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 (fads2) family. The present study applied whole transcriptome analysis on two CRISPR-mutated salmon strains (crispants), 1) Δ6abc/5Mt with mutations in Δ5fads2, Δ6fads2-a, Δ6fads2-b and Δ6fads2-c genes, and 2) Δ6bcMt with mutations in Δ6fads2-b and Δ6fads2-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 Δ6abc/5Mt and Δ6bcMt crispants demonstrated high percentage of indels within all intended target genes, though different indel types and percentage were observed between individuals. The Δ6abc/5Mt 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 Δ6bcMt 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-HUFAAs [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 elovl5b 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 C18 and C20 fatty acids, while elovl2 and elovl4 are involved in elongating C20 and C22 [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 C18 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 mosaicism 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/5Mt salmon targeted Δ6fads2-a, Δ6fads2-b, Δ6fads2-c and Δ5fads2 genes, while the gRNA of Δ6bcMt 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/5Mt and Δ6bcMt strains were confirmed by using AmpliSeq. All fish injected with CRISPR/Cas9 carried structural variants at the respective gRNA target sites (Fig. 1b). For all individuals from both CRISPR 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/5Mt gRNA targeted sequence right after the cytochrome b5-like domain of fads2 genes, while Δ6bcMt gRNA targeted sequences on exon 1 before all protein domains. Therefore, the out-of-frame mutations in Δ6abc/5Mt and Δ6bcMt 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/5Mt fish [22].

![Fig. 1](image_url)

**Fig. 1** a Circos plot showing the different target sites of the CRISPR gRNAs. Gene Δ6fads2, 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 Δ6fads2-a exon skipping events

Interestingly, we found that CRISPR/Cas9 induced mutations of Δ6abc/5Mt 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/5Mt 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 Δ6abc/5 but not Δ6bc 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/5Mt salmon compared to WT regardless of the dietary treatment (Fig. 3). In Δ6bcMt 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/5Mt salmon. Surprisingly, the expression of Δ5fads2 and Δ6fads2-a genes was also down-regulated in Δ6bcMt salmon, though both genes were not targeted by Δ6bcMt 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.
mutations (Fig. 3). The expression of other genes in the LC-HUFA synthesis pathway, elovl2, elovl5-a and elovl5-b, was stable between Δ6abc/5Mt, Δ6bcMt 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/5Mt 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/5Mt samples between PC2 and PC3 (6.8%) (Fig. 4). Although not as strong, we also found a clear tendency for separation of WT and Δ6bcMt 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/5Mt 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 Δ6bcMt 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
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 Δ6abc/5Mt and Δ6bcMt 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 Δ6abc/5Mt 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 consequent 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,
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 Δ6bcMt 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 Δ6bcMt 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 Δ6bcMt salmon and their enriched pathways needs to be further investigated.

**Expression of lipid metabolism genes in response to Δ6abc/5 mutation**

Due to many unexpected and lipid metabolism unrelated DEGs found in Δ6bcMt salmon, only Δ6abc/5Mt fish were included for further transcriptomic analysis to understand the transcriptional regulation of lipid metabolism after disrupting LC-HUFA synthesis genes. Here we

![Fig. 5](image-url)
discussed DEGs of lipid metabolism pathways that were enriched in Δ6abc/5Mt versus WT salmon, aiming to understand the regulatory network of lipid metabolism genes in response to Δ6abc/5Mt. 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/5Mt 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/5Mt 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/5Mt 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 levels have little effect on the expression of elovl5 and elovl2 genes as shown in the present Δ6abc/5Mt 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 contain lower DHA and EPA, our data has shown that the expression of elovl2 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/5Mt 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 elov genes were stable in Δ6abc/5Mt 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 acshb 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 acshb genes are likely the key target genes of salmon Srebp-1. The same acshb, 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
A. Expression change of lipid metabolism genes

| Lipoprotein metabolism | Cholesterol metabolism | Triacylglycerol and phospholipids metabolism | Fatty acid transport | Fatty acid synthesis | Fatty acid β-oxidation | Transcriptional regulators |
|------------------------|------------------------|---------------------------------------------|---------------------|---------------------|------------------------|---------------------------|
| plpp2b-b*              | agpat3a-a              | acc1-b*                                     | cpt1-b*             | srebpc1             | srebpd1                | srebpb2*                  |
| sqlea-b                | hmgcrab                | fasn-a*                                     | acc2                | fasn-a*             | rsa-rb                  | rsa-rb                    |

Log2 Fold change: $\Delta 6abc/5$-mutant vs. WT plant oil

$\Delta 6abc/5$-mutant vs. WT fish oil

plant oil vs. fish oil WT

B. Correlation between gene expression and DHA content

For each gene, the correlation coefficient ($R$) and p-value are provided. The gene expression is measured in TPM (transcripts per million).

Fig. 6 (See legend on next page.)
transcription factors, likely Srebp-2 [32] or Ppar-γ [37]. Genes of cholesterol metabolism including \textit{hmgcrab}, \textit{mvd-a} and \textit{sqlea-a} were only highly up-regualted in WT fed plant oil diet versus fish oil, while no transcription change was observed in $\Delta6abc/S^{Mt}$ versus WT salmon. Several studies have found up-regulation of cholesterol biosynthesis and \textit{srebp2} genes in salmon fed plant oils [12, 13, 15]. The present study has supported that the relationship between \textit{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 \textit{srebp2}-specific (Fig. 7) [32].

CRISPR/Cas9-mediated mutation of \textit{fads2} genes in $\Delta6abc/S^{5}$ also affected the fatty acid β-oxidation pathway in salmon. This was indicated by a strong down-regulation of \textit{acc2} gene following $\Delta6abc/S^{5Mt}$ (Fig. 5). Unlike the \textit{acc1} gene which is mostly involved in de-novo fatty acid synthesis in the cytosol, the \textit{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-regulation of \textit{acc2} in $\Delta6abc/S^{5Mt}$ salmon could suggest an increased fatty acid β-oxidation after disruption of LC-HUFA synthetic pathway. This could be regulated by PPAR which is key regulator of fatty acid catabolism [28]. Similar to \textit{srebp1}, we also found a negative correlation between DHA level and two \textit{ppara-a} genes, though their expression levels were not changed after $\Delta6abc/S^{5}$ 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 transcription 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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Transcription regulation of lipid metabolism genes after $\Delta6abc/S^{5Mt}$ or after feeding plant oil diet. Up red arrow indicates increased transcription of genes in $\Delta6abc/S^{5Mt}$ compared to WT (green line) and in plant oil compared to fish oil (orange line).}
\end{figure}
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 inducedcpt1a 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-regulation 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/SMt 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/S-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/SMt 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/SMt and was likely regulated by PPAR. The CRISPR-mutation of Δ6bcMt 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 Δ6bcMt 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/S-mutated (Δ6abc/SMt) 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 (Δ6bcMt) 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 (N = 108) of approximately 85 ± 25 g for Δ6abc/SMt salmon (N = 36), 104 ± 25 g for Δ6bcMt 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/SMt, Δ6bcMt 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/SMt salmon, 281 ± 52 g for Δ6bcMt salmon and 250 ± 62 g 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 (165 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...
Coulter Life Sciences, Indianapolis, IN, USA) before running index-PCR using the Nextera XT Index Kit (Illumina, A5, 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 cutadapt [41] before aligning them to the salmon genome (ICSAGenome) 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/803_0/100/). 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, \( \Delta 6abc/5 - \mu - \Delta 6abc/5 - \mu \) associated salmon versus WT fed plant oil diet, \( \Delta 6abc/5 - \mu - \Delta 6abc/5 - \mu \) associated 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 \((|\text{Log2FC}|) > 0.5 \) were considered to be differentially expressed genes (DEGs) between the two test conditions. Subsequently, a KEGG ontology enrichment analysis (KOEAS) 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].

### Table 1 CRISPR gRNA target sequences and AmpliSeq primer sequences

| CRISPR gRNA | Target Gene | CRISPR targets (5' > 3')a | AmpliSeq primer sequences |
|-------------|-------------|---------------------------|---------------------------|
| Delta6abc/5 | \( \Delta 6fads-2-a \) | GGACCGACGACGCGCC | Forward (5' > 3'): TTTTGGAGCACCATTTGTCG |
|             | \( \Delta 6fads-2-b \) | GCAGGGCCGACGACGCC | Reverse (5' > 3'): AGATGCACACTCTTTTTCAGGAG |
|             | \( \Delta 6fads-2-c \) | GCGGAGAACCGACGACGC | Forward (5' > 3'): CCCCGGTCTACTTGCTCCAAC |
|             | \( \Delta 6fads-2-d \) | CCAAGGTCGCGCTGTC | Reverse (5' > 3'): AGGGGTAGTGAGGAGGACGAG |
|             | \( \Delta 6fads-2-e \) | CCAAGGTCGCGCTGTC | Forward (5' > 3'): TGATCACCAGCGTGAGGAAAT |
|             | \( \Delta 6fads-2-f \) | CCAAGGTCGCGCTGTC | Reverse (5' > 3'): AGGGGTAGTGAGGAGGACGAG |
|             | \( \Delta 6fads-2-g \) | CCAAGGTCGCGCTGTC | Forward (5' > 3'): ACAAGACTGGACAGAGCG |
|             | \( \Delta 6fads-2-h \) | CCAAGGTCGCGCTGTC | Reverse (5' > 3'): ACAAGACTGGACAGAGCG |
| Slc45a2     | slc45a2     | GGGGAACAGCGCCG | Forward (5' > 3'): TGATGAGCAGCATCACAGGAG |

\( a \) Underlined trinucleotides are the CRISPR protospacer adjacent motif (PAM) sites

\( b \) The CRISPR target sites was published in Datsomor et.al [2019].

### RNA extraction and library preparation

Total RNA was extracted from liver of 36 individual fish by using RNaseasy 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/803_0/100/). 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.

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The authors declare that they have no competing interests.

Norwegian Animal Welfare Act of 19th of June 2009. Experiments carried out activated receptor; ELOVL5: Elongase 5; ELOVL2: Elongase 2; ΔLC-HUFA: Long-chain highly unsaturated fatty acids; FADS2: Fatty acid

Abbreviations

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

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Supplementary Information

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Additional file 1

Additional file 2

Additional file 3

Additional file 1: XLSX 608 kb

Additional file 2: CSV 61 kb

Additional file 3: CSV 3 kb

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