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Dietary fat and fiber interact to uniquely modify global histone post-translational epigenetic programming in a rat colon cancer progression model

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Abstract

Dietary fermentable fiber generates short-chain fatty acids (SCFA), e.g., butyrate, in the colonic lumen which serves as a chemoprotective histone deacetylase inhibitor and/or as an acetylation substrate for histone acetylases. In addition, n-3 polyunsaturated fatty acids (n-3 PUFA) in fish oil can affect the chromatin landscape by acting as ligands for tumor suppressive nuclear receptors. In an effort to gain insight into the global dimension of post-translational modification of histones (including H3K4me3 and H3K9ac) and clarify the chemoprotective impact of dietary bioactive compounds on transcriptional control in a preclinical model of colon cancer, we generated high-resolution genome-wide RNA (RNA-Seq) and “chromatin-state” (H3K4me3-seq and H3K9ac-seq) maps for intestinal (epithelial colonocytes) crypts in rats treated with a colon carcinogen and fed diets containing bioactive (i) fish oil, (ii) fermentable fiber (a rich source of SCFA), (iii) a combination of fish oil plus pectin or (iv) control, devoid of fish oil or pectin.

In general, poor correlation was observed between differentially transcribed (DE) and enriched genes (DERs) at multiple epigenetic levels. The combinatorial diet (fish oil + pectin) uniquely affected transcriptional profiles in the intestinal epithelium, e.g., upregulating lipid catabolism and beta-oxidation associated genes. These genes were linked to activated ligand-dependent nuclear receptors associated with n-3 PUFA and were also correlated with the mitochondrial L-carnitine shuttle and the inhibition of lipogenesis. These findings demonstrate that the chemoprotective fish oil + pectin combination diet uniquely induces global histone state modifications linked to the expression of chemoprotective genes.

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Introduction

"Nutri-epigenetics" describes the influence of dietary components on mechanisms modulating epigenetic programming. These processes are typically mediated by specific histone modifications (methylation and acetylation) and thus serve as a promising new target for cancer prevention strategies[1, 2]. Recent evidence indicates that epigenetic alterations contribute to cancer-related cellular defects. For example, epigenetic silencing of critical genes, e.g., detoxifying enzymes, tumor suppressor genes, cell cycle regulators, apoptosis-inducing and DNA repair genes, nuclear receptors such as NRF2, PPARs, FXR, HNF4A, mediated by promoter methylation and modification of histones and non-histone proteins by acetylation or methylation, drives malignant transformation[1, 3].

With respect to dietary chemoprevention, a plethora of published reports indicate a protective effect of fish oil and its bioactive components, n-3 polyunsaturated fatty acids (PUFA) e.g., eicosapentaenoic acid (20:5Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6Δ 4,7,10,13,16,19), with respect to colon cancer risk[4–6]. In contrast, dietary lipids rich in n-6 PUFA [found in vegetable oils; e.g., linoleic acid (LA;18:2Δ 9,12) and arachidonic acid (20:4Δ 5,8,11,14)] have been linked to an increase in colon tumor development[7–10]. It has also been previously demonstrated that the chemopreventive effect of fish oil is due to the direct action of n-3 PUFA and not to a reduction in the content of n-6 PUFA[11] .

Nuclear receptors function as ligand-activated transcription factors capable of regulating the expression of target genes considered vital to gut cell development and metabolism[1, 12]. With respect to diet-derived ligands, DHA and EPA and their oxidative metabolites have been shown to interact with specific ligand dependent nuclear receptors including CAR, HNF4A, PPARG, PXR and RXRA[12]. Since the original description of dietary fat as a regulator of gene expression over a decade ago, many transcription factors have also been identified as prospective indirect targets for n-3 PUFA regulation. For example, DHA can increase the activity of CREBBP, EP300, and MYC, while inhibiting NF-kB (NFKB1) and STAT3 activity[1, 12, 13]. Thus, n-3 PUFA function as nuclear receptor ligands with the potential to modulate transcriptional processes.

There are also many studies linking dietary fiber, gut microbiota and colon cancer prevention[14, 15]. The major metabolites produced by gut microbiota from readily fermentable fiber include short-chain fatty acids (SCFAs), such as butyrate, which has multiple beneficial effects at the intestinal and extra-intestinal level[14–17]. As more dietary fiber is ingested, SCFA production increases[18]. Even though some controversy remains concerning the in vivo responses of colonocytes to butyrate exposure, butyrate has been shown to impact cell kinetics, lumen pH, and epigenetics which modulate risk of developing colon carcinogenesis[19, 20]. There are at least 2 epigenetic mechanisms by which butyrate can increase histone acetylation in the colonic mucosa[21]. Bacteria in the gut generate SCFAs including butyrate, which can act as a histone deacetylase (HDAC) inhibitor or alternatively, in colonocytes, butyrate can be metabolized to acetyl-CoA and used for energy or transported to the nucleus and act as a histone acetyl transferase (HATs) substrate[18].

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Animals fed diets containing n-3 PUFA (fish oil) and pectin (which is fermented to SCFAs) as a fiber source maximally promote apoptosis and reduce cancer incidence in the colon compared with diets high in other dietary lipids and poorly fermentable fiber, e.g., n-6 (corn oil)[11, 20, 22]. In a follow-up study, the administration of butyrate-containing pellets for targeted release in the colon was used to demonstrate that butyrate and fish oil work coordinately in the colon to promote apoptosis and reduce aberrant crypt foci levels[9]. Subsequently, DHA and butyrate were shown to synergistically enhance apoptosis in colonocyte cultures compared with butyrate alone[23, 24]. A clinical validation of the chemoprotective effects of fat × fiber interaction was recently reported by Orlich et al., who demonstrated that the pescovegetarian diet is a highly protective regimen in terms of colorectal cancer prevention[25].

From a preclinical perspective, the azoxymethane (AOM) chemical carcinogenesis murine model serves as one of the most definitive means of assessing human colon cancer risk[26, 27]. We have previously demonstrated that at 10 weeks post AOM injection, the colonic mucosa is precancerous, e.g., high multiplicity aberrant crypt foci are apparent. Macroscopic tumors are not detectable until ~34 weeks post AOM injection[11]. From a temporal perspective, major cellular functions and pathways, including drug metabolism, cell cycle regulation, DNA damage repair and targeted apoptosis, response to inflammatory stimuli, cell signaling, and cell growth control and differentiation are progressively dysregulated in this model[28, 29].

In this study, we employ state-of-the-art technologies and bioinformatics algorithms in order to explore 'nutri-epigenomics' at a genome-wide level and document epigenetic mechanisms related to dietary chemoprevention. By determining transcriptional levels of regulation (H3K4me3, H3K9ac and mRNA expression) in colonocytes from the same animals, we were able to gain a greater understanding of the epigenome associated with the interaction of fish oil (rich in DHA/EPA n-3 PUFA), pectin (a readily fermentable fiber) and AOM (colon carcinogen) treatments.

Methods

Animals

Sixty-eight weanling male Sprague Dawley rats (Harlan, Houston, TX) were individually housed and acclimated for 1 week in the same room, maintained in a temperature and humidity-controlled animal facility with a daily 15 h light/9 h dark photoperiod. The animal use protocol was approved by the Animal Care Committee of Texas A&M University and conformed to NIH guidelines. In this study, the combinatorial effects of four experimental diets and two treatments (injection of AOM or saline control) were examined (Supplementary Methods Figure 1 and 2). Animals were stratified by body weight across quartiles and sampled after the acclimation period so that mean initial body weights did not differ[30] (Supplementary Methods Figure 1). Body weight and food intake were monitored throughout the study.

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Diets

After 1-week acclimation on standard pelleted diet, rats were assigned to one of four diet groups, which differed in the type of fat and fiber. The diets contained (g/100 g diet): dextrose, 51.06; casein, 22.35; D,L-methionine, 0.34; AIN-76 salt mix, 3.91; AIN-76 vitamin mix, 1.12; and choline chloride, 0.22. The total fat content of each diet was 15% by weight as follows: n-6 fat diet, 15.00 g corn oil (Dyets Inc.)/100 g diet; n-3 fat diet, 11.50 g fish oil/100 g diet (OmegaPure TE from Omega Protein Inc); and 3.50 g corn oil/100 g diet. The total fiber content of each diet was 6% by weight of pectin (fermentable fiber from Gum Technology) or cellulose (non-fermentable fiber from Bio-Serv). To prevent formation of oxidized lipids, diets were stored at −20°C and provided daily to the animals. To protect against lipid oxidation during storage, 0.025% tertiary butylhydroquinone and mixed tocopherols (MTS-50; ADM, Decatur, IL) were added to the oils prior to mixing. For quality control purposes, a fatty acid analysis of the experimental diets was performed by gas chromatography (see Supplementary Methods Table 1 for details).

Carcinogen Treatment

After 2 weeks of feeding, 24 rats were injected with saline (control), and 43 rats were AOM (Sigma, St. Louis, MO) injected s.c. at 15 mg/kg body weight. Each rat subsequently received a second AOM or saline injection 1 week later and animals were terminated 10 weeks after the first AOM injection.

Aberrant Crypt Foci Scoring

Immediately after removal, the colon lengthwise (11 per diet group with AOM injection and 2 per diet with saline injection) was flattened between Whatman 1 filter paper and fixed in 70% ethanol for 24 h. Subsequently, the whole mount colon was stained with 0.5% methylene blue in PBS for 30 sec, placed on a plastic sheet with a 5-mm grid, and examined under the microscope at 400×. The number of aberrant crypts (putative colon cancer precursors), as singlets and multiples was determined. Crypts were classified as aberrant using the morphologic characteristics described previously[31]. The number of high multiplicity aberrant crypt foci (HM-ACF) (more than three aberrant crypts per foci) was scored on half of the total colon as previously described.

Isolation of Colonic Crypts

The large intestine was resected from the junction between the cecum and the rectum, and was opened longitudinally and washed in 1× PBS. Subsequently, the visible “herringbone” folds were used to identify the proximal colon. Colonic crypts were extracted from the distal region (distal colon) as previously described[32]. Following incubation, tissue sections were placed in a petri dish on ice, and the colonic crypts isolated by scraping with a rubber policeman. Isolation of crypts was verified by histological examination to ensure that epithelial cells were removed and the lamina propria and muscle layers remained intact. Cells were washed with HBSS and centrifuged at 100 × g for 15 min. The pellet was resuspended in HBSS and an aliquot of the isolated crypts was subsequently used to generate mRNA expression profile libraries. The remaining crypts were immediately crosslinked for ChIP analysis.

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Western Blotting

Colonic crypt nuclear protein was analyzed by immunoblot as previously described[32] Colonic crypts were rocked in 50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100 with protease inhibitors for 10 min at 4°C, followed by centrifugation at 1,350 × g, 4°C for 7 min. The crypt-containing pellet was subsequently resuspended in 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA with protease inhibitors and incubated by gently rocking at room temperature for 10 min. Nuclei were pelleted by centrifugation at 1,350 × g, 4°C for 7 min and resuspended in RIPA buffer (50 mM HEPES-KOH, pKa 7.6, 500 mM LiCl, 1 mM EDTA, 1.0% NP-40, 0.7% Na-deoxycholate with protease inhibitors). Nuclear lysates (2 ug protein) were treated with 1× pyronin sample buffer and subjected to SDS polyacrylamide gel electrophoresis (PAGE) in precast 4–20% Tris-glycine mini gels (Invitrogen). After electrophoresis, proteins were electroblotted onto a polyvinylidene fluoride membrane with the use of a Hoefer Mighty Small Transphor unit at 400 mA for 90 min. Following transfer, the membrane was incubated in 5% milk and 0.1% Tween 20 in TBS (TBST) at room temperature for 3 h with shaking, followed by incubation with shaking overnight at 4°C with primary antibody diluted in 5% milk in TBST. Membranes were washed with TBST and incubated with secondary peroxidase conjugated secondary antibody as per manufacturer's instructions. Bands were developed using Pierce SuperSignal West FemtoTM maximum sensitivity substrate. Blots were scanned using a Fluor-S Max MultiImager system (Bio-Rad, Hercules, CA). Quantification of bands was performed using Quantity One software (Bio-Rad). Primary antibodies used to detect histones included H3 tri methyl K4 (Active Motif 39160), H3 acetyl K9 (ab10812), H4 acetyl K16 (Active Motif 39929), pan-acetylated histone H3 (ab47915) and histone H3 (ab1791) levels. Peroxidase conjugated goat anti-rabbit IgG was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Chromatin Immunoprecipitation

ChIP-seq analyses were performed in order to determine global histone mapping in crypts isolated from the distal colon. The ChIP protocol described by Triff et al was utilized[32] with one modification, cells were cross-linked by adding freshly prepared formaldehyde at 1% concentration for 15 min at room temperature. Cells were then lysed and sheared in 3 mL tubes at 4°C using a Covaris S2 sonicator to obtain DNA distributions of ~300 bp (range of 200–400 bp): duty cycle −20%, intensity −8, cycles/burst −200 and time 25 min. ChIP antibodies included: ChIP Grade (Active Motif 39160) anti-histone H3 (tri methyl K4) antibody, ChIP Grade anti-histone H3 acetyl K9 antibody (ab10812). Antibody–chromatin complexes were captured using Dynabeads G Protein coupled (Dynal) and eluted with 1% SDS in 50 mM Tris-HCl pH 8.0, and 10 mM EDTA, after extensive washing. Cross-linking between DNA and chromatin proteins was reversed by incubation at 65°C overnight. DNA was purified by treatment with RNAseA, proteinase K and QIAquick PCR Purification Kit (Qiagen 28004) and dissolved into 50 μL EB (10 mM Tris pH8.0) buffer per sample for immunoprecipitation purposes. The specificities of all antibodies were validated by Western blot and ChIP-qPCR. Equal amounts of (200–500 bp) ChIPed DNA from 2-3 AOM injected rats from the same dietary treatment (biological replicates) were pooled into 16 barcoded groups (representing 43 individual rats), and the saline biological replicates were similarly

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pooled into 12 barcoded groups (representing 24 individual rats) prior to high throughput sequencing (Supplementary Methods Figure 2).

ChIP Sequencing

BioScientific NETflex (ChIPseq kit 5143-01, Barcodes kit 514120) multiplex libraries from ChIPed DNA (10 nM) were sequenced using an Illumina HiSeq 2000 DNA Sequencer. Sequence reads with poor quality bases and with adaptors or other contaminants were filtered. The remaining reads (>290 million total per sample) were mapped to the reference rat genome (rn4) with commonly used BWA for Illumina (version 1.2.3) settings and only non-identical uniquely mapped reads were retained. The peak caller program MACS (version 1.4.1)[33] was used. Islands (enriched regions) were defined as the genomic areas enriched with the ChIPed protein (peaks aka enriched regions) in at least one sequenced sample (using merge function of BEDTools[34]), and reads were quantified using coverageBed function of BEDTools[34]. The UCSC Genome Browser was used to visualize bigwig data tracks. The nearest gene to each island, i.e., within 5 kb of the island was identified using closestBed from the BEDTools software suite[34] and the refGene table downloaded from the UCSC Genome Browser for the Baylor 3.4/rn4 assembly files.

Regions showing differences in histone modification were identified using the Bioconductor-edgeR package[35, 36] for the R software environment[33, 35]. In order to increase statistical power (higher number of samples per treatment) and focus on key dietary and AOM effects associated with cancer progression, rats were pooled across the various treatment groups described above. Read counts per gene were normalized using the scaling factor method of Anders and Huber[37]. Differential expression testing of genes was performed using likelihood ratio tests on the negative binomial GLMs estimated by edgeR[35, 36] (Supplementary Methods Figures 4 and 5). Regions with FDR < 0.1 and a minimal threshold of one count per million mapped reads in at least four samples were selected as differentially enriched regions (DERs) (Supplementary Table 2). ChIPseq data was validated by qPCR on all 68 rat samples. See Supplementary Methods Figure 6 and 7 for additional details. See Supplementary Methods for additional details.

RNA Isolation

For total RNA isolation, colonic crypts were homogenized on ice in lysis buffer (RNAqueous Isolation kit, Ambion) and frozen at −80°C until RNA was isolated. Subsequently, total RNA was isolated using the RNAqueous kit, followed by DNase treatment. RNA integrity was analyzed on an Agilent Bioanalyzer to assess RNA integrity.

RNA Sequencing

Total RNA (1000 ng) was used to generate multiplex libraries for whole-transcriptome analysis following Illumina's TruSeq RNA v2 sample preparation protocol. Libraries from 24 individual rats per treatment were sequenced on an Illumina HiSeq 2000. At least 151 million, 50 bp single-end reads per treatment were obtained for each sample. Reads were mapped with the STAR aligner using the default parameters and rat genome assembly[38]. Greater than 85% of reads aligned uniquely to the rat genome. Genes that did not have at least one read count per million mapped reads in at least four samples were removed. Read

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counts per gene were normalized using the scaling factor method of Anders and Huber[37]. The read counts were modeled directly using negative binomial generalized linear models accounting for the differences in diet and subsequently fit with the R programming language[33] and Bioconductor package edgeR[35, 36]. Differential expression was then tested using likelihood ratio tests involving the fitted models (Supplementary Methods Figures 4 and 5). Genes with false discovery rate adjusted p-values (FDR) less than 0.1 were selected as differentially expressed transcripts (DE) (Supplementary Table 2). See Supplementary Methods for additional details.

Ingenuity Pathway Analysis

"Functional enrichment" analysis was performed using Ingenuity Pathway Analysis (IPA) version 2.0 software (Ingenuity Systems Inc., Redwood City, CA) as we have previously described[39].

Data Access

Sequencing data have been deposited in the GEO database under the accession number GSE87525.

Results

We have demonstrated that dietary n-3 PUFA and pectin synergistically suppress colon tumorigenesis[9, 23, 24, 40]. To elucidate the epigenetic mechanisms related to the unique properties of this chemoprotective combination, experiments were designed to contrast the chemoprotective components (fish oil and/or pectin) against the control diet (corn oil and cellulose) lacking chemoprotective bioactives in the presence and absence of carcinogen. Three main biological comparisons were examined: 1) dietary fat effects (fish oil vs corn oil), 2) dietary fiber effects (pectin vs cellulose) and *3*) dietary fat × fiber interaction in the presence of carcinogen [fish oil + pectin + AOM (FPA) vs corn oil + cellulose + AOM (CCA)].

Enumeration of aberrant crypts

Whereas AOM-treated rats developed aberrant crypts, their saline-treated counterparts did not. Therefore, all aberrant crypt data represent AOM-injected groups only. No HM-ACF were detected in the proximal colon, independent of the diet (data not shown). Rats fed the corn oil + cellulose (control) diet exhibited a greater number of total HM-ACF compared with other treatment groups. In addition, the fish oil + pectin-fed rats had the lowest incidence of HM-ACF compared with all other groups (Figure 1.A and Supplementary Figure 1.A).

Overall effects of fish oil and pectin feeding on transcription and histone tail modifications

In an effort to identify key, genome-wide, bioactive effects associated with fish oil and pectin feeding, gene expression profiling by Next Generation Sequencing of RNA, H3K4me3 and H3K9ac ChIPed DNA were performed. Experimental groups are referred to by 3-letter acronyms based on type of fat, fiber, and carcinogen treatment: fish oil + pectin + AOM (fpa), corn oil + pectin + AOM (cpa), fish oil + cellulose + AOM (fca), corn oil +

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cellulose + AOM (cca), fish oil + pectin + saline (fps), corn oil + pectin + saline (cps), fish oil + cellulose + saline (fcs), corn oil + cellulose + saline (ccs). For dietary lipid comparisons, fish oil induced differentially expressed transcripts (DE) and differentially enriched chromatin regions (DERs) were determined by pooling sequence data from individual rats fed a fish oil diet across the various treatment groups in comparison to rats fed a corn oil diet (fcs+fps+fca+fpa vs ccs+cps+cca+cpa; n=34 fish oil fed rats vs n=33 corn oil fed rats). Similarly, pectin induced DEs and DERs were determined by pooling rats fed a pectin diet across the various treatment groups and comparing them against rats fed a cellulose diet (cps+fps+cpa+fpa vs ccs+fcs+cca+fca; n=34 pectin fed rats vs n=33 cellulose fed rats). To assess the consequence of each bioactive treatment, we used the corn oil (rich in n-6 PUFAs) + cellulose (rich in poorly fermentable fiber) diet as a control[4]. Data for AOM: fpa vs cca, fca vs cca, cpa vs cca, and for saline (control): fps vs ccs, fcs vs ccs, cps vs ccs) are presented in Figure 1.B and Supplementary Table 1. Figure 1.C shows the distribution of expression strength (x-axis) relative to the log-ratio of DE and DERs (y-axis) as an MAplot. Included above each MAplot are the total number of DE genes (including different isoforms) and the total number of DERs (annotated and un-annotated). Figure 1.B summarizes the total number of genes with differentially expressed (DE) transcripts and differentially enriched peaks (DERs) with an FDR<0.1 and a p-value<0.01 in fish oil vs corn oil and readily fermentable (pectin) versus poorly fermentable (cellulose) fiber. Fish oil feeding altered the transcription of 163 genes along with 58 K9ac and 5 K4me3 regions with DERs (FDR<0.1) (Figure 1.C), with similar numbers of up- and downregulated genes detected at each level (Supplementary Table 1).

Based on previous studies indicating that histone tail modifications regulate gene expression at the transcriptional level[41], we expected genes with K4me3 and K9ac DERs to correlate with differentially expressed (DE) genes at the RNA level. Generation of a global plot of all the K4me3 and K9ac changes (DER) against the DE for the same gene revealed poor correlation between histone marks and RNA expression, regardless of p-values (the axes include total number of genes with an FDR<0.1 (Figure 2.A). Similarly, a poor correlation was observed between annotated K9ac and K4me3 DERs (Figure 2.A and Supplementary Figure 1.B). Interestingly, there were no genes with an FDR<0.1 that were simultaneously modulated at the RNA, K9ac and K4me3 level by fish oil. A more relaxed filtering parameter using genes with a Fisher's Exact test p-value<0.01 revealed only 4 genes that were simultaneously affected at all epigenetic and transcriptional levels tested (Supplementary Figure 1.B). This gene set included upregulated tumor suppressors CDH11 (cadherin 11) and SCD2 (stearoyl-Coenzyme A desaturase 2) and downregulated oncogenes CERS4 (ceramide synthase 4) and PDE4B (cAMP-specific phosphodiesterase 4B) (Supplementary Table 1). Very few genes with an FDR<0.1 were modulated by pectin. Specifically, no genes with an FDR<0.1 were simultaneously affected at the RNA, K9ac and K4me3 level by pectin, and only 2 genes with p<0.01 were simultaneously affected between levels. For example, EGFLAM (EGF-like, G domains) was upregulated and oncogene ANXA3 (annexing 3) was downregulated (Supplementary Figure 1.D). Among the fish oil DE genes, 8 have been previously associated with DHA and lipid metabolism, including the upregulated transporter FABP1 (Supplementary Figure 1.D), a fatty acid binding protein often downregulated in colon cancer and upregulated by DHA[42, 43]. Four mitochondrial

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enzymes previously shown to be modulated by DHA were also upregulated, acyl-CoA synthetase ACSBG1[44], carnitine palmitoyltransferases CPT1 and CPT2[45], and the kinase MAP2K1[40]. Stearoyl-CoA desaturase SCD was also upregulated and phospholipase A2 PLA2G1B was downregulated (Supplementary Table 1).

Since genes typically function as part of intricate networks, prior biological knowledge greatly facilitates the meaningful interpretation of the gene-expression changes associated with large datasets. Therefore, we used pathway analysis to help interpret the data in the context of biological processes, pathways and networks. Functional analysis of DEs and DERs using Ingenuity Pathway Analysis (IPA) software was performed in an effort to better understand the biological relevance of the genes modulated by fish oil feeding. Pathway analysis of the 32 genes with K9ac DERs (FDR<0.1) revealed 23 genes related to Metabolic Disease, Lipid Metabolism, and Cell Death and Survival networks (Supplementary Table 2). Network analysis using a less stringent filtering parameter on K9ac DERs ($p<0.01$) revealed similar types of biological processes affected by fish oil at the RNA and K4me3 levels (Supplementary Table 2). Therefore, although the RNA, K9ac, and K4me3 networks were mostly comprised of different genes, the top affected metabolism pathways were correlated with respect to Lipid Metabolism, Small Molecule Biochemistry, and Vitamin and Mineral Metabolism (Supplementary Table 2).

Assessment of nuclear levels of H3 and H4 acetylation

Fermentable fiber supplementation had no effect on total histone acetylation when corn oil was the lipid source during cancer progression (AOM). In contrast, when fish oil was the lipid source, fermentable fiber enhanced histone H3 pan-acetylation and K9 acetylation (Figure 3.A) along with H4K16 acetylation (Figure 3.B) while total levels of nuclear H3K4me3 remained unchanged (Figure 3.C). In contrast, there was no effect of diet on protein levels of H3ac, H3K9ac, H4K16ac or H3K4me in saline-injected rats (Supplementary Figure 2).

Context specific epigenetic effects of fish oil and pectin

Differentially transcribed genes (DEs) and K4me3 and K9ac differentially enriched (DERs) genes in common between saline and AOM injected rats fed the same diet were compared to determine whether dietary bioactives exhibit the same effects on a healthy colon (fps vs ccs; fcs vs ccs; cps vs ccs) versus a precancerous colon (fpa vs cca; fca vs cca; cpa vs cca) (Figure 2.B). Dietary effects differed in healthy (saline) vs carcinogenic (AOM) conditions at all transcriptional and epigenetic levels. Only in the fish oil + pectin treatment, at the transcription level, was a slightly higher number of common genes detected, with 42 genes differentially expressed in both saline and AOM treated animals (Figure 2.B). Among these genes, 17 were upregulated and 18 were downregulated in both datasets. In contrast, 7 additional genes were upregulated in AOM injected animals but downregulated in saline injected animals fed the same diet (Supplementary Table 1). Poor correlation between transcriptional and epigenetic levels (RNA, K4me3 and K9ac) for all treatment comparisons (listed under “treatment comparison” in Figure 1.B) was observed. Furthermore, the epigenetic and functional response of colonocytes to fish oil and pectin individually were distinct relative to the combination fish oil + pectin diet (Figure 2.C and Supplementary

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Figure 1.C). Among these 6 individual comparisons, the most noteworthy changes (FDR<0.1) were the transcriptional effects of fish oil + pectin feeding in the presence of carcinogen (fpa vs cca) with 83 DEs (Supplementary Figure 1.C).

Identification of upstream regulators modulated by dietary fat and fiber interaction

Upstream Regulator (URs) analysis was used to identify the transcriptional regulators linked to fish oil + pectin, i.e., (fpa vs cca). Causal networks constructed from individual relationships curated from the literature were used to create mechanistic hypotheses that explain changes in DE and DER gene expression. Statistical approaches were used to identify and score those Upstream Regulators whose connections to our dataset genes were unlikely to occur in a random model[46]. Initially, we quantified known targets of transcriptional regulators present in our dataset and compared their direction of change (over- or under-expression) to predict likely relevant regulators including transcription factors, nuclear receptors and enzymes (Supplementary Figure 3). The analysis was performed on the gene sets of all 6 individual comparisons listed in Figure 1.B at each transcriptional and epigenetic level (total of 18 gene sets with $p<0.01$) as well as on fpa vs cca DE genes with an FDR<0.1 (Supplementary Table 3). Ligand dependent nuclear receptors were prevalent among the top modulated URs linked to the greatest numbers of differentially transcribed (DE) genes in fpa vs cca (Figure 4.A). These nuclear receptors included activated PPARs alpha, delta, and gamma along with LXR (NR1H), FXR (NR1H4), PXR (NR1I2), GCR (NR3C1) and HNF4A. Some of the receptors were also activated, to a lesser extent, in other fish oil containing diets under saline and carcinogenic conditions at the transcriptome and K9ac levels. Except for glucocorticoid receptor (NR3C1), HNF4A and FXR (NR1H4), the nuclear receptors were predicted to be activated only at the RNA and K9ac states (Supplementary Figure 3). Furthermore, 39 of the 68 fpa vs cca DE genes associated with lipid metabolism were also part of the 58 genes directly connected to the nuclear receptors. The lipid metabolism related genes with K9ac differential enrichment (DERs) were distinct from lipid metabolism DE genes (Supplementary Table 2).

With regard to the unique properties of the combination diet, we also assessed which biological functions were modulated at the histone modification levels (K4me3 and K9ac) during carcinogenesis. Pathway analysis revealed 29 genes were associated with biological processes linked to lipid metabolism, especially increased beta-oxidation of fatty acids (Figure 4.B). Nineteen of these genes were linked to cellular functions associated with a decreased accumulation of lipids (such as cholesterol, acylglycerols and fatty acids) ($p<0.0001$, z-score −1.91) and 17 were linked to increased fatty acid metabolism ($p<0.001$, z-score 2.37). More specifically, these genes included ABCD3 and CPT2, vital to the mitochondrial l-carnitine shuttling process, beta-oxidation acyl-CoA enzymes, ACSBG1 (which also plays a role in the activation of DHA) and ACADM. In addition, genes linked to the metabolism of acyl-CoA (DBI and ACOT1) and transporters that regulate beta-oxidation of very long chain fatty acids (ABCD3 and FABP1) were detected (Figure 4.B). Examination of the 267 DE genes with a $p<0.01$ (fpa vs cca) revealed an increase in the number (68) of lipid metabolism affiliated genes (Supplementary Table 2). Among lipid metabolism associated genes, only 6 (ACSBG1, AQP8, CPT2, CYP1A1, ENTPD5, and

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SCD) were upregulated in fish oil + pectin fed rats following saline injection, and only the cytochrome p450 subfamily member CYP1A1 was upregulated by the chemoprotective diets (Supplementary Table 1). Genes differentially expressed following FCA vs CCA comparison are described in Figure 4.B.

Discussion

To our knowledge, this is the first in vivo study to globally assess changes in histone post-translational modifications and the transcriptome in colonocytes during colon cancer progression. In this study, we employed novel technologies and bioinformatics algorithms, such as next-generation sequencing, in order to explore “nutri-epigenomics” at a genome-wide level and further elucidate epigenetic mechanisms related to chemoprevention. By determining multiple epigenetic levels of regulation (H3K4me3, H3K9ac) and mRNA expression in colonocytes from the same animals, we were able to gain a greater understanding of the histone states associated with the interaction of fish oil (rich in DHA/EPA n-3 PUFAs), pectin (a readily fermentable fiber) and AOM (colon carcinogen) treatments.

The data presented in our study show that pectin preferentially affects H3 acetylation status in the presence of fish oil and synergistically enhances transcription of lipid metabolism associated genes. Interestingly, the physiological and epigenetic stress induced by AOM was uniquely associated with the modulation of fatty acid metabolism during cancer progression (Figure 4 and Supplementary Tables 2 and 3). It is possible that the accumulation of acetyl-CoA associated with increased beta-oxidation activity, observed in AOM treated rats fed fish oil and pectin (Figure 4), promotes protein acetylation in colonocytes. This is consistent with previous findings indicating that excess acetyl-CoA is transported to the nucleus, where it acts as a histone acetyl transferase (HATs) substrate[18]. This could explain why the nuclear levels of H4K16ac, H3ac and K9ac increased only in rats fed the fish oil + pectin diet during cancer progression (Figure 3), suggesting that the nutritional combination of n-3 PUFA and fermentable fiber is being processed distinctly by colonocytes in response to biological stress[1, 12, 13, 18, 39, 44, 47, 48] (Figure 5). It is noteworthy, that a poor correlation was observed between the differential gene expression profiles of the transcriptome and the differential genomic enrichment profiles of H3K4me3 and H3K9ac (Figure 2 and Supplementary Figure 1). This, in part, may be explained by the fact that, regardless of diet, colon cancer progression is associated with global perturbations in histone state modifications linked to the expression of chemoprotective genes[39] (Supplementary Methods Figure 3). Collectively, these data reveal unique fish oil + pectin effects at the physiological (Figure 1.A), epigenetic (Figures 1.B and 3) and transcriptional (Table 1 and Figures 1.A and 4) levels. These biological responses are multifaceted and not necessarily straightforward, therefore further studies are required to delineate precisely how these phenotypes are linked.

Mounting evidence indicates that the pescovegetarian diet, high in n-3 PUFA and fiber, is a highly protective regimen in terms of colorectal cancer prevention[25]. Interestingly, we noted that fish oil + pectin feeding predominantly induced transcriptional changes in many genes associated with lipid metabolism (Figure 4), including enzymes and transporters. Specifically, a large cluster of upregulated genes were associated with increased fatty acid

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catabolism and a decreased accumulation of lipids such as cholesterol, triacylglycerols, acylglycerols and fatty acids (Figure 4.B). These transcriptional changes are consistent with previous studies by Mori et al., who assessed the effect of fish oil on the small intestine of obese mice and identified many of the same lipid metabolism related genes, e.g., ACOT1, ACADM, CAT, CPT1A, CPT2, MGLL, PDK4, PEX11A[47]. From a mechanistic perspective, the n-3 PUFA induced downregulation of de novo fatty acid synthesis, which is required for membrane biosynthesis, may inhibit cell growth and proliferation[1, 10] and therefore can be chemoprotective[13]. This effect may include the concurrent enhancement of beta-oxidation and the induction of futile mitochondrial respiration (proton leak) that leads to the uncoupling of ATP synthesis, resulting in nutrient wasting and apoptosis[19, 48]. Interestingly, the gut is kept hypoxic by beta-oxidation, and this maintains growth of obligate anaerobic bacteria and inhibits dysbiotic microbial expansion[49].

Previous studies have demonstrated that colon cancer progression suppresses chemoprotective nuclear receptors, perturbs innate immunity responses[14,16,] and the gut experiences dysbiotic microbial expansion[39,49]. We postulate that the addition of a fish oil + pectin diet during this phenomenon concurrently facilitates the stimulation of DHA ligand-activated nuclear receptors associated with lipid metabolism[1, 3, 16, 17, 48], while butyrate triggers oxygen consumption via the beta-oxidation metabolic pathway[15, 16, 49] (Figure 5). The augmented beta-oxidation associated with fish oil + pectin (fpa) feeding may also promote the ability of n-3 PUFA to activate ligand dependent nuclear receptors (Figure 4.A and Supplementary Table 3). Under these conditions, we envision that enhanced butyrate entry, a preferential source of energy in colonocytes, into the mitochondria (from pectin) may allow a greater proportion of the n-3 PUFA to avoid catabolism and thereby act as nuclear receptor ligands (Figure 5). This would then maximally induce the transcription of beta-oxidation and other n-3 PUFA associated genes, e.g., PPARgamma and HNF4alpha[48] (Figure 4 and Supplementary Tables 2 and 3).

In order to further explore how fish oil and pectin synergistically promote ligand dependent nuclear receptor activation, we assessed the activity of predicted upstream transcriptional regulators. Among the many top Upstream Regulators detected in fish oil containing diets under carcinogen exposure conditions were ligand dependent nuclear receptors (Figure 4 and Supplementary Figure 3). Previous reports supporting our predictions suggest that fish oil-derived n-3 PUFAs modulate the function of the nuclear receptors (NRs) presented in Figure 4 and therefore can modulate colon cancer and colitis in a chemoprotective manner[1, 3]. The majority of nuclear receptors known to be induced by n-3 PUFA (PPARs, LXRs, FXRs, HNF4A and SREBPs), were predicted to be induced by fish oil + pectin fed rats in the presence of carcinogen (Figure 4 and Supplementary Table 3). This is noteworthy because PPARgamma and HFN4alpha activate beta-oxidation and suppress intestinal permeability, respectively[49, 50].

In summary, our results document for the first time the chromatin structure associated with the feeding of a highly chemoprotective diet containing fish oil (rich in n-3 PUFAs) and fermentable fiber (rich in short chain fatty acids) under normal (saline control) and carcinogenic conditions. Activation of ligand dependent nuclear receptors and transcriptional upregulation of genes associated with enhanced beta-oxidation was primarily

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observed in fish oil + pectin (fpa vs cca) fed rats. We demonstrate that the combination of fish oil + pectin generates unique epigenetic modifications and transcriptional profiles in a context (AOM vs saline) specific manner. These results support our hypothesis that, in colonocytes, fish oil related effects are synergistically enhanced by the inclusion of pectin to the diet during the onset of carcinogenesis. In conclusion, our data contribute to the understanding of the chemotherapeutic properties of fish oil and pectin (n-3 PUFAs and SCFAs) in colonic crypts and provide critical mechanistic insight into recently reported epidemiological findings[25].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty

We describe how combined exposure to the chemoprotective bioactive compounds, fish oil and fermentable fiber, during malignant transformation of colonocytes uniquely induces global transcriptional and epigenetic modifications linked to chemoprotective genes. Combination dietary chemoprevention up-regulates lipid catabolism and beta-oxidation associated genes. These genes are linked to activated ligand-dependent nuclear receptors associated with n-3 polyunsaturated fatty acids and to enhanced metabolic oxidation via fiber fermentation.

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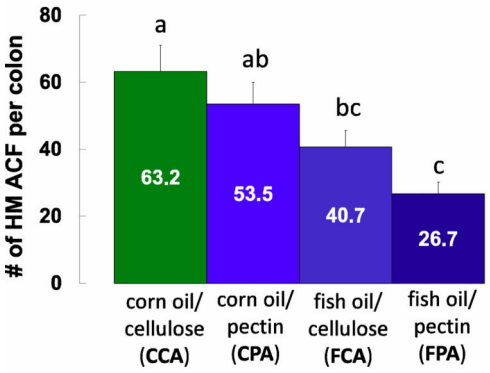
A



B

treatment comparison	RNA FDR<0.1	RNA p<0.01	K9ac FDR<0.1	K9ac p<0.01	K4me3 FDR<0.1	K4me3 p<0.01
fish oil overall	160	367	32	217	3	430
pectin overall	2	102	9	432	6	222
fpa vs cca	83	278	0	400	21	228
fca vs cca	1	90	0	432	0	172
cpa vs cca	0	18	0	480	15	189
fps vs ccs	14	145	15	272	3	89
fcs vs ccs	63	302	4	303	0	133
cps vs ccs	0	55	24	703	0	38

C

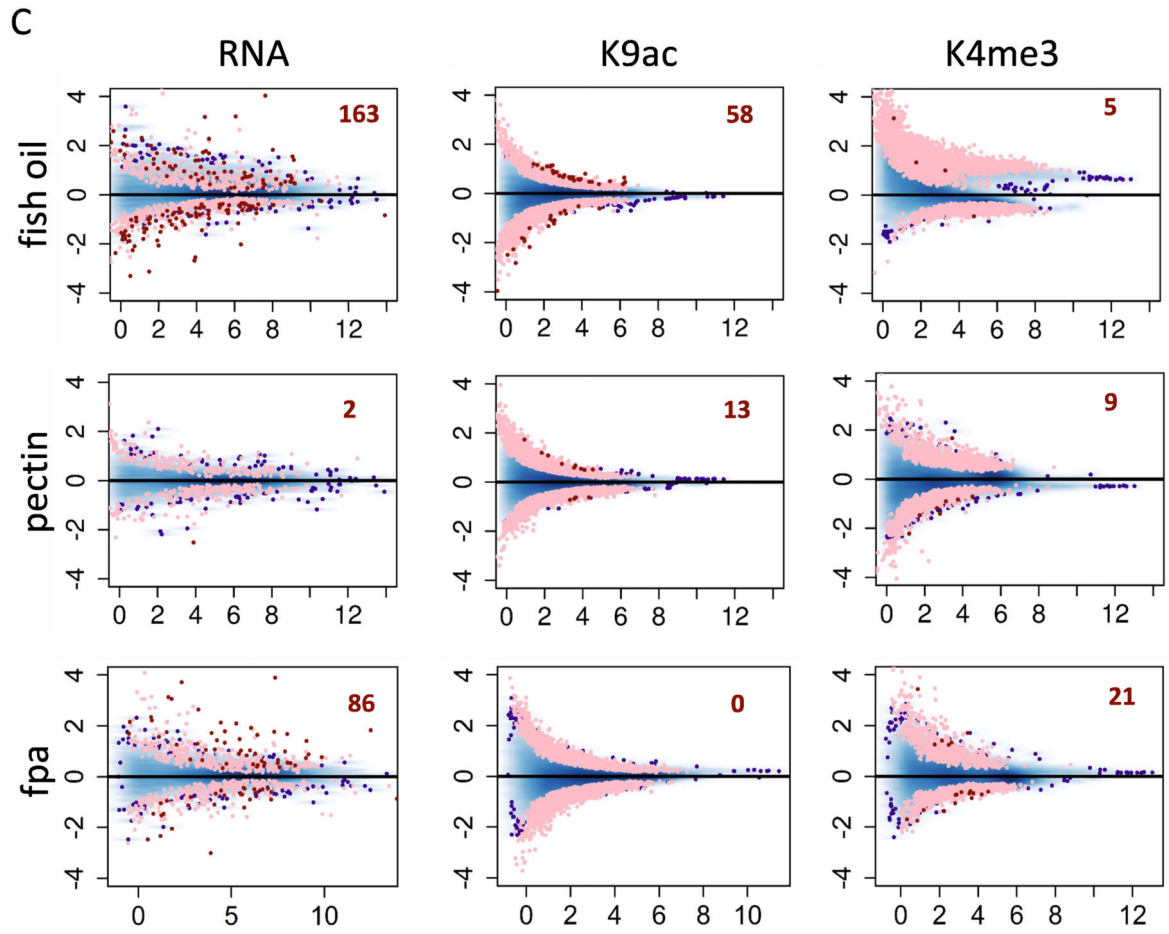


Figure 1. Fish oil and pectin synergistically suppress malignant transformation in the colon

A) High multiplicity aberrant crypt foci (HM ACF) incidence per rat colon is shown. Bars not sharing the same superscript letters are significantly different, $p<0.05$. **B)** Summary of diet effects on differentially expressed and differentially enriched genes. **C)** Differential expression of all transcribed genes or histone tail differentially enriched regions illustrated in MAplots indicate the differential expression of all transcribed genes or histone tail enriched regions (y-axis, log-ratio of difference in intensity of histone tail modifications enriched regions) vs their overall intensity of expression (x-axis, log-average of read counts)

following chemoprotective vs control treatment. Pink highlights represent differentially expressed transcripts and differentially enriched regions (with p-values < 0.05. Genes (and total number of genes) with an FDR<0.1 are highlighted in red and all other detected genes are blue highlighted.

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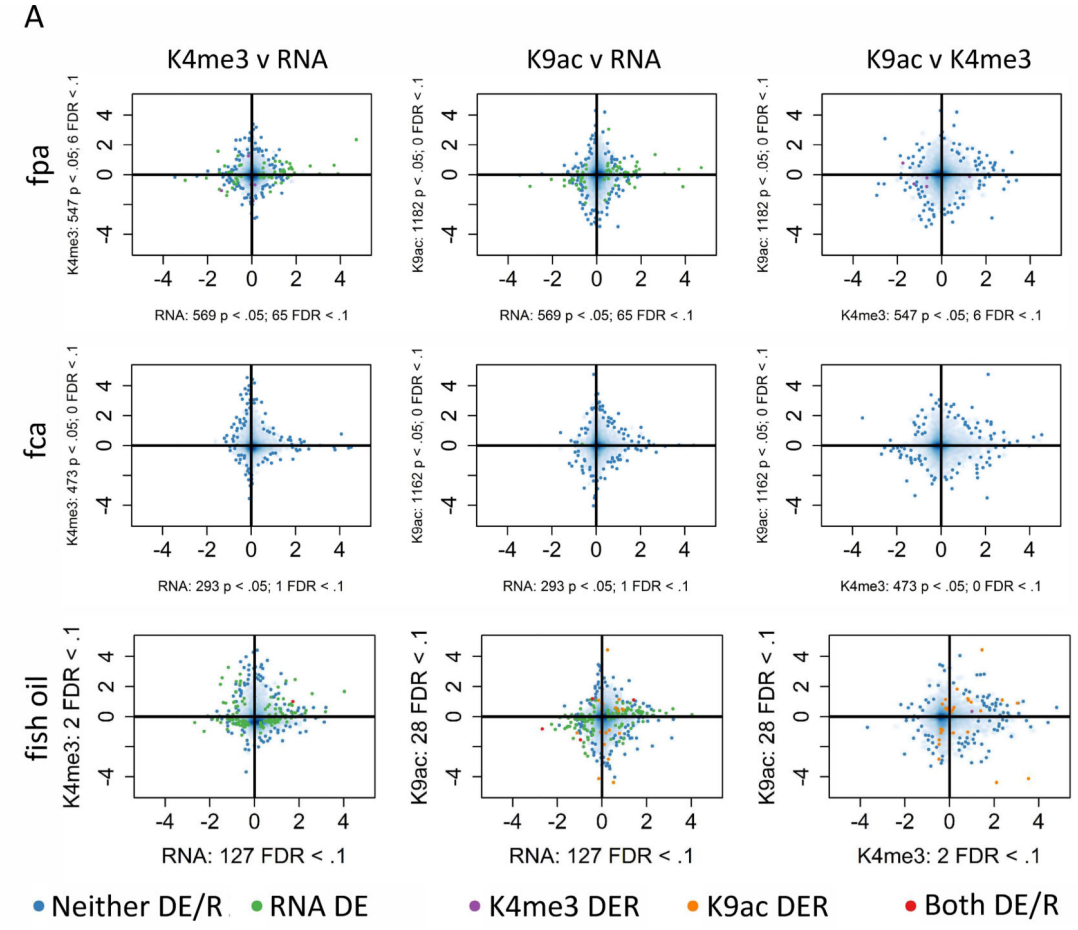

B

bioactive	unique saline	both aom & saline	unique aom	epigenetic level	treatments compared
pectin	35	3	186	K4me3	cp vs cc
fish oil	127	6	166	K4me3	fc vs cc
fish oil + pectin	78	11	217	K4me3	fp vs cc
pectin	53	1	17	RNA	cp vs cc
fish oil	280	15	74	RNA	fc vs cc
fish oil + pectin	105	42	225	RNA	fp vs cc
pectin	641	62	418	K9ac	cp vs cc
fish oil	270	33	399	K9ac	fc vs cc
fish oil + pectin	244	28	372	K9ac	fp vs cc

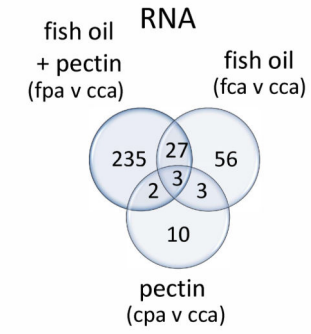

Figure 2. Overall poor correlation between differentially transcribed (DE) and enriched gene regions (DERs) at multiple epigenetic levels

A) Comparison between epigenetic states across dietary treatments is shown. Scatterplots reveal low correlation between DE (transcripts) and DER (histone tail modifications) upon comparison of the gene log2(fold changes) from fpa vs cca, fca vs cca, and fish oil vs corn oil, treatment at different epigenetic stages. The total number of genes with FDR<0.1 is listed in each axis. Contrasts between epigenetic stages for the same treatment include number of genes with FDR<0.1. Blue dots represent genes with FDR>0.1 at both DE and

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DER epigenetic stages. **B)** The majority of AOM regulated genes are unique for each diet. Genes in common at $p<0.01$ between saline and AOM injected rats fed the same diet are shown by epigenetic level. **C)** Diet combination vs individual treatments. Intersection between diets containing the fish oil + pectin combination and each individual bioactive compound with respect to differentially transcribed genes ($p<0.01$).

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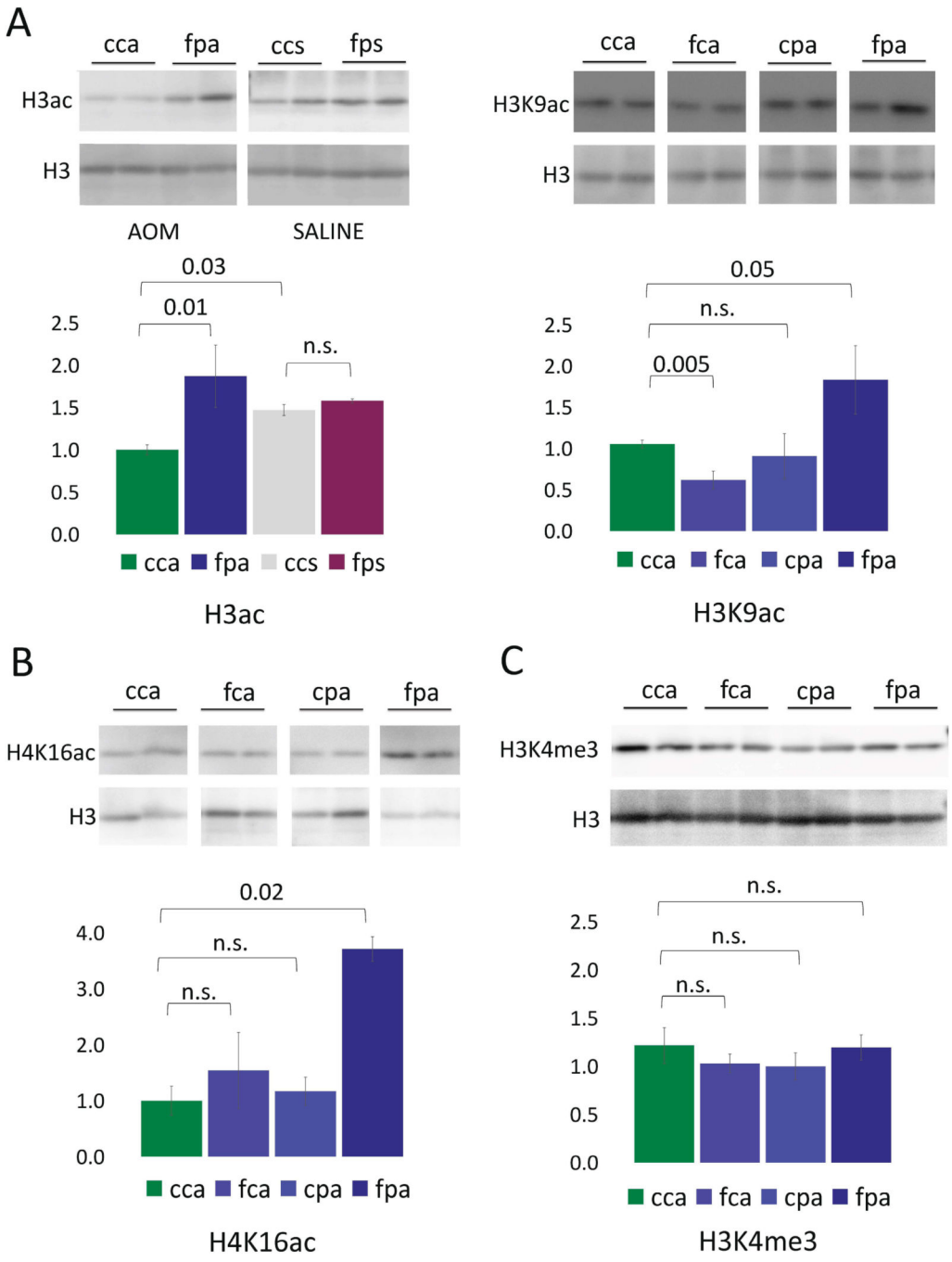

Figure 3. Levels of acetylated H3 increase following chemoprotective in fish oil and pectin fed rats following carcinogen exposure

Summary of western blot analysis of nuclear protein extracts from diet and carcinogen treated rats, total H3 serves as loading control. **A**) Comparison of acetylated H3 and H3K9 levels following feeding of fish oil and pectin containing diets in the presence and absence of carcinogen exposure. **B**) H4K16ac levels increased following fish oil + pectin feeding during cancer progression. **C**) Trimethylated H3K4 levels were unchanged by dietary and carcinogen treatments. Samples were compared to a saline control and probed for histone

post-translational modification and H3. Data are expressed as mean ± SD normalized to total H3 with significant differences relative to control conditions (cca) as indicated by p values. At least 2 independent assays were conducted in (n = 6) rats, n.s. denotes p > 0.05.

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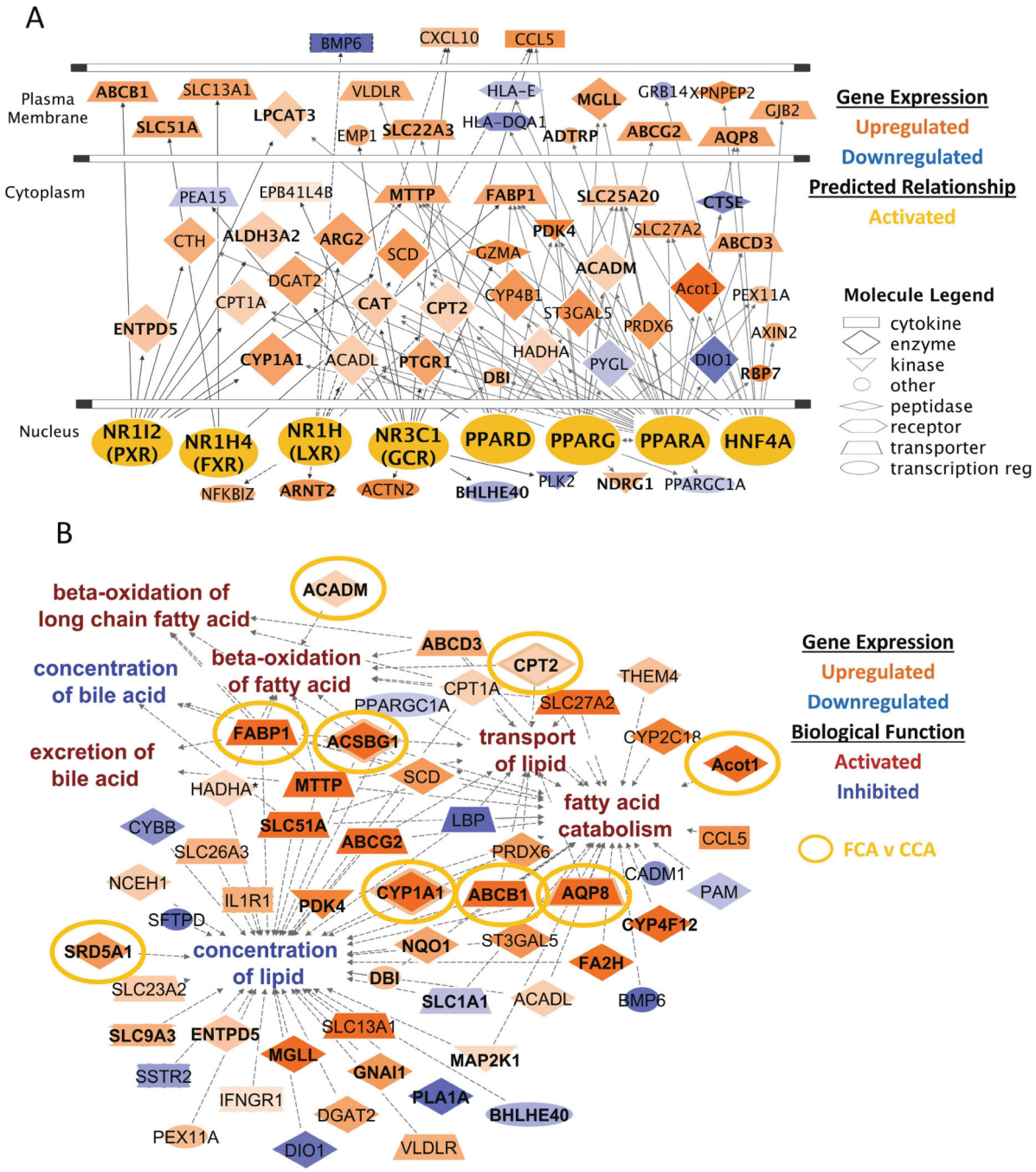

Figure 4. Upstream regulators modulated by dietary fat and fiber interaction

A) Representative networks of genes transcriptionally regulated in fish oil and pectin fed rats following carcinogen exposure by top ranked upstream key regulators (URs). Yellow fill represents the projected increase in UR activity. Blue fill indicates decreased gene activity (DE) and orange fill indicates increased gene activity (DE), deeper color hue indicates genes with greater |log2(FoldChange)|, and genes with FDR<0.1 are bolded. Solid lines represent direct and dashed lines represent indirect gene interaction. P-values and activity (z-scores) of URs were determined by Ingenuity Pathway Analysis of genes with $p < 0.01$. **B)** Fish oil +

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pectin diet transcriptionally enhanced beta-oxidation and decreased lipid metabolism associated genes during colon cancer progression. The dashed lines indicate aspects of lipid metabolism associated with a specific gene and the activation/inhibition of a biological function. Only a small subset of these genes were differentially transcribed in fish oil + cellulose fed rats following carcinogen injection, as labeled by yellow ovals.

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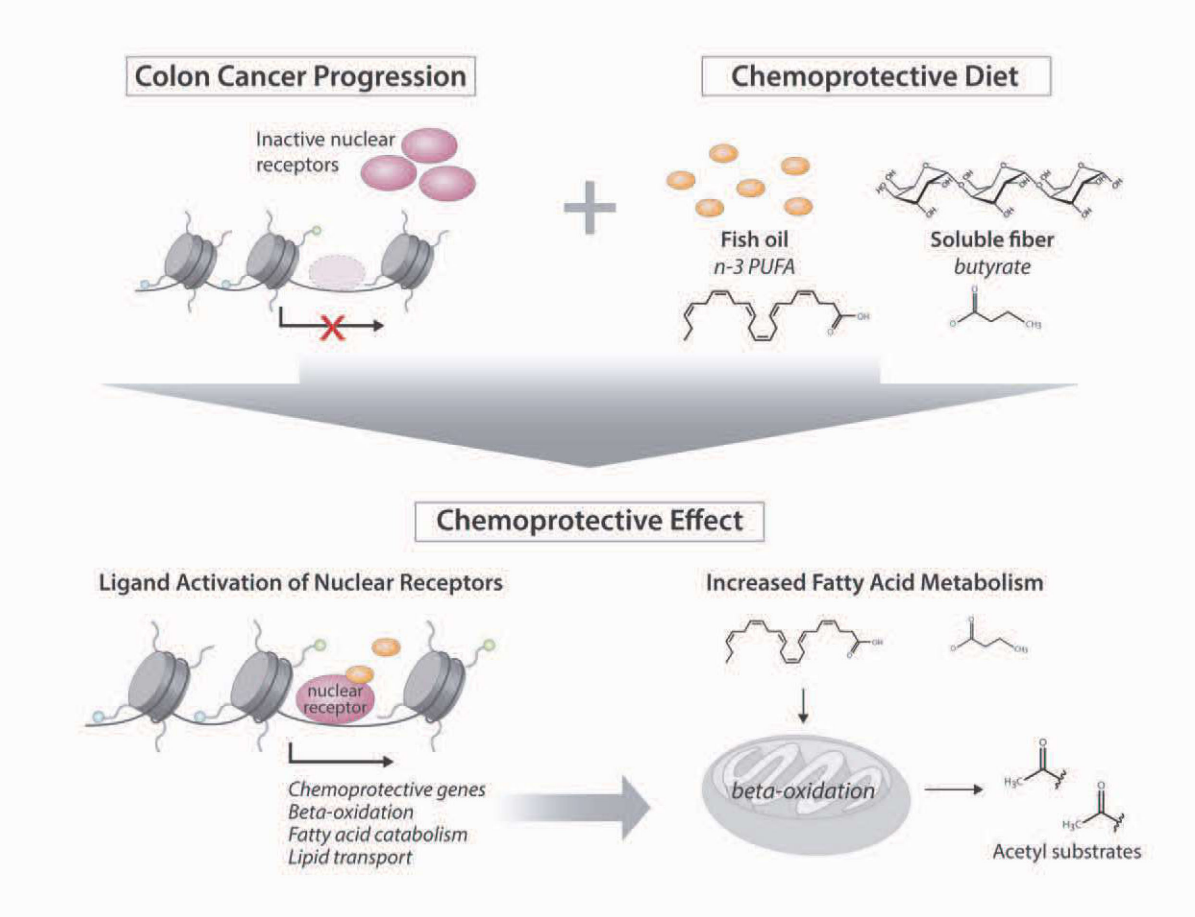

Figure 5. Proposed molecular model of the chemoprotective synergistic effects of fish oil and soluble fiber diet in colonocytes

The combinatorial diet (fish oil + pectin) activates ligand-dependent nuclear receptors associated with n-3 PUFA, affecting transcriptional profiles in the intestinal epithelium, e.g., upregulation of lipid catabolism and beta-oxidation associated genes, while butyrate triggers oxygen consumption via beta-oxidation. Overall, the combinatorial “pesco-vegetarian” diet modulates the function of the mitochondrial L-carnitine shuttle, inhibiting lipogenesis and promoting the accumulation of acetyl-CoA by increased beta-oxidation of both n-3 PUFA and short chain fatty acids.

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Table 1

Differentially transcribed genes by fish oil + pectin treatment during colon cancer progression.

Symbol	Entrez Gene Name	Refseq	FDR	log2(FC)		
1810065E05Rik	RIKEN cDNA 1810065E05 gene	NM_001109633	**4.3E-03**	**3.89**	*	+
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP),1	NM_133401	**1.6E-03**	**1.68**	*	+
ABCD3	ATP-binding cassette, sub-family D (ALD),3	NM_012804	**7.9E-02**	**0.88**	*	
ABCG2	ATP-binding cassette, sub-family G (WHITE),2 (Junior blood group)	NM_181381	**3.3E-03**	**1.64**	*	
ACAD11	acyl-CoA dehydrogenase family,11	NM_001108181	**7.1E-05**	**0.99**	*	
ACADM	acyl-CoA dehydrogenase, C-4 to C-12 straight chain	NM_016986	**8.2E-03**	**0.45**	*	+
Acot1	acyl-CoA thioesterase 1	NM_031315	**1.5E-02**	**1.57**	*	+
ACSBG1	acyl-CoA synthetase bubblegum family1	NM_134389	**1.8E-04**	**2.13**	*	+
ADTRP	androgen-dependent TFPI-regulating protein	NM_001014144	**7.0E-03**	**0.83**	*	
ALDH3A2	aldehyde dehydrogenase 3 family,A2	NM_031731	**1.5E-02**	**0.53**	*	+
ANXA3	annexin A3	NM_012823	**4.6E-05**	**−3.01**	*	+
AQP8	aquaporin 8	NM_019158	**9.2E-02**	**1.92**	*	
ARG2	arginase 2	NM_019168	**7.1E-05**	**1.78**	*	
ARHGAP20	Rho GTPase activating protein 20	NM_213629	**5.9E-02**	**3.06**	*	+
ARNT2	aryl-hydrocarbon receptor nuclear translocator 2	NM_012781	**4.2E-02**	**1.06**	*	
B4GALT4	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4	NM_001012018	**5.1E-02**	**−1.23**	*	
BACE2	beta-site APP-cleaving enzyme 2	NM_001002802	**4.5E-02**	**−0.50**	*	+
BHLHE40	basic helix-loop-helix family,e40	NM_053328	**1.3E-02**	**−0.61**	*	
BMPER	BMP binding endothelial regulator	NM_001135799	**3.2E-03**	**6.27**	*	+
BTC	betacellulin	NM_022256	**3.0E-02**	**−0.96**	*	
CA1	carbonic anhydrase I	NM_001107660	**7.0E-03**	**1.83**	*	
CAT	catalase	NM_012520	**4.0E-02**	**0.54**	*	
CDC45	cell division cycle 45	NM_001105866	**1.7E-02**	**0.65**	*	
CLCA1	chloride channel accessory 1	NM_001107449	**6.2E-02**	**−0.88**	*	
CLCA4	chloride channel accessory 4	NM_201419	**3.6E-02**	**1.41**	*	
COQ6	coenzyme Q6 monooxygenase	NM_001011983	**4.8E-02**	**0.40**	*	
CPT2	carnitine palmitoyltransferase 2	NM_012930	**3.4E-02**	**0.46**	*	

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Symbol	Entrez Gene Name	Refseq	FDR	log2(FC)	
CREG1	cellular repressor of E1A-stimulated genes 1	NM_001105966	**4.0E-02**	**−0.71**	*
CTSE	cathepsin E	NM_012938	**1.4E-02**	**−0.91**	*
CTSH	cathepsin H	NM_012939	**1.4E-02**	**−0.47**	*
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	NM_012540	**5.9E-03**	**5.71**	*
CYP4F12	cytochrome P450, family 4, subfamily F, polypeptide 12	NM_019623	**1.3E-02**	**1.77**	*
DAP	death-associated protein	NM_022526	**2.9E-03**	**−0.50**	*
DBI	diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein)	NM_031853	**2.1E-03**	**0.65**	*
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	NM_001135802	**8.3E-02**	**0.51**	*
DUSP3	dual specificity phosphatase 3	NM_001173376	**3.3E-03**	**−0.74**	*
ENTPD4	ectonucleoside triphosphate diphosphohydrolase 4	NM_001108384	**2.5E-03**	**−0.71**	*
ENTPD5	ectonucleoside triphosphate diphosphohydrolase 5	NM_199394	**6.0E-02**	**0.56**	*
FA2H	fatty acid 2-hydroxylase	NM_001135583	**5.9E-04**	**1.48**	*
FABP1	fatty acid binding protein 1, liver	NM_012556	**9.1E-06**	**4.72**	*
FAM129A	family with sequence similarity 129,A	NM_022242	**2.9E-03**	**−0.67**	*
FAM81A	family with sequence similarity 81,A	NM_001108163	**9.2E-02**	**−1.53**	*
GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	NM_013145	**1.0E-01**	**1.03**	*
GPSM2	G-protein signaling modulator 2	NM_001191962	**4.0E-02**	**0.43**	*
LBH	limb bud and heart development	NM_001129880	**4.0E-02**	**−0.91**	*
LHFPL1	lipoma HMGIC fusion partner-like 1	NM_181085	**1.6E-03**	**−1.13**	*
LPCAT3	lysophosphatidylcholine acyltransferase 3	NM_001012189	**3.6E-03**	**0.54**	
MAP2K1	mitogen-activated protein kinase kinase 1	NM_031643	**5.8E-02**	**0.38**	
MGLL	monoglyceride lipase	NM_138502	**6.9E-03**	**1.72**	
MTTP	microsomal triglyceride transfer protein	NM_001107727	**1.3E-02**	**1.75**	
MYOF	myoferlin	NM_001191636	**6.1E-03**	**−0.62**	
NAPSA	napsin A aspartic peptidase	NM_031670	**9.0E-02**	**3.71**	
NDRG1	N-myc downstream regulated 1	NM_001011991	**3.2E-03**	**0.73**	
NQO1	NAD(P)H dehydrogenase, quinone 1	NM_017000	**1.5E-02**	**0.90**	+
NSL1	NSL1, MIS12 kinetochore complex component	NM_001109083	**3.7E-02**	**0.68**	
Oasl2	2′-5′ oligoadenylate synthetase-like 2	NM_001009682	**7.2E-02**	**−1.10**	

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Symbol	Entrez Gene Name	Refseq	FDR	log2(FC)	
PDK4	pyruvate dehydrogenase kinase, isozyme 4	NM_053551	**5.9E-02**	**1.30**	
PLA1A	phospholipase A1A	NM_138882	**1.5E-02**	**−1.47**	
PTGR1	prostaglandin reductase 1	NM_138863	**5.5E-02**	**0.90**	
RBP7	retinol binding protein 7, cellular	NM_001108693	**8.8E-03**	**1.33**	
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	NM_001013232	**6.9E-03**	**−2.34**	
ROR1	receptor tyrosine kinase-like orphan receptor 1	NM_001108671	**4.7E-03**	**−1.47**	
RT1-S2	RT1 class Ib, locus S2	NM_001008857	**9.9E-03**	**2.34**	
SFXN3	sideroflexin 3	NM_022948	**5.7E-03**	**−0.65**	
SGK2	serum/glucocorticoid regulated kinase 2	NM_134463	**7.0E-03**	**1.07**	
SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag),1	NM_013032	**1.3E-02**	**−0.48**	
SLC22A3	solute carrier family 22 (organic cation transporter),3	NM_019230	**2.5E-03**	**1.43**	
SLC23A1	solute carrier family 23 (ascorbic acid transporter),1	NM_017315	**7.2E-02**	**2.21**	
SLC25A20	solute carrier family 25 (carnitine/acylcarnitine translocase),20	NM_053965	**1.6E-03**	**0.62**	
SLC39A2	solute carrier family 39 (zinc transporter),2	NM_001107260	**2.7E-02**	**−2.48**	
SLC51A	solute carrier family 51, alpha subunit	NM_001107087	**4.4E-02**	**2.09**	+
SLC9A3	solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3),3	NM_012654	**5.8E-02**	**0.77**	+
SRD5A1	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	NM_017070	**4.3E-03**	**1.04**	
SYTL4	synaptotagmin-like 4	NM_080410	**1.5E-02**	**−0.45**	
TM4SF4	transmembrane 4 L six family4	NM_053785	**1.4E-02**	**3.13**	+
TRIM6	tripartite motif containing 6	NM_001170461	**4.8E-02**	**−0.81**	

*gene differentially expressed at the fish oil overall effect level.

+gene differentially expressed in at least one other fish oil containing pairwise comparison (fca vs cca and/or fps vs ccs and/or fcs vs ccs).

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