVDLELEDAIHTAILTLK** YNEDLELEDAIHTAILTLK KLAQQYFLVYQEPIPTAQLVQR
GM10	kcrt Creatine kinase, testis isozyme	Ssa.31750	6.85/59.0	615	56	7/7	615	**ILTPAIYER ELLDPIIEDR** **GQSIDNIMPSQK MSVEALDSLSGDLK** **GGDDLDPNYVLSSR** **LGFSEVELVQMVVDGVK** **GTGGVDTAAVGGTFDISNADR**

[a] Significant threshold score; [b] Total matched peptides / total unique peptides; [c] Total score of unique peptides; [d] Unique peptide sequences are in bold.

Table 3

List of proteins that are over- and under-expressed in the distal intestine of Atlantic salmon orally intubated with beta 1,3/1,6 glucan

Spot No.	Protein Name	Fold change
GM1	Apolipoprotein A-IV precursor, Apoa4	2.69 ↑
GM2	Ribonuclease UK114, Uk114	0.55 ↓
GM3	60S ribosomal protein L9, Rpl9	0.43 ↓
GM4	Cathepsin B precursor, Ctsb	0.50 ↓
GM5	Transgelin, Tagln	1.77 ↑
GM6	Actin cytoplasmic 1, Beta actin, Actb	1.86 ↑
GM7	Actin cytoplasmic 1, Beta actin, Actb	2.22 ↑
GM8	Galectin, Lgal	0.64 ↓
GM9	Proteasome subunit alpha type 2, Psma2	2.59 ↑
GM10	Creatine kinase, testis isozyme, Ckt	1.58 ↑

↑ indicates overexpression and ↓ indicates underexpression

582 **Supplementary material**

583 **Supplementary figure 1. 2-DE gels of Atlantic salmon from the CO and NL groups.** Gels
584 were generated using the samples collected at 7 dpi.

585 **Supplementary figure 2. The volumes of the protein spots in the gels of the CO and NL**
586 **groups.** * indicates statistically significant differences of a protein in NL compared to that in
587 CO. Values are presented as mean ± s.e.m

588 **Supplementary figure 3. Photomicrographs of the distal intestine of Atlantic salmon.**
589 The images show PAS positive acid and neutral regions in the distal intestine of Atlantic
590 salmon orally intubated with buffer saline (CO) and or beta 1,3/1,6 glucan at 15 mg/kg fish
591 (NL). Yellow arrows point to goblet cells and blue arrows indicate intraepithelial
592 lymphocytes. Comparisons of the number of goblet cells within the similar sized boxes
593 indicate an abundance of goblet cells in NL (a). Comparisons of the number of intraepithelial
594 lymphocytes within the boxes indicate an abundance of the immune cells in NL (c). Scale:
595 100 μm (a), 20 μm (b).

596 **Supplementary figure 4. Photomicrographs of the distal intestine of Atlantic salmon.**
597 The images show PCNA immunopositive regions of the distal intestine of Atlantic salmon
598 orally intubated with buffer saline (CO) and or beta 1,3/1,6 glucan at 15 mg/kg fish (NL)
599 (n=6, data from 4 fish is presented). Intense nuclear staining are considered positive for
600 PCNA. Scale: 100 μm (a), 20 μm (b).

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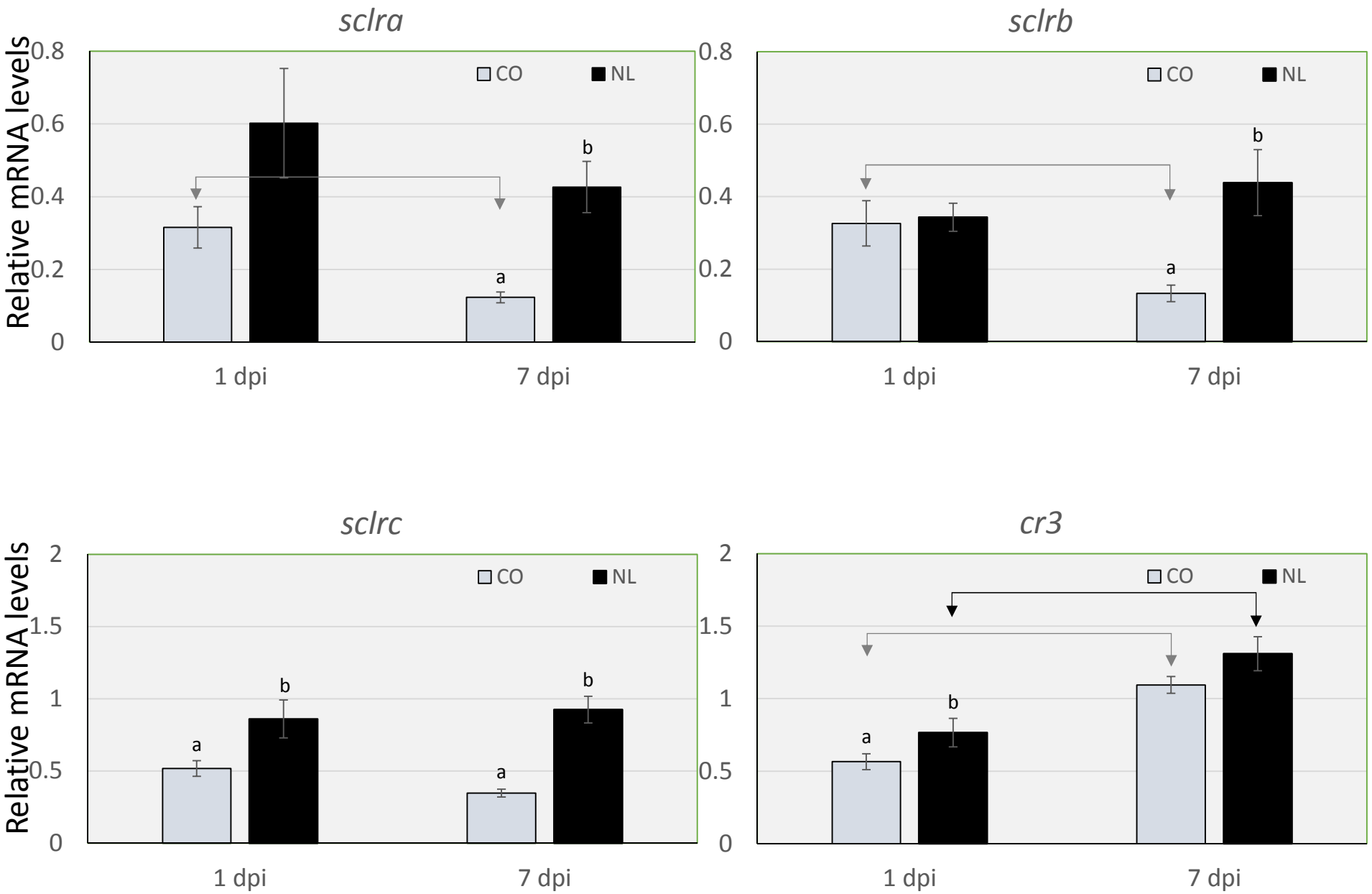
Figure 1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sclrc
cr3
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NL
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b
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7 dpi
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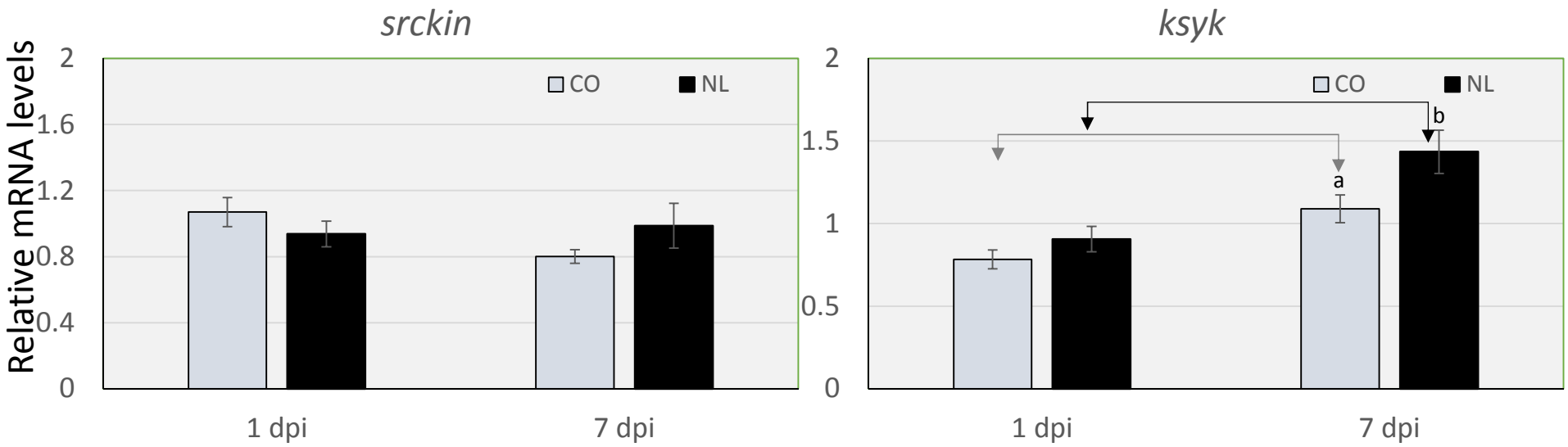
0.6
0.8
0.5
1
1.5
2

Figure 2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ksyk
Relative mRNA levels
CO
NL
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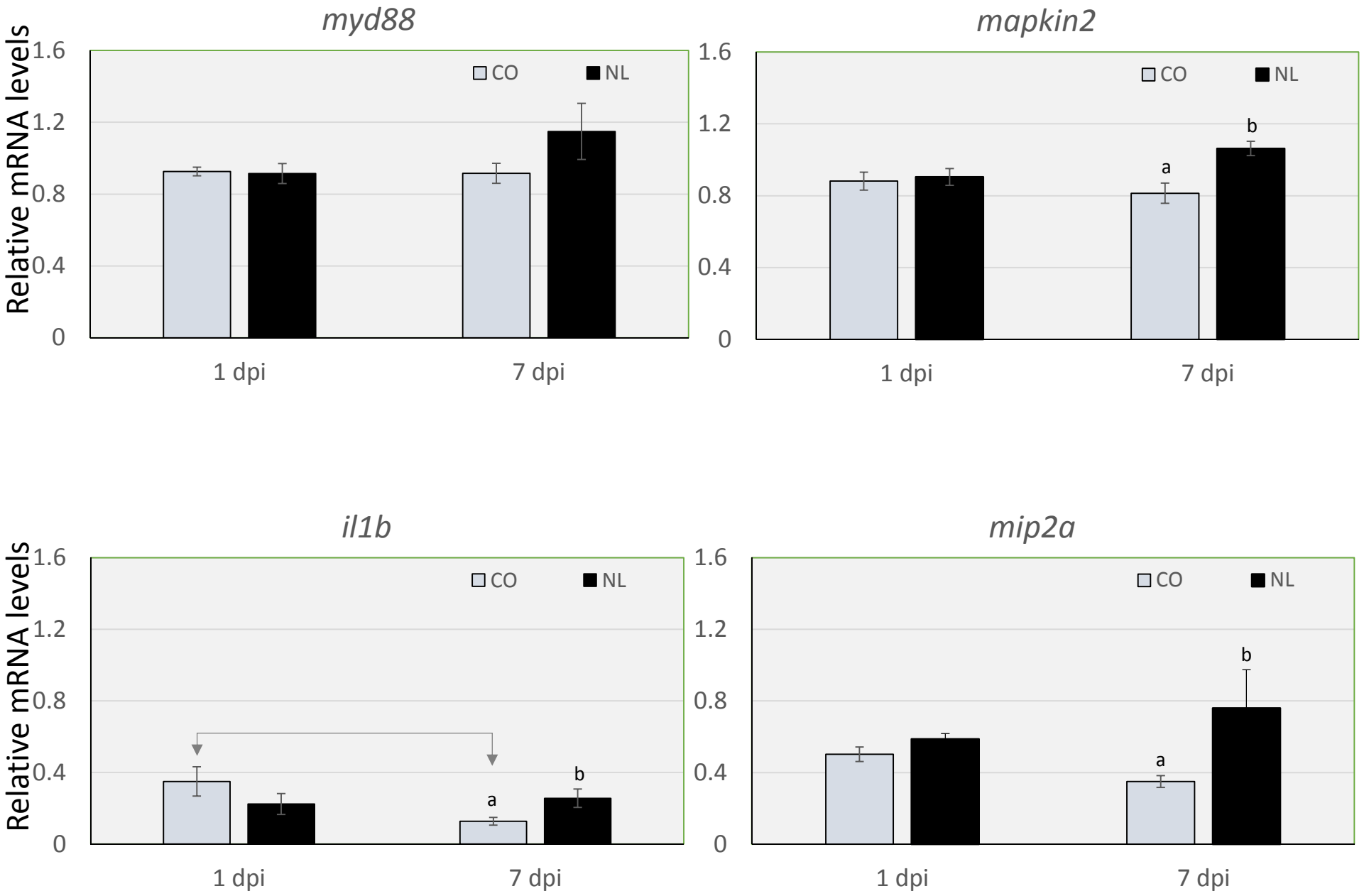
a
b
1 dpi
7 dpi

Figure 3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a
b
1 dpi
7 dpi

Figure 4

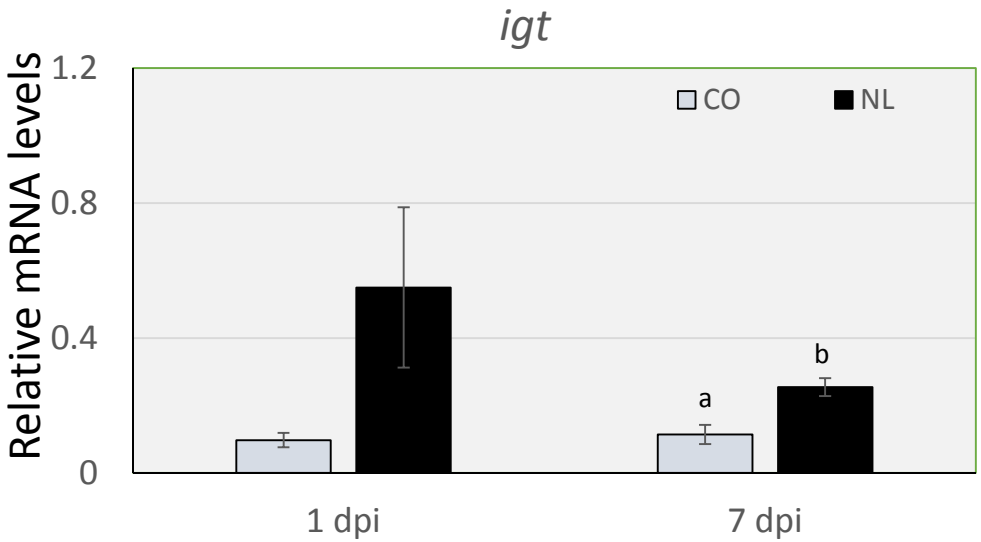
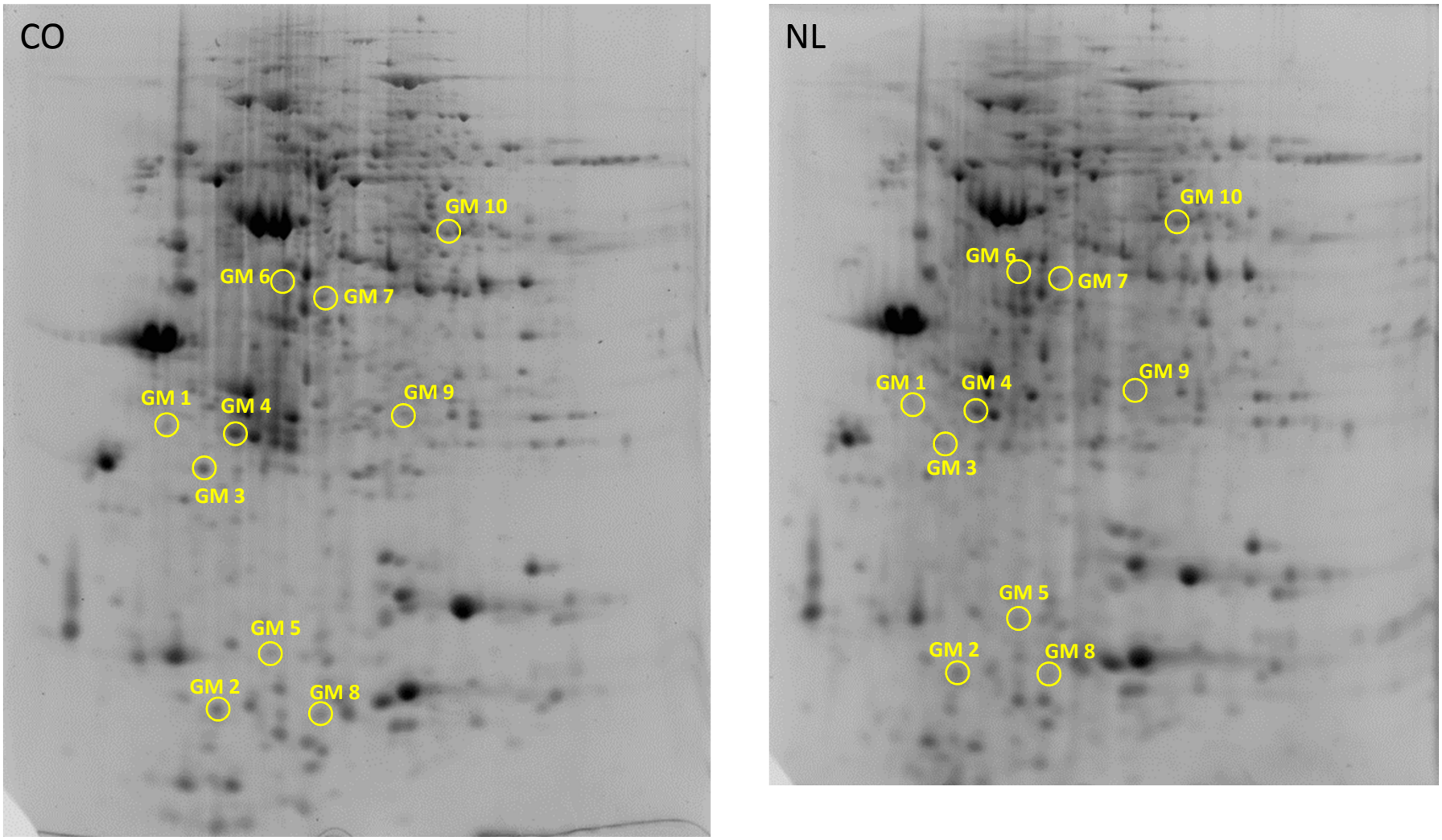


Figure 5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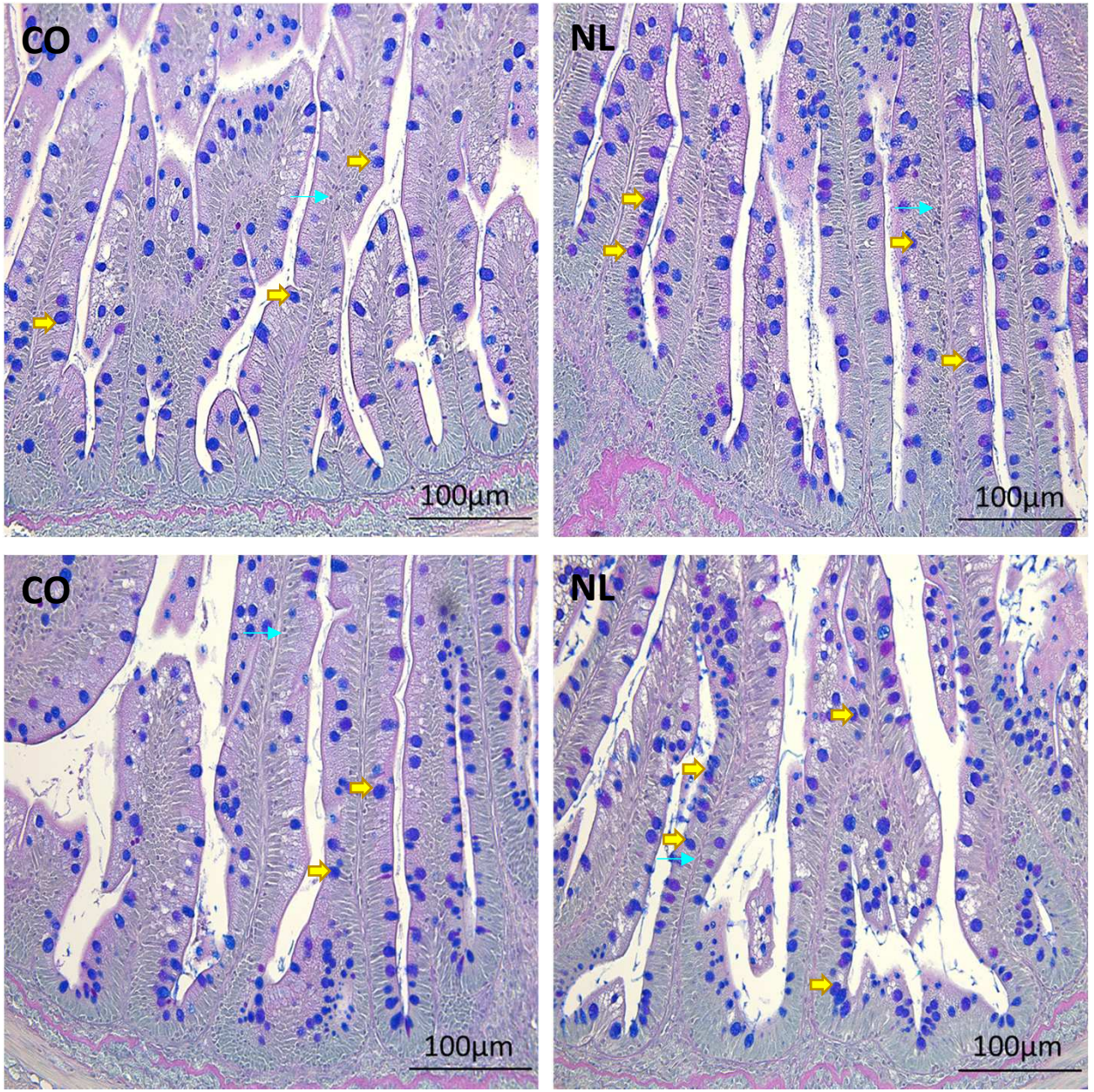
GM 2
GM 8

Figure 6a



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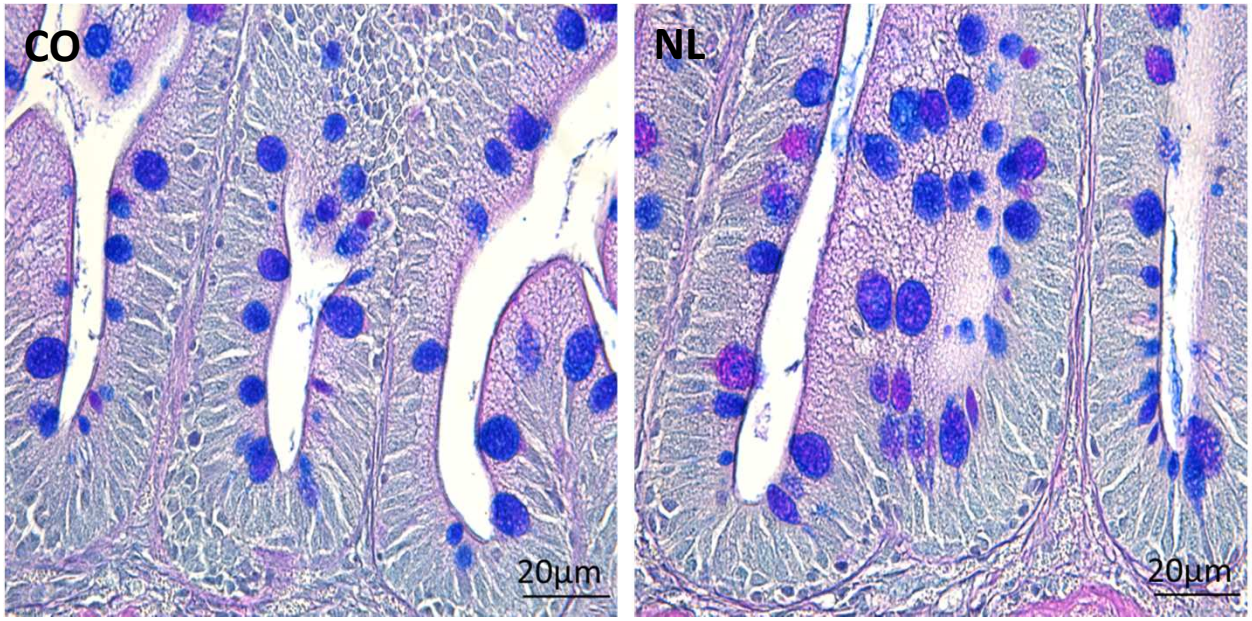
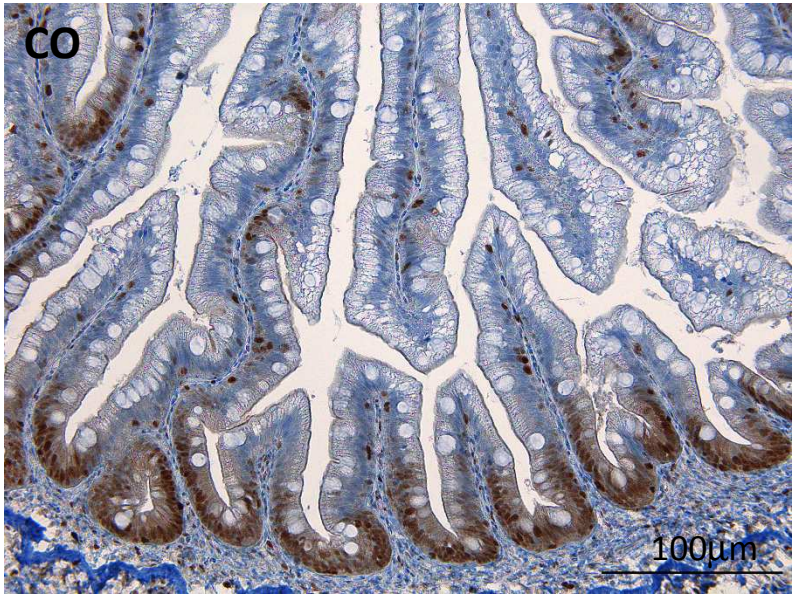


Figure 7a



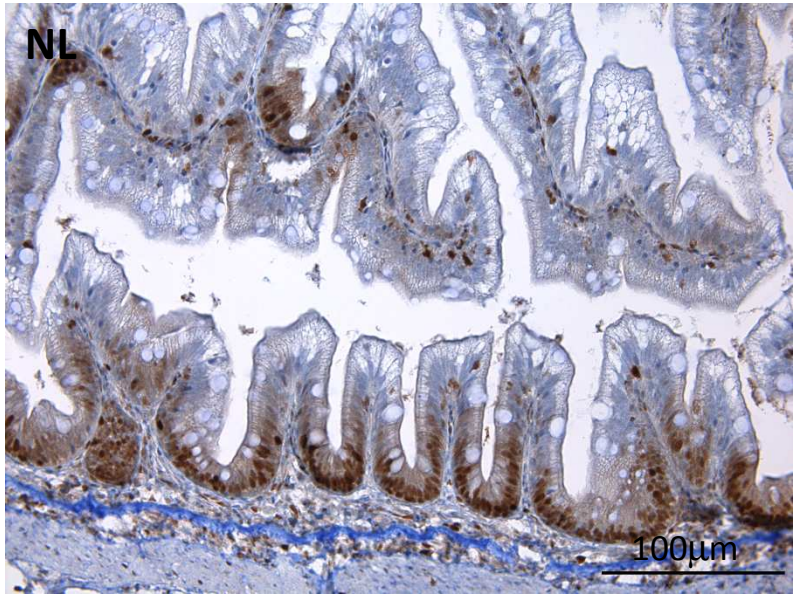
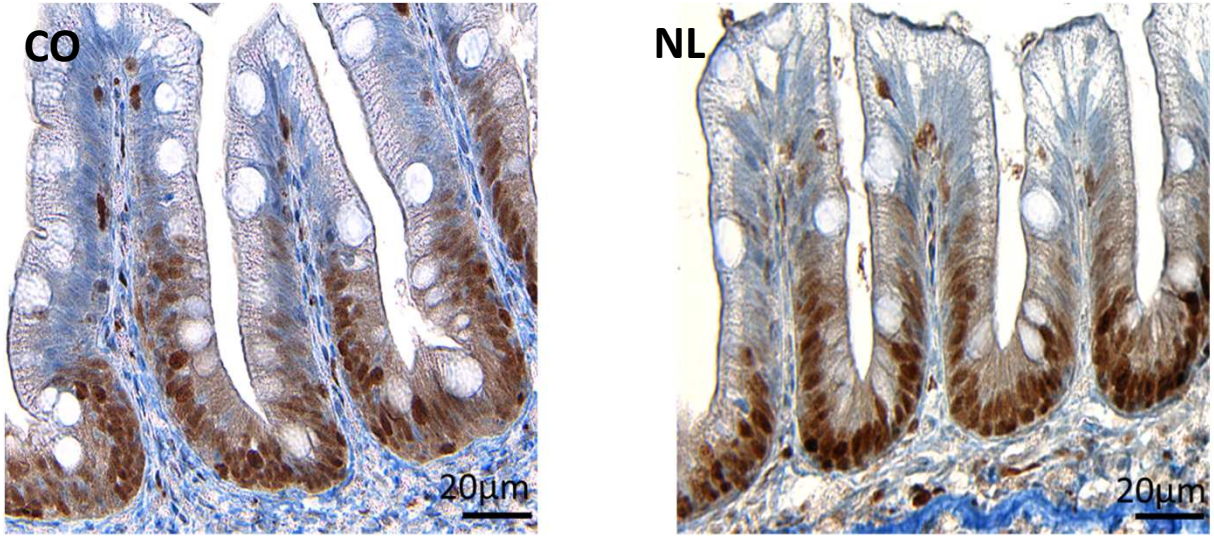
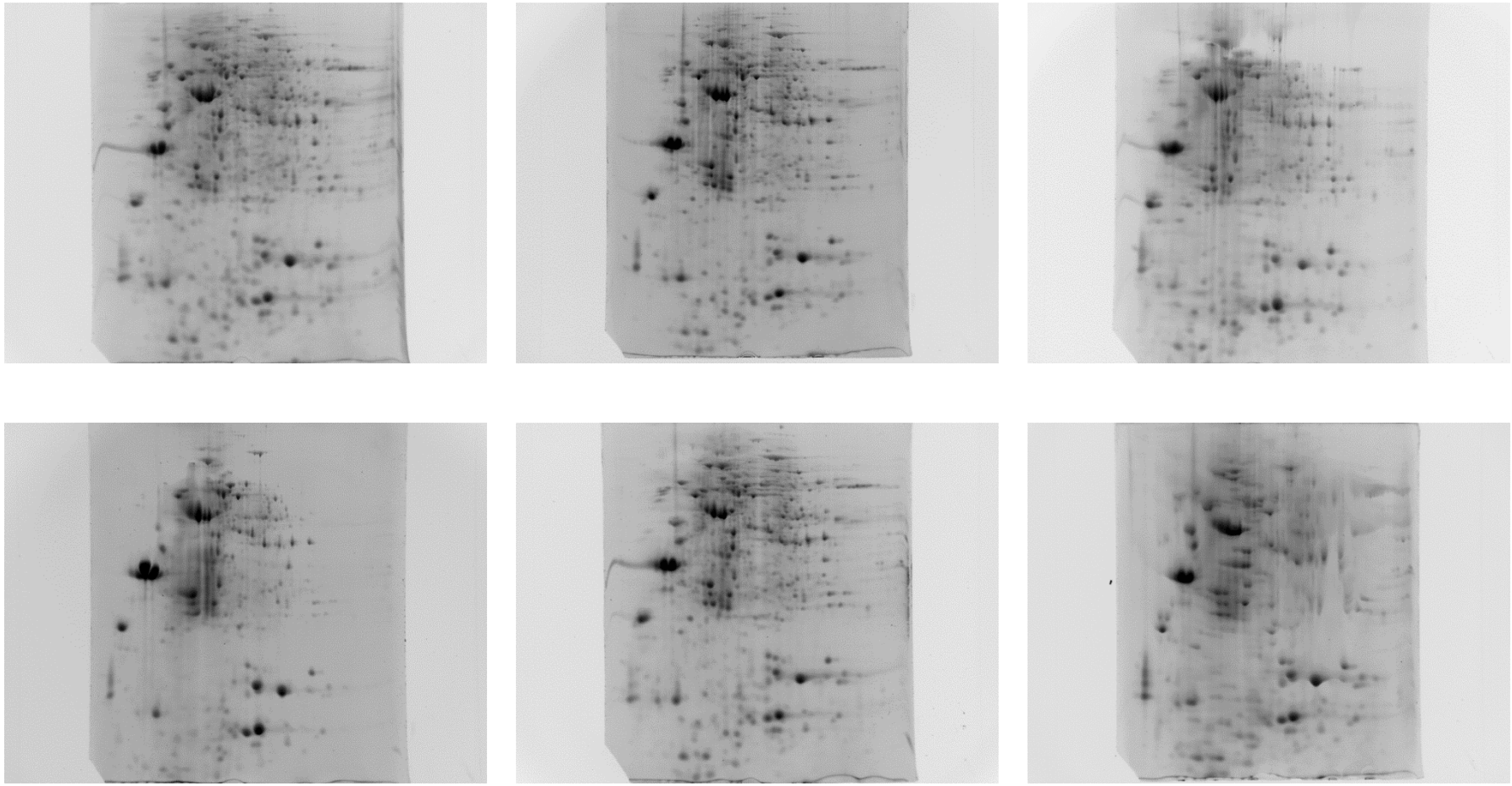


Figure 7b



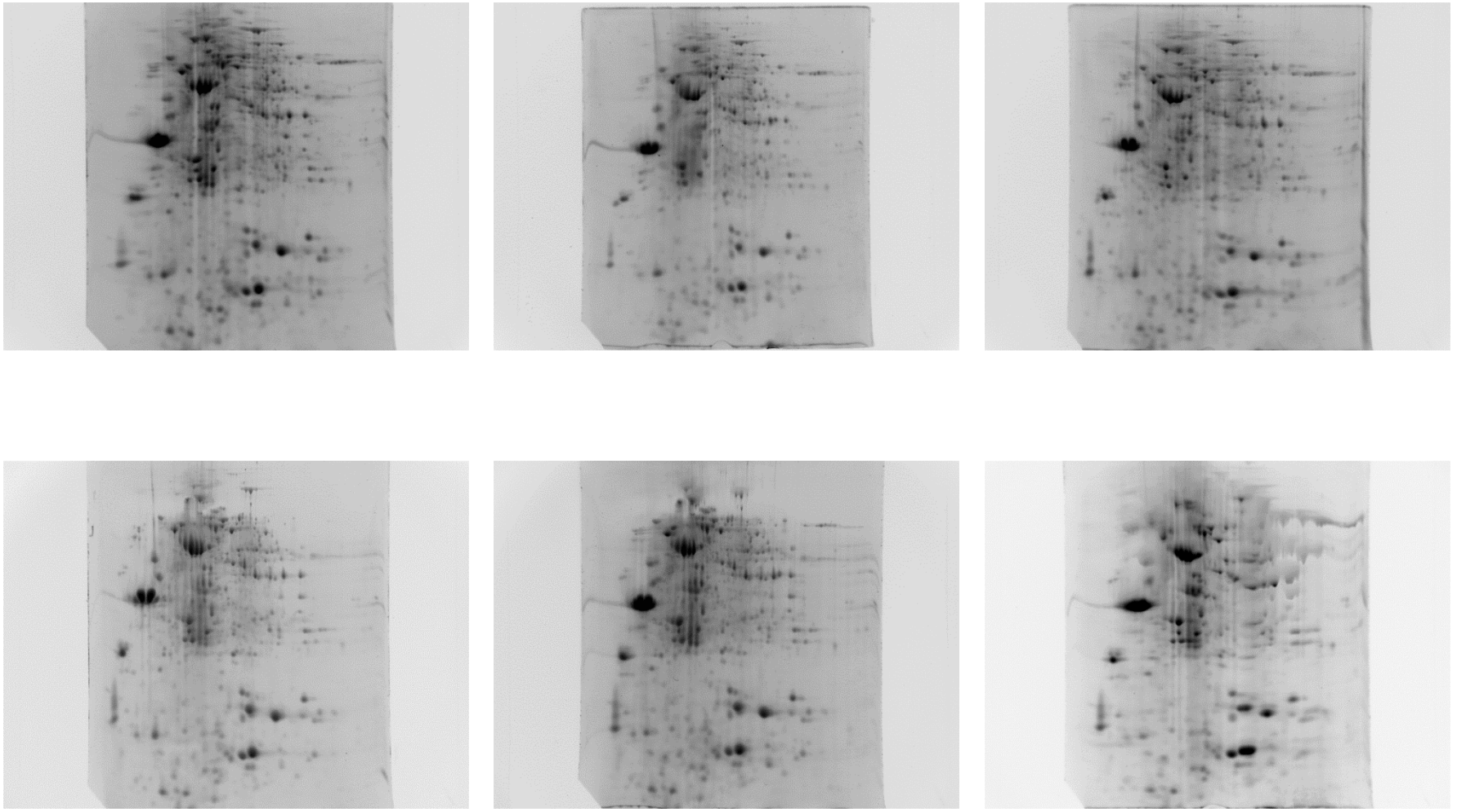
Supplementary figure 1

Control

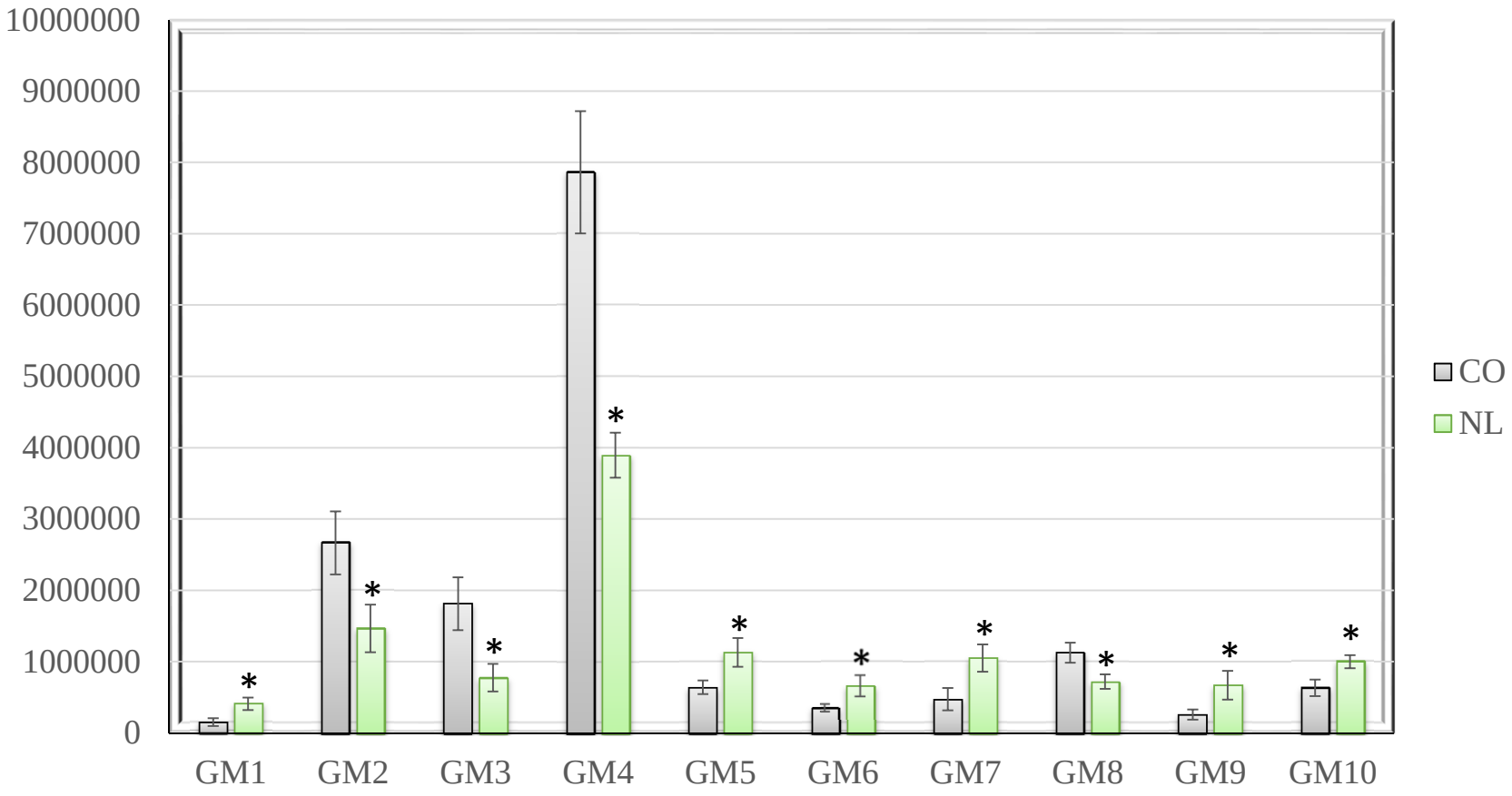


Supplementary figure 1

NL

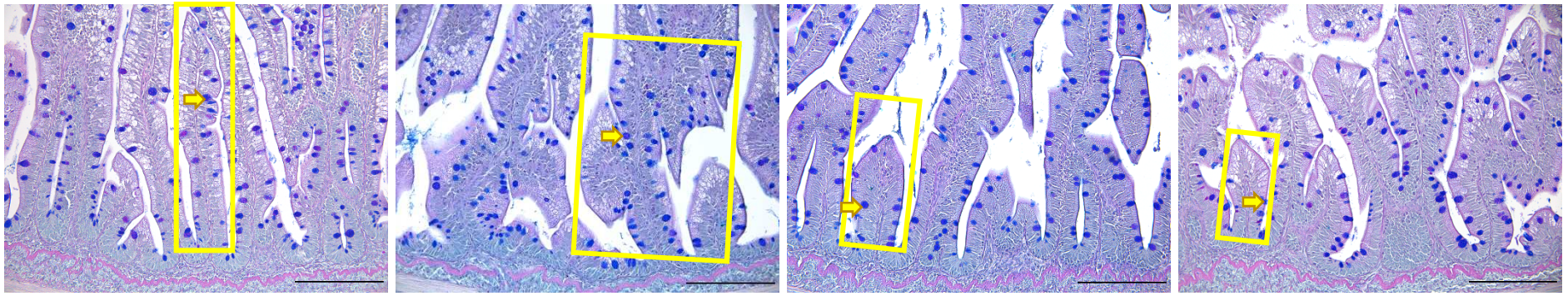


Supplementary figure 2



Supplementary figure 3a

CO



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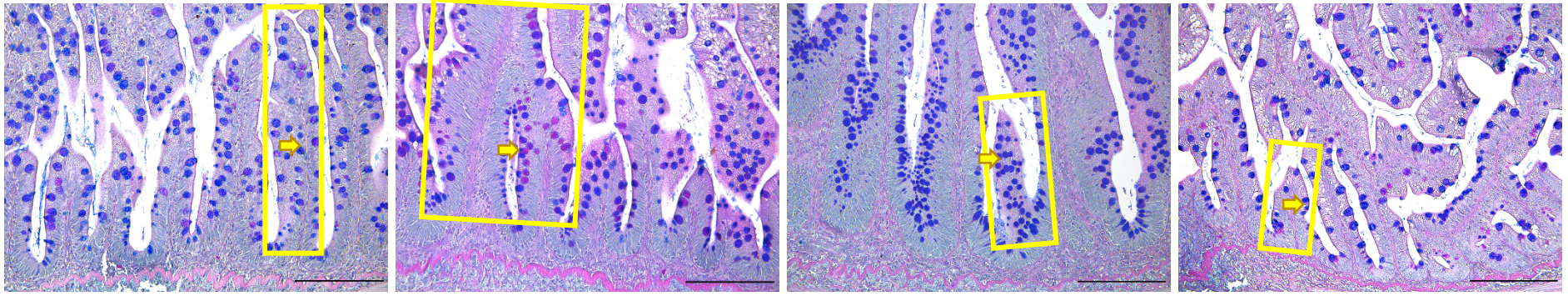
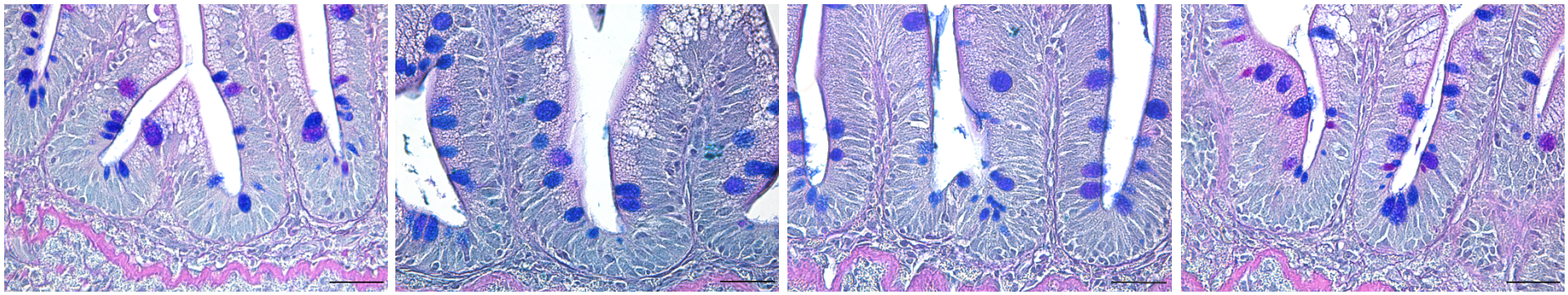


Supplementary figure 3b

CO



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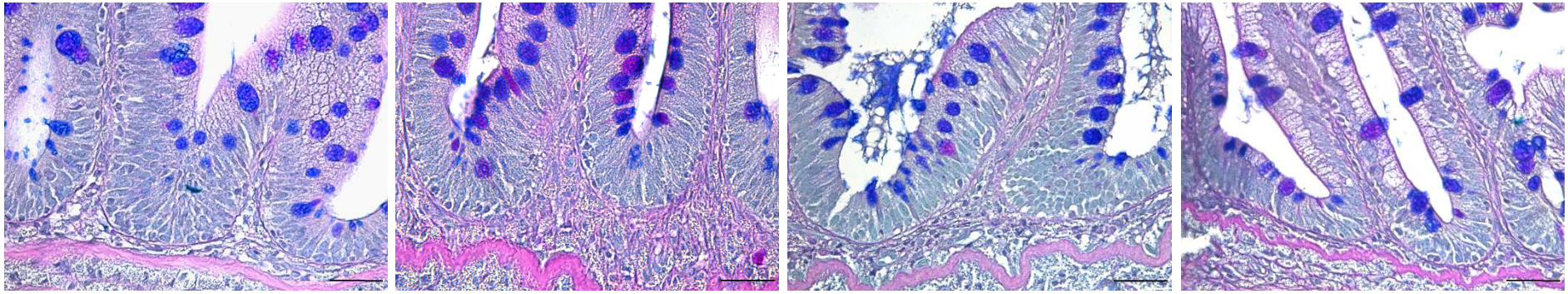
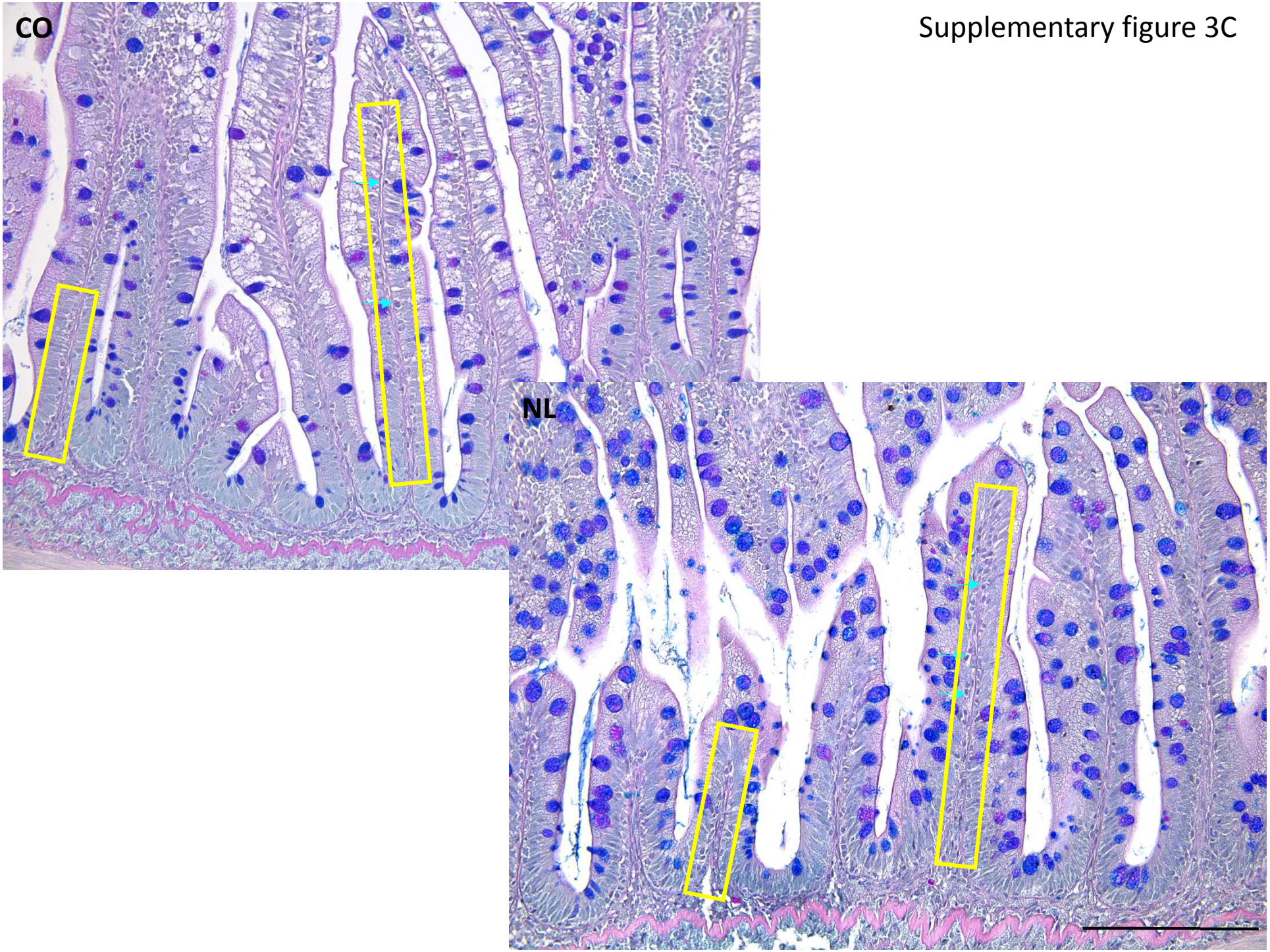
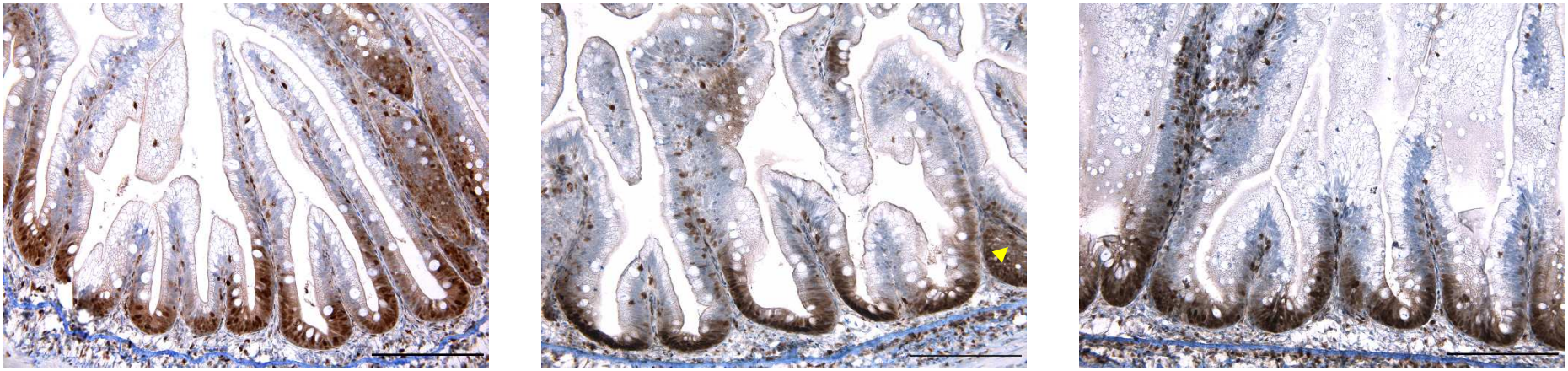


Supplementary figure 3C



Supplementary figure 4a

CO



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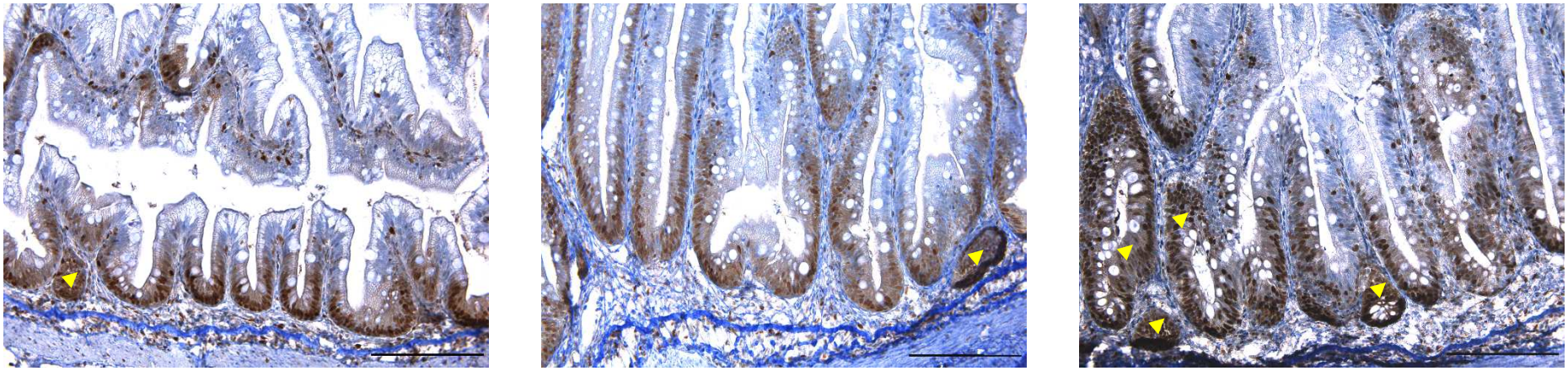
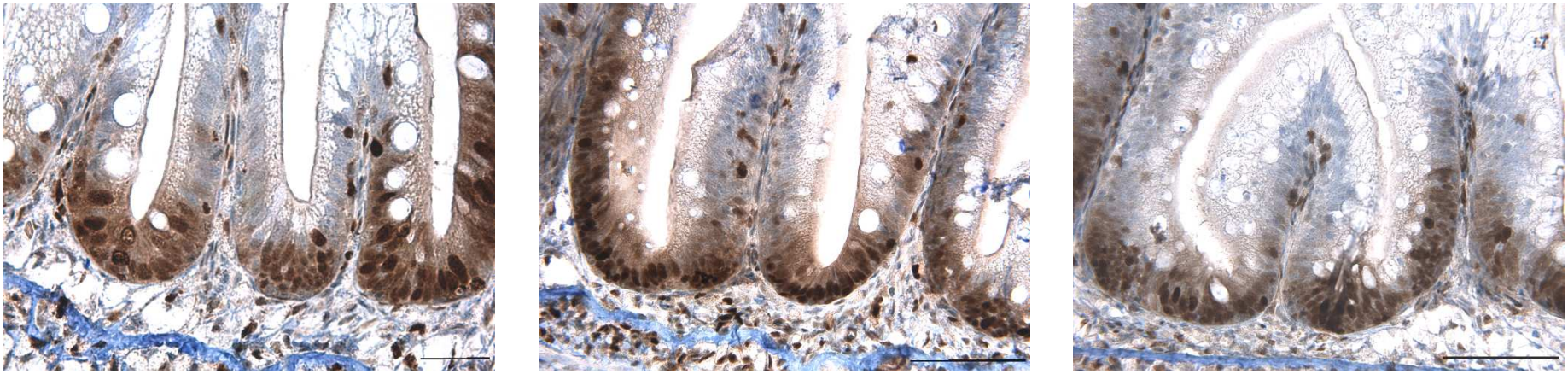


Supplementary figure 4b

CO



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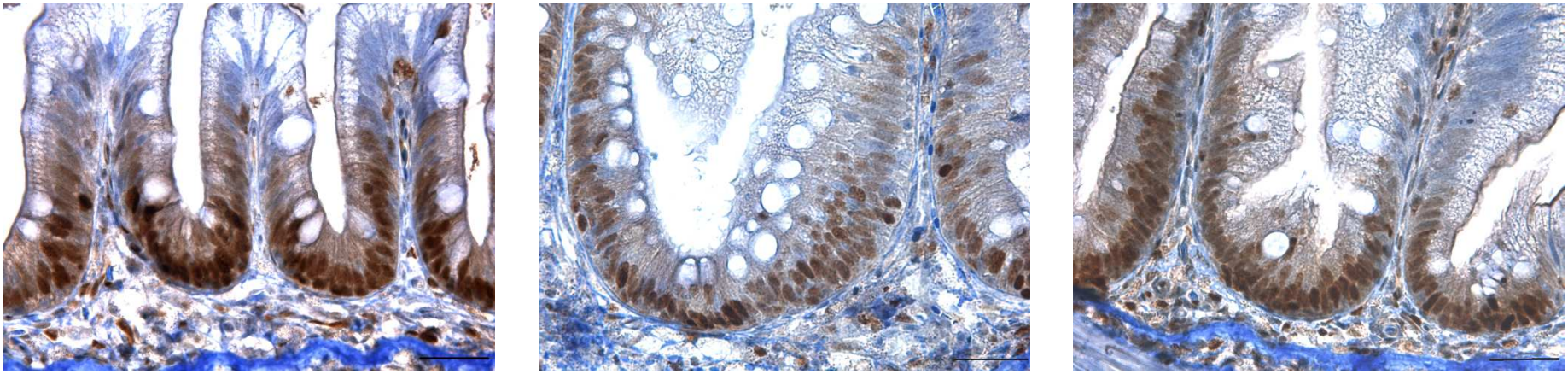

- Recognition and responses of purified β-glucan product at the intestinal level
- Upregulation of genes of C-type lectin receptors
- Overexpression of proteins linked to uptake and substrate recognition
- Presence of more immune cells