# REGULATION OF FATTY ACID DESATURASE EXPRESSION AND ALTERNATIVE SPLICING

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# REGULATION OF FATTY ACID DESATURASE EXPRESSION AND ALTERNATIVE SPLICING

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The *FADS1* and *FADS2* genes encode fatty acid desaturases required for synthesis of omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA) important in the central nervous system, inflammatory response, and cardiovascular health. The adjacent *FADS3* gene is a putative desaturase, and both *FADS2* and *FADS3* have alternative transcripts (AT) of unknown function. Here, genetic, dietary and molecular factors regulating expression and alternative splicing of FADS genes were identified. A *FADS2* haplotype was significantly associated with *FADS1* expression in lymphoblasts from Japanese International HapMap participants, and overlapped with a predicted sterol response element (SRE). Minor haplotype homozygotes had significantly higher induction of *FADS1* and *FADS2* after treatment with a statin drug or an LXR agonist, both activators of SREBP-1c. Sequencing identified 2 deletions within 150 bp of the putative SRE only present in minor haplotype carriers, suggesting this region controls response to SREBP-1c. We next examined the effect of dietary LCPUFA levels on baboon liver FADS gene expression. *FADS1* and both *FADS2* transcripts were downregulated by

dietary LCPUFA, but *FADS3* AT were upregulated, both in baboon liver and human liver-derived HepG2 cells. A PPARγ antagonist prevented *FADS3* upregulation, while *FADS1* and *FADS2* were unaffected. In HepG2 cells, knockdown of the polypyrimidine tract binding protein (PTB) modulated alternative splicing of *FADS2* and *FADS3*. *PTB* and *FADS2AT1* were inversely correlated in neonatal baboon tissues, implicating PTB as a major regulator of tissue-specific *FADS2* splicing. Omega-3 fatty acids decreased by nearly one half relative to omega-6 fatty acids in PTB knockdown cells, with a particularly strong decrease in eicosapentaenoic acid concentration and its ratio with arachidonic acid. The baboon results showing an opposing pattern and mechanism of regulation suggested a dissimilar function for *FADS3* AT compared to classical desaturases, and PTB knockdown results suggested possible dominant negative inhibition by FADS splice variants. This is a rare demonstration of a mechanism specifically altering the cellular omega-3 to omega-6 fatty acid ratio without any change in diet/media. These findings reveal how genotype and alternative splicing affect response to diet and medication, and alter availability of eicosanoid precursors for cell signaling.

## BIOGRAPHICAL SKETCH

Holly Turner Reardon grew up in North Hollywood, California. She began attending the University of California, Los Angeles part-time at the age of 15, and continued there full-time after graduating as valedictorian of her class at North Hollywood High School. She paid for college entirely with merit scholarships, including a National Merit Scholarship and a California Robert C. Byrd Scholarship. During college she read an article about a biotechnology start-up company called Tularik (now part of Amgen), which was seeking to discover drugs targeting transcription factors (a novel idea in those days.) Intrigued, she wrote to the company and asked if she could work there for a summer; in response, the company created a summer internship for her and later hired her full-time. She graduated *magna cum laude* from UCLA in 1995 with a B.S. in Molecular, Cellular and Developmental Biology, and moved to the San Francisco bay area. After Tularik, she worked in cancer drug discovery at Axys Pharmaceuticals (now part of Celera Genomics), and later developed microfluidic assays and instrumentation at Caliper Life Sciences. She was promoted six times in ten years, eventually reaching Ph.D.-level positions of Scientist I and Scientist II for her last four years in industry. Seeing the first signs of an economic downturn ahead, she began to worry about holding positions without the necessary credentials, and decided to go back to school for a Ph.D. As a strong believer in the Japanese saying, “food is medicine,” she decided she would like to bring her pharmaceutical industry perspective to the study of bioactive components in food. Eventually choosing the Molecular Nutrition program at Cornell, she became interested in omega-3 fatty acids and entered the laboratory of J. Thomas Brenna.

For my late father, Frank Turner.

He showed me which weeds in our yard were edible, how to tell the direction the Earth was moving by the path of stars in the sky, how to grow hydroponic strawberries on a smoggy rooftop, how to diagnose car trouble, and many other interesting things. In short, he taught me to think like a scientist. In his life, he was a counter-intelligence agent in World War II, a tractor operator, a mechanical engineer who designed jet engines, a mailman and later a postal supervisor, and a chemistry hobbyist who started his own company converting solid silver into a sprayable form. But most of all, he was an amazing and inspiring parent. I wish he were still here.

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I am tremendously grateful to my advisor, Tom Brenna, for giving me an amazing amount of freedom in choosing my research topics and designing my studies. I am also very thankful for funding support in my 4th year through an NRSA training grant in Reproductive Sciences and Genomics (Grant Number T32HD052471), and I thank Cornell for funding me in my first year through a Presidential Life Sciences Fellowship. I am indebted to the other members of the Brenna lab for creating a wonderful work environment. In particular, I am especially grateful to Woo Jung Park and Kumar Kothapalli for teaching me to work with RNA and do PCR, and for many helpful talks and lots of teamwork. I was especially lucky to have Jimmy Zhang, a very talented undergraduate, to work with me in the lab. Pete Lawrence was incredibly helpful with fatty acid analysis. My office mate, Rinat Ran-Ressler, was great company and helped preserve my sanity. Outside our lab, I am grateful to Magnolia Ariza-Nieto for helpful advice and gracious access to instruments.

I could not have got through this graduate school adventure without my caring family and the support of my wonderful husband, Brian Reardon. Also, kudos to my baby Helen for sleeping occasionally over the past five months so I could get some work done; heartfelt thanks to my mom for the glider, and to Lily and Paul Wallace for the baby swing, that helped to make the sleeping possible.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACTH | adrenocorticotropic hormone |
| ALA | alpha-linolenic acid, 18:3n-3 |
| ARA | arachidonic acid, 20:4n-6 |
| AT | alternative transcript |
| BSA | bovine serum albumin |
| CS | classically spliced |
| DHA | docosahexaenoic acid, 22:6n-3 |
| DPA | docosapentaenoic acid, 22:5n-3 |
| ELOVL | elongation of very long chain fatty acids |
| eQTL | expression quantitative trait locus |
| EPA | eicosapentaenoic acid, 20:5n-3 |
| ESS | exonic splicing silencer |
| FADS | fatty acid desaturase (FADS1, FADS2, FADS3) |
| FBS | fetal bovine serum |
| HDL | high density lipoprotein |
| hnRNP | heteronuclear riboprotein |
| InDel | Insertion/Deletion |
| IQ | intelligence quotient |
| JPT | Japanese in Tokyo International HapMap Project population |
| LA | linoleic acid, 18:2n-6 |
| LCPUFA | long-chain polyunsaturated fatty acid |
| LD | linkage disequilibrium |
| LDL | low density lipoprotein |
| LXR | liver X receptor |

| | |
|---|---|
| mut | mutant |
| PPAR | peroxisome proliferator-activated receptor |
| PPRE | peroxisome proliferator response element |
| PTB | polypyrimidine tract binding protein (also, hnRNP I) |
| qRT-PCR | quantitative real-time PCR |
| RNAi | RNA interference |
| RT-PCR | reverse transcriptase PCR |
| RXR | retinoid X receptor |
| siRNA | small interfering RNA |
| SNP | single nucleotide polymorphism |
| SR | serine-arginine |
| SRE | sterol response element |
| SREBP | sterol response element binding protein |
| wt | wild type |

# CHAPTER 1

# REGULATION OF LCPUFA BIOSYNTHESIS

## *Introduction*

The omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA) play important roles in many physiological processes. Omega-3 and omega-6 LCPUFA are precursors for oxygenated lipid mediators known as eicosanoids and docosanoids, which convey signals controlling inflammation and blood clotting [1], and affect gene expression directly through interaction with nuclear receptors [2]. The LCPUFA docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are major components of the central nervous system, comprising 25% of structural lipid in gray matter [3].

## *Dietary sources*

DHA and ARA can be obtained pre-formed from dietary sources, or can be synthesized starting from alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively (Figure 1.1). ALA and LA are considered essential fatty acids because humans cannot synthesize them, so they must be obtained from plant foods. The most recent dietary reference intakes for macronutrients from the Institute of Medicine defined Adequate Intakes as 12-17 g/day for LA, and 1.1 – 1.6 g/day for ALA [4]. Flax and canola oil are good sources of ALA, and nearly all vegetable and nut oils are abundant sources of LA. DHA and the eicosanoid precursor EPA (eicosapentaenoic

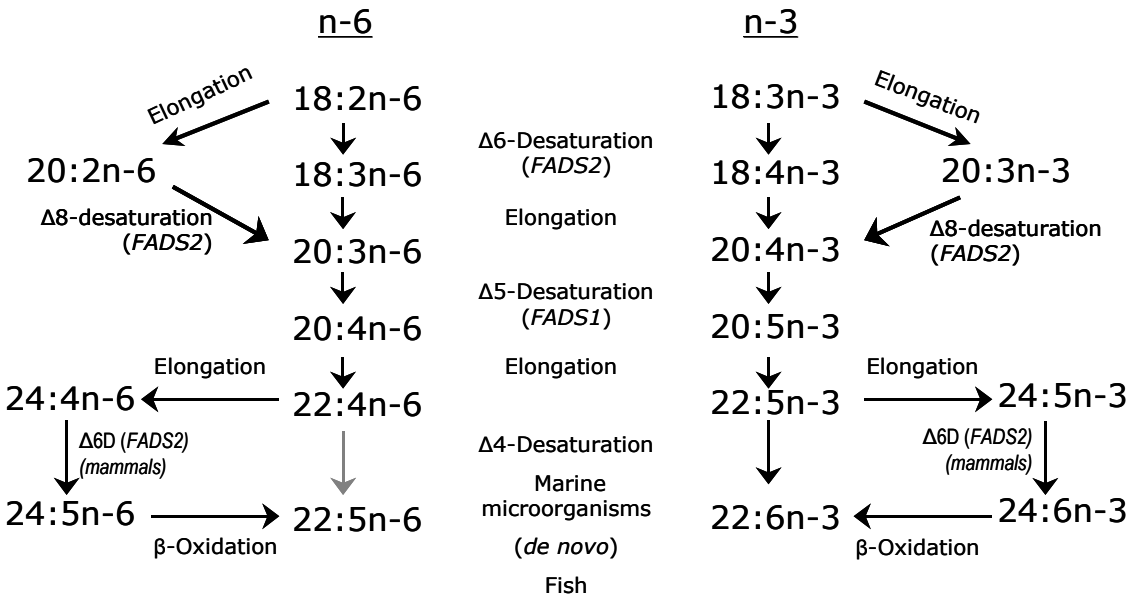


**Figure 1.1. Biosynthesis of omega-3 (n-3) and omega-6 (n-6) LCPUFA.** Nomenclature refers to the number of carbons and double bonds (e.g., 20:5n-3 has 20 carbons and 5 double bonds, with n-3 describing distance of the double bonds from the terminal methyl carbon of the fatty acid.) Fatty acids of special interest include linoleic acid (LA, 18:2n-6), alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), arachidonic acid (ARA, 20:4n-6), and docosahexaenoic acid (DHA, 22:6n-3).

acid, 20:5n-3) are mainly found in oily fish such as salmon and mackerel. No natural foods are especially rich in ARA, but it can reach high levels in farmed fish fed corn [5]. All dietary PUFA, including ALA and LA, have one of three fates, depending on physiological needs: incorporation into membrane phospholipids, beta-oxidation to yield energy, or biosynthesis of longer-chain polyunsaturated fatty acids.

### *Biosynthesis*

The biosynthetic pathway depicted in Figure 1.1 occurs through alternating steps of desaturation (addition of double bonds) and elongation (addition of two carbons) of fatty acyl-CoA substrates. Most of these steps have been reproduced in microsomal reactions, so the majority of the pathway is thought to occur in the endoplasmic reticulum. There are sporadic reports of desaturase activity in other membranes, for instance, in nuclei [6].

#### *Desaturation Reactions*

Desaturase enzymes introduce double bonds into the fatty acid chain, according to the general reaction:

$$R_1\text{-}CH_2\text{-}CH_2\text{-}R_2 + O_2 + 2e^- + 2H^+ \longrightarrow R_1\text{-}CH{=}CH\text{-}R_2 + 2H_20$$

LCPUFA biosynthesis requires the activity of two desaturases: the delta-6/delta-8 desaturase, encoded by *FADS2*, and the delta-5 desaturase, encoded by *FADS1*. The “delta” terminology refers to placement of the double bond by the number of carbons from the carboxyl end of the fatty acid (i.e., delta-5 introduces a double bond 5 carbons

from the carboxyl terminus.) Both enzymes are considered “front-end” desaturases due to their ability to introduce a double bond between the omega-9 position and the carboxyl (“front”) end of the fatty acid. A histidine repeat motif common to desaturase sequences is thought to coordinate two iron atoms in the active site for some desaturase enzymes [7,8], but details of the catalytic mechanism remain to be elucidated. Protein has never been purified reproducibly for these integral membrane enzymes, and no crystal structures have been reported. Both enzymes contain an N-terminal fused cytochrome *b5* domain, believed to act as an electron donor for the desaturation reaction. In a plant homolog of the delta-6 desaturase, mutagenesis of the heme binding domain of the cytochrome *b5* region results in loss of enzymatic activity [9].

The FADS2 enzyme carries out the rate-limiting first step in the biosynthetic pathway, converting the essential fatty acids to long-chain PUFA. In addition, FADS2 has been shown to accept 24-carbon fatty acids as substrates, as shown in Figure 1.1. Delta-8 desaturation by FADS2 results in the rescue of 20-carbon fatty acids once thought to be dead-end products [10]. The FADS1 enzyme is responsible for the step generating the important eicosanoid precursors ARA and EPA. Northern analysis has demonstrated that the delta-6 desaturase is most highly expressed in liver, followed by brain, heart, and lung; the delta-5 desaturase is most highly expressed in the adrenal gland, followed by liver and brain [11].

*Elongation Reactions*

The overall reaction for elongation involves the incorporation of two carbons from malonyl-CoA into the fatty acyl-CoA substrate at the carboxyl end, with NADPH as the reducing agent. This occurs by four separate enzymatic reactions, with elongases catalyzing only the initial, rate-controlling condensation step combining malonyl-CoA with the fatty acyl-CoA to generate 3-ketoacyl-CoA. This product is then reduced to 3-hydroxyacyl-CoA, followed by dehydration to produce trans-2-enoyl-CoA, then a second reduction to the final acyl-CoA product. The mammalian gene family containing the elongases is designated ELOVL, for elongation of very-long-chain fatty acids. ELOVL2, ELOVL4, and ELOVL5 have been shown to prefer PUFA substrates, with ELOVL5 acting on 18- and 20-carbon PUFA, and ELOVL2 and ELOVL4 acting on >20 carbon PUFA [12]. ELOVL5 is expressed ubiquitously, but is highest in liver, testis, and adrenal gland [13]. ELOVL2 and ELOVL4 are expressed in only a few tissues, particularly liver and testis for ELOVL2 [14,15], and retina, brain, skin and testis for ELOVL4 [16].

***Efficiency/Rate of Biosynthesis***

During the first 10 weeks of pregnancy, blood levels of DHA and ARA increase while LA decreases, consistent with enhanced biosynthesis [17]. Beginning in the 17$^{th}$ week of gestation, human fetal livers have significant delta-5 and delta-6 desaturase activities in microsomal assays [18]. Studies of mouse and bovine mammary gland have demonstrated dramatic upregulation of *FADS1* and *FADS2* at the commencement of lactation [19,20], suggesting that nursing mothers have increased biosynthetic capability. However, supplementation of 10 g/day ALA from flaxseed oil in lactating

women raised breast milk EPA and DPA (22:5n-3, docosapentaenoic acid), but not DHA; the lack of breast milk DHA response was unexpected and seemingly paradoxical [21]. DHA levels can be raised by dietary ALA in preterm newborns and young infants, at rates indicating far more efficient biosynthesis than in adults [22,23,24], consistent with direct measurements showing biosynthesis is far more efficient in fetal [25], compared to neonatal [26] primates. However, a wide range of DHA biosynthetic capability has been observed in human infants, with DHA biosynthesis actually undetectable in some cases [27]; it is unclear whether these individual differences are due to genotype, environmental influences, or some combination of the two. Adults have much lower biosynthetic activity, and numerous studies have shown that less than 5% of dietary ALA is converted to DHA; consuming pre-formed DHA is the only effective way to raise blood levels in adults [28,29,30]. Omega-3 fatty acid biosynthesis is especially compromised, in part because about 60% of dietary ALA is oxidized, the highest rate known for fatty acids [28]. However, the situation is more complex, since supplemental ALA in adults increases blood levels of EPA and DPA, but not DHA; DHA derived from biosynthesis can only be increased if dietary LA is decreased at the same time as ALA increases [5].

Because the biosynthetic enzymes described above act on both omega-3 and omega-6 fatty acids, final levels of LCPUFA of each class depend on the ratio of omega-6 to omega-3 fatty acids consumed in the diet. ALA and LA compete for the same biosynthetic enzymes to be desaturated and elongated into bioactive end-products such as ARA, EPA and DHA. Humans consuming experimental diets with identical total PUFA, but differing in LA/ALA ratio, had significantly lower platelet aggregation as the omega-6/omega-3 ratio decreased [31]. Within the past 100 years, adoption of new

food technology and practices of large-scale agribusiness have resulted in an omega-6/omega-3 ratio of about 10-15:1 in the modern western diet, compared to an estimated ratio of 2:1 or 3:1 throughout the rest of human history [32,33]. In particular, advances in production of vegetable oils from seeds have led to dramatic increases in LA consumption, at the same time that consumption of wild game and pasture-raised meat (with omega-6/omega-3 ratio close to 1) has been replaced by mass-produced farming of animals fed grain high in LA [33]. Because the modern Western diet provides primarily omega-6 precursors, biosynthesis is more likely to account for ARA levels than EPA or DHA. ARA gives rise to eicosanoids with functions generally opposing those of lipid mediators derived from EPA or DHA, so a high omega-6/omega-3 ratio tilts the balance toward pro-inflammatory, pro-thrombotic, and pro-arrhythmia effects [2]. In addition, the omega-6/omega-3 ratio in phospholipids composing cell membranes alters function of the G-protein coupled receptor, rhodopsin [34]. It should be noted, however, that effects of differential regulation of biosynthetic pathway enzymes depend very much on dietary context; for example, higher desaturase activity might result in higher inflammation from ARA-derived eicosanoids in western countries, but not in cultures with lower dietary omega-6/omega-3 ratios.

### *Dysregulation of Biosynthesis in Disease*

From a public health standpoint, the current emphasis is on the need for increased consumption of all omega-3 fatty acids, and especially DHA, but relatively little attention has been paid to biosynthesis. However, genomic studies suggest biosynthesis may be more important than previously appreciated. Single nucleotide polymorphisms (SNPs) in enzymes in the biosynthetic pathway have been associated with various physiological

conditions, and several diseases are associated with altered expression of desaturases and/or elongases. SNPs in *FADS2* have been associated with allergy and atopic eczema, total cholesterol, LDL, C-reactive protein levels, and coronary artery disease risk, as well as cognitive outcomes such as attention-deficit hyperactivity disorder and intelligence quotient (IQ) in children [35,36,37,38,39,40]. SNPs in *FADS1* have been associated with altered glucose metabolism, LDL, HDL, total cholesterol, triglycerides, age-related macular degeneration, coronary artery disease, and IQ [36,41,42,43,44,45,46,47,48,49,50]. Minor alleles of SNPs in *ELOVL2* are associated with reduced DHA and higher EPA and DPA (docosapentaenoic acid, 22:5n-3) levels [51]. In addition, several forms of inherited macular degeneration are caused by mutations in the *ELOVL4* gene [12]. Rats with streptozotocin-induced diabetes have reduced expression of both *ELOVL2* and *ELOVL4* in retina [52], as well as lower expression of *FADS1* and *FADS2* in liver, adrenal gland, and testes [53]. *FADS1, FADS2,* and *ELOVL5* were downregulated in skin biopsies from atopic eczema patients [54]. Downregulation of *FADS1* expression has been observed in the brain frontal cortex of suicide completers [55], whereas upregulation of *FADS2* was found in the prefrontal cortex in autopsy samples from patients with schizophrenia, even in patients free of antipsychotic medication [56]. Overexpression of *FADS2* was also observed in ERBB2-positive breast cancer [57]. Conversely, *FADS1* has been reported to be silenced by methylation in gastric cancer, but not in normal gastric mucosa [58]. The environmental, genetic, or physiological factors that may have produced these changes in gene expression have not been elucidated, but identifying regulatory mechanisms would improve understanding of these complex disorders.

***Regulation by Diet, Hormones and Drugs***

Because delta-6 desaturation is known to be rate-limiting, most studies of regulation of LCPUFA biosynthesis have focused on modulation of desaturase activity by diet, hormones and drugs. Significant downregulation of *FADS1* and *FADS2* has been observed in adipocytes following weight loss on diets restricted by 600-1100 kcal per day, regardless of the percent total fat [59]. The same effect was observed even after a 3 week isocaloric adjustment period designed to account for changes due solely to PUFA composition [60], before commencing the energy restricted diet.

Dietary PUFA have been shown to suppress SREBP-1 expression [61]. The end-product DHA is known to down-regulate expression of both *FADS1* and *FADS2* by lowering activity of the sterol response element binding protein 1c (SREBP-1c) [62]. Other transcription factors that have been implicated in regulation of FADS genes include the peroxisome proliferator-activated receptors (PPARs) [63]. *FADS1* is strongly upregulated in livers of vitamin A-deficient rats, and it is downregulated as retinoic acid dose increases [64]. The exact mechanism is unknown, but it is thought that retinoic acid may exert its effects by altering RXR binding to LXR, and subsequent binding of the heterodimer to the SREBP gene promoter. *ELOVL5* is regulated by SREBP-1c in mouse liver [65], and is downregulated by LCPUFA [14]. Both SREBP-1a and SREBP-1c have been implicated in regulation of *ELOVL2*, but the exact mechanism is still unclear [12].

Many hormones are believed to affect desaturases by genomic mechanisms [53], acting on steroid regulatory elements (SREs) [63] or through Peroxisome Proliferator response elements (PPREs) [66]. Most hormones decrease desaturase activity, as measured by

studies with radiolabeled fatty acids, usually in rodent models. It should be noted, however, that rodents at all ages have much higher constitutive desaturase activity than humans, so there may be species-specific differences in desaturase regulation [67]. Insulin has been demonstrated to increase desaturase activity for both the Δ5- and Δ6-desaturases in rodent models of diabetes [53]. Glucagon and epinephrine/adrenaline decrease desaturase activity by a mechanism involving induction of cAMP [53]. Adrenocorticotropic hormone (ACTH) also decreases desaturase activity, by both direct and indirect modes that are not well understood [68]. Steroid hormones categorized as glucocorticoids decrease desaturase activity by directly altering gene expression [69]. Evidence from controlled feeding studies of humans, including transgender individuals (female to male and male to female), have confirmed that females have higher DHA status due to estrogenic effects, likely through modulation of desaturases [70]. Higher levels of *FADS1* and *FADS2* mRNA and activity have been observed in livers of female rats compared to male rats [71].

Several pharmacological agents have been shown to affect desaturase activity. Although statin drugs primarily are known as inhibitors of HMG-CoA reductase, they also have important pleiotropic effects beyond cholesterol reduction, including improved endothelial function and reduced inflammation and thrombosis. It has been hypothesized that an increase in LCPUFA levels resulting from increased desaturase activity is primarily responsible for statins' pleiotropic effects [72,73]. Intriguingly, high omega-3 LCPUFA diets decreased risk for overall and cardiac mortality by the same amount as statin treatment [74]. Simvastatin, a statin drug, is known to upregulate SREBP-1c levels [75], so the likely mechanism increasing LCPUFA levels is by induction of desaturase gene expression via SREBP-1c. Similarly, antipsychotic drugs

of the so-called typical and atypical classes, such as haloperidol and clozapine, respectively, upregulate *FADS1* and/or *FADS2* expression by an SREBP-dependent mechanism in human glioma cells [76]. Interestingly, atypical antipsychotic medications, approved for schizophrenia treatment but also used off-label for numerous mental disorders, are now the top-selling drugs in the United States [77]. In 2005, the two best-selling drugs in North America were atorvastatin and simvastatin [78]. Thus, the most popular drugs in America are likely to upregulate desaturases as part of their effects. Research is needed to determine whether enhanced LCPUFA biosynthesis contributes to the therapeutic benefits of these medications.

***Areas for Future Research***

Several aspects of the LCPUFA biosynthetic pathway remain to be understood. *FADS2* is alternatively spliced to generate an alternative transcript (*FADS2 AT1*) with a different expression pattern compared to the classical form in primate tissues [79]. The function of this alternative splice variant is unknown, but its expression is conserved across numerous species [80], suggesting an important physiological role.

*FADS1* and *FADS2* form part of a 100kb gene cluster on chromosome 11, together with a third member of the gene family, designated *FADS3*. *FADS3* is a putative fatty acid desaturase gene due to extensive sequence homology with *FADS2* (62%) and *FADS1* (52%), but no function for *FADS3* has been found [81]. However, genetic evidence suggests *FADS3* plays an important role in lipid metabolism and diseases. For example, SNPs in *FADS3* are associated with plasma sphingolipids and triglyceride

levels, and with risk of myocardial infarction [82,83]. Expression of *FADS3* is dysregulated in familial combined hyperlipidemia [83], and *FADS3* is one of the six most highly expressed genes at the embryo implantation site in mouse uterus [84]. Uncovering a function for *FADS3* is made more challenging because *FADS3* has seven splice variants, each with distinctive patterns of expression in primate tissues [85]. As with *FADS2 AT1*, at least five of these variants were conserved from chickens to humans [80], underscoring their importance. Because of the sequence similarity of *FADS3* to the other FADS genes, it is reasonable to suppose that its function may involve LCPUFA biosynthesis. However, efforts in our laboratory to find a substrate for FADS3 or any of the splice variants have so far been unsuccessful.

***Summary of our Approach***

The three papers included in this dissertation all are related to the overall topic of desaturase regulation. In the first paper, we adopt a genomics approach to study regulation of *FADS1* and *FADS2*. Since there are no known non-synonymous SNPs in coding regions of *FADS2*, we reasoned that a likely mechanism of action by which disease-associated SNPs might function would be by altering FADS gene regulation. Accordingly, we investigated genetic variants in the FADS gene cluster for association with expression of FADS genes, and differences in response to modulators of SREBP-1c and PPAR γ. The second paper examines regulation of *FADS3* splice variants in response to dietary LCPUFA in infant baboons. We studied regulation in order to obtain clues to FADS3 function. If the function of FADS3 were similar to those of FADS1 and FADS2, then like them, FADS3 should be downregulated by LCPUFA. We also investigated molecular mechanisms by which LCPUFA affected gene expression for the

three genes. Similarly, splicing regulation is examined in the third paper, in which we test our hypothesis of a role for a splicing factor in regulating *FADS2* and *FADS3* alternative mRNA splicing, and examine fatty acid changes associated with an altered splicing pattern.

***Conclusion***

In recent years, increased attention has focused on the importance of LCPUFA biosynthesis, after numerous studies have found SNPs and dysregulated expression of desaturases associated with diseases. Diets lacking DHA and EPA are commonplace in western cultures, increasing dependence on biosynthesis compared to cultures with high seafood consumption. Genetic and molecular studies are needed to identify individuals most at risk for deficiency, and to uncover mechanisms underlying desaturase associations with diseases. Much is still not understood about regulation of biosynthesis, and the functions of desaturase alternative splice variants and *FADS3* remain to be found. Deeper understanding of the remaining mysteries in this field will help reveal how genetics and environment interact to bring about individual differences in nutritional requirements for the essential omega-3 and omega-6 fatty acids, and may lead to strategies for optimizing biosynthesis as an alternative or complement to supplementation.

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# CHAPTER 2

# A FATTY ACID DESATURASE EXPRESSION QUANTITATIVE TRAIT LOCUS MODULATES RESPONSE TO SIMVASTATIN


## Abstract

The *FADS1* and *FADS2* genes encode fatty acid desaturases required for synthesis of omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA) important in the central nervous system, inflammatory response, and cardiovascular health. Single nucleotide polymorphisms (SNPs) in these genes are associated with numerous health outcomes, but it is unclear how genetic variation affects desaturase enzyme function. Here, lymphoblasts obtained from Japanese in Tokyo participants in the International HapMap Project were evaluated for association of expression microarray results with SNPs in the FADS gene cluster. Six SNPs in the first intron of the *FADS2* gene were significantly associated with *FADS1* expression after Bonferroni correction. A 10-SNP haplotype in *FADS2* (rs2727270 to rs2851682, permuted p = 0.028) present in 24% of the population was significantly associated with lower expression of *FADS1*, confirmed by qRT-PCR. A highly conserved region coinciding with the most significant SNPs contained predicted binding sites for PPARγ and SREBP. Lymphoblasts homozygous for either the major or minor haplotype were treated with agonists for these transcription factors, and expression of *FADS1* and *FADS2* measured. There was no difference by genotype in response to the PPARγ agonist rosiglitazone. The statin drug simvastatin and the LXR agonist GW3965 both upregulated expression of *FADS1* and *FADS2* through activation of SREBP-1c. Surprisingly, minor haplotype homozygotes had 20-40% higher induction of *FADS1* and *FADS2* after simvastatin or GW3965 treatment,

suggesting enhanced responsiveness to SREBP-1c. Sequencing of the region flanking the putative sterol response element (SRE) in *FADS2* identified two deletion mutations within 150 bp of the putative SRE in all minor haplotype carriers, and none among the major haplotype carriers studied. Individuals carrying the minor haplotype may be particularly vulnerable to alterations in diet that reduce LCPUFA intake, such as low fish consumption, and may be especially responsive to statin or marine oil therapy.

## Introduction

The delta-5 and delta-6 desaturases (encoded by *FADS1* and *FADS2*, respectively) are essential for biosynthesis of long-chain omega-3 and omega-6 fatty acids. These long-chain polyunsaturated fatty acids (LCPUFA), such as docosahexaenoic acid (DHA) and arachidonic acid (ARA), are major components of cell membranes in the central nervous system, act as precursors for signaling molecules such as eicosanoids and docosanoids, and directly affect gene expression to influence important physiological functions such as inflammation and blood clotting [1,2,3]. Although both ARA and DHA can be obtained pre-formed through diet, most populations do not consume enough to completely remove any dependence on biosynthesis. Indeed, genetic studies have underscored the importance of *FADS1* and *FADS2* by linking single nucleotide polymorphisms (SNPs) in these genes with numerous health outcomes, including coronary artery disease, total cholesterol, LDL, C-reactive protein levels, allergy and atopic eczema, as well as cognitive outcomes such as attention-deficit hyperactivity disorder and IQ in children [4,5,6,7,8,9].

However, mechanisms describing how gene variants affect FADS gene function are lacking, so it is difficult to translate SNP associations into disease prevention or treatment. Despite numerous SNPs in *FADS2* associated with health outcomes, there are no validated non-synonymous SNPs in the *FADS2* gene. This suggests that mechanisms are likely to be regulatory rather than through protein structural changes for any causal SNPs in *FADS2*. A causal SNP in the promoter region of *FADS2* has been reported [10], but this SNP is not polymorphic in the Han Chinese, Japanese, and Yoruba International HapMap project populations (HapMap Genome browser release #28). Thus, this one SNP does not explain all associations found in other world

populations. In addition, this SNP only affects *FADS2* expression, so it does not explain results in European or majority European-descendent populations suggesting lower Δ5-desaturase activity associated with some SNPs [11,12,13].

Some key molecules involved in regulation of *FADS1* and *FADS2* gene expression have been identified, but regulatory regions and functional binding sites within these genes have not been fully elucidated. The FADS genes reside as a cluster on chromosome 11 consisting of *FADS1*, *FADS2* and a third putative desaturase designated *FADS3*. All reported mechanisms for regulation, such as dietary fatty acid or hormonal responses, affect both *FADS1* and *FADS2* in concert. For example, the end-product DHA is known to down-regulate expression of both *FADS1* and *FADS2* by lowering activity of the sterol response element binding protein 1c (SREBP-1c) [14]. Other transcription factors that have been implicated in regulation of FADS genes include the peroxisome proliferator-activated receptors (PPARs) [15]. However, no information is available to link specific SNPs and phenotypes with response to specific transcription factors.

Here, we have taken advantage of dense genotyping conducted for the International HapMap Project to achieve fine mapping of an expression quantitative trait locus (eQTL) in the FADS gene cluster. Because previous studies have focused mostly on European-derived populations, the Japanese in Tokyo (JPT) population was chosen for study here. The JPT population has a different linkage disequilibrium (LD) block structure from Europeans, and SNPs have different minor allele frequencies, so that results are likely to provide new information complementary to existing studies in Europeans. We evaluated gene expression in lymphoblast cell culture, which controls

for environmental and hormonal influences that would otherwise reduce power to detect effects; this approach is ideal for a Japanese population, where exposure to pre-formed DHA from high seafood consumption in the traditional diet would otherwise confound any study of FADS gene expression. Single SNPs and haplotypes were evaluated for association with FADS gene expression, and the region of highest association was searched for putative transcription factor binding sites. Cells were treated with transcription factor agonists to test hypotheses, and a novel eQTL associated with SREBP-1c response was identified. Finally, sequencing of the region flanking the putative SREBP-1c binding site revealed two nearby deletion mutations specific to minor haplotype carriers.

## Materials and Methods

*Single SNP association analysis*

SNP asociations were carried out for SNPs in the FADS gene cluster using publicly available data from 46 International HapMap lymphoblast cell lines for the Japanese in Tokyo (JPT) population. Normalized expression data from the Illumina Sentrix Human-6 Expression BeadChip Microarray were obtained from the Gene Expression Omnibus (Series GSE6536) [16,17]. Genotype information for lymphoblast cell lines was obtained from the Coriell Institute for Medical Research SNP Browser. All genotyped SNPs were used, excluding those non-polymorphic in the JPT population. Single SNP associations and multiple test correction were carried out in PLINK v1.07 [18] using linear regression, with a dominant model chosen because of lack of power in this small sample size to detect additive effects. Individuals or SNPs missing more than 30% of genotypes were excluded, as well as SNPs with minor allele frequency below 1%, or p-

value < 0.001 for deviation from expected frequency of genotypes in the population (Hardy-Weinberg equilibrium).

*Haplotype association analysis*

Phase was imputed and haplotypes identified in the Japanese and European (CEU) HapMap populations using Haploview v4.2 [19]. Linkage disequilibrium blocks were defined by the confidence interval-based algorithm of Gabriel et al. [20]. Identical parameters were used to calculate blocks in both populations. Haplotype associations were carried out in PLINK v1.07 by linear regression with an additive model, using the max(T) permutation procedure with 10,000 permutations to correct for multiple testing. Exclusion criteria were the same as for individual SNP associations, except that SNPs or individuals missing more than 10% of genotypes were excluded. Haplotype allele frequencies lower than 5% were also excluded from analysis.

*Conserved regions and transcription factor binding predictions*

Genomic sequence alignment calculating percent identity of multiple species compared with the human sequence was carried out with mVISTA (http://genome.lbl.gov/vista/mvista/about.shtml) [21]. Predicted transcription factor binding sites were identified by TRANSFAC Professional 9.2 database search combined with comparative sequence analysis, using rVISTA (regulatory VISTA, http://genome.lbl.gov/vista/rvista/about.shtml) [22].

*Lymphoblast cell culture, treatments, and RNA extraction*

Immortalized B-lymphocyte (lymphoblast) cell lines from Japanese HapMap participants were obtained from the Coriell Institute for Medical Research, and used within 10 passages of receipt from the repository. Cells were grown in RPMI 1640 with 2 mM L-alanyl-glutamine (Sigma) and 15% fetal bovine serum (media and serum obtained from HyClone) in a humidified environment at 37°C with 5% $CO_2$. All experiments were conducted on cells grown and treated in parallel in identical media and growth conditions. Cells were treated with 5 μM simvastatin (Sigma), 1 μM GW3965 (Sigma), or 20 μM rosiglitazone (Cayman Chemicals), or vehicle for 24 hours before collecting lysates. RNA was extracted using the RNeasy kit (Qiagen), and RNA quality was checked by agarose gel electrophoresis to verify RNA integrity and by 260/280 nm ratios on a NanoDrop 2000 (Thermo Scientific). cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol.

*Quantitative real-time PCR*

Quantitative real-time PCR was carried out using SYBR Green Master Mix (Roche) on a LightCycler 480 instrument (Roche). PCR primers were obtained from Integrated DNA Technologies (sequences available upon request), except for 18S, which was obtained from Qiagen as a QuantiTect Primer Assay. PCR reaction efficiency was calculated from standard curves, and reactions were assessed by both melting curves and by running on agarose gels to verify reaction products and the absence of primer-dimers. Quantitative cycle (Cq) values were determined using LightCycler 480 SW1.5.0SP3 software, version 1.5.0.39 (Roche). Relative quantification was carried out using the

Pfaffl method [23], taking into account reaction efficiency and using multiple reference genes for greater accuracy (β-actin, GAPDH, and 18S). Before proceeding with analysis, basal expression was shown to be invariant by vehicle-only treatment for all genes studied. Statistical significance of differences in fold changes between genotypes in response to cell treatments (normalized to vehicle treatment for each genotype) was assessed by the Mann-Whitney U test. Bootstrapping and randomization techniques were used in REST 2009 software (Qiagen) to calculate significance of fold changes normalizing both genotypes to basal expression in major allele homozygotes.

*Sequencing of candidate sterol response element regions*

A total of about 5 million cells from lymphoblast cultures were harvested for DNA extraction. DNA extraction was performed using DNeasy Blood & Tissue Kit (Qiagen). A 629 base pair portion (bases 6908015-6908643; GenBank Accession Number NT_167190.1) of *FADS2* intron 1 and a 291 base pair portion of *FADS1* intron 5 (bases 6882783-6882505; GenBank Accession Number NT_167190.1) flanking the sterol regulatory element (SRE) DNA sequence were amplified using the following primer pairs: *FADS2* forward primer 5' TTTCTCAAAGGCCGTGGTGT 3', *FADS2* reverse primer 5' AGTGCTAACCACTCCTGGAA 3' and *FADS1* forward primer 5' ACAGAGAAT GAAGGGACGCA 3', *FADS1* reverse primer 5' ACCCGAAGGAGGCCATATCT 3'. The PCR reactions were carried out using GeneAmp High Fidelity PCR System (Invitrogen). Thermal cycling conditions were: initial denaturation at $94^0$C for 5 min followed by 35 cycles of denaturation at $94^0$C for 30 s, annealing at $60^0$C for 45 s and extension at $72^0$C for 1 min, with a final extension at $72^0$C for 5 min. PCR products were separated

on 2% agarose gels, and the DNA bands were gel eluted and purified using PureLink Quick Gel Extraction Kit (Invitrogen, USA). The purified products were sequenced at the Cornell University Life Sciences Core Laboratories Center.

## Results

*Identification of eQTL associated with FADS1 expression*

Lymphoblast cell cultures derived from all 46 Japanese participants in the International HapMap Project were used to search for genetic variants associated with FADS gene expression. Archived Illumina expression microarray data was obtained from the Gene Expression Omnibus database repository [16,17]. Analysis was carried out on 41 SNPs densely covering the FADS gene cluster. The Manhattan plot shown in Figure 2.1A demonstrated a highly significant region located in *FADS2* intron 1 that was associated with lower *FADS1* expression for the minor allele. Within this region, six SNPs passed Bonferroni correction for multiple testing, as shown in Table 2.1. There were a few nominally significant SNPs associated with *FADS2* or *FADS3* expression, but none of these passed correction for multiple testing (not shown). Analysis of the linkage disequilibrium (LD) block structure for the Japanese HapMap population revealed two LD blocks in the region of interest, with the most highly significant SNPs primarily in the second block. Three haplotype alleles were observed within Block 2, as shown in Figure 2.1B. SNPs in this block were: rs2727270, rs2727271, rs174576, rs2524299,

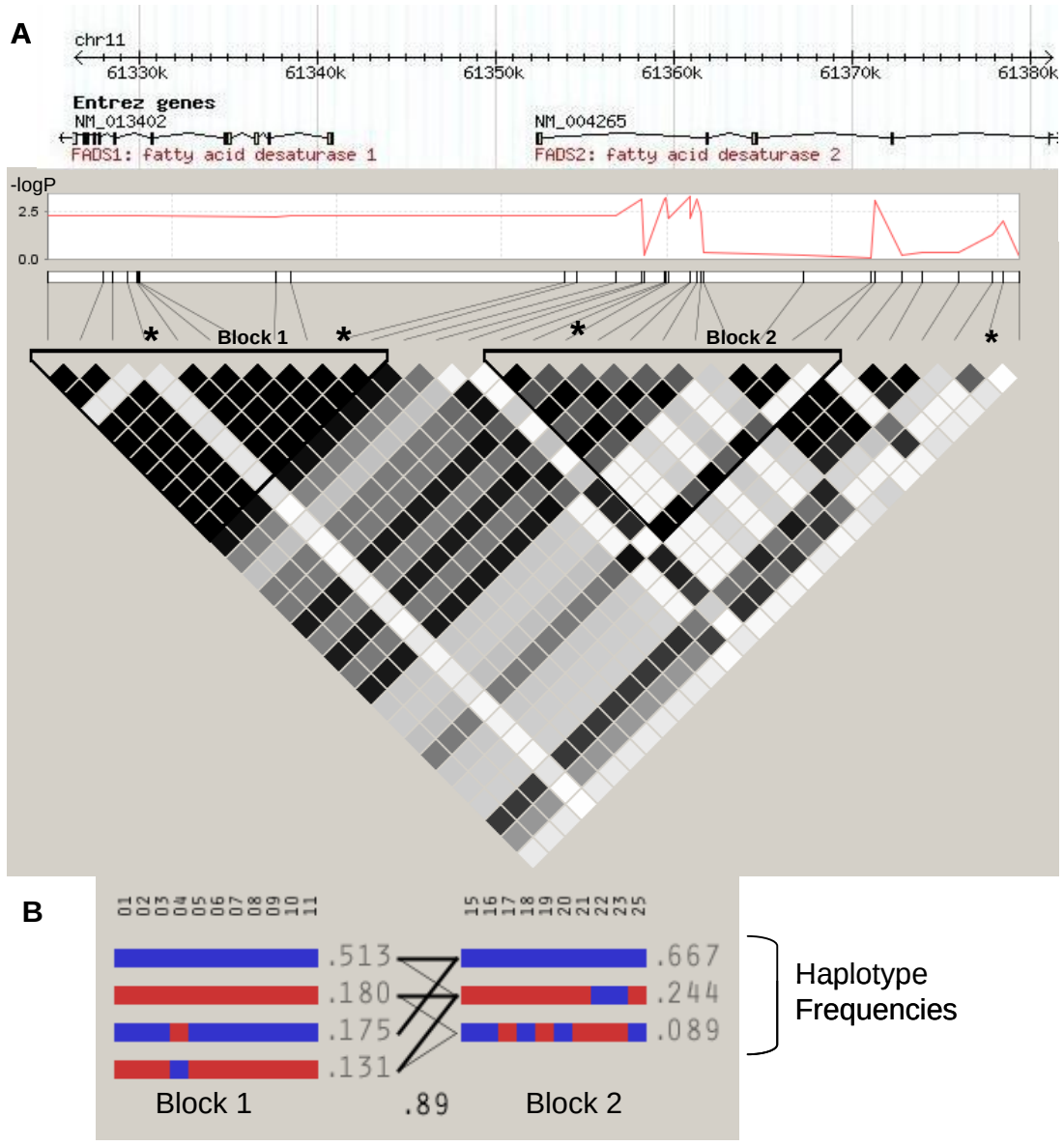


**Figure 2.1. Expression quantitative trait locus in *FADS2* intron 1 and associated haplotype blocks in the Japanese HapMap population.** (A) A Manhattan plot of -log(p-value) vs. distance in Kb is shown for the association of individual SNPs with *FADS1* expression, with gene annotations above. Two LD blocks for this population are shown, with darkness of shaded cells representing the degree of correlation ($R^2$) between pairs of SNPs. * Marked SNPs were associated with the ratio of arachidonic to linoleic acid (a measure of apparent total desaturase activity) in other Asian populations [11,24]. (B) Haplotypes for each LD block are shown, with major alleles for each SNP in blue, and minor alleles in red. Recombination between blocks is depicted by the density of lines connecting individual haplotypes, and the multiallelic D' (0.89).

**Table 2.1. Single SNPs in *FADS2* intron 1 significantly associated with *FADS1* expression.**

| SNP | Base position[a] | p-value | Beta[b] | Bonferroni[c] | FDR[d] |
|---|---|---|---|---|---|
| rs2845573 | 61358484 | 0.00073 | -0.21 | 0.0299 | 0.00597 |
| rs2727270 | 61359813 | 0.00073 | -0.21 | 0.0299 | 0.00597 |
| rs2727271 | 61359934 | 0.00065 | -0.21 | 0.0265 | 0.00597 |
| rs2524299 | 61361358 | 0.00053 | -0.23 | 0.0216 | 0.00597 |
| rs2072114 | 61361791 | 0.00073 | -0.21 | 0.0299 | 0.00597 |
| rs2851682 | 61372588 | 0.00091 | -0.21 | 0.0374 | 0.00623 |

[a] Base positions from NCBI Build 36
[b] Linear regression coefficient
[c] Bonferroni adjusted p-value
[d] False discovery rate (Benjamini-Hochberg method)

rs174577, rs2072114, rs174578, rs174579, rs174585, and rs2851682. The minor haplotype present in about one quarter of the population was significantly associated with lower *FADS1* expression, as summarized in Table 2.2.

To estimate relevance of these results to studies in other populations, the LD block structure of the European (CEU) HapMap population is visualized in Figure 2.2. Previous large-scale studies, with associated SNPs marked in Figure 2.2, found a variety of outcomes associated with the first intron of the *FADS2* gene in European-derived populations. In particular, fatty acid changes suggestive of lower FADS1 enzyme activity were found for SNPs in close proximity and in LD with the most highly significant region in the Japanese population.

*Predicted transcription factor binding sites*

To investigate possible causal mechanisms for the lower *FADS1* expression associated with this minor haplotype, the area immediately surrounding the most strongly associated region, and thus most likely to contain the actual causal locus, was examined for predicted regulatory elements. A region highly conserved from zebrafish to humans was identified that overlapped the area containing the most highly significant associated SNPs, as shown in Figure 2.3. TRANSFAC database search using the bioinformatics tool rVISTA revealed predicted consensus binding sites in the conserved region for two transcription factors: peroxisome proliferator-activated receptor gamma (PPARγ) and sterol response element binding protein (SREBP). We hypothesized that the significant SNPs identified above could be associated with a genetic variant within

**Table 2.2. Haplotype testing for association with *FADS1* expression.**

| Haplotype | Frequency | Beta[a] | p-value | Empirical p-value[b] |
|---|---|---|---|---|
| CACACATCGA | 0.67 | 0.090 | 0.061 | 0.15 |
| TTATAGACGG | 0.24 | -0.12 | 0.010 | 0.028 |
| CAAAAAATAA | 0.089 | 0.068 | 0.36 | 0.64 |

[a] Linear regression coefficient
[b] Corrected empirical p-value from max(T) permutation testing, controlling familywise error rate

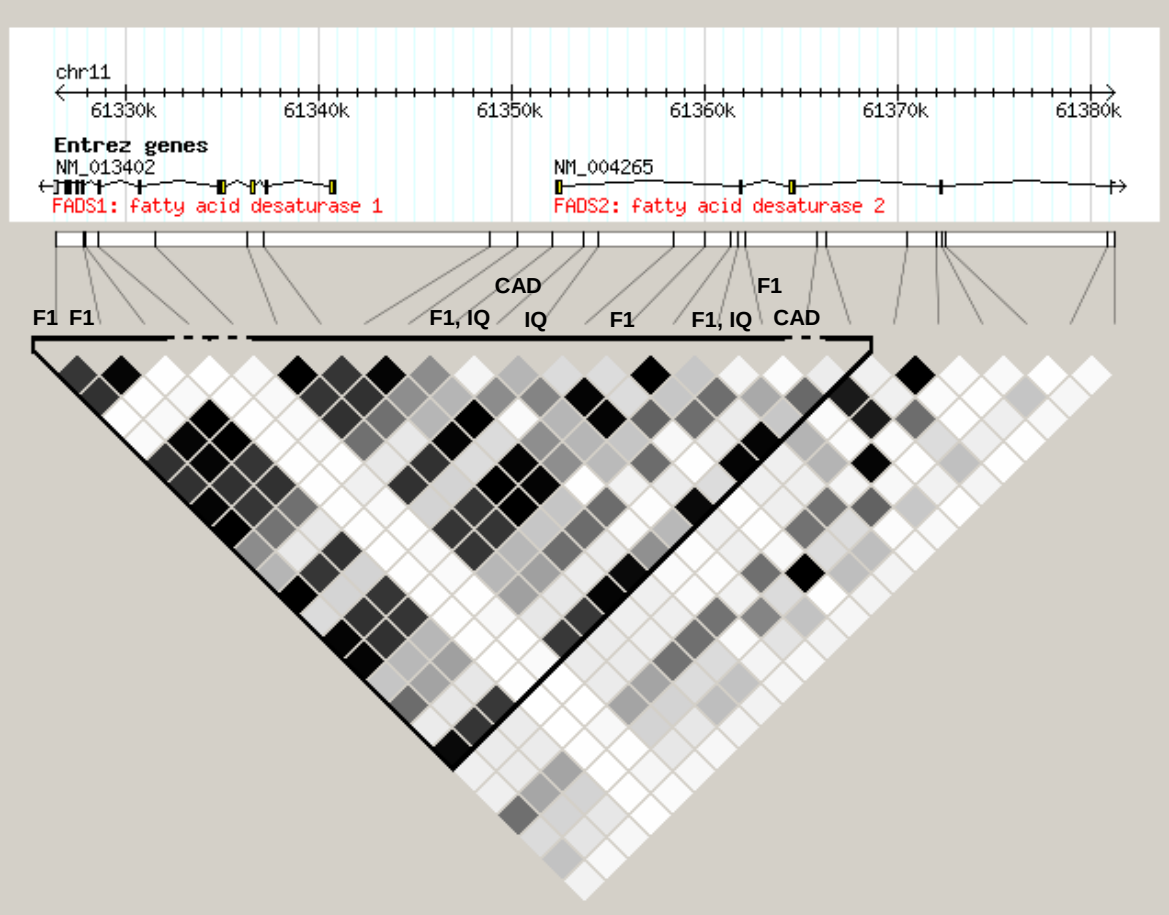


**Figure 2.2. Haplotype block in European (CEU) HapMap population.** *FADS2* intron 1 is contained within a large single LD block in the European HapMap population. SNPs associated with apparent *FADS1* activity are in close proximity and in LD with the region highly associated with *FADS1* expression in the Japanese population. Abbreviations: F1 = SNPs associated with apparent *FADS1* activity, inferred from fatty acid product/substrate ratios [11,12,13]; IQ = SNPs associated with IQ in breastfed children [9,25]; CAD = SNPs associated with coronary artery disease and c-reactive protein levels [7].

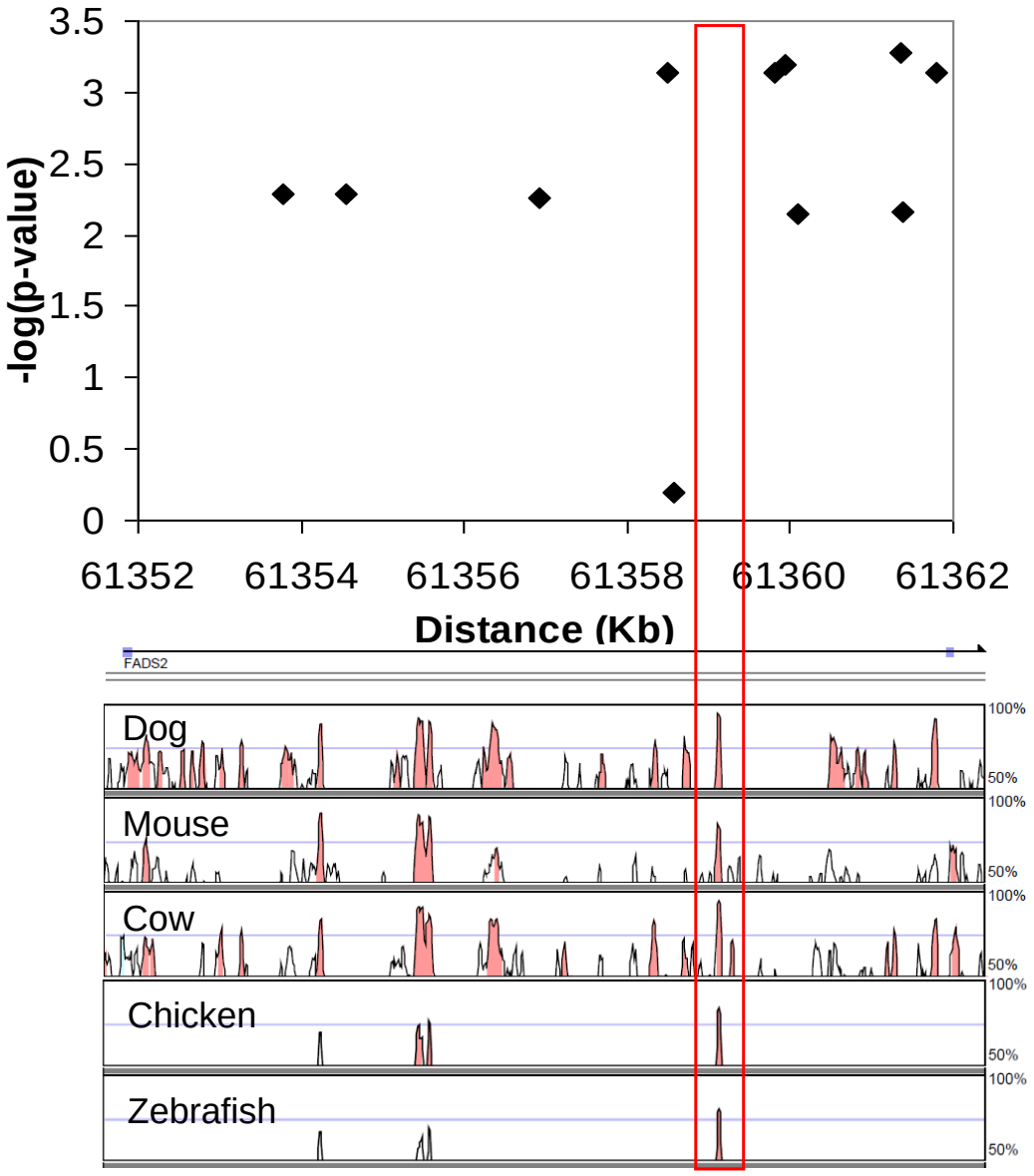


**Figure 2.3. Conserved region overlapping with significant SNPs in *FADS2* intron 1.** Zoomed-in detail of *FADS2* intron 1 in the Manhattan plot of -log(p-value) vs. base position depicts association of SNPs in the Japanese HapMap population with *FADS1* expression, with conserved regions depicted below as percent identity with the human sequence. The red box outlines a region overlapping with the area of highest significance that is conserved from zebrafish to humans, and contains predicted binding sites for SREBP and PPARγ.

one of these binding sites, resulting in altered binding of a transcription factor responsible for regulating *FADS1* expression.

*Lymphoblast FADS1 and FADS2 expression and transcription factor agonists*

Follow-up experiments were carried out in Japanese HapMap lymphoblast cell lines homozygous for the minor haplotype associated with *FADS1* expression, or homozygous for the major haplotype. All available minor haplotype homozygote cell lines were used (n = 5), as well as randomly chosen major allele homozygote cell lines (n =11). *FADS1* expression was examined by quantitative real-time PCR, confirming the initial microarray results showing lower *FADS1* expression for minor haplotype homozygotes (Figure 2.4A).

To investigate the possibility of a causal genetic variation in a transcription factor binding site, cells were treated with drugs acting directly or indirectly on PPARγ or SREBP-1c. The PPARγ agonist rosiglitazone produced identical modest *FADS1* upregulation responses in major and minor allele homozygotes, so the basal difference in *FADS1* expression was maintained even after rosiglitazone treatment, as shown in Figure 2.4. Next, two drugs of different classes that alter SREBP-1c by distinct mechanisms were examined: the statin drug simvastatin, and the LXR agonist GW3965. Both treatments upregulated *FADS1* by about two-fold in major haplotype homozygotes. Unexpectedly, minor haplotype homozygotes responded even more strongly to modulators of SREBP-1c; upregulation of *FADS1* was 40% higher in response to simvastatin, and 25% higher in response to GW3965 for the minor haplotype (Figure

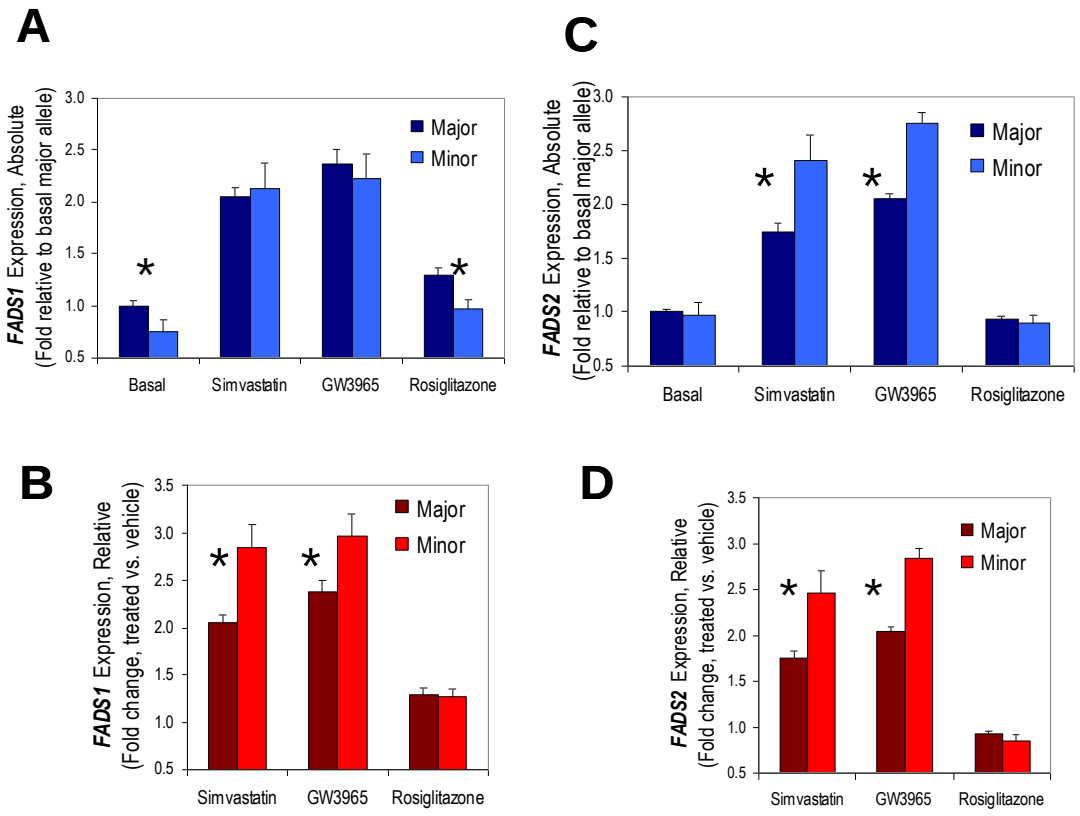


**Figure 2.4. Basal *FADS1* and *FADS2* expression and drug response in lymphoblasts homozygous for major or minor haplotypes. (A)** *FADS1* expression normalized to the major haplotype cells grown under basal conditions (ordinary growth media = 1). Under basal conditions, the minor haplotype homozygotes had significantly lower basal *FADS1* expression than the major haplotype homozygotes, and this pattern persisted with rosiglitazone treatment. In contrast, simvastatin or GW3965 treatment upregulated expression in both genotypes such that they were no longer significantly different. **(B)** The same data as in (A) expressed as the change in *FADS1* expression relative to drug treatment, normalized to vehicle treatment (not shown) for each genotype. Minor haplotype homozygotes confirm the findings in (A), showing significantly greater fold increase in *FADS1* in response to simvastatin and GW3965 and no difference from major haplotype homozygotes in response to rosiglitazone. **(C)** *FADS2* expression normalized to basal condition for the major haplotype is equivalent for the two haplotypes under basal conditions and for rosiglitazone. Simvastatin and GW3965 differentially upregulate haplotype *FADS2* expression so that the minor haplotype is greater than the major haplotype. **(D)** The change in *FADS2* expression relative to each genotype's vehicle control is significant for simvastatin and GW3965. *p<0.05

2.4B). These strong responses to SREBP-1c eliminated the difference in *FADS1* expression observed in untreated cells, so that the two genotypes did not significantly differ in final *FADS1* expression after treatment with simvastatin or GW3965 (Figure 2.4A).

Although no difference in basal *FADS2* expression for each genotype was observed in the archived microarray data (not shown), both PPARγ and SREBP-1c have previously been implicated as regulators of *FADS2* expression. Thus, *FADS2* expression with and without the same drug treatments was evaluated. As shown in Figure 2.4C, the qRT-PCR results confirmed the microarray results for basal *FADS2*, with no difference between genotypes, and neither genotype showed any response to rosiglitazone treatment. As with *FADS1*, SREBP-1c modulators produced about a two-fold increase in *FADS2* levels for major haplotype homozygotes, and minor haplotype homozygotes increased *FADS2* expression even more in response to simvastatin or GW3965 (Figure 2.4D). Because there was no difference in basal *FADS2* levels by genotype, final levels of *FADS2* were at least 20% higher after statin or LXR agonist treatment in minor haplotype homozygotes compared to the major haplotype group (Figure 2.4C). The pattern of response for *FADS1* and *FADS2* is remarkably consistent (Figures 2.4B and 2.4D) and the differences in the pattern of total expression (Figures 2.4A and 2.4C) are due to differences in basal expression.

*Sequence differences near the putative SREBP-1c binding site in FADS2 intron 1*

Based on the difference in response to SREBP-1c, we hypothesized that a sequence variation between the major and minor haplotypes might exist within or in close proximity to a putative sterol response element (SRE) binding site for SREBP-1c.

Analysis of *FADS1* and *FADS2* genes with the rVISTA program revealed two decamer SREs, one in *FADS2* intron 1 (5'-ATCACCCCAC-3'), and another in *FADS1* intron 5 (5'-ATCACGCCAC-3'). The sequencing of a 291 base pair (bp) fragment flanking the SRE DNA sequence of *FADS1* showed no differences between the major and minor haplotypes. However, sequencing of a 629 bp fragment flanking the SRE DNA sequence of *FADS2* showed two insertion/deletion (InDel) variants exclusively in the minor haplotype. As shown in Table 2.3, a 22 bp deletion (rs66698963) was identified by sequencing in all five minor haplotype homozygotes within a variable number tandem repeat minisatellite sequence located 137 bases downstream of the putative SRE. In addition, a 3 bp deletion (-CCA, rs138766446) located 81 bases downstream of the putative SRE was observed in all but one of the minor haplotype homozygotes, with the remaining one individual missing only one of the 3 bp (-A, rs149597144). Interestingly, the individual with the -A deletion instead of -CCA consistently had the highest basal *FADS1* expression of all of the minor haplotype homozygotes (data not shown). None of these deletions were observed in any of the eleven major haplotype homozygotes.

**Table 2.3. InDel Mutations near the putative SRE in FADS2 intron 1.**

| ID number | Sequence | Distance to SRE (bp) | Minor haplotype |
|---|---|---|---|
| rs66698963 | - / ACTTCTCCCTGCCTCCCCAGGG | 137 | Deletion |
| rs138766446 or rs149597144 | - / CCA or - / A | 81 or 83 | Deletion |

## Discussion

Here, single SNPs and a haplotype in *FADS2* intron 1 were found to be significantly associated with *FADS1* expression, measured by both microarray and independently by qRT-PCR. The *FADS2* gene is positioned immediately adjacent at a distance of 11.3 kb to *FADS1* in a head-to-head orientation [26], so that *FADS2* intron 1 is upstream of the transcription start site for *FADS1*. Thus, the existence of an important regulatory region for *FADS1* transcription in intron 1 of the *FADS2* gene is quite plausible. It has been shown that *CYP1A1* and *CYP1A2* genes that are adjacent to each other in head-to-head orientation on human chromosome 15 share common bidirectional regulatory regions [27]. The region most highly associated with *FADS1* expression overlapped with a conserved region containing predicted binding sites for PPARγ and SREBP. In follow-up experiments, no difference was observed in response to a PPARγ agonist, rosiglitazone, but homozygotes for a minor haplotype were significantly more sensitive to expression regulation by SREBP-1c modulation. The enhanced response to SREBP-1c was consistently observed for drugs activating SREBP-1c by two different mechanisms: the statin simvastatin upregulates SREBP-1c levels as part of its pleiotropic effects [28], and the LXR agonist GW3965 stimulates the LXR/RXR heterodimer to activate SREBP-1c [29]. Both genotypes upregulated both *FADS1* and *FADS2* in response to the drugs, but homozygotes for the minor haplotype exhibited a significantly greater increase in expression of both genes after drug treatment. Minor haplotype homozygotes had final *FADS1* levels equivalent to major haplotype homozygotes, and 20% higher *FADS2* levels. The minor haplotype was thus associated with two paradoxical states: lower *FADS1* expression in the basal state, and stronger upregulation of *FADS1* and *FADS2* in response to SREBP-1c. The results

suggest that the minor haplotype is associated with enhanced response to SREBP-1c in a binding site with shared regulatory activity for both *FADS1* and *FADS2*. These findings do not explain the reason for lower *FADS1* expression in the basal state, but it is possible that a mutation that enhances SREBP-1c binding may be mutually exclusive to binding of another transcription factor in a shared binding region. Sequencing of the candidate region in *FADS2* led to the identification of two deletion mutations present only in the minor haplotype homozygotes, located 81 and 137 bases downstream of the putative SREBP-1c binding site. Although the close proximity to the SRE is highly suggestive of a causal role, further *in vitro* experimentation is needed to determine whether these InDel mutations affect SREBP-1c binding and activity.

LCPUFA biosynthesis via FADS-encoded desaturase activity is redundant with direct dietary intake of preformed LCPUFA, specifically 20:4n-6, 20:5n-3 and 22:6n-3. Fish intake is recommended in part for its high content of LCPUFA. The traditional Japanese diet delivers on average more than one gram of LCPUFA daily through regular fish consumption [30]. At these levels, only minimal biosynthesis of LCPUFA from linoleic and linolenic acids is metabolically necessary at any life stage, and any differences in desaturase expression and consequent LCPUFA biosynthetic activity between major and minor haplotypes would be masked and presumably of little health consequence. In contrast, typical North American diets provide, on average, less than 300 mg LCPUFA cholesterol total, and thus biosynthesis may be much more important.

Our *in vitro* results on human lymphoblasts appear to be relevant to free living humans. Previous studies in Asian populations have linked SNPs in this region with serum fatty acid changes consistent with lower total desaturase activity (noted in Figure 2.1),

suggesting that the genetic associations observed here can be replicated in human populations. These previous studies suggest that the usual state for individuals carrying these genetic variants is to have basal *FADS1* levels so low that they become rate-limiting, reducing overall synthesis of fatty acid end-products. If this is the case, individuals with the minor haplotype may particularly benefit from diets incorporating higher levels of preformed LCPUFA from fatty fish or marine oil supplements to augment biosynthesis. Thus, although the minor haplotype is common (present in about one quarter of the Japanese HapMap population), any detrimental effects of the minor genotype are likely to be masked by the large amounts of fatty fish in the traditional Japanese diet. Importantly, the prevalence of this genetic variant may represent an additional risk to adopting a western diet for many individuals of Japanese descent. Put another way, individuals with the minor haplotype are predicted to be particularly vulnerable to ill-health when adopting diets that severely reduce preformed LCPUFA intake.

Further studies are needed to determine whether the expression results extend to other populations. Our data suggest the hypothesis that in populations with low LCPUFA intake, possibly due to low fish consumption, individuals with these genetic variants may have a conditional requirement for LCPUFA. At least some of these populations, such as Americans, derive cardiovascular benefits from statins. A number of previous studies have associated SNPs in *FADS2* intron 1 with apparent *FADS1* enzyme activity in large studies of European-derived populations (Figure 2.2), suggesting that the same causal locus may exist in Europeans as well as Japanese. Figure 2.2 also shows that the same region has been associated with IQ in breastfed children, but several studies in various European populations have produced conflicting and sometimes paradoxical

results that were not accounted for by differences in fish consumption between populations [6,9]. However, these studies did not take into account cholesterol, an activator of SREBP-1c [29] present in breastmilk but not in infant formula; population differences in maternal dietary fatty acids and phytosterols may affect breastmilk and infant plasma cholesterol and phytosterol levels [31,32,33]. In addition, individuals with the minor haplotype might have lower *FADS1* expression in the absence of SREBP-1c activators (such as with infant formula, which does not contain cholesterol), but higher total FADS gene expression in the presence of cholesterol (breastmilk); a similar trend of significantly lower IQ in formula-fed, and almost-significant higher IQ in breastfed children, was observed in one IQ study for several SNPs in *FADS2* intron 1 [9].

SNPs nearby and in LD with the SNPs most strongly associated with *FADS1* expression here have also been associated with coronary artery disease in European studies (Figure 2.2). Thus, the finding of enhanced responsiveness to simvastatin for the minor haplotype may be of special interest. Statins have long been known to increase LCPUFA levels, and there is some previous evidence of changes in apparent desaturase activity in response to statin treatment [34,35]. Although statins primarily are known for lowering cholesterol by inhibiting HMG-CoA reductase, they also have numerous pleiotropic effects that contribute to their medical benefits, including improved endothelial function and reduced inflammation and thrombosis. It has been theorized that statins' pleiotropic effects may be primarily due to alterations in LCPUFA levels [35]. Interestingly, diets high in omega-3 LCPUFA were found to reduce overall and cardiac mortality by the same amount as statin treatment [36]. Here, we show that simvastatin upregulates both *FADS1* and *FADS2*. The minor haplotype identified here was associated with an especially strong response to simvastatin. Assuming living

humans have similar responses as observed in lymphoblasts, individuals with the minor haplotype may have lower basal *FADS1* expression, leading to lower LCPUFA production. Fortunately, simvastatin is shown here to correct the basal deficiency in *FADS1* expression, and to produce *FADS2* expression that would be higher than in other statin users; our results predict that minor haplotype carriers would have especially high LCPUFA synthesis after simvastatin treatment, and thus would especially benefit from pleiotropic effects attributed to LCPUFA production.

Our results highlight an extreme example of gene-environment interaction, in which a minor haplotype is associated with either lower, or higher, desaturase expression depending on levels of SREBP-1c or LXR agonists. Environmental factors can be adjusted to mitigate potential harm for minor haplotype carriers: diet can easily be modified to enhance SREBP-1c or LXR activity via natural ligands, or to take in pre-formed LCPUFA and minimize the need for biosynthesis. Moreover, carriers of this minor haplotype who are candidates for statin treatment may especially benefit from these drugs, as well as possibly from future LXR agonist drugs currently under development.

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# CHAPTER 3

# DIETARY LONG-CHAIN POLYUNSATURATED FATTY ACIDS UPREGULATE EXPRESSION OF *FADS3* TRANSCRIPTS


## Abstract

The fatty acid desaturase gene family at 11q12-13 includes *FADS1* and *FADS2*, both known to mediate biosynthesis of omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA). *FADS3* is a putative desaturase due to its sequence similarity with *FADS1* and *FADS2*, but its function is unknown. We have previously described 7 *FADS3* alternative transcripts (AT) and 1 *FADS2* AT conserved across multiple species. This study examined the effect of dietary LCPUFA levels on liver FADS gene expression *in vivo* and *in vitro*, evaluated by qRT-PCR. Fourteen baboon neonates were randomized to three diet groups for their first 12 weeks of life: C: Control, no LCPUFA; L: 0.33% docosahexaenoic acid (DHA)/ 0.67% arachidonic acid (ARA) (w/w); and L3: 1.00% DHA/ 0.67% ARA (w/w). Liver *FADS1* and both *FADS2* transcripts were downregulated by at least 50% in the L3 group compared to controls. In contrast, *FADS3* AT were upregulated (L3>C), with four transcripts significantly upregulated by 40% or more. However, there was no evidence for increased liver fatty acid desaturation to coincide with increased *FADS3* expression. Significant upregulation of *FADS3* AT was also observed in human liver-derived HepG2 cells after DHA or ARA treatment. The PPARγ antagonist GW9662 prevented *FADS3* upregulation, while downregulation of *FADS1* and *FADS2* was unaffected. Thus, *FADS3* AT were directly upregulated by LCPUFA by a PPARγ-dependent mechanism unrelated to regulation of

other desaturases.  This opposing pattern and mechanism of regulation suggests a dissimilar function for *FADS3* AT compared to other FADS gene products.

## Introduction

Biosynthesis of long-chain polyunsaturated fatty acids (LCPUFA) requires introduction of *cis* double bonds by the ∆5 and ∆6 desaturases, encoded by the *FADS1* and *FADS2* genes, respectively. *FADS1* and *FADS2* span a 100kb cluster on the long arm of chromosome 11 (11q12-13.1), together with a third member of the gene family, designated *FADS3*. *FADS3* is a putative fatty acid desaturase gene due to its high degree of sequence homology with *FADS2* (62%) and *FADS1* (52%), but no function for *FADS3* has been demonstrated experimentally [1].

Although its exact function is unknown, genetic evidence suggests *FADS3* plays an important role in lipid metabolism and diseases. For example, single nucleotide polymorphisms in *FADS3* have been associated with plasma sphingolipids and triglyceride levels, and with risk of myocardial infarction [2,3]. Expression of *FADS3* is altered in familial combined hyperlipidemia [3], and *FADS3* is one of the six most highly expressed genes at the implantation site in mice at the initiation of pregnancy [4].

Early attempts in our laboratory to characterize *FADS3* expression resulted in the discovery of seven alternative transcripts (AT) of *FADS3* with distinctive patterns of expression in primate tissues [5]. In addition, we recently reported an alternative splice variant for *FADS2* [6]. *FADS2 AT1* and at least five of the *FADS3* AT were conserved from chickens to humans [7]. Despite this evidence of crucial roles in essential processes, functions of the splice variants remain unclear.

Patterns of regulation can often provide clues to function; we reasoned that if the *FADS3* AT encoded functional fatty acid desaturases, they were likely to be regulated

similarly to the classical desaturase genes, *FADS1* and *FADS2*. These two genes encode desaturases required for biosynthesis of the omega-3 and omega-6 LCPUFA docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6). These two products of the biosynthetic pathway have been shown to decrease the classical transcripts of *FADS1* and *FADS2* [8]. DHA and ARA are both known to bind members of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors (especially PPARα and PPARγ), which form heterodimers with the retinoid X receptor (RXR) and influence gene expression [9,10]. The effect of dietary LCPUFA on *FADS1* and *FADS2* gene expression has been shown to occur via PPARα and the sterol response element binding protein, SREBP-1c [11]. Nutrients, hormones, and drugs regulating *FADS1* and *FADS2* are known to regulate both in concert, with the same directionality of change [12], as would be expected for genes functioning in the same biosynthetic pathway.

Here we asked whether dietary LCPUFA affect expression of *FADS3* AT and *FADS2 AT1* similarly to classical *FADS1* and *FADS2,* both *in vivo* and *in vitro*. Neonatal baboons were fed infant formula with varying levels of DHA and ARA for 12 weeks, and liver fatty acids and FADS gene expression examined. In vitro, human liver-derived HepG2 cells were studied to determine whether the observed effects were reproducible in human cells, and if it was a direct response to a fatty acid or an endocrine response.

## Materials and Methods

*Animals and diets*

All baboon work was carried out at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX. Animal protocols were approved by the SFBR and Cornell University Institutional Animal Care and Use Committee (IACUC, protocol # 02-105.) Diets and feeding protocols were described in detail previously [13]. Briefly, fourteen baboon neonates were randomized to one of three diet groups with varying concentrations of ARA and DHA, as described in Table 3.1. The infant formulas used for C and L groups correspond to the human infant formulas Enfamil and Enfamil LIPIL, respectively, and the L3 group was targeted to have three-fold higher DHA concentration, corresponding with the upper end of DHA levels found in human breast milk worldwide [14]. These diets were identical to a subset of those used in recently reported human studies [15,16]. As shown in Table 3.1, analysis of the prepared diets showed that the actual concentrations used were slightly higher than target values, since the diets were prepared within tolerances designed to account for losses and variation during manufacturing and storage. Infant baboons consumed the experimental diets until 12 weeks of life, when tissues were harvested for lipid and RNA extraction.

*Quantitative real-time PCR*

Baboon liver RNA was extracted as described previously [5]. For HepG2 cells, RNA was extracted using the RNeasy kit (Qiagen), and RNA quality was checked by agarose gel electrophoresis to verify RNA integrity and by 260/280 nm ratios on a NanoDrop

**Table 3.1. Characteristics of experimental groups and diets**

| | C | L | L3 |
|---|---|---|---|
| Number of animals (n) | 5 | 4 | 5 |
| Female | 4 | 3 | 3 |
| Male | 1 | 1 | 2 |
| DHA (% w/w) | 0 | 0.42 ± 0.02 [0.33] | 1.13 ± 0.04 [1.00] |
| ARA (% w/w) | 0 | 0.77 ± 0.02 [0.67] | 0.71 ± 0.01 [0.67] |

Analyzed concentrations are shown as mean ± SD; target values are shown in brackets.

2000 (Thermo Scientific). cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time PCR was carried out using SYBR Green Master Mix (Roche) on a LightCycler 480 instrument (Roche). Human and baboon PCR primers were obtained from Integrated DNA Technologies (sequences available upon request), except for 18S, which was obtained from Qiagen as a QuantiTect Primer Assay. PCR primers designed for FADS splice variants were validated by cloning and sequencing PCR products. PCR reaction efficiency was calculated from standard curves, and reactions were assessed by both melting curves and by running on agarose gels to verify reaction products and the absence of primer-dimers. Quantitative cycle (Cq) values were determined using LightCycler 480 SW1.5.0SP3 software, version 1.5.0.39 (Roche). Relative quantification was carried out using the method of Pfaffl [17], taking into account reaction efficiency and using multiple reference genes for greater accuracy (β-actin and GAPDH for baboon experiments; β-actin, GAPDH, and 18S for HepG2 cell experiments).

*Fatty acid analysis*

Lipids from baboon liver samples were extracted and fatty acids analyzed by covalent adduct chemical ionization tandem mass spectrometry as described in detail previously [13]. Percent conversion of substrates (S) to products (P) was calculated as: [(P) / (S + P)] * 100, and normalized to the control group.

*Cell treatments*

For all experiments, human HepG2 hepatocellular carcinoma cells were maintained

within ten passages of the original passage received from the ATCC. HepG2 cells were grown in MEM with 10% FBS (media and serum obtained from HyClone) in a humidified environment at 37°C with 5% $CO_2$. For fatty acid treatment, free fatty acids were first non-covalently bound to fatty-acid free bovine serum albumin (BSA). Fatty acid sodium salts were suspended in PBS, then mixed with fatty-acid free bovine serum albumin (U.S. Biologicals) in a 3:1 ratio of fatty acid to albumin, and incubated for 5 hours at 37°C. Fatty acids conjugated to albumin were sterilized by passage through a syringe filter before cell treatments. Cells were treated with 100 µM of DHA-BSA, ARA-BSA, palmitic-BSA, and/or 2 µM GW9662 (Sigma) for 78 hours in media containing 0.5% FBS.

*Statistical methods*

Data are presented as mean ± standard deviation. Bootstrapping and randomization techniques were used in REST 2009 software (Qiagen) to calculate significance of fold changes in expression for qRT-PCR experiments. Statistical analysis of changes in fatty acid conversion was conducted using Student's t-test to compare LCPUFA supplementation with control. Linear regression analysis of fatty acid data was carried out in SAS v.9.2.

## Results

*FADS expression in baboon liver*

The splicing and expression patterns of the seven alternative transcripts (AT) of *FADS3* and the one splice variant for *FADS2* have been described in detail previously [5,6]. Because of shared sequences across transcripts, it was not possible to design PCR primers unique to the full-length *FADS3* classical transcript. However, the splice variants could each be assessed by quantitative real-time PCR (qRT-PCR), with the exception of *FADS3 AT2*, which was present at levels too low to quantify accurately. Thus, to assess the effect of dietary LCPUFA on *FADS3* expression, six of the alternative transcripts were evaluated by qRT-PCR in samples obtained from baboon livers after 12 weeks on diets L3, L, or C (described in Materials and Methods). As shown in Figure 3.1, *FADS3 AT1, AT3, AT4,* and *AT7* were about 40% upregulated in the highest LCPUFA group, L3, relative to control. *FADS3 AT5* and *AT6* also had apparently elevated mean expression in L3, but it was not statistically significant. However, *FADS3 AT5* was significantly upregulated in the intermediate group L compared to control. Interestingly, *AT1* and *AT7* had a significant U-shaped expression response, with lower expression in L compared to control, but higher expression in L3.

As shown in Figure 3.2, the other two members of the FADS gene cluster had an entirely different pattern of regulation. *FADS1*, *FADS2*, and the alternative transcript *FADS2 AT1* were all downregulated in both the L and the L3 groups relative to control. The magnitude of expression change was similar for both genes; *FADS1* and total *FADS2* transcripts were reduced by at least 50% for both L and L3. These data are

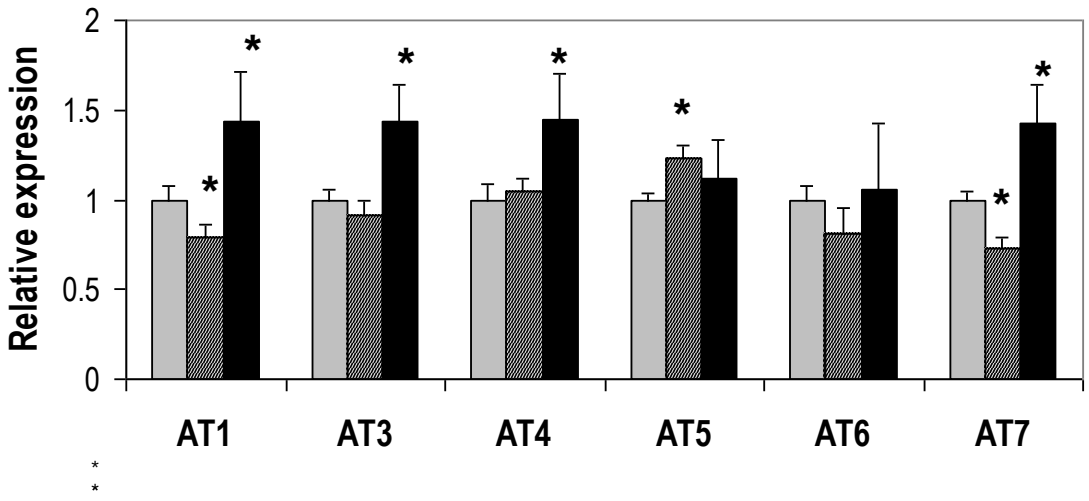


**Figure 3.1. *FADS3* isoform expression is altered by dietary LCPUFA.** *FADS3* splice variant expression in livers from baboons fed diet C (gray bars), L (striped bars), or L3 (black bars) was measured by qRT-PCR, with GAPDH and beta-actin as reference genes. Most isoforms were significantly upregulated in the L3 diet relative to C (* $p < 0.05$ compared to C). Downregulated AT1 and AT7, and upregulated AT5, were observed for the intermediate diet, L.

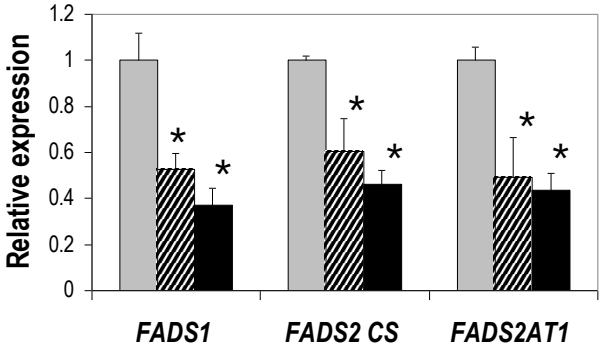


**Figure 3.2. *FADS1* and both *FADS2* isoforms are downregulated by dietary LCPUFA.** Expression in baboon liver from animals fed diet C (gray bars), L (striped bars), or L3 (black bars) was measured by qRT-PCR, with GAPDH and beta-actin as reference genes. * $p < 0.05$ compared to C group.

consistent with previous studies showing lower appearance of labeled 22:6n-3 from labeled 18:3n-3 in liver and blood pools 22:6n-3 in a diet with DHA-ARA similar to the present L diet compared to the C diet [18].

*Liver PUFA substrate-product ratios*

To investigate whether *FADS3* upregulation affected desaturase reactions, levels of fatty acid substrates and products in baboon liver were evaluated as indicators of apparent desaturase activity. DHA and ARA were not included in ratios because their concentrations reflected both biosynthesis and incorporation pre-formed from the experimental diets. Instead, several other substrate/product pairings were used to infer activity, as shown in Figure 3.3. *FADS2*-encoded Δ6-desaturase activity catalyzes the desaturation of 18:2n-6 to 18:3n-6. The percent conversion to the 18:3n-6 product was significantly decreased in L compared to C, but L3 was not significantly different from control, though the L and L3 means were similar. Diet 20:4n-6 is zero in the C treatment and 0.64 % (w/w) in both the L and L3 treatments, consistent with the concept that ARA downregulated both L and L3.

Conversion of 22:4n-6 to 22:5n-6 is presumed to proceed via *FADS2*-mediated Δ6-desaturation. A linear regression against diet DHA yielded a significant slope ($p<0.05$) and thus a significant downward trend for decreasing percent conversion with increasing LCPUFA. The pairing of 18:3n-3 with the downstream product 20:5n-3 was used as an aggregate measure of total desaturase activity, since both *FADS1* and *FADS2* gene products are required for 20:5n-3 synthesis. Substrate conversion

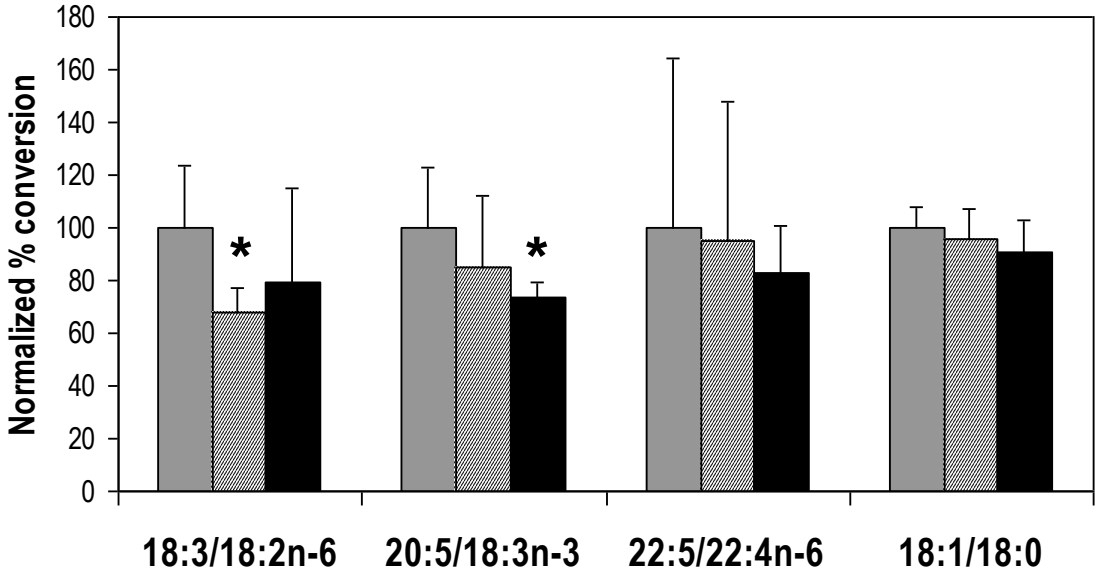


**Figure 3.3. Apparent desaturase activity is consistent with downregulation.** Percent conversion of fatty acid substrates into desaturated products (ratios shown, calculated as product/[substrate + product]) was measured in baboon liver and normalized to C group (gray bars). Group L is shown as striped bars, and L3 as black bars. * $p < 0.05$ compared to C group.

resulting in 20:5n-3 was significantly decreased in L3 compared to C. Finally, a reaction catalyzed by a desaturase outside the FADS cluster, the conversion of 18:0 to 18:1 by stearoyl-coA desaturase, was also examined since this conversion is sensitive to overall dietary unsaturation. The downward trend in mean conversion with increasing LCPUFA was not significant. Thus, significant differences in pairwise comparisons and trends associated with more unsaturation implied steadily decreasing desaturation activity with increasing dietary LCPUFA. No substrate-product pairs were significantly increased by dietary LCPUFA, leaving no obvious candidates for FADS3 substrates.

*FADS expression in response to LCPUFA supplementation in HepG2 cells*

To investigate the mechanism for the effect of LCPUFA on *FADS3* transcripts, human liver-derived HepG2 cells were grown in low-serum media supplemented with albumin-bound fatty acids. Cells were treated either with docosahexaenoic acid (DHA, 22:6n-3) alone, arachidonic acid (ARA, 20:4n-6) alone, or palmitic acid (16:0) as a control. As summarized in Table 3.2, DHA alone significantly upregulated all *FADS3* transcripts except *AT7*. ARA alone significantly upregulated *FADS3 AT1* and *AT3*, while *FADS3 AT4*, *AT6*, and *AT7* increases were comparable to those observed with DHA treatment, but were not statistically significant. As in baboon livers, both fatty acids also significantly downregulated *FADS1* and both *FADS2* transcripts.

To understand the role of transcription factors in mediating these expression changes, the effect of co-incubation of fatty acids with the PPARγ antagonist GW9662 was evaluated. GW9662 treatment completely blocked the upregulation of *FADS3*

**Table 3.2. Fold changes in FADS gene expression in HepG2 cells for LCPUFA treatments with or without the PPARγ antagonist GW9662, shown relative to control (palmitic acid).**

| Gene | DHA | DHA + GW9662 | ARA | ARA + GW9662 |
|---|---|---|---|---|
| *FADS3 AT1* | 1.2** | 1.1 | 1.3** | 1.1 |
| *FADS3 AT3* | 1.2** | 1.0 | 1.2** | 1.1 |
| *FADS3 AT4* | 1.2* | 0.93 | 1.2 | 1.1 |
| *FADS3 AT5* | 1.4* | 0.92 | 0.91 | 1.1 |
| *FADS3 AT6* | 1.6** | 0.85 | 1.4 | 0.92 |
| *FADS3 AT7* | 1.2 | 0.90 | 1.2 | 0.93 |
| *FADS2* | 0.59** | 0.37** | 0.47** | 0.22** |
| *FADS2 AT1* | 0.47** | 0.34** | 0.32** | 0.31** |
| *FADS1* | 0.81** | 0.50** | 0.65** | 0.49** |

* $p < 0.05$, ** $p < 0.01$ for being different from 1 (control = palmitic acid)

transcripts observed with both DHA and ARA treatment, but had no effect on the downregulation of *FADS1* or *FADS2* transcripts.

**Discussion**

We have evaluated transcriptional changes in FADS genes in infant baboon liver in response to diets corresponding to the physiological range of LCPUFA found in human breastmilk worldwide. *FADS3* transcripts followed a pattern of regulation opposite to the other members of the FADS gene cluster, *FADS1* and *FADS2*. *FADS3* transcripts were upregulated by approximately 40% in livers of animals fed the highest LCPUFA diet, L3, compared to control. In contrast, the same diet downregulated *FADS1*, *FADS2*, and the alternative transcript *FADS2 AT1*, by at least 50%. The similarity of the downregulation of *FADS2* and *FADS2 AT1* suggests transcription-level control rather than any change in splicing regulation.

The expression changes observed in baboon liver were reproduced in human liver-derived HepG2 cells treated with either DHA or ARA. DHA produced statistically significant fold changes in *FADS3* isoforms, while ARA treatment produced similar magnitude fold-changes, though most were not statistically significant. Thus, there was no obvious difference in sensitivity of the *FADS3* response to DHA or ARA, but the DHA response had greater precision in this experiment. Moreover, because the cell treatments did not differ in caloric or fat content from the control (palmitic acid treatment), the response was a specific, direct response to DHA or ARA rather than an endocrine mechanism or a general response to energy density of the diet/media. Both

DHA and ARA have previously been shown to bind and activate the transcription factor PPARγ [19]. Co-incubation with the PPARγ antagonist GW9662 prevented *FADS3* upregulation by DHA or ARA, suggesting that the fatty acids acted by a PPARγ-dependent mechanism. In contrast, GW9662 did not prevent the downregulation of *FADS1* and both *FADS2* isoforms by DHA or ARA. Thus, *FADS3* expression is regulated in opposite sense to the other FADS genes, and occurs by a different mechanism.

Extensive substrate screening in our laboratory has so far failed to uncover a substrate for any *FADS3* isoforms (unpublished data). Investigation of baboon liver fatty acids as evidence of desaturase enzyme activity produced evidence for downregulation of *FADS1* and *FADS2*, but no evidence for increased desaturation of any substrate/product pair to correspond with the upregulation of *FADS3* isoforms. This observation could be explained if *FADS3* isoforms function as desaturases on non-LCPUFA substrates. Alternatively, they may act as non-functional dominant negative inhibitors by binding non-productively to LCPUFA substrates, and removing them from availability for desaturase reactions. In favor of the latter theory, Δ5-desaturase, Δ6-desaturase, and stearoyl-coA desaturases are all downregulated by dietary unsaturated fatty acids [20,21], probably as a mechanism to maintain the unsaturation index of cell membranes within certain limits. It is difficult to reconcile the opposite pattern of regulation of *FADS3* with a putative function as a desaturase. Moreover, dominant negative inhibition is a common mode of action for splice variants of other enzymes [22], and binding specificity for different fatty acids could explain the large number of splice variants for FADS3. Further studies are required to test this hypothesis.

We have demonstrated that *FADS3* is regulated by a different mechanism from other members of the FADS gene cluster, and expression is upregulated when other desaturases are downregulated. These results suggest that, despite a high degree of sequence similarity, *FADS3* isoforms have a function quite distinct from *FADS1* and *FADS2*, and may not encode functional desaturases at all. *FADS3* has been implicated in cardiovascular conditions of enormous public health import, so determining the true function of *FADS3* and its alternative transcripts should be a high priority. Further work characterizing regulation of *FADS3* transcription and alternative mRNA splicing may yield more clues to functional roles.

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# CHAPTER 4

# THE POLYPYRIMIDINE TRACT BINDING PROTEIN REGULATES DESATURASE ALTERNATIVE SPLICING AND PUFA COMPOSITION


## Abstract

The delta-6 desaturase, encoded by *FADS2*, plays a crucial role in omega-3 and omega-6 fatty acid synthesis. These fatty acids are essential components of the central nervous system, and act as precursors for eicosanoid signaling molecules and as direct modulators of gene expression. The polypyrimidine tract binding protein (PTB, or hnRNP I), is a splicing factor that regulates alternative pre-mRNA splicing. Here, PTB is shown to bind an exonic splicing silencer element and repress alternative splicing of *FADS2* into *FADS2 AT1*. *PTB* and *FADS2AT1* were inversely correlated in neonatal baboon tissues, implicating PTB as a major regulator of tissue-specific *FADS2* splicing. In HepG2 cells, PTB knockdown modulated alternative splicing of *FADS2,* as well as *FADS3*, a putative desaturase of unknown function. Omega-3 fatty acids decreased by nearly one half relative to omega-6 fatty acids in PTB knockdown cells compared to controls, with a particularly strong decrease in eicosapentaenoic acid concentration and its ratio with arachidonic acid. This is a rare demonstration of a mechanism specifically altering the cellular omega-3 to omega-6 fatty acid ratio without any change in diet/media. These findings reveal a novel role for PTB, regulating availability of membrane components and eicosanoid precursors for cell signaling.

## Introduction

The delta-6/delta-8 desaturase, encoded by *FADS2*, is the rate-limiting enzyme in synthesis of long-chain (20-carbon and above) omega-3 and omega-6 polyunsaturated fatty acids (PUFA) (Figure 4.1). These fatty acids constitute about 25% of the structural lipid of central nervous system gray matter [1], and must be supplied in especially large quantities for the brain growth spurt during perinatal development [2,3]. Long chain omega-3 and omega-6 fatty acids are also precursors for lipid mediators known as eicosanoids and docosanoids, which convey signals controlling inflammation and blood clotting, among many other physiological conditions [4]. Together with their fatty acid precursors, they also affect gene expression directly by interaction with nuclear receptors [5]. Although long-chain omega-3 and omega-6 fatty acids can be obtained directly from diet, genetic evidence suggests that biosynthesis is also important for optimal health throughout life. Single nucleotide polymorphisms (SNPs) in *FADS2* have been associated with allergy and atopic eczema, total cholesterol, LDL, C-reactive protein levels, and coronary artery disease risk, as well as cognitive outcomes such as attention-deficit hyperactivity disorder and IQ in children [6,7,8,9,10,11].

Recently, we discovered that *FADS2* is alternatively spliced to generate an alternative transcript (*FADS2 AT1*) with a pattern of expression that differs from the classical form in primate tissues [12] . No function has yet been found for this alternative splice variant, but its expression is conserved across numerous species [13]. We have also identified 7 alternative transcripts for *FADS3*, an adjacent gene thought to encode a desaturase due to its sequence homology to other FADS (fatty acid desaturase,

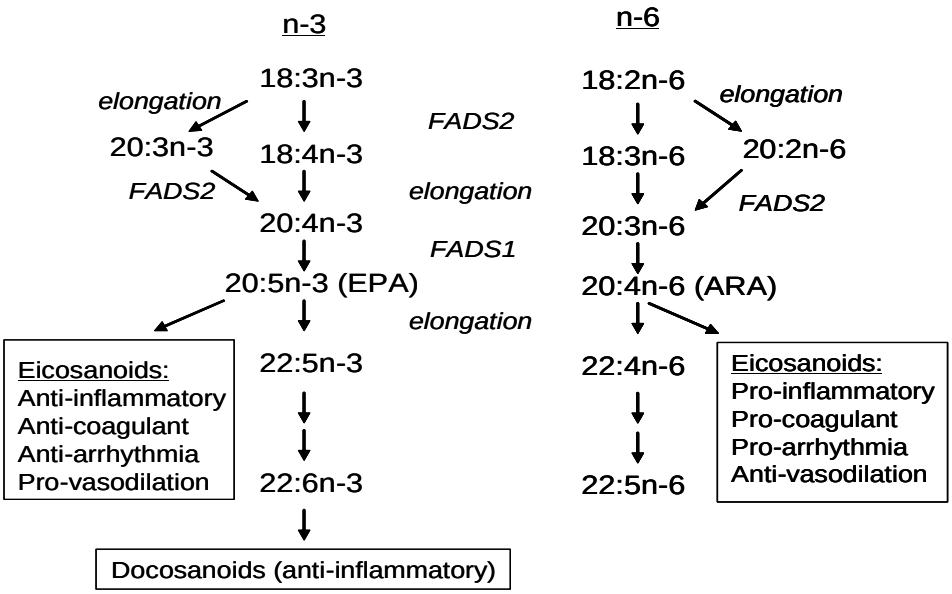


**Figure 4.1.** *FADS2* codes for a desaturase that adds cis double bonds to the growing fatty acid chains in omega-3 (n-3) and omega-6 (n-6) long chain polyunsaturated fatty acid synthesis. Nomenclature refers to the number of carbons and double bonds (e.g., 20:5n-3 has 20 carbons and 5 double bonds, with n-3 describing distance of the double bonds from the terminal methyl carbon of the fatty acid.) The end-products of the biosynthetic pathway give rise to eicosanoids and docosanoids with opposing effects.

referring specifically to the cluster of genes comprised of *FADS1, FADS2,* and *FADS3*) genes [14]. Although the function of *FADS3* and its alternative splice variants is unknown, SNPs in this gene are associated with plasma sphingolipids, triglycerides, and myocardial infarction, and expression of *FADS3* is altered in familial combined hyperlipidemia, and in mouse uterus at the site of embryo implantation [15,16,17]. Although the genetic evidence suggests that *FADS3* is important for lipid metabolism, mechanisms by which genetic variants in *FADS3* affect blood lipids and disease have not been identified.

Here, we investigated regulation of fatty acid desaturase alternative pre-mRNA splicing. Two major classes of splicing factors are known to function in opposing ways to regulate splicing: the serine-arginine (SR) proteins, which typically enhance splicing, and the heteronuclear riboprotein (hnRNP) family, known for splicing repression [18]. Polypyrimidine tract binding protein (PTB, also known as PTBP1 or hnRNP I) is a member of the hnRNP family that usually acts as a repressor, but also sometimes enhances splicing, depending on the location it binds pre-mRNA and proximity to binding sites for other splicing factors [19]. The majority of PTB protein is normally found in the nucleus, but under some circumstances it can relocalize to the cytoplasm, where it affects mRNA stability and translational efficiency [20]. Although sometimes described as ubiquitous because it is expressed by most or all cell types at some point during development, PTB protein levels vary in different tissues, and control of function by phosphorylation allows for dynamic temporal and spatial adjustment of PTB activity.

PTB is known to be a primary regulator of alternative splicing for several genes [20],

and presumably affects numerous others. However, relatively little is known about high-level functional changes resulting from PTB activity. PTB appears to promote growth in some cancer cell types [21], and it binds pyrimidine tracts in several growth-promoting genes, encouraging transcription [22]. Cytoplasmic PTB has been shown to enhance production of both insulin and insulin secretory granules by stabilizing mRNA, leading to increased translation [23]. These functions are consistent with switching from lipid utilization to glucose as an energy source, and enhancing fatty acid biosynthetic pathways, but to our knowledge PTB has never been directly linked with lipid metabolism.

Preliminary bioinformatics predictions suggested that PTB might function in regulation of *FADS2* alternative splicing. To investigate this, we examined binding of PTB *in vitro* to a putative exonic silencer site, effects of PTB knockdown with siRNA, and expression of PTB relative to FADS alternative transcripts in baboon tissues. Finally, we demonstrated changes in the ratio of omega-3 to omega-6 fatty acids with PTB knockdown in liver-derived cells.

## Materials and Methods

### *Cell culture*

For all experiments, human HepG2 hepatocellular carcinoma and SK-N-SH neuroblastoma cells were maintained within ten passages of the original passage received from the ATCC. HepG2 cells were grown in MEM with 10% FBS, and SK-N-SH cells were grown in DMEM/F-12 with 10% FBS (media and serum obtained from HyClone) in a humidified environment at 37°C with 5% $CO_2$.

*In vitro RNA binding assay*

HepG2 nuclear extracts were prepared from cell lysates using the Qproteome Nuclear Protein Kit (Qiagen). Protein concentration was determined using a BCA Protein Assay Kit (Pierce). Biotin labeled RNA oligos (Integrated DNA Technologies) were suspended in nuclease-free PBS. The sequences used were: FADS2wt GAUUAUGGCCACCUGUCUGUCUACAGAAAA, and FADS2mut GAUUAUGGACAGAGAUAGACGGACAGAAAA. PTB binding to wild type (wt) but not to the mutant version was predicted using the online tool Splicing Rainbow (EMBL-EBI Alternative Splicing Workbench, <http://www.ebi.ac.uk/asd-srv/wb.cgi>) [24]. For each binding reaction, 1 mg of magnetic streptavidin-labeled M-280 Dynabeads (Invitrogen) were first pre-washed to remove nucleases, following the manufacturer's recommended protocol. Beads were mixed with 27 µg of biotin-RNA and 15 µg of nuclear extract in PBS for 30 minutes with gentle rotation at 4°C. Beads were washed four times with PBS, then bound proteins were eluted with the addition of Laemmli sample buffer and boiling for 5 minutes. Eluted proteins were loaded onto a Mini-Protean pre-cast Any KD SDS-PAGE gel (Bio-Rad) along with 15 µg nuclear extract as a positive control. After separation, proteins were transferred to a nitrocellulose membrane (Li-Cor). The membrane was probed with antibodies at the following dilutions: mouse SH54 anti-PTB (EMD), 1:100; rabbit anti-beta-actin (Li-Cor) as a negative control, 1:1000; goat anti-mouse 680LT (Li-Cor), 1:10,000; goat anti-rabbit 800CW, 1:15,000. The blot was visualized on a Li-Cor Odyssey two-color fluorescence imager.

*siRNA*

PTB siRNA (100 nM) and control non-targeting siRNA (Dharmacon siGENOME SMARTpool) were used in triplicate treatments with Dharmafect 4 (HepG2) or Dharmafect 1 (SK-N-SH), following the manufacturer's protocol. SMARTpool reagents contained four different siRNA, allowing lower concentrations of individual siRNA to be used in order to prevent off-target effects. In addition, a combination of sense and antisense strand chemical modifications that reduce off-target effects were incorporated when needed [25]. Cells were treated for 72 hours before RNA or lipid extraction. For fatty acid treatment, omega-3 docosapentaenoic acid (22:5n-3) was first non-covalently bound to fatty-acid free bovine serum albumin (BSA). Fatty acid sodium salts were suspended in PBS, then mixed with fatty-acid free bovine serum albumin in a 3:1 ratio of fatty acid to albumin, and incubated for 5 hours at 37°C. RNAi was carried out as usual, with the addition of 25 μM BSA-bound 22:5n-3 or vehicle (BSA only) in the media for the entire 72 hour incubation.

*RNA extraction and PCR*

Banked neonate baboon tissues were obtained in a previous study from our laboratory [26], and stored from time of necropsy at -80°C until RNA was extracted. All tissues were from a 12-week old control animal that had been fed an infant formula containing no long chain PUFA. Live baboon work was carried out at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX. Animal protocols were approved by the SFBR and Cornell University Institutional Animal Care and Use Committee (IACUC, protocol # 02-105.) Baboon tissue and cell culture RNA was extracted, RNA integrity assessed, cDNA prepared, and semi-quantitative RT-PCR carried out as

described previously [14]. PCR primers designed for FADS splice variants (Integrated DNA Technologies, sequences available upon request) were validated by cloning and sequencing PCR products. Gel bands were analyzed by densitometry using ImageJ software (National Institutes of Health). Quantitative real-time PCR was carried out using SYBR Green Master Mix (Roche) on a LightCycler 480 instrument (Roche), with β-actin chosen from a panel of candidate reference genes because it was not affected by cell treatments. PCR reaction efficiency was calculated from standard curves, and reactions were assessed by both melting curves and by running on agarose gels to verify reaction products and the absence of primer-dimers. Quantitative cycle (Cq) values were determined using LightCycler 480 SW1.5.0SP3 software, version 1.5.0.39 (Roche). Analysis was carried out with REST 2009 software (Qiagen), which employs the Pfaffl method for relative quantification [27], and uses bootstrapping and randomization techniques to calculate fold changes and statistical significance.

*Fatty acid analysis*

Cells were washed with HBSS, then trypsinized to remove from growth surfaces. After centrifugation and removal of supernatant, lipid extraction was carried out on the cell pellets. Fatty acid methyl esters (FAME) were prepared using a modification of the one-step method of Garces and Mancha [28]. FAME were analyzed in triplicate and quantified by gas chromatography-flame ionization detection (GC-FID), using an equal weight FAME standard mixture to verify response factors daily [29]. Peak identities were confirmed by GC-covalent adduct chemical ionization tandem mass spectrometry (GC-CACI-MS/MS) [30,31,32]. The standard deviation for ratios (fold change in treated/control) was calculated using a propagation of error approach.

## Results

*PTB is predicted to bind FADS2 and FADS3 exonic splice sites*

To identify putative binding sites for splicing factors that might regulate alternative splicing of FADS genes, we investigated alternative exonic splice sites in *FADS2* (NCBI accession no. EU780003) and *FADS3* (NCBI accession no. EU780002) using the bioinformatics tool Splicing Rainbow (EMBL-EBI Alternative Splicing Workbench, <http://www.ebi.ac.uk/asd-srv/wb.cgi>) [24]. The alternative transcripts (AT) and *in vivo* expression patterns of *FADS2* and *FADS3* splice variants have been described in detail previously [12,14]. Approximately 30nt regions flanking splice sites identified from our previous work were examined for predicted binding sites. Numerous splicing enhancer binding sites were predicted, but there was also a striking pattern of predicted PTB binding directly on splice sites identified from baboon cDNA for *FADS2 AT1* (NCBI accession no. FJ901343) and *FADS3 AT7* (NCBI accession no. FJ641203). The alternative splice site in *FADS2* Exon 4 occurred at base 7 of the 14-mer predicted PTB binding site. *FADS3* Exon 8 included a 35 nt stretch predicted to bind PTB, with the alternative splice site located at nucleotide 19 of the 35-mer. Replacing the sequences with human cDNA sequences yielded identical binding predictions. In addition, *FADS3 AT1* (NCBI accession no. EU780004) exhibited a predicted PTB binding site near the splice site, but not overlapping with it.

*PTB is associated with the FADS2 alternative splice site in vitro*

To test the validity of the predicted binding sites, we carried out an *in vitro* RNA pull-down assay to determine whether the putative exonic splicing silencer (ESS) in *FADS2* could pull down PTB from nuclear extracts. Biotinylated RNA oligos were designed containing either the intact 14-mer human predicted binding site, or a mutated binding site of equal length, with pyrimidines in the binding site replaced by purines in the mutant, but retaining the same flanking sequences (oligo sequences shown in Materials and Methods section). The mutant sequence was checked with Splicing Rainbow to ensure that no consensus binding sites for other splicing factors were introduced in the process of altering the predicted PTB binding site. Each oligo was incubated with nuclear extract, and RNA-bound proteins were eluted and detected by Western blotting. Whole nuclear extract was run in parallel as a positive control, and the blot was probed for β-actin as a negative control to detect non-specific protein binding. As shown in Figure 4.2, PTB bound preferentially to the predicted ESS rather than the mutant version.

*PTB knockdown upregulates FADS2 AT1 and FADS3 AT7*

Because PTB has previously been shown to repress splicing by sterically hindering binding of splicing enhancers [20], we hypothesized that PTB might function as a repressor for *FADS2 AT1* and *FADS3 AT7* expression. To investigate this hypothesis, siRNA against *PTB* was used to knockdown PTB in human neuronal (SK-N-SH) and

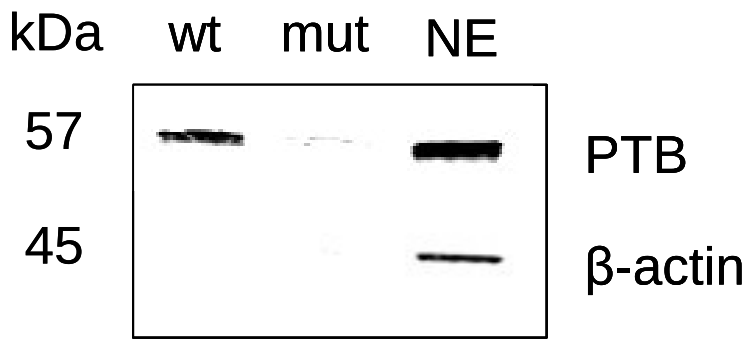


**Figure 4.2.** Western blot showing eluted proteins from an RNA pull-down assay (first two lanes) run in parallel with whole nuclear extract (NE) as a positive control. PTB was pulled down by an RNA oligo containing the *FADS2* putative binding site (wt), but not by a mutated version (mut), with no non-specific binding of β–actin.

liver-derived (HepG2) cells. Expression of the alternative transcripts was examined by RT-PCR using primers designed to bridge sequences unique to each splice variant. In a preliminary time course experiment, *FADS2 AT1* levels rose with time, concomitant with reductions in *PTB* mRNA levels in SK-N-SH cells (Figure 4.3A). Changes in expression were investigated in more detail in HepG2 cells with quantitative real-time PCR for transcripts in the FADS gene cluster (Figure 4.3B). With > 90% knockdown of *PTB*, *FADS2 AT1* expression increased 1.48 fold, while *FADS3 AT7* increased 1.39 fold, consistent with splicing repression by PTB. Interestingly, *FADS3 AT1* relative mRNA abundance decreased slightly, suggesting PTB might play a role in stabilizing splicing of this transcript. There were no significant changes in *FADS1*, *FADS2* (classically spliced, CS), or any other *FADS3* transcripts (data not shown). The concentration of *FADS2 AT1* is about 10-fold lower than *FADS2 CS* in HepG2 cells (estimated from Cq values for identical cDNA dilutions), so even a 50% increase in *FADS2 AT1* levels would decrease *FADS2 CS* by only 5%, well below the detection limit for real-time PCR; hence no reduction in observed *FADS2 CS* is expected. Thus, in this liver cell model, PTB knockdown was sufficient to regulate splicing of *FADS2 AT1* and *FADS3 AT7* transcripts.

*PTB is inversely correlated with FADS2 AT1 in baboon tissues*

To understand the role of PTB in regulating tissue-specific splicing of FADS genes *in vivo*, we examined levels of *FADS2 AT1, FADS3 AT7*, *FADS3 AT1*, and *PTB* mRNA in neonatal baboon tissues. Eight of 11 tissues evaluated followed an inverse correlation of *FADS2 AT1* with *PTB* mRNA levels (Figure 4.4A, $R^2 = 0.87$), suggesting that PTB

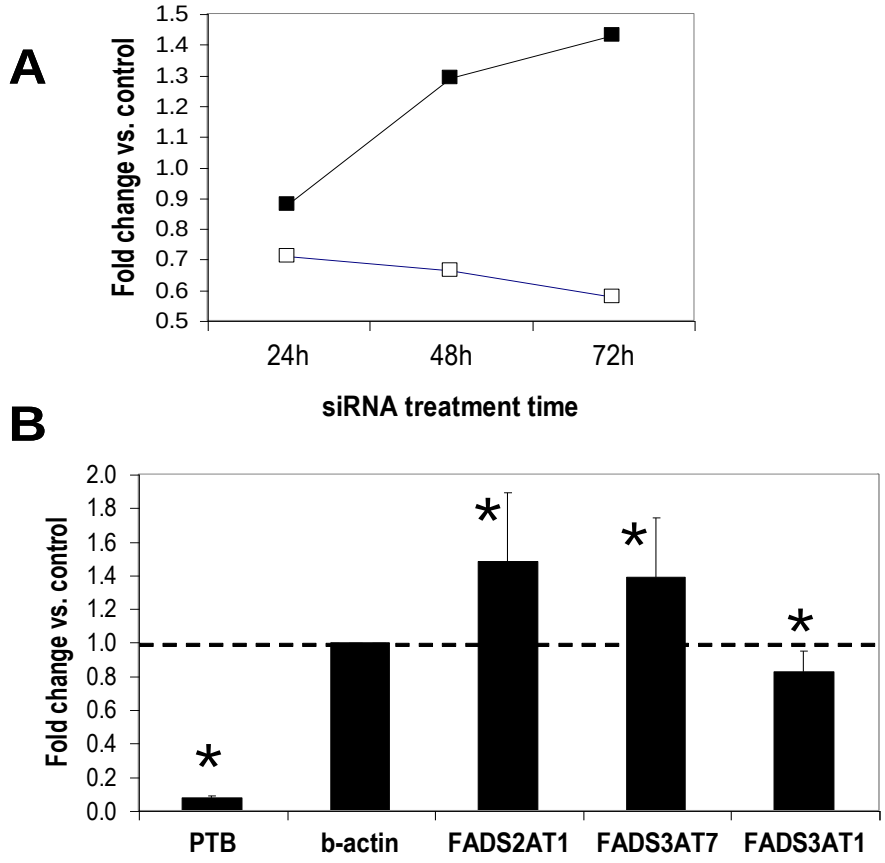


**Figure 4.3**. (A) RT-PCR results for a time-course of PTB siRNA knockdown in SK-N-SH neuroblastoma cells, showing increasing *FADS2 AT1* (■) with decreasing *PTB* (□). Integrated densities of gel bands were normalized to actin and expressed as the ratio of knockdown/control. (B) Quantitative real-time PCR showing fold change in FADS gene transcript expression in HepG2 cells for PTB knockdown compared to non-targeting siRNA control, n=3 biological and technical replicates. * $p < 0.01$

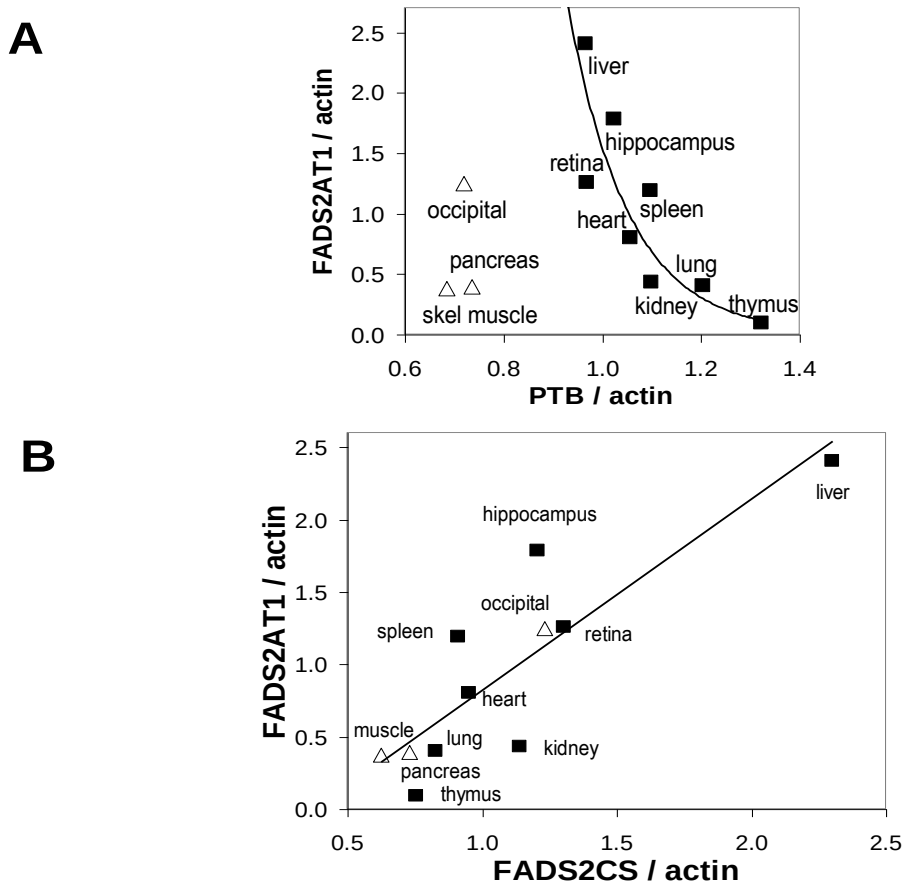


**Figure 4.4.** The combination of splicing and transcriptional regulation determines *FADS2 AT1* levels *in vivo*. (A) Splicing regulation is suggested by inverse correlation of *PTB* and *FADS2 AT1* in the majority of neonatal baboon tissues examined (■), with $R^2$ = 0.87 for trend excluding tissues with low *PTB* (Δ). Axes represent RT-PCR integrated densities normalized to β-actin. (B) Tissues with low *PTB* levels (Δ) closely follow the trendline relating *FADS2 AT1* with *FADS2* CS ($R^2$ = 0.74 for trend including all points), suggesting transcription-level regulation (pre-mRNA abundance) is the primary determinant of *FADS2 AT1* levels in the three tissues with low PTB.

may play an important role in regulating *FADS2* tissue-specific splicing *in vivo*. The three tissues with the lowest levels of *PTB* did not fit the pattern; these results are consistent with predominant transcription-level control in these tissues, since levels of *FADS2 AT1* corresponded closely with *FADS2 CS* levels in tissues with relatively low *PTB* (Figure 4.4B). Unlike *FADS2 AT1*, there was much lower correlation between *PTB* levels and *FADS3 AT7* or *FADS3 AT1* in baboon tissues (Figure 4.5). There was a slight inverse correlation of *PTB* and *FADS3 AT7* (Figure 4.5A, $R^2 = 0.44$) in the same eight tissues examined above, while *FADS3 AT1* showed very little correlation with *PTB* (Figure 4.5B, $R^2 = 0.26$).

*PTB knockdown specifically decreases omega-3 fatty acid content*

To determine functional consequences of upregulation of *FADS2 AT1* and *FADS3 AT7*, the fatty acid composition of HepG2 cells was analyzed after PTB knockdown or control siRNA treatment (Figure 4.6, gray bars). Both omega-3 and omega-6 fatty acids decreased significantly after PTB knockdown, offset by a slight increase in monounsaturates and no change in saturated fatty acids. Interestingly, in all cases, omega-3 fatty acids declined more than omega-6 fatty acids (23% decrease overall compared to 13%, respectively, $p < 0.01$ for the difference). There was a 12% reduction ($p = 0.005$) in the ratio of total omega-3 to omega-6 fatty acids with PTB knockdown.

The most dramatic change with PTB knockdown was a 50% reduction in eicosapentaenoic acid (EPA, 20:5n-3) content coinciding with upregulation of *FADS2 AT1* and *FADS3 AT7*. Although both EPA and arachidonic acid (ARA, 20:4n-6) levels

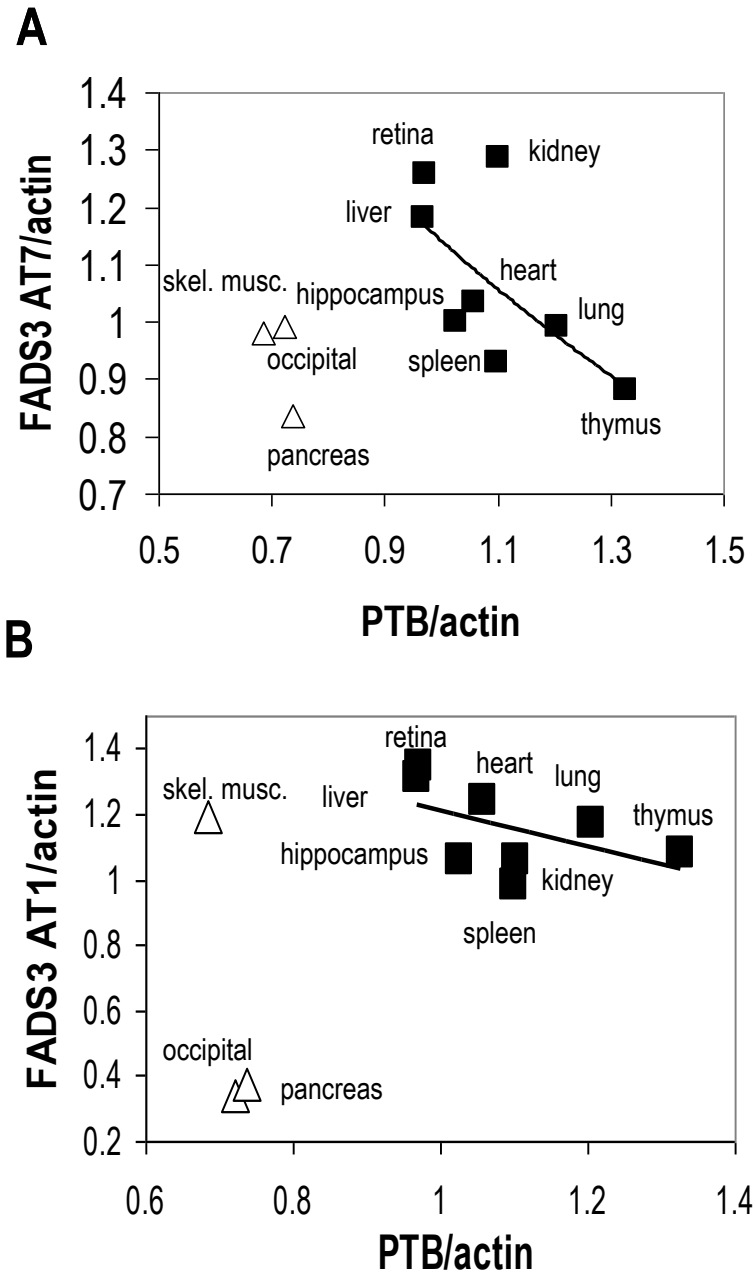


**Figure 4.5.** Lower correlation between *PTB* and *FADS3* transcripts. (A) Slight inverse correlation of *FADS3 AT7* and *PTB* in the majority of neonatal baboon tissues examined (■), with $R^2 = 0.44$ for trend excluding tissues with low *PTB* (Δ). Axes represent RT-PCR integrated densities normalized to β-actin. (B) Low correlation of *FADS3 AT1* and *PTB*, with $R^2 = 0.26$ for trend excluding tissues with low *PTB* (Δ).

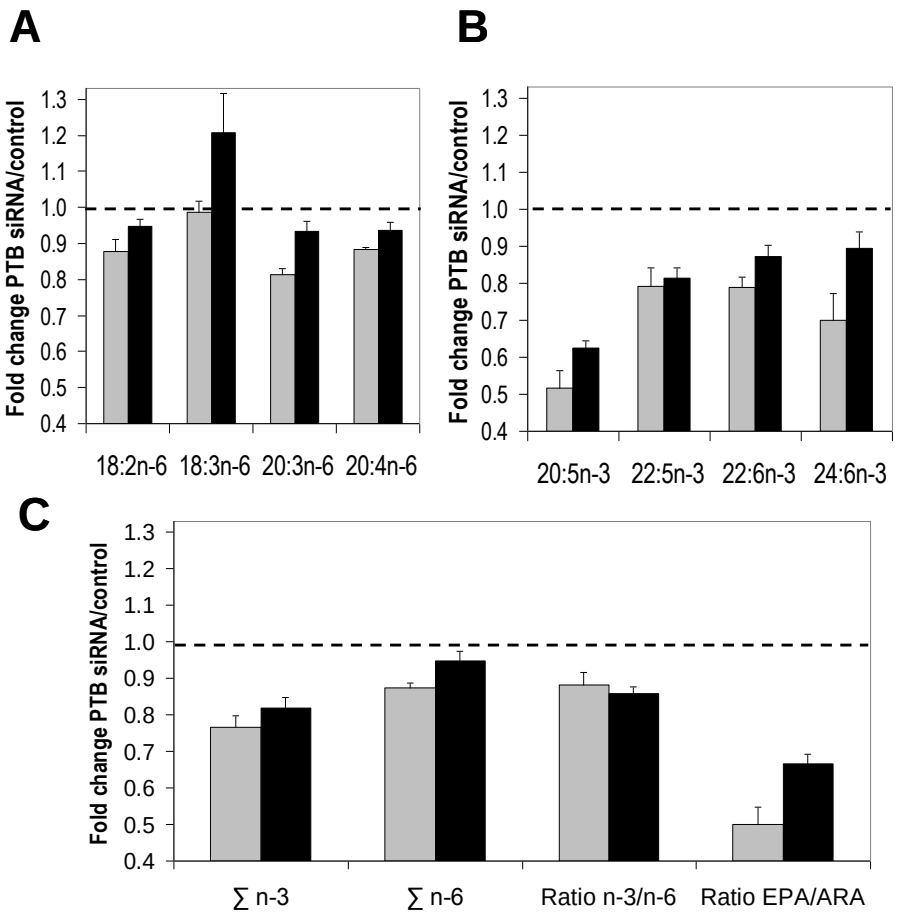


**Figure 4.6**. Fatty acid composition of liver-derived HepG2 cells, showing fold change in concentration of each fatty acid with PTB siRNA compared to non-targeting control siRNA. Gray bars represent treatments in regular media, and black bars are media supplemented with 25 μM 22:5n-3. All changes shown were significant at $p < 0.05$, except for 18:3n-6 in regular media. (A) Omega-6 (n-6) fatty acids, (B) Omega-3 (n-3) fatty acids, (C) Summary of total fatty acids and ratios of fatty acids with opposing effects.

declined with PTB knockdown, EPA levels went down much more than ARA levels; the ratio of EPA/ARA was reduced by 43% ($p = 0.0002$) with PTB knockdown.

The EPA precursors 20:4n-3 and 18:3n-3, normally at very low levels in most cells, were below quantifiable limits, and thus the substrates were not accumulating as EPA product decreased. However, the ratio of 20:3n-6 with its precursor, 18:3n-6, decreased 17% with PTB knockdown ($p = 0.0005$), consistent with inhibition of the elongation step from 18-carbon to 20-carbon polyunsaturated fatty acids.

To determine how specific the inhibitory activity was for EPA synthesis compared to longer chain omega-3 fatty acids, we asked whether adding 22:5n-3, an elongation product of EPA, would rescue levels of the desaturation product 22:6n-3 (docosahexaenoic acid, or DHA). To investigate this, PTB knockdown was repeated in HepG2 cells in the presence of media supplemented with 22:5n-3 (Figure 4.6, black bars). As before, EPA levels were sharply reduced with PTB knockdown, as was the ratio of EPA to ARA, and the ratio of total omega-3 to omega-6 fatty acids. Despite 22:5n-3 supplementation, levels of 22:5n-3, 22:6n-3, and the elongation product 24:6n-3 were still significantly reduced with PTB knockdown, though the differences from control cells tended to be less obvious than without supplementation. For example, 22:6n-3 concentration declined 21% in PTB knockdown compared to control ($p = 0.001$) in unsupplemented cells, whereas the decrease was only 13% ($p = 0.002$) in cells supplemented with 22:5n-3. Thus, the reduction in total omega-3 levels with PTB knockdown cannot be entirely explained by reduction in EPA, since 22:5n-3 supplementation failed to completely rescue levels of downstream desaturation

products.

## Discussion

Here, we have demonstrated that PTB plays a role in repressing expression of FADS splice variants and altering cellular omega-3 to omega-6 fatty acid ratio. Knockdown of PTB in liver-derived cells led to upregulation of both *FADS2 AT1* and *FADS3 AT7,* and an RNA pulldown assay showed that PTB was associated with the putative binding site in *FADS2 in vitro.* Further studies are needed to determine whether PTB binds directly to the predicted binding site, or whether other proteins are necessary for PTB binding and splicing repression. Our RNAi results showing roughly equivalent induction of *FADS2 AT1* in liver and neuronal-derived cells, despite very different levels of *PTB* silencing, corroborate the idea of additional, tissue-specific factors contributing to splicing regulation in different cell types. However, the fact that the putative PTB binding sites overlap with the alternative splice sites in *FADS2* and *FADS3* strongly suggests PTB binding sterically hinders access of splicing machinery proteins to the splice sites. Moreover, the high correlation of the expression pattern of *FADS2* and *PTB* in baboon tissues suggested that PTB alone may be an important regulator of *FADS2* splicing *in vivo*. *FADS3* splicing was not as closely correlated with *PTB* mRNA levels in baboon tissues. It is possible that post-transcriptional regulation of PTB is more important than mRNA level in determining splicing repression of *FADS3*. There also are likely to be other important regulators of *FADS3* splicing that vary by tissue type, and interact with PTB to determine the final splicing pattern. Alternatively, the paralog PTBP2 may replace PTB in repressing *FADS3* splicing in some tissues, since PTBP2 has some

functional redundancy with PTB [33]. However, the increase in *FADS3 AT7* levels in HepG2 cells in response to PTB knockdown may reflect liver-specific regulation, where PTB alone may play a major role. The 40-50% changes in expression we observed *in vitro* were relatively subtle compared to changes often observed in disease states, but similar fold changes have routinely produced important biochemical changes in studies of nutritional effects on metabolic genes [24,34,35,36].

Knockdown of PTB in liver-derived cells also resulted in decreased cellular omega-3 and omega-6 fatty acid content. Omega-3 fatty acids decreased by nearly one half relative to omega-6 fatty acids in PTB knockdown cells compared to controls. These results were surprising because the same enzymes operate on both omega-3 and omega-6 fatty acids (Figure 4.1), so it has been accepted that synthesis of both occur largely in concert. Intervention studies to raise the proportion of omega-3 relative to omega-6 fatty acids in cell membranes have always focused on dietary modifications, since it was presumed that consuming altered ratios of substrates or end-products was the only way to change cellular ratios of omega-3 to omega-6 fatty acids. However, puzzling biochemical evidence has occasionally emerged suggesting the possibility of separate delta-6 desaturase enzymes for different fatty acids [37,38]. The idea of separate enzymes lost favor after the discovery of a single *FADS2* gene, which was found to code for a desaturase with dual substrate activity toward both omega-3 and omega-6 fatty acids [39]. Since classical enzymes in the biosynthetic pathway accept both classes of fatty acids as substrates, it is difficult to explain how the omega-3 to omega-6 fatty acid ratio could change without any alteration in media components.

A reasonable alternative is that desaturase splice variants may be able to preferentially bind and sequester omega-3 fatty acid substrates and remove them from availability for enzymatic reactions, leading to a greater reduction in omega-3 than in omega-6 content. Our results provide only circumstantial evidence linking desaturase alternative splicing with fatty acid changes. However, this hypothesized role for FADS splice variants is appealing because dominant negative inhibition is a common role for alternative splice variants; splicing can generate variants that are able to bind substrates, but do not contain domains necessary for enzymatic reactions [40]. *FADS2 AT1* lacks a conserved cytochrome b5 domain and the first histidine motif, and *FADS3 AT7* lacks the last histidine motif characteristic of front end desaturases [12]. Binding non-productively to substrates reduces the effective concentration of substrate available for reactions, and thus limits product formation in biosynthetic pathways. Sequestration of fatty acids would affect both elongation and desaturation reactions, while also disrupting processes such as fatty acid incorporation into phospholipids. The resulting change in omega-3 to omega-6 ratio alters cell membrane structure, and is known to influence function of rhodopsin, a G-protein coupled receptor [41], as well as changing physiological responses such as inflammation through the production of lipid mediators with opposing functions [5].

The desaturase splicing pattern resulting from PTB knockdown was associated with a particularly strong decrease in EPA concentration, and the ratio of EPA with ARA. Although EPA is present as only a small percentage of total fatty acids, it is a precursor to several families of bioactive eicosanoids, including prostaglandins, thromboxanes, and leukotrienes. Products of EPA tend to act in direct opposition to eicosanoids

derived from ARA; EPA-derived eicosanoids have anti-inflammatory, anti-arrhythmia, anti-coagulant, and pro-vasodilation effects [5]. Because levels of ARA in tissues tend to be tightly regulated [42], specific modulation of EPA may be an effective way to adjust ARA net activity without a large change in ARA concentration. Moreover, because eicosanoids are paracrine species that function with high potency but very short half-life [43], a mechanism to specifically modulate EPA levels in different cell types would allow for flexible localized responses.

The ratio of 20:3n-6 with its precursor, 18:3n-6, decreased significantly with PTB knockdown, suggesting that the elongation step from 18-carbon to 20-carbon polyunsaturated fatty acids is inhibited. Sequestration of 18-carbon fatty acids by dominant negative desaturase splice variants, with a preference for omega-3 over omega-6, may be expected to decrease EPA levels by effectively suspending metabolic transformations, including acylation and recycling pathways. This, in turn, would decrease levels of all longer-chain omega-3 fatty acids in the biosynthetic pathway, consistent with our observations. However, the failure of 22:5n-3 supplementation to rescue levels of longer chain fatty acids in the pathway suggests binding affinity for both 18-carbon and >20-carbon fatty acids, as is observed in functional FADS2CS [44]. Long chain omega-3 products such as 22:6n-3 may also be important in mediating PTB's high-level functions.

Fatty acid composition is well known to change in many tissues and cells during development, as well as during disease states and nutrient deficiency conditions. However, the results reported here represent a rare demonstration of a mechanism

specifically altering the cellular omega-3 to omega-6 fatty acid ratio in well nourished cells without any change in diet/media, and the first associated with a splicing factor. Moreover, the sensitivity of PTB to extracellular stimuli and developmental stage provides a means to fine-tune cellular desaturase activity and the ratio of EPA to ARA, as needed for different tissue types or physiological states. These findings reveal a novel role for PTB as a modulator of the availability of key membrane components and eicosanoid precursors, with the potential to propagate extracellular signals to affect the circulatory system and inflammatory responses.

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# CHAPTER 5

# CONCLUSION AND FUTURE DIRECTIONS

The three studies presented here have in common an underlying theme of desaturase regulation. Several facets of regulation were examined, from transcriptional control to mRNA splicing regulation, and from these a model of the overall effects on LCPUFA metabolism is beginning to emerge.

In the first study, SNPs and a minor haplotype were associated with lower *FADS1* expression in the Japanese HapMap population. We found two deletion mutations near a putative sterol response element in individuals carrying the minor haplotype. This genotype was associated with greater response to desaturase transcription activation by SREBP-1c modulators, including a statin drug and an LXR agonist. We have identified a locus in *FADS2* intron 1 that appears to be a major bidirectional regulatory region affecting both *FADS1* and *FADS2* expression. These results may provide a mechanism to explain other studies finding associations of SNPs in this region with diseases and other phenotypes.

Further research is required to determine whether these deletion mutations cause the altered response to SREBP-1c, or if they are markers for a causal genetic variation that has yet to be found. The reason for lower basal *FADS1* also remains a mystery, but lower desaturase expression implies that individuals with this genotype would be especially good candidates for recommendations of increased fish consumption or supplementation with marine oils. It would also be of interest to establish the

prevalence of these variants in other populations besides the Japanese, particularly in European-derived populations that derive major benefits from statin use. The discovery of this genetic association provides an opportunity to study the extent to which induction of LCPUFA mediates the medical benefits of statin drugs. Namely, do people carrying these deletions have reduced cardiac mortality on statin drugs compared to others on statins?

Several other chemical entities are known to affect desaturase activity by modulating SREBP-1c, and these are likely to show differences by genotype similar to those observed for the statin and LXR agonist. These include the top-selling antipsychotic drugs, but it is unclear whether LCPUFA contribute to the medical benefits of these drugs. Similarly, insulin has recently been shown to activate SREBP-1c [1], suggesting that the induction of desaturases by insulin may occur via this mechanism, and may show individual differences by genotype. It would also be of great interest to know how dietary cholesterol affects LCPUFA levels in individuals carrying these deletions, and whether differences in dietary cholesterol could explain the genetic associations of nearby SNPs with IQ in studies comparing formula fed with breastfed children. The exclusion of cholesterol from infant formula, despite evidence that it is universally present in breast milk, has long been controversial, and our results may cause this argument to be revisited. As we demonstrate here, LCPUFA (present in breast milk and in most modern formula products) downregulate *FADS1* and *FADS2*, but cholesterol (in breast milk only) in theory counteracts this effect through SREBP-1c.

The second study addressed regulation of the putative desaturase, *FADS3*. We found

that infant formula containing LCPUFA upregulated several *FADS3* alternative transcripts while downregulating *FADS1* and *FADS2* in infant baboon liver. Reproducing the effect in liver-derived cells showed that LCPUFA act directly rather than via an endocrine response, and that the mechanism involves PPARgamma. These results, showing an opposite pattern of regulation and different mechanism for response to LCPUFA, suggested that FADS3 and/or its splice variants had a unique function rather than being similar to FADS1 and FADS2.

We explored this idea further in the third study, examining the role of PTB in repressing alternative mRNA splicing of *FADS2* and *FADS3*. PTB knockdown upregulated *FADS2 AT1* and *FADS3 AT7* and specifically decreased the n-3/n-6 ratio in liver-derived cells. There was an especially dramatic decrease in levels of EPA relative to ARA, suggesting that fatty acid changes in response to PTB activity might impact eicosanoid levels, affecting inflammation and blood clotting, among other processes. These results suggested that these splice variants might act as dominant negative inhibitors of classical desaturases.

The hypothesis of dominant negative inhibition by splice variants is also consistent with our results showing upregulation of *FADS3* AT in response to LCPUFA. Cells decrease desaturase expression in response to LCPUFA as a mechanism of feedback inhibition, preventing excessive desaturation of cellular lipids. Simultaneous upregulation of inhibitors of LCPUFA biosynthesis would be an efficient way to slow down biosynthesis without waiting for protein turnover to decrease desaturase levels. Our results suggest splice variants have specificity for preferred fatty acids (implied by the greater decrease

in n-3 compared to n-6 fatty acids), which might explain why we have observed so many different alternative transcripts for *FADS3*.

Feedback loops may also be relevant to function of PTB as a modulator of fatty acid composition. Splicing activity of PTB can change in response to extracellular stimuli [2]. Signaling cascades leading to PTB phosphorylation can change PTB subcellular localization [3]; redistribution of PTB out of the nucleus and into cytoplasm would be expected to replicate the effects of PTB knockdown on desaturase alternative splicing, and possibly cellular fatty acid composition. It has been reported that PTB binding to inducible nitric oxide synthase mRNA can be modulated by cytokine stimulation [4], suggesting the intriguing possibility that the effect of PTB on eicosanoid precursor concentrations could be part of a positive or negative feedback loop responding to extracellular inflammation. In addition, very preliminary evidence in our lab suggests that insulin treatment replicates the effect of PTB knockdown on FADS AT expression in HepG2 cells. Cytoplasmic PTB is known to enhance production of both insulin and insulin secretory granules [5]; if our results can be replicated in pancreatic cells, insulin might stimulate redistribution of PTB out of nucleus and into cytoplasm, in a positive feedback loop promoting more insulin secretion.

The studies reported here suggest that exquisite temporal and spatial control of LCPUFA production is achieved through a combination of transcriptional and splicing regulation of FADS genes. Far from being a static or constitutive process, regulation of LCPUFA biosynthesis may be much more dynamic and responsive to external cues than previously thought. Our genetic evidence further underscores the importance of

regulation, suggesting that dysregulation of expression, rather than faulty enzyme structure, may underlie associations of *FADS2* SNPs with diseases. Understanding mechanisms for dietary, hormonal, and pharmacological regulation of fatty acid desaturases may improve our ability to treat or prevent diseases, and brings us another step closer to personalized medicine and individually-tailored dietary recommendations.

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