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Targeted mutagenesis of Δ5 and Δ6 fatty acyl desaturases induce dysregulation of lipid metabolism in Atlantic salmon (*Salmo salar*)

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Abstract

Background: With declining wild fish populations, farmed salmon has gained popularity as a source for healthy long-chain highly unsaturated fatty acids (LC-HUFA). However, the introduction of plant oil in farmed salmon feeds has reduced the content of these beneficial LC-HUFA. The synthetic capability for LC-HUFAs depends upon the dietary precursor fatty acids and the genetic potential, thus there is a need for in-depth understanding of LC-HUFA synthetic genes and their interactions with other genes involved in lipid metabolism. Several key genes of LC-HUFA synthesis in salmon belong to the fatty acid desaturases 2 (*fads2*) family. The present study applied whole transcriptome analysis on two CRISPR-mutated salmon strains (crispants), 1) Δ*6abc/5*^{Mt} with mutations in Δ*5fads2,* Δ*6fads2-a,* Δ*6fads2-b and* Δ*6fads2-c* genes, and 2) Δ*6bc*^{Mt} with mutations in Δ*6fads2-b and* Δ*6fads2-c* genes. Our purpose is to evaluate the genetic effect *fads2* mutations have on other lipid metabolism pathways in fish, as well as to investigate mosaicism in a commercial species with a very long embryonal period.

Results: Both Δ*6abc/5*^{Mt} and Δ*6bc*^{Mt} crispants demonstrated high percentage of indels within all intended target genes, though different indel types and percentage were observed between individuals. The Δ*6abc/5*^{Mt} fish displayed several disruptive indels which resulted in over 100 differentially expressed genes (DEGs) enriched in lipid metabolism pathways in liver. This includes up-regulation of *srebp1* genes which are known key transcription regulators of lipid metabolism as well as a number of down-stream genes involved in fatty acid *de-novo* synthesis, fatty acid *β*-oxidation and lipogenesis. Both *elovl5* and *elovl2* genes were not changed, suggesting that the genes were not targeted by Srebp1. The mutation of Δ*6bc*^{Mt} surprisingly resulted in over 3000 DEGs which were enriched in factors encoding genes involved in mRNA regulation and stability.

Conclusions: CRISPR-Cas9 can efficiently mutate multiple *fads2* genes simultaneously in salmon. The results of the present study have provided new information on the transcriptional regulations of lipid metabolism genes after reduction of LC-HUFA synthesis pathways in salmon.

Keywords: Atlantic salmon, CRISPR mosaicism, Long-chain highly unsaturated fatty acids, Fatty acid desaturase, Sterol regulatory binding protein, exon skipping, Transcriptional regulation

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Background

Atlantic salmon (*Salmo salar* L.) is a popular fish species for human consumption since it contains high amounts of long-chain highly unsaturated fatty acids (LC-HUFA) such as docosahexaenoic acid (22:6n-3, DHA), eicosapentaenoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, ARA). The high LC-HUFA content in farmed salmon originates mainly from dietary inclusions of marine fish oil and fish meal. However, traditional marine fisheries have been exploited to their limits, and with increasing volume of salmon production, dietary marine oil and meal sources have been gradually diluted over the past decades. Plant oils are used to substitute marine oils in aquaculture diets, with an increasing levels from 0% of total lipids in 1990 to 19.2% in 2013 [1]. This has resulted in a reduction of LC-PUFA levels in salmon flesh since plant oils do not contain LC-PUFA [2].

Salmon are capable of synthesizing LC-HUFA through elongation and desaturation of α-linolenic (18:3n-3) and linoleic (18:2n-6) acids, and the synthesis is often increased when the fish are given a plant oil diet with low LC-HUFA [3]. This explains the fact that salmon can tolerate partial substitution of fish oil with plant oil without negative impact on growth rate, feed conversion or any histopathological lesions [4]. However, the synthesized LC-HUFA in salmon is still not enough to compensate for the reduced LC-HUFA level caused by inclusion of plant oil in diet [2]. Thus, salmon has limited capability in bioconverting the precursors, 18:3n-3 and 18:2n-6 to essential LC-HUFAs [5, 6]. In order to further improve the LC-HUFA synthetic capacity in salmon, a better understanding of the regulation of genes involved in LC-HUFA synthesis is needed.

The pathways of LC-HUFA synthesis in salmon involves 4 elongases encoded by *elovl2*, *elovl4*, *elovl5a* and *elvol5b* and 4 desaturases encoded by *Δ5fads2*, *Δ6fads2-a*, *Δ6fads2-b* and *Δ6fads2-c*. All 8 genes have been cloned and functionally characterised through heterologous expression in yeast (*Saccharomyces cerevisiae*) [7, 8]. Both *elovl5a* and *elovl5b* are mainly involved in elongating C_{18} and C_{20} fatty acids, while *elovl2* and *elovl4* are involved in elongating C_{20} and C_{22} [8–10]. All four *fads* genes in salmon are homologs to the human *FADS2* gene. In salmon they have separate functions where double bonds are introduced at C5 (*Δ5fads2*) or C6 (*Δ6fads2-a*, *Δ6fads2-b* and *Δ6fads2-c*) from the carboxyl end [10, 11]. Feeding of plant oil often leads to up-regulation of both *elovl* and *fads2* genes in salmon, which is likely due to the low LC-HUFA content in the diet [5, 12–14].

In addition to the LC-HUFA synthesis genes, many other genes involved in fatty acid *de-novo* synthesis, fatty acid oxidation and cholesterol biosynthesis are also differentially expressed after feeding plant oil [5, 12–14]. It is difficult to conclude the reason for the differential expression of lipid metabolism genes since plant oils are devoid of cholesterol and LC-HUFA, and contain high amounts of C_{18} PUFA precursors and phytosterols compared to fish oil [15–17]. In a recent study, we disrupted the LC-HUFA synthesis pathway in salmon by mutating *elovl2* gene using CRISPR/Cas9 technology [18]. In addition to a decreased DHA content in mutant fish, we were able to identified up-regulation of *fads2* genes as well as several genes involved in fatty acid biosynthesis and lipogenesis as consequence of the knock out [18]. This suggests a systemic change of lipid metabolism regulation in response to the disruption of LC-HUFA synthesis in salmon.

CRISPR/Cas9 technology has recently been used in salmon to edit genes and generate mutants for *elovl2*, *slc45a2* and *dnd* [18–21]. Both guide RNA (gRNA) and Cas9 mRNA are injected into one-cell stage salmon embryos to induce a targeted double-strand break, followed by non-homologous end joining (NHEJ) which generates random insertions and deletions (indels) at the target sites that can lead to a non-functional protein. However, because of a three-year generation interval, the generation of homozygous edited salmon is too tedious for research projects. Genetic manipulation efficacy in the founder generation largely depend upon target gene and gRNA design, but there is also a need to address how mosaics differ in the tissues and affects function of the encoded gene product. For this species it is therefore necessary to optimize editing efficiency and reduce the problem of mosaicism in the F0 generation. Compared to teleost model species, the Atlantic salmon embryo develops slowly and hatches after about 80 days, or 500-day degrees (days x temperature in °C). This developmental pace may lead to degradation of CRISPR components such as CAS9 mRNA or protein and guide RNA's which may have an impact upon mosaicism.

We have recently used CRISPR/Cas9 to mutate *fads2* genes in salmon which resulted in down-regulation of targeted genes and lower DHA and EPA contents in tissues [22]. However, the impact of impaired LC-HUFA biosynthesis on the regulation of other genes - both from lipid metabolism and globally - was still unclear. In the present study we aimed to further characterize transcriptional regulation of lipid metabolism in *fads2*-mutated salmon by comparing their transcriptomes to wildtype fish. Our study also seeks to provide detailed insights on the effect and distribution of genetic mosaicism in salmon individuals after mutation of *fads2* genes.

Result and discussion

CRISPR/Cas9 induced mutations

The two strains of Atlantic salmon carrying CRISPR/Cas9-mediated mutations were generated as described earlier [22]. In both strains CRISPR/Cas9 mediated mutations were induced using a single CRISPR gRNA

targeting multiple genes (Fig. 1a). The gRNA of *Δ6abc/5*^{Mt} salmon targeted *Δ6fads2-a*, *Δ6fads2-b*, *Δ6fads2-c* and *Δ5fads2* genes, while the gRNA of *Δ6bc*^{Mt} targeted *Δ6fad2s-b* and *Δ6fads2-c*. Both *Δ6abc/5* and *Δ6bc* mutant salmon were co-injected with a CRISPR gRNA targeting *slc45a2* which induces an albino phenotype and served as visual control in our experiment.

CRISPR/Cas9-induced structural mutations at the *fads2* as well as the *slc45a2* genes of fish from both *Δ6abc/5*^{Mt} and *Δ6bc*^{Mt} strains were confirmed by using AmpliSeq. All fish injected with CRISPR/Cas9 carried structural variants at the respective gRNA target sites (Fig. 1 b). For all individuals from both CRIS PR strains we observed a high degree of mosaicism at each of the respective gRNA target sites (Fig. 1b). This suggests that Cas9-induced editing continues after the one-cell stage of the embryos. In order to better understand the consequences of the different structural variants on a phenotypic level, we predicted variant effects using SnpEff and summarised the results according to the impact category (Fig. 1c). The majority of structural variants across all individuals were predicted to have "high" impact, meaning to have a likely disruptive effect on the protein function. Nevertheless, our analysis also showed that many of the individuals from the two CRISPR strains still carried a considerable amount of the WT genotype (non-CRISPR mutated). Therefore, we believe it is more correct to consider the two resulting CRISPR strains as *fads2* knock-downs rather than knockouts. The *Δ6abc/5*^{Mt} gRNA targeted sequence right after the cytochrome b5-like domain of *fads2* genes, while *Δ6bc*^{Mt} gRNA targeted sequences on exon 1 before all protein domains. Therefore, the out-of-frame mutations in *Δ6abc/5*^{Mt} and *Δ6bc*^{Mt} were expected to disrupt characteristic domains identified in fatty acyl desaturases, though our CRISPR-target sites did not specifically fall within protein domains. These out-of-frame mutations identified by Ampliseq could explain the nonsense-mediated decay (NMD) of the mutant mRNA and impaired biosynthesis of LC-PUFA in *Δ6abc/5*^{Mt} fish [22].

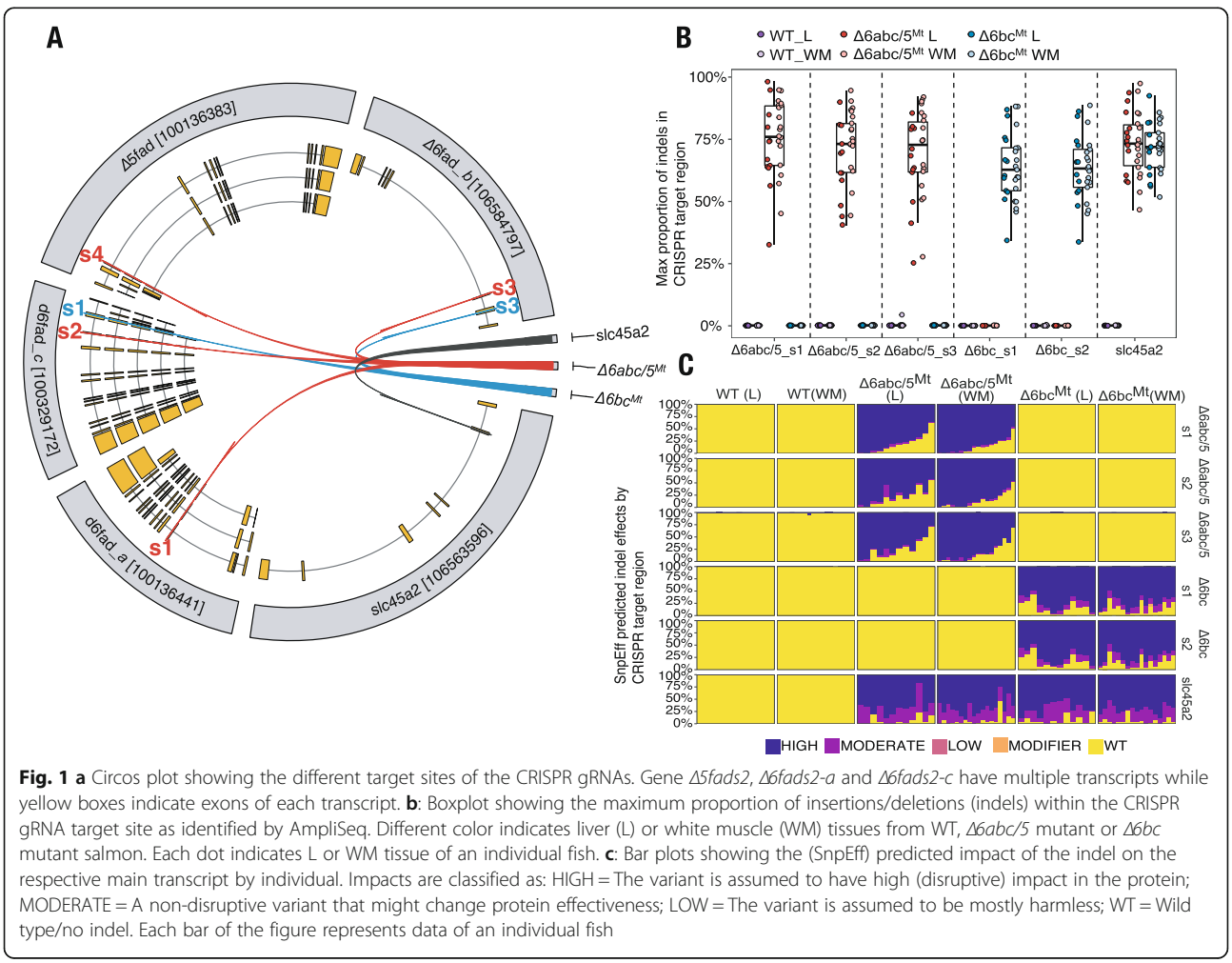

Fig. 1 **a** Circos plot showing the different target sites of the CRISPR gRNAs. Gene *Δ5fads2*, *Δ6fads2-a* and *Δ6fads2-c* have multiple transcripts while yellow boxes indicate exons of each transcript. **b**: Boxplot showing the maximum proportion of insertions/deletions (indels) within the CRISPR gRNA target site as identified by AmpliSeq. Different color indicates liver (L) or white muscle (WM) tissues from WT, *Δ6abc/5* mutant or *Δ6bc* mutant salmon. Each dot indicates L or WM tissue of an individual fish. **c**: Bar plots showing the (SnpEff) predicted impact of the indel on the respective main transcript by individual. Impacts are classified as: HIGH = The variant is assumed to have high (disruptive) impact in the protein; MODERATE = A non-disruptive variant that might change protein effectiveness; LOW = The variant is assumed to be mostly harmless; WT = Wild type/no indel. Each bar of the figure represents data of an individual fish

CRISPR/Cas9-induced indels cause Δ*6fads2-a* exon skipping events

Interestingly, we found that CRISPR/Cas9 induced mutations of *Δ6abc/5*^{Mt} gRNA in the *Δ6fads2-a* gene were affecting splicing of exonic part 6 (harbouring the CRISPR target site; exonic part 6 corresponds to exon 4 in transcript: XM_014170212.1; exon 3 in XM_014170213.1). Analysis of exonic-part 6 retention in *Δ6abc/5*-mutated salmon using RNA-seq data revealed mis-splicing of the *Δ6fads2-a* transcript resulting in the skipping of exonic part 6 (Fig. 2). Exon skipping caused by CRISPR/Cas9-generated mutations was observed previously in both cell lines [23, 24] and genetically modified organisms including zebrafish [25] and salmon [18]. CRISPR induced mis-splicing is mostly caused by one of two mechanisms: i) indels generated by a CRISPR-mutation affects the exon-intron boundaries or ii) indels promote exon skipping by disrupting an exon splicing enhancer or introducing an exon splicing silencer within the targeted exon [26]. However, neither mechanism fits to our study. This was because other *Δ6abc/5*^{Mt} gRNA target sites on *Δ5fads2*, *Δ6fads2-b* and *Δ6fads2-c* genes contained identical sequences and showed the same distance to exon-intron boundaries, but did not affect splicing. Nonetheless, the skipping of exon 6 in *Δ6fads2-a* transcripts will result in the production of truncated proteins that lack 37 amino acids, which suggests deleterious effects on protein structure and functions.

CRISPR-targeted *fads2* genes are down-regulated in the liver of Δ*6abc/5* but not Δ*6bc* salmon

Many of the CRISPR induced structural variants introduce premature termination codons likely to trigger mRNA degradation by nonsense-mediated decay (NMD) [27]. Indeed, we found that CRISPR-targeted *Δ5fads2*, *Δ6fads2-a* and *Δ6fads2-b* genes were strongly down-regulated ($q < 0.05$) in *Δ6abc/5*^{Mt} salmon compared to WT regardless of the dietary treatment (Fig. 3). In *Δ6bc*^{Mt} salmon, the CRISPR-targeted *Δ6fads2-b* gene was down-regulated compared to WT, but the levels of down-regulation were less clear than in *Δ6abc/5*^{Mt} salmon. Surprisingly, the expression of *Δ5fads2* and *Δ6fads2-a* genes was also down-regulated in *Δ6bc*^{Mt} salmon, though both genes were not targeted by *Δ6bc*^{Mt} gRNAs. The expression of *Δ6fads2-c* gene was generally very low, suggesting that it is unlikely to play a major role in salmon liver. This low level expression may also explain that *Δ6fads2-c* was not affected by CRISPR

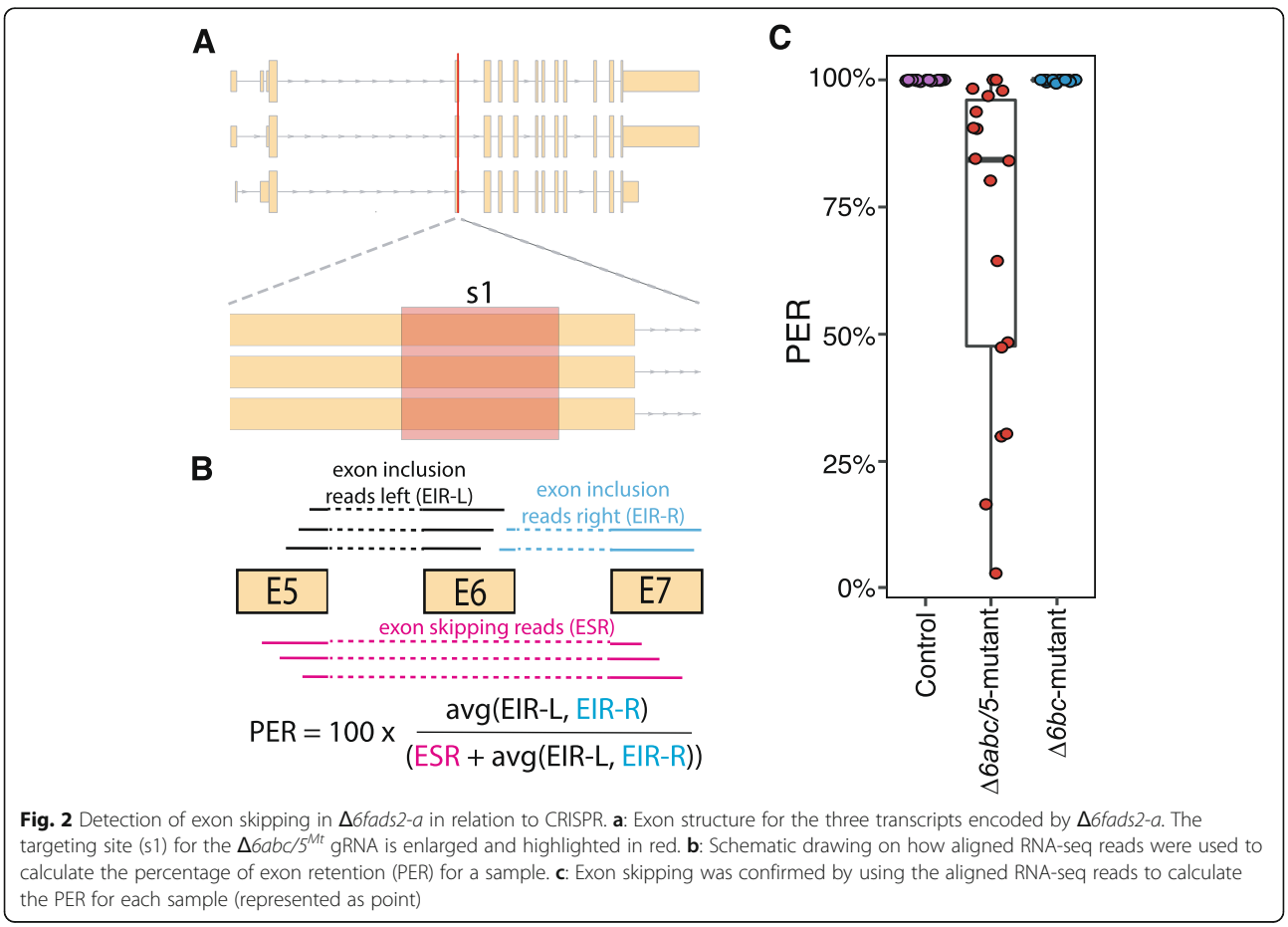

Fig. 2 Detection of exon skipping in Δ*6fads2-a* in relation to CRISPR. **a**: Exon structure for the three transcripts encoded by Δ*6fads2-a*. The targeting site (s1) for the Δ*6abc/5*^{Mt} gRNA is enlarged and highlighted in red. **b**: Schematic drawing on how aligned RNA-seq reads were used to calculate the percentage of exon retention (PER) for a sample. **c**: Exon skipping was confirmed by using the aligned RNA-seq reads to calculate the PER for each sample (represented as point)

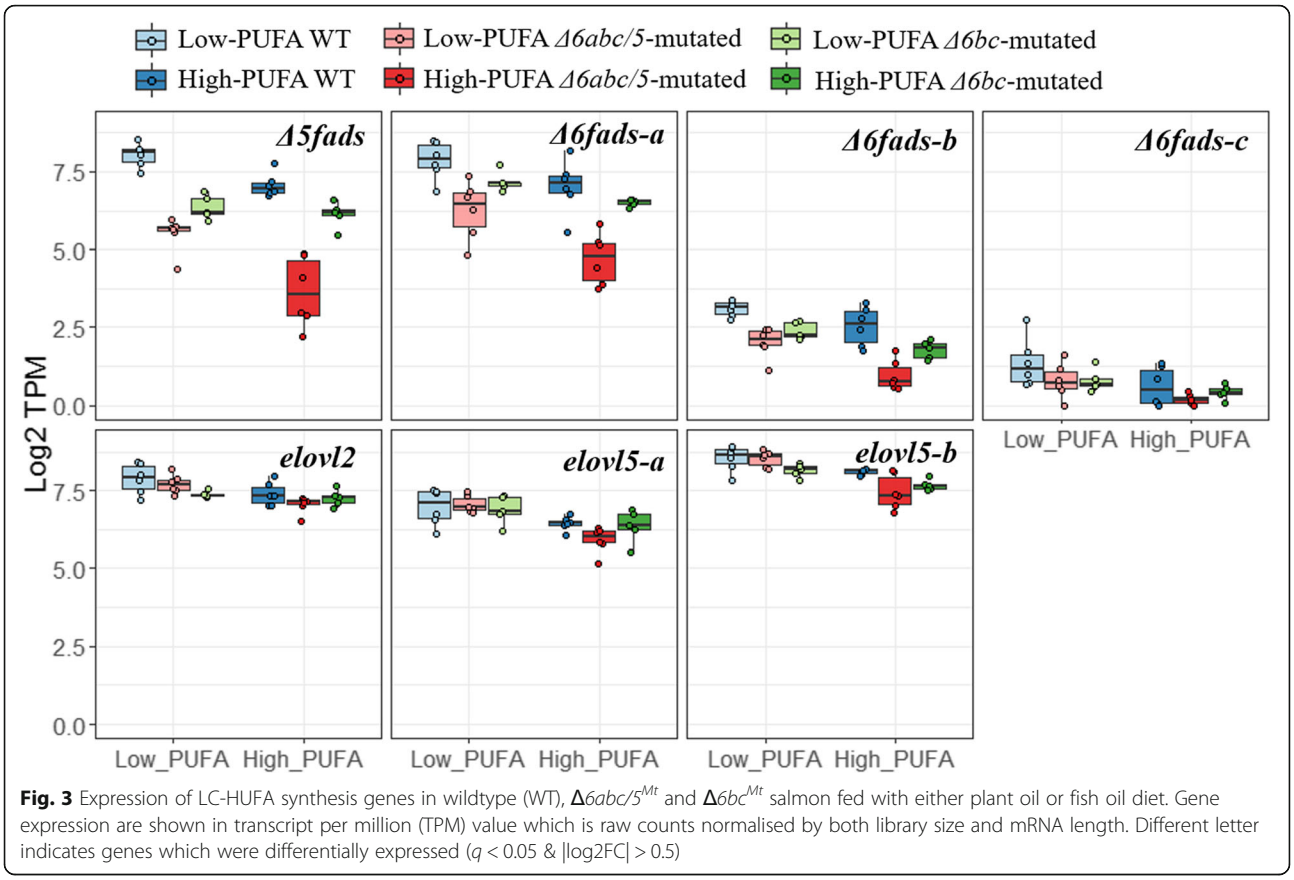

Fig. 3 Expression of LC-HUFA synthesis genes in wildtype (WT), Δ*6abc/5*^{Mt} and Δ*6bc*^{Mt} salmon fed with either plant oil or fish oil diet. Gene expression are shown in transcript per million (TPM) value which is raw counts normalised by both library size and mRNA length. Different letter indicates genes which were differentially expressed ($q < 0.05$ & $|\log2FC| > 0.5$)

mutations (Fig. 3). The expression of other genes in the LC-HUFA synthesis pathway, *elovl2*, *elovl5-a* and *elovl5-b*, was stable between *Δ6abc/5*^{Mt}, *Δ6bc*^{Mt} and WT salmon.

The NMD-mediated mRNA degradation, absence of exon 6 in *Δ6fads2-a* transcripts, and other CRISPR-induced mutations such as out-of-frame mutations are expected to produce non-functional enzyme proteins that would ultimately disrupt LC-HUFA biosynthesis in the fish. Indeed, analysis of tissue composition of LC-HUFA coupled with assays of desaturation and elongation activities in liver showed clear impacts of the CRISPR-mutations. The mutation of *Δ6abc/5* genes in salmon resulted in significant reduction of DHA and EPA in phospholipids compared to WT [22]. On the other hand, we observed effects of background wildtype alleles in the *Δ6abc/5*^{Mt} salmon (Fig. 1b and c) accounting for limited but measurable desaturation activities [22].

Transcriptional changes in liver after mutating *fads2* genes

An average of 29 million reads were mapped on to the salmon genome ICSASG_v2. From a total of 55,304 annotated genes, 23,114 genes had at least 1 count per million (CPM) in 25% of the samples, and were considered for subsequent analysis. By applying principal component analysis (PCA) on Log2 CPM of the top 1000 most variant genes, we identified a clear separation of plant oil and fish oil samples between PC1 (explaining 34.8% of the observed variation) and PC2 (8.3%) as well as a separation of WT and *Δ6abc/5*^{Mt} samples between PC2 and PC3 (6.8%) (Fig. 4). Although not as strong, we also found a clear tendency for separation of WT and *Δ6bc*^{Mt} samples between PC2 and PC3. Plant oil diets and CRISPR-mutation seemed to have different impacts on gene transcription in salmon liver, though both the diet and mutation have generated low levels of LC-HUFA in the fish body. The 20 most variant genes are listed in Supplementary Table 3.

Differential expression analysis (DEA) was done by contrasting crispants and WT salmon separately under plant oil and fish oil diets. This resulted in 121 differentially expressed genes (DEGs, $q < 0.05$ & $|\log2FC| > 0.5$) in *Δ6abc/5*^{Mt} salmon compared to WT when fed a fish oil diet, while 104 DEGs were found between crispant and WT salmon under a plant oil diet (Fig. 5 a). Surprisingly, more DEGs were found in *Δ6bc*^{Mt} salmon compared to WT. This includes 1156 genes identified in crispant salmon when fed a fish oil diet and 1348 DEGs identified in salmon fed a plant oil diet. A total number

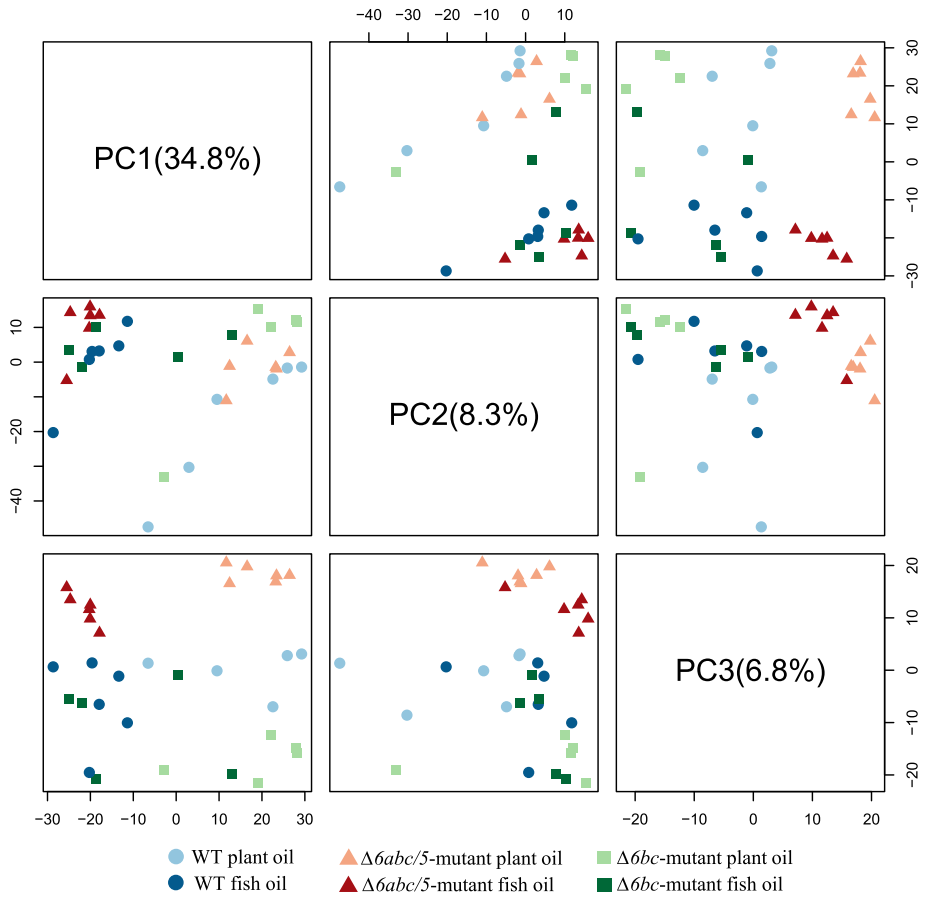

Fig. 4 Principle component analysis (PCA) on Log2 count per million (CPM) of the top 1000 most variant genes between all liver samples. Different colors represents genetic groups of WT, **Δ***6abc/5*-mutated and **Δ***6bc*-mutated salmon, while the color intensity represents different dietary treatments of either plant oil (low HUFA) diet or fish oil diet (high HUFA)

of 3987 DEGs was found in WT salmon fed a plant oil diet compared to fish oil, while the numbers of diet-associated DEGs were 4179 and 2057 in $\Delta 6abc/5^{Mt}$ and $\Delta 6bc^{Mt}$ fish respectively.

To further understand the functions of DEGs between crispant and WT salmon, we conducted a KEGG enrichment analysis by comparing the number of DEGs to the total number of genes in each KEGG pathway (Fig. 5 b). The DEGs of $\Delta 6abc/5^{Mt}$ salmon were not only enriched in the fatty acid metabolism pathway, but also the peroxisome proliferator-activated receptors (PPAR) signalling pathway which is involved in many metabolic pathways including fatty acid synthesis and catabolism [28]. This supports previous studies, indicating PPAR to be the key transcriptional regulator of fatty acid metabolism in salmon [3]. Differential regulation of these pathways was likely caused by decreased EPA and DHA, and consequential accumulation of 18:3n-3 and 18:2n-6 after disruption of the LC-HUFA synthesis pathway [22]. Accumulated 18:3n-3 and 18:2n-6 could not be synthesised further to DHA and EPA after disruption of *fads2* genes. Instead they were most likely consumed by β-oxidation which was activated by the PPAR transcription factor [28]. Similar enrichment of fatty acid metabolism and PPAR signalling pathways was also found in the DEGs between WT salmon fed plant oil and fish oil (Fig. 5 b). Additionally, the sterol biosynthesis pathway was enriched for DEGs between WT salmon fed plant oil and fish oil, but was not enriched for the DEGs between *fads2* mutants versus WT fish (Fig. 5 b). Indicating that the LC-HUFA level and PPAR has little effect on cholesterol biosynthesis in salmon, which is more likely regulated by other biochemical signals such as low cholesterol level and other transcription factors including sterol regulatory binding protein 2 (SREBP2) [12, 13, 15]. Many other pathways were also enriched for the DEGs of WT fed plant oil versus fish oil, such as amino acid biosynthesis and RNA transport. This suggests that dietary inclusion of plant oil has more complex impact on salmon than just reducing LC-HUFA and cholesterol levels in the fish body. Our study has successfully separated the effect of low LC-HUFA level from other effects of plant oil inclusion,

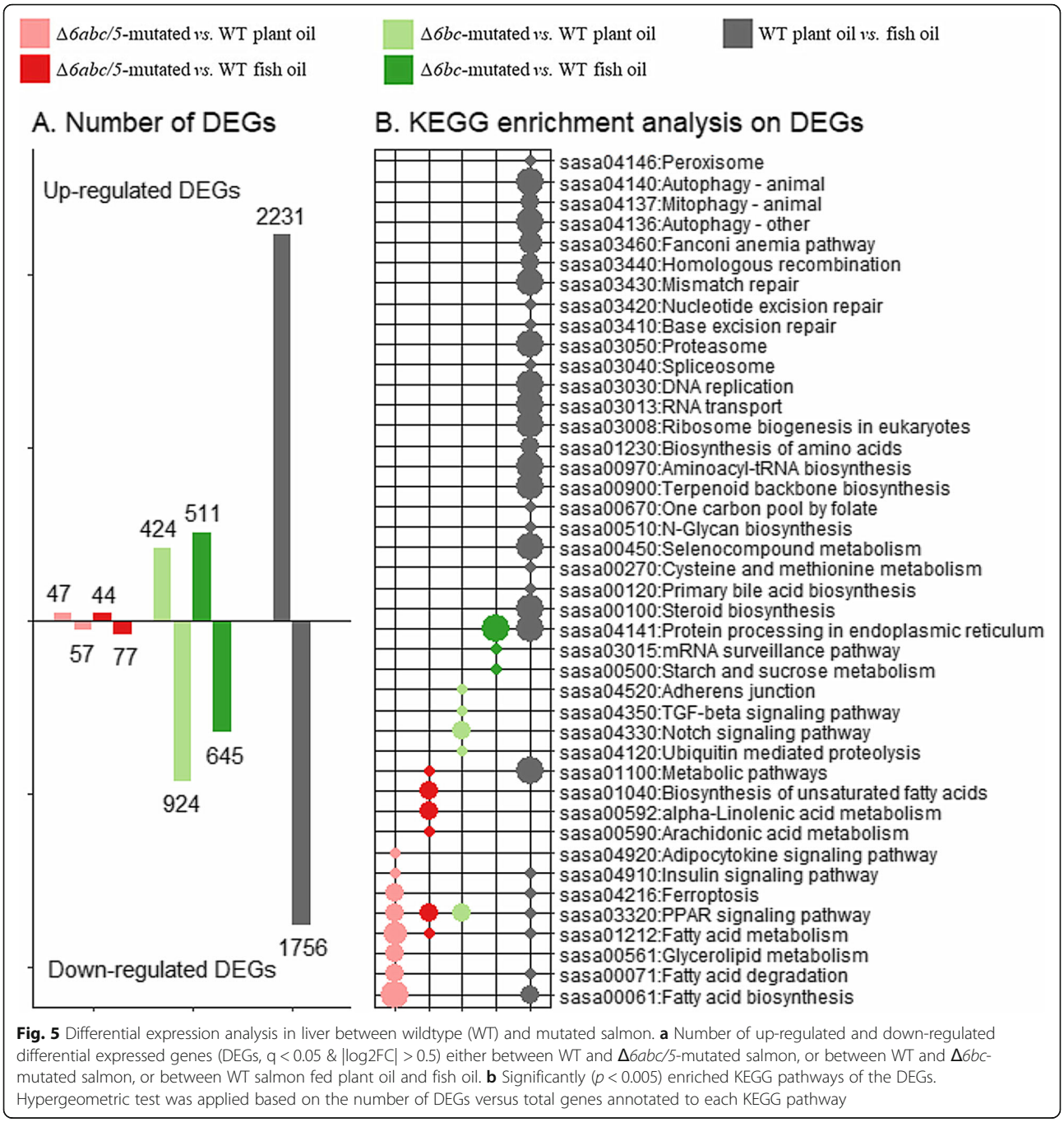

Fig. 5 Differential expression analysis in liver between wildtype (WT) and mutated salmon. **a** Number of up-regulated and down-regulated differential expressed genes (DEGs, q < 0.05 & |log2FC| > 0.5) either between WT and Δ*6abc/5*-mutated salmon, or between WT and Δ*6bc*-mutated salmon, or between WT salmon fed plant oil and fish oil. **b** Significantly ($p < 0.005$) enriched KEGG pathways of the DEGs. Hypergeometric test was applied based on the number of DEGs versus total genes annotated to each KEGG pathway

however more research is required to understand the complete regulatory network in response to the change of plant oil in the diet. Surprisingly, no lipid metabolism pathways were enriched in $\Delta 6bc^{Mt}$ salmon compared to WT, regardless of dietary LC-HUFA level. This was in accordance to the fatty acid composition in liver, where no significant difference was found between $\Delta 6bc^{Mt}$ salmon and WT [22]. The DEGs were likely more enriched in mRNA regulation pathways, including mRNA surveillance and spliceosome pathways. Nevertheless, the reason for the high number of DEGs in $\Delta 6bc^{Mt}$ salmon and their enriched pathways needs to be further investigated.

Expression of lipid metabolism genes in response to Δ*6abc/5* mutation

Due to many unexpected and lipid metabolism unrelated DEGs found in $\Delta 6bc^{Mt}$ salmon, only $\Delta 6abc/5^{Mt}$ fish were included for further transcriptomic analysis to understand the transcriptional regulation of lipid metabolism after disrupting LC-HUFA synthesis genes. Here we

discussed DEGs of lipid metabolism pathways that were enriched in *Δ6abc/5*^{Mt} versus WT salmon, aiming to understand the regulatory network of lipid metabolism genes in response to *Δ6abc/5*^{Mt}. The *Δ6abc/5* mutant showed 14 (13.4%) differentially expressed lipid metabolism genes when fed plant oil diet, while fewer (7 genes, 5.8%) lipid DEGs were identified in salmon fed the fish oil diet (Supplementary Table 1). The higher numbers of DEGs in *Δ6abc/5*^{Mt} salmon fed the plant oil diet suggest a compensatory response to the combined effects of impaired endogenous LC-HUFA biosynthesis and reduced dietary LC-HUFA levels. On the other hand, the reduced number of lipid DEGs in *Δ6abc/5*^{Mt} salmon fed the fish oil diet suggests an impact of dietary LC-HUFA levels on gene transcription, most likely an end-product-mediated inhibition. Nevertheless, 4 lipid DEGs were identified in *Δ6abc/5*^{Mt} fish fed both plant oil and fish oil experimental diets including *Δ5fad*, *Δ6fad-a*, *abcd1* and *acc2*. Besides the two CRISPR-targeted genes, the down-regulation of *acc2* and up-regulation of *abcd1* suggests an increase of the fatty acid *β*-oxidation pathway for energy expenditure after CRISPR-mutation [29].

Low levels of LC-HUFA often induce hepatic expression of *Δ5fads2* and *Δ6fads2-a* genes as shown in our previous *elovl2*-mutated salmon [18]. On the other hand, reduced DHA level has little effect on the expression of *elovl5* and *elovl2* genes as shown in the present *Δ6abc/5*^{Mt} salmon (Fig. 3). However, the expression of *elovl2* and *elovl5* genes are often up-regulated in fish fed plant oil compared to fish oil diets (Fig. 3) [30, 31]. Although plant oil diets also contains lower DHA and EPA, our data has shown that the expression of *elovl* genes was more likely induced by other differences between fish oil and plant oil diets. Sterol regulatory element binding proteins (SREBPs) are suggested to be involved in regulating lipid metabolism in both mammals and fish [32, 33]. Atlantic salmon has four *srebp1* paralogous genes, *srebp1a*, *srebp1b*, *srebp1c* and *srebp1d* which are all orthologs of the zebrafish *srebp1* gene (Supplementary Table 1). Both *Δ6abc/5*^{Mt} and low LC-HUFA diets resulted in increased transcription of all four *srebp1* genes in salmon (Fig. 6 and Supplementary Table 1). The transcription of the *srebp1* genes was negatively ($p < 0.05$) correlated to the DHA level in phospholipids. On the other hand, transcription of *srebp2* genes were not up-regulated in mutated versus WT salmon, and are not correlated to DHA level (Fig. 6 b). The different regulation of *srebp1* and *srebp2* transcription is consistent with previous studies in mammals, suggesting that *srebp1* transcription is regulated by DHA levels in salmon, while *srebp2* transcription is more likely to be induced by low cholesterol levels in the plant oil diet [32].

By comparing salmon gene promoter sequences to 6 transcription factor binding sites databases (CISBP, HUMAN.H10MO.B, HT-SELEX2, HumanTF, JASPAR, TRANSFAC), we identified 235 lipid metabolism genes with potential sterol regulatory elements (SRE), the Srebp binding sites, between 1000 bp upstream to 200 bp downstream from transcription starting sites (Supplementary Table 2). This includes *Δ5fads2*, *Δ6fads2-a*, *elovl5-a*, *elovl5-b* and *elovl2* which are the major genes in LC-HUFA synthesis pathway. A recent study showed that CRISPR/Cas9-mediated editing of *elovl2* in salmon has increased transcription of *srebp1*, *Δ6fads2* and *Δ5fads2* genes together with decreased LC-HUFA content, supporting the regulation of *fads2* genes by the Srebp-1 transcription regulator (Fig. 7) [18]. However, the salmon Srebp-1 transcription factor is unlikely to induce expression of *elovl5* and *elovl2*. This was because the expression of both *elovl* genes were stable in *Δ6abc/5*^{Mt} compared to WT salmon, though *srebp1* expression was upregulated. The *elovl5* genes were also stable in *elovl2*-mutated salmon [18]. One possible reason is that the SRE in promoter regions of *elovl5* and *elovl2* genes may be more efficient for binding Srebp-2 rather than Srebp-1 [34], or that other transcription factors such as liver X receptor (LXR) are responsible stimulation of *elovl* genes in salmon under a plant oil diet. On the other hand, mammalian SREBP-1 can target both fatty acid desaturase (*FADS2*) and elongase (*ELVOL5*) genes and regulate LC-HUFA synthesis [35, 36].

To further investigate the relationship between key transcription factors and lipid metabolism genes, we compared the expression changes of the 230 lipid metabolism genes except LC-HUFA synthesis genes, either between mutated and WT salmon fed plant oil, or between mutated and WT salmon fed fish oil, or between WT salmon fed plant oil and fish oil (Fig. 6a). Several *agpat3* and *acsbg* genes were significantly ($q < 0.05$ & $|\log2FC| > 0.5$) up-regulated in plant oil mutated salmon together with up-regulated *srebp1*. The function of the Srebp-1 transcription factor in salmon is likely similar to its function in mammals, which works as a key transcription factor for hepatic lipogenesis, and *agpat3* and *acsbg* genes are likely the key target genes of salmon Srebp-1. The same *acsbg*, *agpat3* and *srebp1* genes were also up-regulated when the *elovl2* gene was CRISPR-mutated in salmon, confirming an increase of fatty acid acylation and lipogenesis in response to decreased tissue DHA content (Fig. 7) [18]. Other typical mammalian SREBP-1 targets, *fasn*, *acc1* and *elovl6* genes of fatty acid synthesis and elongation pathways were also up-regulated, but not significantly ($q > 0.05$) in mutated salmon compared to WT under the plant oil diet (Fig. 6). However, the transcriptional increase of these genes was much higher and significant ($q < 0.05$) in WT salmon fed the plant oil diet compared to fish oil. This means that the genes of fatty acid synthesis and elongation in salmon were not merely targeted by Srebp-1, but by other

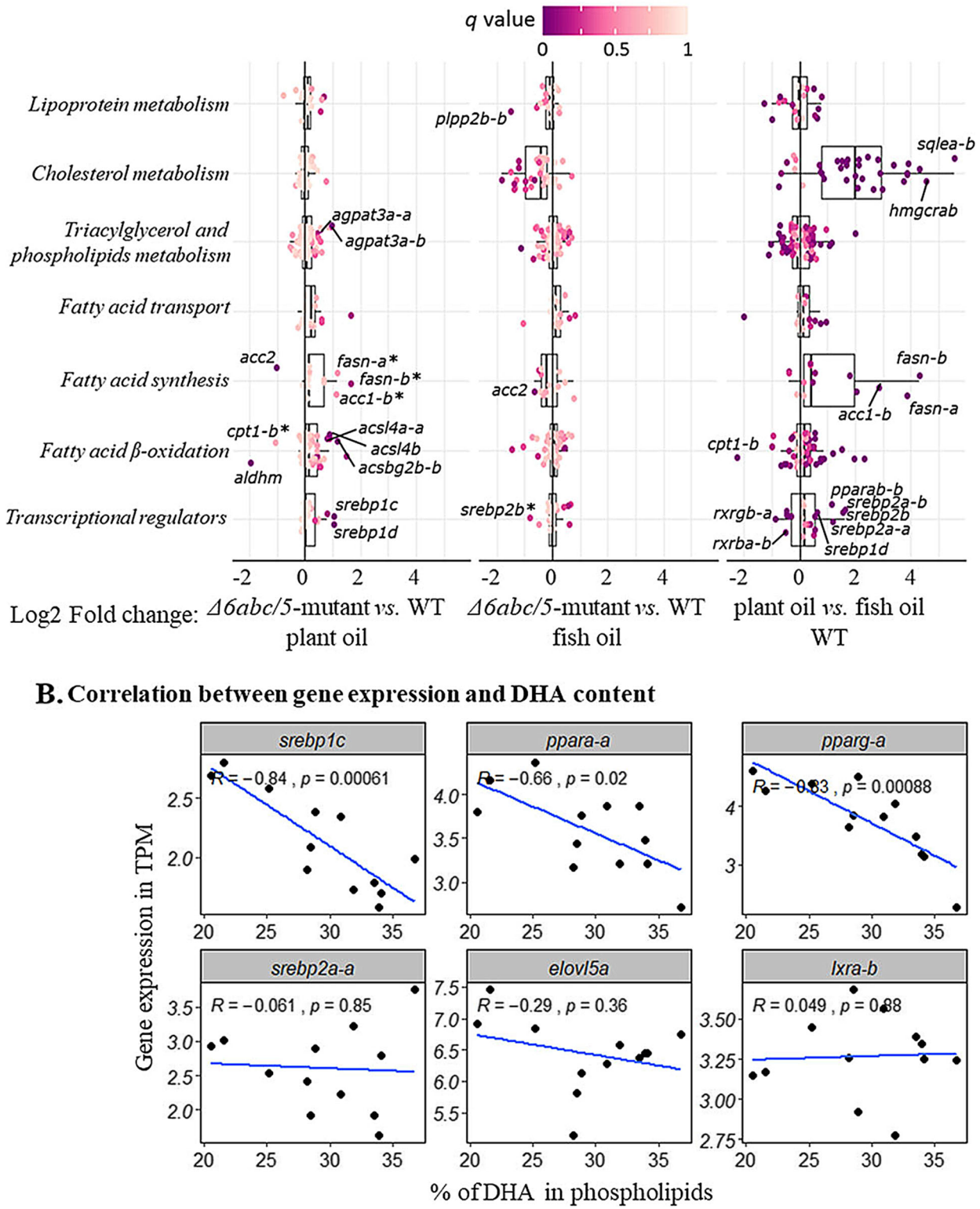

Fig. 6 (See legend on next page.)

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Fig. 6 Expression change of liver genes involved in lipid metabolism after Δ*6abc/5*mutation. **a** Expression changes of genes in Log2 fold change between Δ*6abc/5*^{MT} and WT salmon. Differentially expressed genes (DEGs, $q < 0.05$ & $|log2FC| > 0.5$) are labelled, except three genes with asterix (*) which had high log2 fold change but not significant ($q > 0.05$) **b** Correlation between gene expression and DHA content in phospholipid. Three fish individuals of each diet (plant oil or fish oil) and genetic (WT or Δ*6abc/5*^{MT}) group were included in the analysis. Data of DHA measurement was acquired from Datsomor *et.al*, 2019

transcription factors, likely Srebp-2 [32] or Ppar-γ [37]. Genes of cholesterol metabolism including *hmgcrab*, *mvd-a* and *sqlea-a* were only highly up-regualted in WT fed plant oil diet versus fish oil, while no transcription change was observed in *Δ6abc/5*^{Mt} versus WT salmon. Several studies have found up-regulation of cholesterol biosynthesis and *srebp2* genes in salmon fed plant oils [12, 13, 15]. The present study has supported that the relationship between *srebp2* and cholesterol biosynthesis genes is quite conserved in salmon as in mammals, and suggests that the SREBP binding sites of cholesterol biosynthesis genes were *srebp2*-specific (Fig. 7) [32].

CRISPR/Cas9-mediated mutation of *fads2* genes in *Δ6abc/5* also affected the fatty acid β-oxidation pathway in salmon. This was indicated by a strong down-regulation of *acc2* gene following *Δ6abc/5*^{Mt} (Fig. 5). Unlike the *acc1* gene which is mostly involved in *de-novo* fatty acid synthesis in the cytosol, the *acc2* gene in mammals produces mitochondria-associated malonyl-CoA which is a negative regulator of CPT1 and inhibits mitochondria β-oxidation [38, 39]. Therefore, the down-regualtion of *acc2* in *Δ6abc/5*^{Mt} salmon could suggest an increased fatty acid β-oxidation after disrutpion of LC-HUFA sythetic pathway. This could be regulated by PPAR which is key regualtor of fatty acid catabolism [28]. Similar to *srebp1*, we also found a negative correlation between DHA level and two *ppara-a* genes, though their expression levels were not changed after *Δ6abc/5* mutation. As PUFA and their derivatives are known natural ligands of PPAR, the activation of PPAR and their target genes including fatty acid β-oxidation may not rely on increased transcirption of PPAR genes [40]. The increased β-oxidation was probably due to accumulation of 18:3n-3, 18:2n-6, and other intermediate fatty acids in the LC-HUFA synthesis pathway which cannot be synthesised further to DHA and EPA after

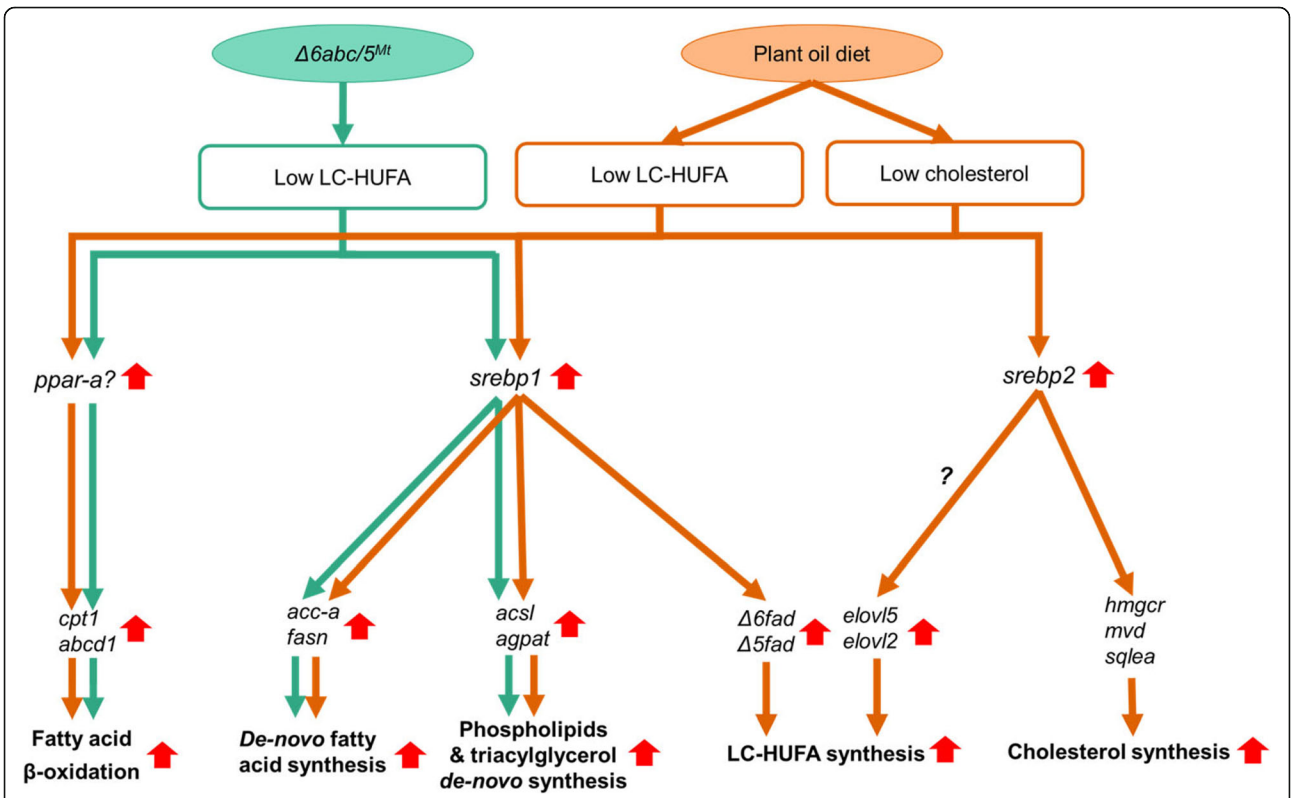

Fig. 7 Transcription regulation of lipid metabolism genes after Δ*6abc/5*^{MT} or after feeding plant oil diet. Up red arrow indicates increased transcription of genes in Δ*6abc/5*^{MT} compared to WT (green line) and in plant oil compared to fish oil (orange line)

disruption of *fads2* genes. These fatty acids were most likely consumed alternatively in *β*-oxidation which was activated by the PPAR transcription factor [22]. Feeding of plant oil diets also induced *cpt1a* and *abcd1*, which are key genes involved in import of fatty acids into mitochondria and peroxisomes for catabolism (Fig. 7). However, a paralog gene *cpt1b* was down-regulated both after *fads2*-mutation and feeding plant oil diet. The reason for the down-regualtion is unclear and whether it would affect fatty acid *β*-oxidation needs to be further investigated. One possible explanation is that malonyl-CoA produced by *acc1* or *acc2* is less organelle-specific in salmon, and that the *cpt1b* gene could be inhibited by malonyl-CoA produced by *acc1* in *de-novo* fatty acid synthesis.

Conclusions

CRISPR-Cas9 can be employed efficiently to mutate multiple *fads2* genes simultaneously in salmon. However, mosaic effects are common, embodied by different indels among tissues and individuals. Exon skipping found in the *Δ6fads2-a* gene during transcription was predicted to result in the production of truncated proteins and strengthen the CRISPR-induced disruption of LC-HUFA synthesis in *Δ6abc/5*^{Mt} salmon. Down-regulation of the targeted *Δ5fads2*, *Δ6fads2-a* and *Δ6fads2-b* genes were found in liver, which likely cause a decrease of LC-HUFA synthesis. On the other hand, the transcription of *elovl5a*, *elovl5b* and *elovl2* genes in the LC-HUFA synthesis pathway was not affected. Since *srebp1* genes were up-regulated in *Δ6abc/5*-mutated salmon the *elovl* genes were not likely regulated by this transcription factor. Increased *de-novo* fatty acid synthesis and lipogenesis was observed after *Δ6abc/5*^{Mt} and could also be regulated by SREBP1. In addition, the level of transcriptional changes of *fasn* and *acc1* genes involved in fatty acid synthesis were much higher when the fish was fed plant oil as compared to fish oil. This suggests that these genes were regulated by one or more transcriptional factors in addition to SREBP1. PPAR or SREBP2 are likely candidates. Increased fatty acid β-oxidation was also observed after *Δ6abc/5*^{Mt} and was likely regulated by PPAR. The CRISPR-mutation of *Δ6bc*^{Mt} genes surprisingly revealed over 3000 DEGs in liver of salmon, and the DEGs were not enriched in any lipid metabolism pathways. The reason for the high number of DEGs in *Δ6bc*^{Mt} salmon was unclear and needs to be further investigated.

Methods

Generation of CRISPR/Cas9-mediated mutated salmon and feeding experiment

The generation of CRISPR/Cas9-mediated mutated salmon and the corresponding feeding trial was previously published in [22]. In brief, two types of *fads2* mutants were generated with CRISPR/Cas9 injection into embryos, sperm and eggs were provided by AquaGen (Trondheim, Norway). Both times a single CRISPR guide RNA (gRNA) was used to target different combinations of *fads2* genes simultaneously: A *Δ6abc/5*-mutated (*Δ6abc/5*^{Mt}) salmon strain was generated using a gRNA targeting *Δ6fads2-a* (NCBI Gene ID 100136441), *Δ6fads2-b* (100329172), *Δ6fads2-c* (106584797) and *Δ5fads2* (100136383). A *Δ6bc*-mutated (*Δ6bc*^{Mt}) salmon strain was generated targeting *Δ6fads2-b* and *Δ6fads2-c*. Both strains were co-injected with a gRNA targeting the *slc45a2* (NCBI Gene ID gene 106563596), involved in melanin synthesis [19]. Target sequences of gRNAs were published in Datsomor *et.al*, 2019.

The feeding trial was performed on Atlantic salmon parr ($N = 108$) of approximately 85 ± 25 g for *Δ6abc/5*^{Mt} salmon ($N = 36$), 104 ± 25 g for *Δ6bc*^{Mt} salmon ($N = 36$), and 176 ± 34 g for wildtype controls (WT; $N = 36$) at the Institute of Marine Research (Matre, Norway). Fish were initially fed a standard commercial diet until start of the experiment. A total of six experimental tanks were used with a common-garden approach, each containing 18 fish consisting of 6 Pit-tagged fish of the *Δ6abc/5*^{Mt}, *Δ6bc*^{Mt} and WT. Three tanks were then fed a plant oil diet containing 5% LC-HUFA of total fatty acids, while the remaining three tanks were fed a fish oil diet with 20% LC-HUFA. The fatty acid composition of the diets was shown in detail in [22]. After 54 days of feeding, fish under plant oil diet reached 203 ± 51 g for *Δ6abc/5*^{Mt} salmon, 281 ± 52 g for *Δ6bc*^{Mt} salmon and 250 ± 62 for WT, while the fish under fish oil diet reached 171 ± 36 g, 191 ± 69 g and 241 ± 47 g for the three groups respectively. Liver and muscle tissues from 6 fish per dietary treatment/strain were then sampled and tissues were flash frozen on dry ice and subsequently stored at − 80 °C. During tissue sampling, unnecessary pain was avoided by anesthetizing all fish by placing in freshwater containing 100 mg/L Finquel MS-222 (Tricaine Methanesulfonate) buffered with 100 mg/L sodium bicarbonate (Scan Vacc AS, Hvam, Norway) which caused rapid loss of consciousness (no body or opercula movement), this was followed by euthanasia using a blow to the head.

AmpliSeq

To confirm CRISPR/Cas9-induced mutations, AmpliSeq was conducted according to the Illumina protocol (16S Metagenomic Sequencing Library Preparation # 15044223 Rev. B, Illumina AS, San Diego, CA, USA). DNA was isolated from selected individuals from both liver and muscle using DNeasy blood and tissue kits (Qiagen, Hilden, Germany). Primers were designed to specifically amplify the regions around the CRISPR gRNA target sites (Table 1). For each sample the amplicons were generated in singleplex reactions, pooled and then purified using AMPure beads (Beckman

Table 1 CRISPR gRNA target sequences and AmpliSeq primer sequences

CRISPR gRNA	Target Gene	CRISPR targets (5′- > 3′)[b]	AmpliSeq primer sequences
Delta6abc/5	Δ*6fads2-a*	GGCACCGACAGAGCC CAGCCAGG[a]	Forward (5′- > 3′): TTTGTAGGACGCATTTGTCGC Reverse (5′- > 3′): AGATGACACACTACTTTTCTAGGAG
Delta6abc/5	Δ*6fads2-b*	GGCACCGACAGAGCCCAG CCAGG[a]	Forward (5′- > 3′): CCCGGGTCCCTACCTAAACCTA Reverse (5′- > 3′): CTCCTCCCCTTCATCAGGTGAC
Delta6abc/5	Δ*6fads2-c*	GGCACCGACAGAGCCCAG CCAGG[a]	Forward (5′- > 3′): GAGACGCTCTAGGCTTCACA Reverse (5′- > 3′): TCCCAGCGGTTTGGATCATTC
Delta6bc	Δ*6fads2-b*	[a]CCAAGGGTGGCGTGG TTGGGCCC	Forward (5′- > 3′): TGATCCAAACCGCTGGGAAAT Reverse (5′- > 3′): ACGGTGTGAGTGGAGCAGAG
Delta6bc	Δ*6fads2-c*	[a]CCAAGGGTGGCGTGG TTGGGCCC	Forward (5′- > 3′): AGAGTCCATTCCCAGGACGAA Reverse (5′- > 3′): ACAGACTGGACAGAGCGTAG
Slc45a2	*slc45a2*	GGGGAACAGGCCGAT AAGACTGG[a]	Forward (5′- > 3′): TGTATGAGCTACAGACAGGTGG Reverse (5′- > 3′): AGGGGCTCTACTTC GTAGGAT

Forward overhang: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[sequence]
Reverse overhang: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[sequence].
[a] Underlined trinucleotides are the CRISPR protospacer adjacent motif (PAM) sites
[b] The CRISPR target sites was published in Datsomor *et.al*, 2019

Coulter Life Sciences, Indianapolis, IN, USA) before running index-PCR using the Nextera XT Index Kit (Illumina AS, San Diego, CA, USA). AmpliSeq libraries were subsequently normalized before sequencing the libraries as 300 bp paired-end reads on Illumina MiSeq (Illumina, San Diego, CA, USA) at Centre of Integrative Genetics (CIGENE, Ås, Norway). Raw .fastq reads were quality trimmed using *cutdapt* [41] before aligning them to the salmon genome ICSA SG_v2 (Accession Number GCF_000233375.1, available for download at NCBI database https://www.ncbi.nlm.nih.gov/assembly/GCF_000233375.1/) using *bwa mem* [42] and saving files in .bam format. For each sample the proportion of indels for each base in a 25 bp window around the target sites was determined using the python3 coverage.py (https://gitlab.com/fabian.grammes/crispr-indel). Additionally we predicted the effect of each indel on the main transcript/protein using *SnpEff* [43].

RNA extraction and library preparation

Total RNA was extracted from liver of 36 individual fish by using RNeasy Plus Universal Mini kit (Qiagen AS, Hilden, Germany), according to manufacturer's instruction. The 36 fish comprised 6 fish by group (strain by dietary treatment; two fish / tank). The RNA concentration and quality were assessed by Nanodrop 8000 (Thermo Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples had RIN values > 8.5. RNA-seq libraries were prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina AS, San Diego, CA, USA). The libraries were subsequently sequenced using 100 bp single-end high-throughput mRNA sequencing (RNA-seq) on an Illumina Hiseq 2500 (Illumina AS, San Diego, CA, USA) at Norwegian Sequencing Centre (Oslo, Norway).

Data analysis and statistics

Read sequences were processed using the *bcbio-nextgen* pipeline (https://github.com/bcbio/bcbio-nextgen). In brief reads were aligned to the salmon genome (ICSASG_v2) using *STAR* [44]. The resulting .bam files were subsequently used to generate i) raw gene counts using *featureCounts* (v1.4.4) [45] using the NCBI *Salmo salar* Annotation Release 100 (available for download at https://ftp.ncbi.nlm.nih.gov/genomes/all/annotation_releases/8030/100/). ii) exon counts using *DEXSeq* (dexseq_count.py) [46]. In addition reads were mapped directly to the transcriptome using Salmon (v0.10.2) [47]. Gene IDs from NCBI GeneBank database (https://www.ncbi.nlm.nih.gov/) were used to identify genes in this study.

Expression analysis of the genes was performed using R (v3.4.1). Only genes with a minimum counts level of at least 1 count per million (CPM) in 75% of the samples were kept for further differential expression analysis (DEA). DEA was performed between groups (strain by dietary treatment, $n = 6$), using the generalized linear model (GLM) method in R package edgeR [48]. The present study focuses on three contrasts, *Δ6abc/5*-mutated salmon versus WT fed plant oil diet, *Δ6abc/5*-mutated salmon versus WT fed fish oil diet, and WT salmon fed plant oil versus fish oil diet. Genes with a false discovery rate (FDR), an adjusted p value (q) < 0.05 and absolute log2 fold change (|Log2FC|) > 0.5 were considered to be differentially expressed genes (DEGs) between the two test conditions. Subsequently, a KEGG ontology enrichment analysis (KOEA) was conducted using edgeR. A hypergeometric test was applied based on number of DEGs compared to total genes annotated to each KEGG pathway, and differences were considered significant when $p < 0.005$. All figures were made by using R package ggplot2 [49].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-020-07218-1.

Additional file 1 (XLSX 608 kb)
Additional file 2 (CSV 61 kb)
Additional file 3 (CSV 3 kb)

Abbreviations

LC-HUFA: Long-chain highly unsaturated fatty acids; FADS2: Fatty acid desaturases 2; DEGs: Differentially expressed genes; SREBP1: Sterol regulatory binding protein 1; LXR: Liver X receptor; PPAR: Peroxisome proliferator-activated receptor; ELOVL5: Elongase 5; ELOVL2: Elongase 2; Δ5fad: Δ5 fatty acid desaturase; Δ6fad: Δ6 fatty acid desaturase; CPT1: Carnitine palmitoyltransferase I; ACC: Acetyl-CoA carboxylase; Indels: Insertions and deletions; gRNA: Guide RNA

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Authors' contributions

Conceptualization: YJ, AD, RE, JT, PW, FG, REO. Data Curation: YJ, FG. Formal Analysis: YJ, FG. Funding Acquisition: RE, JT, PW, JOV, AW. Methodology: YJ, FG. Resources: AD, RE, AW, PW, FG, REO. Visualization: YJ, FG. Writing – Original Draft Preparation: YJ, FG. Writing – Review & Editing: YJ, JOV, AW, AD, RE, JT, PW, FG, REO. All authors have read and approved the manuscript.

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Availability of data and materials

Raw fastq files and raw gene counts table are publicly available under the accession: E-MTAB-8319 at the ArrayExpress Archive (https://www.ebi.ac.uk/arrayexpress/).
The R code which used to generate DEA and KOEA is publicly available at Fairdomhub (https://fairdomhub.org/investigations/242).
The protocol for 16S Metagenomic Sequencing Library Preparation is publicly available at Illumina (https://emea.support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html).
Gene IDs from NCBI GeneBank database (https://www.ncbi.nlm.nih.gov/) were used to identify genes in this study. For example, Δ*6fads2-a* 100136441 (https://www.ncbi.nlm.nih.gov/gene/100136441), Δ*6fads2-b* 100329172 (https://www.ncbi.nlm.nih.gov/gene/100329172), Δ*6fads2-c* 106584797 (https://www.ncbi.nlm.nih.gov/gene/106584797) and Δ*5fads2* 100136383 (https://www.ncbi.nlm.nih.gov/gene/100136383).
The salmon genome ICSASG_v2 (Accession Number GCF_000233375.1) is publicly available for download at NCBI database (https://www.ncbi.nlm.nih.gov/assembly/GCF_000233375.1/).
The *Salmo salar* Annotation Release for ICSASG_v2 (Annotation release ID 100) is used in this study and it is publicly available for download at NCBI database (https://ftp.ncbi.nlm.nih.gov/genomes/all/annotation_releases/8030/100/).

Ethics approval and consent to participate

All experiments on animals were performed in strict accordance with the Norwegian Animal Welfare Act of 19th of June 2009. Experiments carried out in this study were approved by the Norwegian Animal Research Authority (NARA 5741).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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