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Liver Fatty Acid Binding Protein and Obesity

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Abstract

While low levels of unesterified long chain fatty acids (LCFAs) are normal metabolic intermediates of dietary and endogenous fat, LCFAs are also potent regulators of key receptors/enzymes, and at high levels become toxic detergents within the cell. Elevated levels of LCFAs are associated with diabetes, obesity, and metabolic syndrome. Consequently, mammals evolved fatty acid binding proteins (FABPs) that bind/sequester these potentially toxic free fatty acids in the cytosol and present them for rapid removal in oxidative (mitochondria, peroxisomes) or storage (endoplasmic reticulum, lipid droplets) organelles. Mammals have a large (15 member) family of FABPs with multiple members occurring within a single cell type. The first described FABP, liver-FABP (L-FABP, or FABP1), is expressed in very high levels (2-5% of cytosolic protein) in liver as well as intestine and kidney. Since L-FABP facilitates uptake and metabolism of LCFAs *in vitro* and in cultured cells, it was expected that abnormal function or loss of L-FABP would reduce hepatic LCFA uptake/oxidation and thereby increase LCFAs available for oxidation in muscle and/or storage in adipose. This prediction was confirmed *in vitro* with isolated liver slices and cultured primary hepatocytes from L-FABP gene-ablated mice. Despite unaltered food consumption when fed a control diet *ad libitum*, the L-FABP null mice exhibited age- and sex-dependent weight gain and increased fat tissue mass. The obese phenotype was exacerbated in L-FABP null mice pair-fed a high fat diet. Taken together with other findings, these data suggest that L-FABP could have an important role in preventing age- or diet-induced obesity.

Keywords

Liver; fatty acid binding protein; fat; oxidation; body weight; obesity

1. Introduction

Although intracellular fatty acid binding proteins (FABPs) were first discovered over 30 years ago [1], physiological functions of this 15 member protein family are not well elucidated. FABPs are the single most abundant proteins in the cytosol of cells most active in long chain fatty acid (LCFA) uptake and metabolism (liver, intestine), oxidation (kidney, heart, skeletal muscle), and storage (adipose) (Fig. 1) (rev. in [2-6]. Several excellent reviews have previously addressed: (i) native and recombinant FABPs with regards to tissue occurrence, intracellular distribution, isolation, ligand specificity, structure, and potential functions *in vitro* [4,7-11]; (ii) gene structures, regulation, and functions of FABPs in cultured cells (rev. in [6,12-16]; (iii) roles in regulation of nuclear receptors [e.g. peroxisome proliferator activated receptors-α

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(PPARα), hepatocyte nuclear factor-4α (HNF-4α)] (rev. in [17,18]; and (iv) physiological functions of FABPs in genetically engineered mice (rev. in [19-21]. Of these FABPs, liver fatty acid binding protein (L-FABP, also called FABP1) is the most broadly distributed mammalian FABP, is expressed at very high levels in tissues most active in LCFA metabolism [liver (2-5% of cytosolic protein, 0.1-0.4 mM), intestine, kidney].

The current review focuses on L-FABP contributions to LCFA uptake, metabolism, and obesity. An integrated model suggesting the physiological roles of L-FABP is presented in Fig. 2, which incorporates functions first suggested by *in vitro* studies of the pure protein, supported by findings in living cultured cells overexpressing L-FABP, and finally established *in vivo* through the use of L-FABP gene-ablated mice. In multiple steps, L-FABP: (i) enhances cellular LCFA uptake; (ii) binds LCFAs and LCFA-CoAs to minimize toxic effects (detergent properties, inhibition of enzymes) of these poorly soluble lipids; (iii) enhances intracellular transport/diffusion through the cytoplasm; (iv) targets LCFA to peroxisomes for β-oxidation (straight-chain LCFAs) and α-oxidation (branched-chain LCFAs); (v) delivers LCFAs and LCFA-CoAs to mitochondria for oxidation; (vi) targets LCFA and LCFA-CoA to endoplasmic reticulum (ER) for transacylation to complex lipids for membrane synthesis (phosphatidic acid, phospholipids), and storage (triacylglycerides, cholesteryl esters) in multiple tissues wherein L-FABP is expressed as well as for hepatic secretion in VLDL (triacylglycerides, cholesteryl esters); (vii) transports LCFAs and LCFA-CoAs to the nucleus for regulation of nuclear receptors important in transcription of genes encoding proteins involved in LCFA and glucose metabolism [PPAR-α, HNF-4α, liver X receptor (LXR), thyroid hormone receptor (THR)]. Consistent with this model (Fig. 2), ablation of L-FABP inhibits LCFA uptake, reduces LCFA intracellular transport/diffusion, inhibits LCFA esterification, inhibits LCFA oxidation, and inhibits LCFA targeting to the nucleus to thereby redirect dietary LCFA for storage in adipose phenotypically evident as sex-, age-, and high-fat diet dependent obesity. Similarities and differences in phenotype of an independently generated L-FABP gene-ablated mouse underscore the importance of understanding the impact of GFP knock-in strategy, construct design, backcrossing, age, sex, appropriate control diets, and composition of high-fat diets.

2. L-FABP gene

2.1. Gene structure

By chromosomal mapping, the human L-FABP gene was identified and localized to the centromeric p12-q11 region of chromosome 2. The mouse and rat L-FABP genes were localized to chromosome 6 and 4, respectively [22]. Overall, the L-FABP gene structure (four exons and three introns) is identical to other members in the FABP family. For L-FABP, exon 1 encodes amino acids (aa) 1-22, exon 2 (aa 23-79), exon 3 (aa 80-112), and exon 4 (aa 113-126)] [23]. The primary structure of L-FABP in several mammalian species (rat, mouse, human, bovine) displays 79-90% amino acid identity (Fig. 3A), indicating a highly conserved function across several species.

2.2. Transcriptional regulation: peroxisome proliferator activated response element (PPRE)

The promoter region of the L-FABP gene contains several response elements involved in fat metabolism (Fig. 3B). The most well understood of these is the peroxisome proliferator activated response element (PPRE) located between nucleotides -68 and -56. This PPRE contains an imperfect direct repeat sequence that binds and is activated by peroxisome proliferator-activated receptor-α (PPAR-α) (rev. in [24,25]. Consistent with this finding, L-FABP is upregulated by peroxisome proliferator-activated receptor-α ligands (rev. in [17]. Expression of L-FABP is regulated by dietary LCFA as well as by its intracellular activated form—i.e. LCFA-CoA. By transporting LCFAs/LCFA-CoAs to the nucleus for interaction with PPARα, L-FABP regulates its own transcription (Fig. 3B) (rev. in [15,17,18,26]. Dietary

LCFAs induce the expression of L-FABP in liver, but their potency is highly dependent on structure, chain-length, and unsaturation—the branched-chain LCFAs being the most potent as shown by transactivation in cultured cells and dietary studies in animals (rev. in [15,24, 26-35]. Peroxisome proliferator drugs also bind to L-FABP and induce expression of L-FABP (rev. in [26,34,36-39].

While these data suggest free LCFAs (and peroxisome proliferator drugs) could regulate expression of L-FABP and LCFA oxidative enzymes through PPARα, earlier radioligand binding studies requiring separation of bound from free LCFAs yielded only very weak binding affinities of PPARα for *LCFAs (Kds in the μM range)—much higher than nucleoplasmic levels of LCFAs which* are in the nM range [17,30,31]. Thus induction of PPARα by dietary LCFAs was attributed to LCFA metabolite(s) such as LCFA-CoAs, leukotrienes, prostaglandins, and others. However, it is now known that radioligand binding assays requiring separation of bound from free fatty acids seriously underestimate affinity of binding proteins for LCFA and LCFA-CoA by 2-3 orders of magnitude (rev. in [6,40]. To resolve this issue, more recent studies took advantage of the L-FABP intrinsic aromatic amino acid properties (fluorescence) and peptide interaction with circularly polarized light (circular dichroism, CD), fluorescent ligands (LCFAs, LCFA-CoAs), and fluorescence resonance energy transfer (FRET) (rev. in [17,18, 30,31,41]. These assays, not requiring separation of bound from free LCFAs or LCFA-CoAs, established that PPARα exhibits high affinity (low nM Kds) for free LCFAs and LCFA-CoAs in a ligand-dependent manner: (i) branched-chain LCFAs are high affinity (nM Kds) PPARα ligands; (ii) unsaturated LCFAs are also high affinity (nM Kds) PPARα ligands; (iii) both saturated and unsaturated LCFA-CoAs are high affinity (nM Kds) PPARα ligands; and (iv) saturated, straight-chain LCFAs are poor PPARα ligands [17,30,31,41,42]. LCFA and LCFA-CoAs that bind with high affinity alter PPARα structure, DNA binding, coactivator recruitment, and coactivation—with the branched-chain LCFA being the most potent [17,30,31,42]. In addition, nucleoplasmic levels of unesterified LCFAs and LCFA-CoAs are in the same range as PPARα levels in the nucleus of living cells (rev. in [17,43-46]. Thus, many free LCFAs, as well as their CoA thioesters, are physiologically important endogenous ligands of PPARα which in turn induces formation of L-FABP itself in a positive feed-back loop (rev. in [17, 47-50]. The potential roles of LCFAs and LCFA-CoAs as functional ligands of other PPAR subtypes (e.g. PPARγ and PPARδ) are further addressed elsewhere [15,51].

Taken together with the fact that L-FABP transfers LCFAs into the nucleus [43-45,52], these findings suggest a model wherein L-FABP functions in nuclear receptor regulation by binding LCFAs to alter L-FABP conformation, trafficks into the nucleus to bind with PPARα, and transfers bound LCFA ligands to PPARα (Fig. 3) (rev. in [17]. Similar models have been proposed for other FABPs (A-FABP, E-FABP, and others) interacting with other PPAR subtypes (PPARγ, PPARδ) [15] and for other members of the fatty acid binding protein family (cellular retinoic acid binding protein-2) and retinoic acid-mediated regulation of the retinoid X receptor (RXR) in the nucleus [53,54].

2.3. Transcriptional regulation via other response elements involved in fat metabolism

Although the L-FABP gene promoter region also contains two putative sterol response elements SRE1 and SRE2 at -801 to -790 and -245 to -233 respectively [55], L-FABP and PPAR-α expression are only slightly affected by cholesterol-rich diet [56]. In addition, the L-FABP promoter has sites for the following transactivators known to control a number of cellular processes involved in differentiation, proliferation, and apotosis: CCAAT/enhancer binding protein (C/EBP) at -318 to -305 and -177 to -163 and activator protein-1 (AP-1) at -338 to -330 and -236 to -226 (Fig. 3B). The fact that hepatic levels of L-FABP are sex-specific (higher in male than female mice; higher in female than male rats) (rev. in [24,37,57-59], increase during pregnancy and lactation [60], are regulated by growth hormone [61,62], and decrease with age

[62] suggests the existence of additional response elements that influence the sex, age, and obesity phenotype of mice—especially in response to potent PPARα agonists such as phytanic acid and clofibrate (rev. in [24,37,57-59].

2.4. Isoforms and posttranslational regulation

An Asn^{105}/Asp^{105} substitution in bovine liver L-FABP (rev. in [63,64] implies the existence of different isoforms of L-FABP, but the possibility of post-translational modification (such as deamidation) must also be considered. More recent reports have not found such isoforms in rat liver L-FABP. For example, isoelectric focusing of native L-FABP isolated from rat liver resolves several different bands that did not differ in amino acid composition but differed in the amount of bound LCFAs (rev. in [63]. Biochemical fractionation and mass spectrometry of two native rat liver L-FABP subfractions also shows differences in bound LCFA content, but not amino acid structure [65]. Interestingly, the delipidated forms of these subfractions differed markedly in structure/folding (fluorescence, CD), ligand specificity, and ability to stimulate LCFA-CoA transacylation by microsomal glycerol-3-phosphate acyltransferase [63-67]. Since recombinant L-FABP is not resolvable into two fractions differing in structure and function, these data suggest the existence of an as yet unresolved mechanism that may regulate the proportion of conforms (differ in structure/folding) for the native L-FABP in liver *in vivo*.

In contrast to rat liver native L-FABP, post-translational modification (S-thiolation, N-acetylation) of L-FABP has been reported for several other species—suggesting the formation of L-FABP conformers differing in structure/folding may be species dependent (rev. in [63, 65]. The functional significance of L-FABP putative isoforms or post-translational modifications is not well understood. S-thioloation of rat liver L-FABP selectively decreases affinity for unsaturated, but not saturated, LCFAs [68].

3. L-FABP protein

3.1. Surface domains

While L-FABP is largely regarded as a soluble protein that binds LCFAs within its capacious binding cavity for intracellular transfer through the cytoplasm, growing evidence indicates that L-FABP directly binds to membranes and to proteins that utilize L-FABP bound ligands. For example, L-FABP has an α–helical surface domain that interacts with anionic-phospholipid rich membranes to facilitate ligand transfer *in vitro* [69]. L-FABP also directly binds to the surface-exposed ligand binding domain of carnitine palmitoyl transferase 1—the key rate limiting enzyme in mitochondrial β-oxidation of LCFAs [70]. In addition, L-FABP physically interacts with PPARα [71,72] and functionally interacts with both PPARα and PPARγ--key nuclear receptors involved in LCFA metabolism [26,71].

3.2. Interior core ligand binding domain

The amino acid sequence of L-FABP, established over 20 yrs ago, predicted a protein with high content of β–sheet (rev. in [10,13,73], a prediction that was subsequently confirmed by CD and fourier transform infrared spectroscopy of native rat liver L-FABP conformers [65, 66] and recombinant L-FABPs [66,71,74,75]. Low resolution tertiary structure by differential polarized phase fluorometry and time-resolved fluorescence showed that recombinant L-FABP was ellipsoidal in shape [66,75-77]. While detailed high resolution crystal (x-ray) and solution (NMR) structures of native L-FABP have not yet been reported, recombinant L-FABP reveals a barrel-like ligand binding domain comprised of β-sheets with a cavity sufficiently large to accommodate at least two LCFAs—one oriented with a carboxyl interacting with arg and two ser residues in the interior of the binding pocket, and the other oppositely oriented with the carboxyl at the opening of the binding pocket and exposed to the aqueous environment [78,

79]. Second-derivative absorption spectra, tyrosine emission spectra, acrylamide quenching, CD, and differential polarized phase fluorometry all indicated that LCFA binding elicits subtle alterations in conformation and/or tertiary structure of L-FABP in solution [66,75,77]. Although the x-ray crystal structure of recombinant holo-L-FABP is known, difficulty in crystallizing the apo-L-FABP has precluded direct comparison to determine if LCFA binding alters conformation/structure by x-ray crystallography [78]. However, this issue was recently addressed by the NMR solution structures of apo-L-FABP and holo-L-FABP (containing bound LCFA) [79]. While LCFA binding did not alter the overall types and locations of secondary structural elements in L-FABP as determined from chemical shift indices, the LCFA entry portal region of L-FABP exhibits considerable conformational variability and an unusual "open cap" orientation with respect to the β-barrel [79]. The conformational flexibility of this region suggests that LCFA binding may occur through adjustments in the helix-turn-helix motif to open or close the top of the β-barrel [79].

Finally, it is important to note that the volume of the L-FABP ligand binding pocket is the largest of any of the intracellular fatty acid binding protein family, 440 $Å^3$ [78]. Consequently, L-FABP is rather promiscuous in not only being able to accommodate LCFAs and LCFA-CoAs, but also other lipids that also impact development of obesity.

3.3. L-FABP binds up to two LCFAs or LCFA-CoAs—key lipidic ligands in fatty acid metabolism and obesity

L-FABP is unique among the FABP family in its ability to bind more than one LCFA/LCFA-CoA and in the size of ligand that can accommodate. L-FABP contains a high affinity site (K_ds 4-60 nM) and a lower affinity binding site (K_ds 0.3-12 μM) [34,66,80-82]. In the higher affinity site, the LCFA is oriented with carboxyl buried deep in the interior, interacting with Arg^{122} [78,79]. In the weaker affinity site, an LCFA is oriented with carboxyl facing the surface of the protein binding pocket opening [78,79]. Occupancy of the lower affinity LCFA binding site may depend upon prior binding of an LCFA to the higher affinity site. L-FABP exhibits modestly higher affinity (2-4 fold) for unsaturated (kinked-chain) LCFAs as compared to their saturated (straight-chain) counterparts [34,66,80,81,83].

L-FABP is also unique among this protein family in that it can bind two LCFA-CoAs instead of two LCFAs [66]. While the two LCFA-CoA binding sites differ about 12-18 fold in K_ds, L-FABP bound both with relatively high affinity, as indicated by K_ds of 8-10 nM and 97-180 nM for the high and low affinity sites, respectively [66]. Since the CoA moiety of LCFA-CoAs is too large and polar to be accommodated within the L-FABP binding cavity volume, modeling studies indicate that most likely both acyl chains are buried and both CoA moieties are oriented at the surface opening of the binding pocket (rev. in [84]. This orientation is ideal for presenting the thioester linkage of the L-FABP bound LCFA-CoA to enzymes that transacylate LCFA-CoA to phospholipids and cholesteryl esters.

3.4. L-FABP binds intermediates in LCFA anabolism and catabolism

L-FABP binds a variety of intermediates in LCFA oxidation (e.g. fatty acyl-carnitines) [64, 85-87] and glyceride synthesis (e.g. 1-oleoylglycerol) or up to 2 lysophospholipids (e.g. lysophosphatidylcholine), with K_ds in of 0.08-1 μM, depending upon the specific lysophospholipid species and assay [85,86,88].

3.5. L-FABP binds a variety of steroid-like ligands in fatty acid metabolism and storage

Surprisingly, L-FABP binds several types of ligands with a steroid-like nucleus (cholesterol, bile acids)—apparently at a single binding site. Because of the very poor solubility of cholesterol in water (critical micelle concentration near 30 nM), initial studies using high concentrations of cholesterol in displacement assays or in the Lipidex 1000 assay (requires

separation of bound from free) did not detect cholesterol binding to L-FABP [80,89]. In contrast, many laboratories using more sensitive fluorescence and photo-crosslinking techniques have now demonstrated that L-FABP clearly binds cholesterol and fluorescent cholesterol analogues (NBD-cholesterol, DHE, photoactivatable FCBP) [90-98]. As is the case of LCFA/LCFA-CoA, cholesterol binding also alters L-FABP conformation [98]. Due the higher solubility of bile acids (oxidation products of cholesterol), a variety of methods could readily demonstrate that bile acids, important in both LCFA and cholesterol metabolism, are bound by L-FABP at a single site [88,89,99]. However, bile acids were more weakly bound by L-FABP (K_ds 4-50 μM) [88].

3.6. L-FABP binds ligands involved in lipid signaling, growth regulation, and fat storage

The L-FABP binding site (440 $Å^3$) is sufficiently large to accommodate a variety of lipidic molecules with roles in signaling [78]. L-FABP has high affinity for LCFA-CoAs [66]—ligands that regulate vesicular budding from the Golgi [100,101]. The physiological significance of L-FABP in vesicle budding is demonstrated by studies with L-FABP gene-ablated mice in which the lack of L-FABP reduced budding of pre-chylomicron transport vesicles from intestinal ER [102]. L-FABP exhibits high affinity for products of phospholipid hydrolysis (1-oleoylglycerol, lysophospholipids) released by lipases during signaling (K_ds ~0.08-1 μM) [85,86,88], and it also binds a variety of other fatty acid metabolites (prostaglandins, lipoxygenase products, retinoids) that are involved in lipid signaling [64,87, 103]. L-FABP reduces 15-lipoxygenase-induced oxygenation of linoleic acid and arachidonic acid [104]. L-FABP also binds glycerides such as 1-oleoylglycerol and up to 2 lysophospholipids (e.g. lysophosphatidylcholine) with K_ds ~0.08-1 μM, depending upon the specific lysophospholipid species and assay used [85,86,88].

L-FABP also binds a variety of other unrelated ligands including heme and its degradation product bilirubin [4,105,106], as well as warfarin [107], carcinogens [108-110], and the dietary trace metal selenium [111]. While the physiological significance of these findings is not completely clear, studies in transfected cells in culture indicate that L-FABP binding of carcinogens and certain LCFAs can regulate mitogenesis and carcinogenesis [112-116].

In summary, the finding that L-FABP binds a variety of lipidic molecules involved in signaling suggests additional routes whereby L-FABP may impact lipid metabolism, cell grown, and obesity.

4. L-FABP directly and indirectly regulates enzymes involved in LCFA metabolism

4.1. L-FABPdirectly facilitates LCFA desorption from membranes in vitro

Although cellular membranes exhibit high affinity for LCFAs and LCFA-CoAs, high levels of these ligands inhibit the activities of many membrane bound enzymes/signaling proteins involved in lipid metabolism (rev. in [4]. It has been proposed that, by removing these ligands from membranes and solubilizing them in the cytosol, L-FABP can prevent the potent inhibitory/detergent effects of LCFA/LCFA-CoA and facilitate their utilization inside the cell. Consistent with this possibility, L-FABP markedly enhanced desorption of LCFAs and LCFA-CoAs from membranes (rev. in [6,63,67,117,118]. Based on the high affinity of L-FABP for these ligands and the known concentration of L-FABP in hepatic cytosol, the vast majority of unesterified LCFAs and LCFA-CoAs are likely to be bound by cytosolic proteins such as L-FABP.

4.2. Role of L-FABP in activation of LCFAs to LCFA-CoAs

Once taken up into the cell or membrane, LCFAs are rapidly (<1 min) converted to LCFA-CoAs by ubiquitous fatty acyl CoA synthases, membrane-associated enzymes present in plasma membrane, ER, mitochondria, peroxisomes, and nuclear membranes [119-121]. Earlier *in vitro* studies with L-FABP isolated from liver suggest that L-FABP directly interacts with fatty acyl CoA synthases to donate bound LCFAs for conversion to LCFA-CoAs (rev. in [6, 14]. However, later studies with recombinant L-FABP showed that acyl CoA binding protein (ACBP), a likely contaminant of early native L-FABP preparations, stimulated long chain fatty acyl CoA synthases while highly purified L-FABP does not (rev. in [6]. Nevertheless, L-FABP may still play a role in this stimulation via increasing LCFA-CoAs by inhibiting LCFA-CoA hydrolysis. The thioester linkage in unbound LCFA-CoAs is subject to auto-hydrolysis in aqueous environments as well as to degradation by cellular hydrolases [118,122,123]. L-FABP-bound LCFA-CoAs are protected from microsomal hydrolysis (rev. in [6,63,67,117, 118].

4.3. L-FABP facilitates intermembrane transfer of LCFAs/LCFA-CoAs and cholesterol

Consistent with its ability to interact with membranes (see 3.1) L-FABP stimulates the transfer of bound LCFAs and LCFA-CoAs from model membranes and from the plasma membrane to other membranes (ER) *in vitro* (rev. in [4,6,124]. L-FABP also stimulates transfer of bound cholesterol from the plasma membrane to ER *in vitro* (rev. in [125-127].

4.4. L-FABP facilitates mcirosomal LCFA esterification

L-FABP stimulates two key LCFA-CoA transacylation reactions: 1) by increasing activity of several LCFA anabolic enzymes involved in the synthesis of triglycerides and cholesteryl esters —lipids that accumulate to pathological levels in obesity; and ii) enhancing the rate limiting step in phosphatidic acid synthesis mediated by glycerol-3-phosphate-acyltransferase (Fig. 2, GPAT) (rev. in [63,67,117,118,128]. Phosphatidic acid is the precursor of a variety of membrane phospholipids as well as triacyglycerols either stored in lipid droplets in multiple cells expressing L-FABP or secreted by the liver in very low density lipoproteins (Fig. 2). L-FABP also increases the synthesis of cholesteryl esters mediated by cholesterol acyl CoA acyltransferase (ACAT) and targeted for storage in lipid droplets in multiple cells expressing L-FABP or secreted by the liver in very low density lipoproteins (Fig. 2) [129].

4.5. L-FABP stimulates mitochondrial and peroxisomal oxidation of LCFAs

Purified L-FABP removes substrate inhibition of carnitine palmitoyltransferase-1 (CPT-1, the rate limiting step in mitochondrial LCFA oxidation) by palmitoyl CoA [130,131]. L-FABP functions as a LCFA donor protein for mitochondrial LCFA oxidation [132]. To determine if L-FABP directly binds CPT-1 or simply shuttles bound LCFA-CoAs to CPT-1 located in the outer mitochondrial membrane, a fluorescence resonance energy transfer (FRET) study was performed. FRET is a sensitive technique effective in measuring intermolecular distances between proteins 1-100 Å apart [71,94]. L-FABP directly interacted with CPT-1, exhibited saturable binding for CPT-1 with 1:1 stoichiometry, and bound CPT-1 with high affinity (2.1 ± 0.3nM K_d) [70]. The intermolecular distance between L-FABP and CPT-1 was calculated as 53.1 ± 0.9Å [70]. Thus, data were consistent with a close molecular interaction. To determine if L-FABP binding to CPT-1 altered protein conformation, the two proteins were examined separately (Fig. 4A) and together (Fig. 4B) by CD. The proportion of different secondary structures measured experimentally in the complex differed significantly from the theoretical prediction if the proteins did not interact (Fig. 4C)—indicating that L-FABP directly interacted with CPT-1 These studies thus suggest a model wherein L-FABP binds with CPT-1 to undergo a conformational change that transfers bound LCFA-CoA to CPT-1. Consistent with a potential role for L-FABP in LCFA transfer to mitochondria, both subcellular fractionation and

immunogold electron microscopy (EM) detect small amounts of L-FABP associated with mitochondria [128]. L-FABP also serves as a LCFA donor protein for peroxisomal LCFA oxidation [133]. Consistent with a functional role for L-FABP in LCFA transfer to peroxisomes, small amounts of L-FABP have been detected associated with purified peroxisomes and by immunogold EM [134].

4.6. L-FABP stimulates LCFA transfer into purified nuclei

LCFAs alone poorly enter purified nuclei, while L-FABP facilitates the entry of LCFAs by binding and cotransporting the bound LCFA into the nucleus [52]. Several immunofluorescence and immunogold EM studies detected L-FABP in the nucleoplasm of hepatocytes and in cultured cells overexpressing L-FABP [43,44,72,128].

5. Effect of L-FABP overexpression and ablation on LCFA uptake and transport in cultured cells

5.1. L-FABP overexpression enhances while L-FABP ablation inhibits LCFA and cholesterol uptake in living cells

L-FABP enhances uptake of LCFAs and cholesterol from the medium of transfected L-cells overexpressing L-FABP, hepatoma cells expressing increasing amounts of L-FABP, and primary cultured hepatocytes [84,135-141]. Conversely, L-FABP gene ablation decreases uptake of LCFAs in cultured primary hepatocytes and in freshly isolated hepatocytes [142, 143]. Whether L-FABP facilitates LCFA uptake by enhancing desorption of LCFAs from the plasma membrane, from plasma membrane LCFA translocases, or simply acts as an acceptor/cytoplasmic sink for these LCFAs is not clear.

5.2. L-FABP overexpression enhances while L-FABP ablation inhibits intracellular LCFA transport/diffusion in cell culture

L-FABP exhibits high affinity for a fluorescent LCFA analogue, NBD-stearic acid—a poorly metabolizable LCFA [43,44,144,145]. Advantage was taken of this property to determine if L-FABP enhances LCFA cytoplasmic transport/diffusion measured in real-time in living cells by fluorescence recovery after photobleaching (FRAP) imaging. In hepatocytes differing in L-FABP expression (male versus female) and in cultured hepatoma cells, L-FABP significantly enhanced LCFA cytoplasmic diffusion [146,147]. Similarly, in transfected L-cell fibroblasts overexpressing L-FABP, L-FABP increased the cytoplasmic diffusion of this fluorescent LCFA [6,148]. Cultured primary hepatocytes from L-FABP gene-ablated mice had a significantly decreased rate of diffusion of NBD-stearic acid through the cytoplasm [142]. Since L-FABP (mw 14,286 Da) is much larger than a typical LCFA (300 Da), binding of the LCFA to the L-FABP should slow down, rather than speed up the diffusion of the LCFA. While not intuitively obvious, one explanation for this conundrum can be based on the fact that: (i) the cytoplasmic concentration of unesterified LCFA is very low, (ii) the viscosity of cytoplasm is at least an order of magnitude higher than that of aqueous buffers, and (iii) the path length of LCFA through the cytoplasm is much more ‘tortuous’ due to the presence not only of many other proteins but even more so of a high concentration of membranous organelles and microfilaments that restrict motion [149-151]. The finding that cytosol from hepatocytes of L-FABP null mice has markedly diminished capacity for binding LCFA and LCFA-CoA suggests that L-FABP may enhance cytoplasmic diffusion of LCFA at least in part by facilitating desorption of membrane bound LCFA to increase cytoplasmic concentration of unesterified LCFA [152,153].

6. Effect of L-FABP overexpression and ablation on LCFA metabolic targeting and nuclear regulatory targeting in cultured cells

6.1. Targeting LCFA to ER for esterification

Since intracellular accumulation of unesterified LCFAs and LCFA-CoAs (both potent detergents) and cholesterol (crystallizes even at low concentration) is deleterious, a major function of FABPs is thought to be the removal of these ligands by enhancing their anabolic and catabolic metabolism. A role for L-FABP in anabolic removal of LCFAs is supported by studies with transfected cells overexpressing L-FABP which show that L-FABP facilitates transport of LCFA and cholesterol from the plasma membrane to ER for esterification by ACAT, increasing cellular cholesteryl ester mass [135,154-156]. L-FABP overexpression markedly increased incorporation of LCFA into triglycerides nearly 3-fold, albeit without increasing triglyceride mass—suggesting other mechanisms (e.g. lipase/hydrolase activity) contribute to maintain mass constant [137,155,157]. In transfected L-cells overexpressing L-FABP, incorporation of LCFA into phospholipids increased, but in addition phospholipid mass increased 1.7-fold [155,157,158]. Cultured primary hepatocytes and freshly isolated hepatocytes from L-FABP null mice had reduced incorporation of LCFAs (palmitic acid, oleic acid, phytanic acid) into total lipids—especially the neutral lipids (triglycerides, diglycerides, and/orcholesteryl esters) [142,143]. Total mass of triglycerides was decreased 40-60% while phospholipid and cholesteryl esters were increased or unaltered/spared in these cells [142, 143]. Thus, L-FABP plays an important role in LCFA anabolic metabolism to facilitate incorporation into triacylglycerols important for LCFA storage or secretion (VLDL in liver, chylomicrons in intestine). In triglyceride secretion, increased expression of L-FABP in transfected hepatoma cells increased secretion of apoB-100 and decreased cellular biosynthesis and secretion, and increased PPARα mRNA levels—indicating that L-FABP may interact with PPARα to amplify the effects of endogenous PPARα agonists on the assembly of VLDL [159]. Taken together, these data suggest a potential role for L-FABP in LCFA esterification at the ER

6.2. Targeting LCFA for oxidation (mitochondria, peroxisomes) in cultured cells

L-FABP facilitates removal of LCFAs by catabolic metabolism, stimulating oxidation of straight-chain LCFAs such as palmitic acid (primarily mitochondrial β-oxidation) in cultured fibroblasts overexpressing L-FABP [139]. Likewise, L-FABP overexpression enhanced oxidation of branched-chain LCFAs such as phytanic acid (primarily peroxisomal α- and β-oxidation) in these fibroblasts [139]. Conversely, L-FABP gene ablation inhibits oxidation of both palmitic acid (mitochondrial) and even more so phytanic acid (peroxisomal) in cultured primary hepatocytes and freshly isolated hepatocytes from L-FABP null mice [139,160]. Thus, L-FABP plays an important role in directing LCFAs toward oxidation pathways in cell culture.

6.3. Targeting LCFA to nuclei for interaction with PPARα

By real-time confocal and multiphoton imaging of several non- or poorly-metabolizable fluorescent LCFAs, overexpression of L-FABP increased the distribution of synthetic (NBD-stearic acid, BODIPY-C12, BODIPY-C16) and naturally-occurring (parinaric acid) fluorescent LCFAs to the nucleoplasm in cultured L-cells overexpressing L-FABP [43,44]. Conversely, lack of L-FABP decreased the distribution of LCFAs into the nucleoplasm of cultured primary hepatocytes from L-FABP null mice [45]. As shown by immunocoprecipitation, CD, and FRET *in vitro*, L-FABP binds PPARα with high affinity [26,71]. L-FABP also directly interacts with PPARα in fixed cultured cells and cultured primary hepatocytes as shown by immunofluroescence and immunogold EM [44,71]. Additionally, transactivation and gene ablation studies show L-FABP overexpression enhances while L-FABP gene ablation inhibits PPARα transcription of genes coding for LCFA oxidative

enzymes–thereby showing the functional and physiological significance of L-FABP/PPARα interaction [26,71].

7. In vivo function of L-FABP in long chain fatty acid uptake, oxidation, and esterification

7.1. Generation of L-FABP null mice

The first reported L-FABP null (-/-) C57Bl/6 mice mouse line was generated using a neo construct, ablating most of the 5′ non-coding (promoter) region and all 4 exons of the small L-FABP gene, as well as part of the 3′ noncoding region [152]. This design precluded the possibility of expressing L-FABP fragments (small peptides, truncated protein) [152]. The gene deletion was verified by long PCR and confirmed by nested PCR. On the protein level, Western blotting confirmed there were no detectable L-FABP protein or L-FABP-derived peptides in these L-FABP null mice [152]. The N2 generation L-FABP null (-/-) C57Bl/6 mice were further backcrossed to C57BL/6N mice from Charles River Laboratories (Wilmington, MA) obtained through the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD) in order to obtain at least 99.9% homogeneity (N10) backcross generation. Herein, these mice are referred to as the “L-FABP null” mouse line.

A subsequently reported different L-FABP null mouse line was generated by use of a neo and GFP knock-in that left intact the 5′ noncoding (promoter) region, all of exons 3 and 4, and the 3′ noncoding region [143]. Thus, while Northern blotting did not reveal the presence of the L-FABP transcript, based on the construct design and the results presented, the existence of smaller peptides(s) encoded by exons 3-4 (aa80-126—part of a ligand binding domain) could not be ruled out, especially since the full Northern blot was not shown [124,143,161]. While western blotting revealed the absence of the 14 kDa L-FABP, the blot represented only the 14 kDa band and did not indicate whether or not any smaller fragments were present. The N2 generation of these L-FABP null (-/-) C57Bl/6 mice were further backcrossed to C57BL/6J mice from Jackson Laboratories (Bar Harbor, ME)—a substrain much more susceptible high fat diet induced obesity than the C57BL/6N substrain to which the original “L-FABP null” mouse line (see above) was backcrossed as shown in JAX NOTES, Issue 511, Fall 2008 (http://jaxmice.jax.org/jaxnotes/511/511n.html) from the Jackson Laboratory (Bar Harbor, ME). GFP was prominently overexpressed in these mice are herein referred to as the “L-FABP null/GFP overexpressor” mouse line.

7.2. L-FABP gene ablation does not affect food consumption or intestinal lipid absorption

As compared to wild-type controls, neither L-FABP null mouse line exhibited any significant alterations in consumption of commercial or defined control diets or in energy expenditure [56,59,97,98,143,152,162,163]. While the finding that intestinal enterocytes from the L-FABP null/GFP overexpressor line exhibit reduced budding of prechylomicron transport vesicles from the ER—the rate limiting step in transit of absorbed dietary fat across the enterocyte [21,102], studies with L-FABP null/GFP overexpressor mice fed control chow, high fat diet, or cholestatic diet did not detect any effect on intestinal lipid absorption [163-165].

7.3. L-FABP enhances hepatic uptake and oxidation of long chain fatty acids

Based on the above cultured transfected cells, and cultured primary hepatocytes studies performed *in vitro*, it was expected that L-FABP should enhance LCFA uptake and oxidation in liver. Indeed, hepatic uptake of ^{14}C-oleic acid from serum was reduced not only in fed, but even more so fasted L-FABP null mice [152]. A role for L-FABP in hepatic LCFA oxidation was initially based direct correlation of rat liver L-FABP content with LCFA oxidative capacity [5,166] in that incubation of ^{14}C-palmitic acid with liver homogenates from L-FABP null mice

decreased total oxidation (measured a ^{14}C in CO_2 + acid soluble products) and β-hydroxybutyrate production by 34% and 36%, respectively [160]. L-FABP gene ablation also inhibits hepatic oxidation of LCFAs, but not medium chain fatty acids such as octanoic acid (not bound by L-FABP, freely permeable into cells and mitochondria) *in vivo* as shown by direct correlation of L-FABP expression (Fig. 5B) with serum β-hydroxybutyrate levels (Fig. 5A) in a variety of genetically engineered fasted mice [143,160,167]. In contrast, serum β-hydroxybutyrate levels (Fig. 5A) were inversely correlated with SCP-2 expression (Fig. 5C) and did not correlate with expession of SCP-x (Fig. 5D)—proteins more involved in peroxisomal than mitochondrial LCFA oxidation. Serum β-hydroxybutyrate levels in L-FABP null mice were decreased even further after prolonged fasting (48h), feeding a ketogenic diet, or fasting after a ketogenic diet [160].

7.4. L-FABP enhances hepatic esterification of long chain fatty acids

L-FABP gene ablation in the "L-FABP null" mouse line (see 7.1) decreased hepatic triglyceride accumulation in fasted male, but not female, mice fed standard commercial or defined control diets [56,59,97,143,152,162], while increasing hepatic cholesteryl ester accumulation (and in most cases unesterified cholesterol) in fasted female, but not male mice fed standard commercial or defined control diet at all ages examined [59,97,152,152]. While serum triglyceride levels were unaltered in aged L-FABP null mice, serum triglyceride and cholesteryl ester were decreased in younger males fed a defined control chow, while those in females were increased [142].

Taken together, these studies indicate that L-FABP functions in hepatic LCFA uptake, oxidation, esterification, and secretion.

8. Age- and sex- dependent weight gain and obesity in L-FABP null mice

8.1. Potential confounding effects of phytol and phytoestrogen in standard, low fat commercial rodent chow on weight gain and obesity

Based on the decreased LCFA uptake, intrahepatocyte transport, oxidation, and esterification reported in L-FABP null mice fed normal commercial rodent chow, it was predicted that loss of L-FABP would redirect LCFAs toward utilization by tissues other than liver (muscle, adipose). Whether the modestly decreased intestinal lipid absorption in L-FABP null/GFP overexpressor mice (observed only with control chow, but not high fat chow fed null mice) counteracts this expectation is unclear, because a confounding factor is the normal variation in amount of dietary phytol [58,59,168-170] and phytoestrogen [171,172] in standard commercial rodent chows and, even more so in commercial high fat diets (i.e., breeder chow), which also vary in the source of dietary fat (e.g., butter and lard are much higher in phytol content as compared to vegetable oils) [173]. Metabolites of phytol (e.g. phytanic acid, pristanic acid) are potent activators of PPARα (and other PPARs) which in turn regulate transcription of L-FABP and LCFA oxidative enzymes (rev. in [15,17]. Phytol metabolism is sex-dependent, and accumulation of high levels of phytol metabolites is toxic in animals [58,59,139,142, 168-170], including humans [173-175]. Phytoestrogens exert estrogenic effects in mice [171, 172]. To understand these confounding issues, weight gain and whole body phenotype (fat tissue mass, lean tissue mass) were determined in L-FABP null mice fed two types of control fat (5%) rodent chows—standard commercial versus defined (phytol-free, phytoestrogen-free, low-fat) rodent chow.

8.2. Weight gain and obesity in L-FABP gene-ablated mice fed phytol-free, phytoestrogen-free, low fat rodent chow

Early back-cross generation L-FABP null fed different commercial control rodent chows *ad libitum* did not exhibit any significant difference in weight gain or final body weight as

compared to age- and sex- matched wild-type counterparts [143,152]. However, after 6 back-cross generations (N6), important age- and sex-dependent differences emerged (Table 1). After 6 mo of age, female and male L-FABP null mice exhibited increased body weight and weight gain as compared to age- and sex- matched wild-type counterparts [176,177]. This phenotype emerged at even earlier age when the L-FABP null mice were pair-fed a defined (phytol-free, phytoestrogen-free, low fat) control chow diet (Table 1). When 2 mo-old male and female L-FABP null of this null line (≥N6 back-cross generation) were fed for a short time (18 days) on a defined (phytol-free, phytoestrogen-free, low-fat) control chow diet, weight gain and final body weight did not differ from their age- and sex-matched wild-type counterparts [59,167]. However, after ≥42 days of pair-feeding this defined diet, females (but not males) exhibited increased weight gain and increased final body weight as compared to age- and sex-matched littermates [56,162,167].

To determine if the age- and sex-dependent increase of weight gain in standard commercial or defined diets was associated with obesity, whole body mass as fat tissue (FTM) and lean tissue (LTM) as well as their relative distributions (i.e. %FTM, %LTM) in L-FABP null mice (≥N6 back-cross generation) were determined by dual emission x-ray absorptiometry (DEXA). L-FABP null mice exhibited significant age- and sex-dependent changes in whole body phenotype FTM and lean tissue mass LTM (Table 2). After 6 mo of age, females fed a standard commercial rodent chow *ad libitum* exhibited increases in FTM (but not LTM), while the LTM was unchanged and the % LTM decreased as compared to age- and sex- matched counterparts [176,177]. A basically similar, but milder phenotype was observed in the males (Table 2) [176,177]. The obese phenotype emerged at even earlier age when females and males were pair-fed a defined control phytol-free, phytoestrogen-free, low-fat chow diet (Table 2). When 2 mo old wild-type and L-FABP null females (≥N6 back-cross generation) were fed for a short time (18 days) on a this defined chow, neither the mass nor % of FTM and LTM were altered as compared to age- and sex-matched littermates [59,167]. However, after ≥42 days of pair-feeding of this same diet, the null females had increased their FTM and % FTM (less so in LTM and %LTM) as compared to their age- and sex-matched counterparts [56,162,167]. Under the same conditions, males also had significant but lower increases in FTM and % FTM, while LTM and % LTM were unchanged or decreased as compared to age- and sex-matched counterparts [56,162,167]. Whole body FTM and LTM in L-FABP null/GFP overexpressor mice are not yet available for any type of feeding study.

Thus L-FABP null mice exhibit an age- and sex-dependent weight gain and obesity when fed either of two lower fat (5%) diets—commercial standard chow *ad libitum* or a pair-fed, phytol-free, phytoestrogen-free, low fat defined diet. Null females appeared obese, significantly gaining the most weight as compared to wild-type females, primarily as increased mass and proportion of fat.

9. Whole body phenotype of L-FABP gene-ablated mice: high-fat diet induced weight gain and obesity

9.1. Response of L-FABP null mice to a high-fat diet

Thus L-FABP enhances hepatic uptake of LCFAs, intrahepatic LCFA transport, intrahepatic LCFA targeting for oxidation (peroxisomes, mitochondria) and esterification (ER), and LCFA targeting to the nucleus for regulation of nuclear receptors important in transcription of genes encoding proteins involved in LCFA and glucose metabolism (PPAR-α, HNF-4α, LXR, THR). Since ablation of L-FABP inhibits LCFA uptake, reduces LCFA intracellular transport/diffusion, inhibits LCFA esterification, inhibits LCFA oxidation, and inhibits LCFA targeting to the nucleus, L-FABP null mice fed a high fat diet should redirect LCFAs for utilization in other tissues. However, none of the studies reported to date indicate any increased activity/

LCFA use by muscle. Consequently, is would be expected that the excess LCFAs are redirected to adipose tissue for storage and result in an obese phenotype—especially in response to high-fat diet.

To prevent any complication due to mouse palatability preference for fat, L-FABP null mice and age and sex-matched wild-type counterparts were fed a defined control low fat diet or a high fat (24% fat) diet based upon the consumption of control mice the previous day for 12 wks (i.e., pair-feeding) [167]. Body weight as well as food consumption were measured every 2 days for the entire study [167]. The amount of high fat diet consumed (whether expressed as total g, total kcal, g/day, or kcal/day) over the entire study did not differ between L-FABP null and wild-type mice because of this pair-feeding method [167]. As expected, high-fat fed L-FABP null males and females both had reduced serum β-hydroxybutyrate levels as compared to high fat fed controls—consistent with reduced ability of L-FABP null hepatocytes to take up and/or oxidize LCFA under a high LCFA load [167]. L-FABP gene ablation did not protect either males or females from high-fat induced weight gain and obesity [167]. On the contrary, female (and less so male) L-FABP null mice increased in body weight as compared to controls fed the same high fat diet (Table 3). Dual x-ray emission absorptiometry showed that the modestly higher weight in L-FABP null males was associated with increases in both fat tissue mass (FTM) and lean tissue mass (LTM), but when expressed on a % basis, the % FTM increased more than the % LTM (Table 3) [167]. In contrast, the much greater increase in weight of L-FABP null females was also associated with increased FTM as well as LTM, but when expressed on a % basis, the % FTM increased more than % LTM (Table 3) [167]. Thus in pair-fed mice, the ablation of the L-FABP gene did not protect the mice from high-fat diet induced weight gain and relative obesity as compared to wild-type controls.

9.2. Response of L-FABP null mice to a lithogenic diet

By mapping susceptibility loci in response to diet-induced gallstone formation, genetic studies have mapped a locus with L-FABP as a positional candidate from a quantitative trait locus near D6Mit123 on chromosome 6 [165,178,179]. Consistent with this possibility, significant upregulation of L-FABP (5-6 fold) in SCP-x/SCP-2 gene-ablated mice was associated with biliary hypersecretion of cholesterol [180,181]. These findings suggested that L-FABP may play a role in lipid absorption and whole body phenotype in response to a lithogenic diet. Therefore, the effects of a 4-week defined, lithogenic diet (Research Diet D0208081, containing 4.9% fat, 1.25% cholesterol, and 0.5% cholic acid) on 8 week-old male and female L-FABP null mice was examined. Despite similar food consumption (Fig. 6A), and much like results from the cholesterol diet [56,162], lithogenic diet fed wild-type females but not males exhibited increased weight gain (Fig. 6B), primarily as fat tissue mass (Fig. 6C), as compared to control fed wild type females. Control diet fed L-FABP null female mice, but not males, exhibited increased weight gain (Fig. 6B) as compared to their control fed wild-type counterparts. In contrast, lithogenic diet fed L-FABP null females as well as males exhibited decreased weight gain (Fig. 6B), primarily as decreased fat tissue mass in females (Fig. 6C). There was little to no effect observed with the lean tissue mass when comparing each feeding group (Fig. 6D). It is important to note, however, that while less weight gainoccurred in the L-FABP null than wild-type females on the lithogenic diet, there was still a 6% weight gain in the L-FABP null females. Thus, L-FABP gene ablation reduced, but did not protect, the female mice from lithogenic diet induced weight gain.

9.3. Response of “L-FABP null/GFP overexpressor” mice to high fat and lithogenic diets

The “L-FABP null/GFP overexpressor” mouse line shares in common with the L-FABP null mouse line reduced hepatic uptake of fatty acids, reduced hepatic fatty acid oxidation, and showed no difference in weight gain or final body weight in response to feeding different commercial control rodent chows *ad* libitum [143,152]. However, the phenotype of “L-FABP

null/GFP overexpressor" mice differed significantly from that of L-FABP null mouse strain in response to several different types of high fat diet.

"L-FABP null/GFP overexpressor" mice were fed *ad libitum* several high fat diets from different vendors, body weight was measured weekly, food consumption was measured only for a 3 day period toward the end of the study, fat absorption was measured for a short time toward the end of the study, and whole body fat tissue mass and lean tissue mass were not determined. In an earlier study, L-FABP null/GFP overexpressor mice (N5 backcross generation) were fed *ad libitum* a high-saturated fat, high cholesterol Western diet (21% milkfat, 0.15% cholesterol) from Harlan Teklad (Madison, WI) for 10-12 wk [163]. Food consumption and fat absorption did not differ between L-FABP null/GFP overexpressor females and C57BL/6J controls (Jackson Labs, Bar Harbor, ME). Despite the fact that fat oxidation (measured as serum β-hydroxybutyrate) was reduced nearly 5-fold in L-FABP null/GFP overexpressor females fed this high fat diet, L-FABP null/GFP overexpressor null females and males exhibited reduced weight gain as compared to their C57BL/6J controls also fed the same high fat diet *ad libitum* (i.e., not pair-fed) [163,182]. In a subsequent study, L-FABP null/GFP overexpressor females (apparently N5 backcross generation) control C57BL/6 females (Jackson Labs, Bar Harbor, ME) were fed *ad libitum* either a high saturated fat diet (20% coconut oil) or a high polyunsaturated fat diet (20% safflower oil) from MP Biomedicals (Solon, OH) for 10-12 wks [182]. While L-FABP gene ablation did not alter food consumption or fat absorption with either diet, L-FABP null/GFP overexpressor mice fed the high saturated fat diet (but not high polyunsaturated fat diet) *ad libitum* exhibited a reduced weight gain as compared to C57BL/6 controls [182]. In another study, L-FABP null/GFP overexpressor females (N-10 backcross generation) were again fed *ad libitum* either a high saturated fat diet (20% coconut oil) or a high polyunsaturated fat diet (20% safflower oil) from MP Biomedicals (Solon, OH) for 8-24 wks (not pair fed) [164]. While fat absorption was unaltered with either high fat diet, there was a trend towards decreased food consumption in L-FABP null/GFP overexpressor females fed the high saturated fat diet, but not the high polyunsatured fat diet [164]. The L-FABP null/GFP overexpressor females had a reduced weight gain in response to high saturated fat, but not to high polyunsaturated fat [164]. Thus, when fed high fat diets *ad libitum*, L-FABP null/GFP overexpressor females (males were not reported) exhibited a reduced weight gain or relative weight loss rather than an increased weight gain. This reduced weight gain was somewhat surprising in the face of reduced serum β-hydroxybutyrate (indicative of reduced hepatic uptake/oxidation of LCFAs) and no upregulation of LCFA oxidation in skeletal muscle, which together with the unaltered food consumption and unaltered fat absorption, would have suggested redirection of LCFAs to adipose for storage/obesity.

While the mechanistic basis for the variation in high-dietary fat responses exhibited by "L-FABP null/GFP overexpressor" as compared to L-FABP null mouse lines is not completely clear, detailed comparisions reveal multiple potential factors that may contribute (Table 4). Of these, major differences in construct design, overexpression of GFP, backcrossing to a C57BL6J substrain that is more susceptible to high-fat diet induced obesity, and feeding high fat diets *ad libitum* are very likely contributors. The "L-FABP null/GFP overexpressor" mouse studies used the C57BL/6J (more susceptible to high fat diet induced obesity) mouse strain for backcrossing and as controls and fed high fat diets *ad libitum*. In contrast, L-FABP null mice were backcrossed to C57BL/6N (less susceptible to high fat diet induced obesity) mouse strain, performed with age- and sex-matched littermate controls, and fed high fat diets in a pair feeding regimen. *Ad libitum* feeding high fat diet resulted in 20-37% higher ($p<0.01$) higher total food consumption [163,164,182] as compared to pair-feeding a high fat diet [167]. Finally, it is important to note that dietary LCFAs induce transcriptional activity of PPARα, which in turn increases L-FABP transcription and even more LCFA uptake (rev. in [17,18,47,48,51,183]. *Ad libitum* consumption of diets high in fat increases hepatic L-FABP levels (rev. in [4,15, 24,26-35]. Thus, the higher genetic susceptibility of the background C57BL/6J mouse strain,

ad libitum feeding of high fat diet, and induction of L-FABP in the wild-type L-FABP +/+ control (but not the L-FABP null) mice together likely resulted in dramatically higher total fat consumption and absorption by the control wild-type mice (express L-FABP but *not* GFP) than the "L-FABP null/GFP overexpressor" mice—thereby perhaps accounting for the observed 'protection' of "L-FABP null/GFP overexpressor" mice from high fat diet induced obesity.

9.2. Response of "L-FABP null/GFP overexpressor" mice to a lithogenic diet

In similar fashion to the L-FABP null mice, 8-10 wk old male L-FABP null/GFP overexpressor mice were fed *ad libitum* for 2-8 wks on a high fat lithogenic diet (Research Diet 960393 containing 18.8% fat, 1.25% cholesterol, and 0.5% cholic acid). In contrast to results with male wild-type mice fed lithogenic diet in the preceding study, wild-type mice fed high-fat lithogenic diet exhibited weight loss [165]. Similarly, "L-FABP null/GFP overexpressor" males fed high-fat lithogenic diet also exhibited weight loss, but with the final body weight lower and weight loss greater than wild-type [165]. Male "L-FABP null/GFP overexpressor" mice exhibited increased susceptibility to high-fat lithogenic diet induced gallstone formation [165]. There were no significant differences in fat absorption, cholesterol absorption, and food intake between male wild type and L-FABP null/GFP overexpressor mice [165]. However, the effect of high-fat lithogenic diet on female "L-FABP null/GFP overexpressor" mice was not reported. While it was concluded from these data that L-FABP may partially protect male mice from a high-fat lithogenic diet-induced weight loss, this conclusion was complicated by the fact that the wild type control mice (express L-FABP but *not* GFP) fed high-fat lithogenic diet developed 3-fold hepatic upregulation of L-FABP concomitant with a 3-fold decreased intestinal L-FABP expression [165].

Taken together, these findings underscore the fact that even in wild-type mice, key differences in the fat content of lithogenic diet elicit markedly different effects on weight gain. While this complicates direct comparisons between the 2 different L-FABP null mouse lines fed different types of lithogenic diets, in both cases L-FABP gene ablation exacerbated weight loss or reduced weight gain induced by lithogenic diets. These findings suggest agreement in at least this aspect of diet-induced phenotype between the 2 different L-FABP null lines.

10. Physiological significance of genetic modifications in L-FABP in humans: the $Thr^{94}Ala$ mutation

Since to date there have been no reports of complete loss of the L-FABP protein in humans, it is not yet possible to directly correlate the studies of obesity in L-FABP null mice with complete loss of L-FABP in humans and associated pathology. However, some insights may be gained from single nucleotide polymorphisms (SNPs) of L-FABP. While SNPs of L-FABP are fairly common in humans, they have not resulted in loss of the L-FABP but rather a mutant L-FABP protein is expressed. Unfortunately, it is not yet known if the mutant L-FABP differs in structure/function from the native L-FABP or if the expression of the L-FABP protein is altered by the SNP. While either possibility might yield an altered phenotype, neither equates to complete loss of L-FABP as in the L-FABP null mouse model. Furthermore, the genotype of humans is much more heterogeneous than that of inbred mice such that SNPs resulting on a specific phenotype in one population may not necessarily yield the same phenotype in another population. These possibilities are described by examples from SNPs in the human L-FABP gene as well as in SNPs in the related intestinal fatty acid binding protein (I-FABP).

For example, a single nucleotide missense mutation resulting in substitution of threonine 94 (Thr^{94}) with alanine (Ala^{94}) in L-FABP has been reported in two groups of Caucasians: French Canadians [184,185] and Germans [186,187]. This point mutation is apparently common in these Caucasians, wherein 64% are homozygous for Thr^{94}, 23% are Thr^{94}/Ala heterozygotes,

and 13% are homozygous for Ala^{94}/Ala [184-187]. The fact that 36% of the human population carries the Ala^{94} substitution in L-FABP suggests that there is not a very strong selection for one or the other allele and that, at least by about 50 yrs of age (the age of the individuals in these 2 studies), this mutation alone is not sufficient to be pathologic. Correlative examination, however, showed that the body mass index (BMI) of both male and female humans with the Ala^{94}/Ala polymorphism was 4-6% lower than that of the wild-type Thr^{94}/Thr counterparts [186,187]. There was also a significant association of increased serum LDL-cholesterol and triglycerides in females (but not males) with the Ala^{94}/Ala polymorphism [186]. Covariance analysis suggested that the wild type Thr^{94}/Thr has higher serum apoB levels while the Ala^{94} carriers seem to be protected against high apoB levels when consuming a high fat and saturated fat diet [184]. Other covariance analyses indicate that the Thr^{94}/Ala substitution may influence obesity indices as well as the risk to exhibit residual hypertriglyceridemia following lipid lowering therapy with fenofibrate [185]. In addition, a study of a limited number of subjects indicates that the Thr^{94}/Ala substitution appears to reduce hepatic glycogenolysis and blunted elevation of plasma glucose levels in lipid-exposed subjects [187]. While the above studies show modest effects of the Thr^{94}/Ala substitution in L-FABP of humans, it should be noted that none of the above studies reported any western blotting or rtPCR data showing if the Thr^{94}/Ala substitution altered the expression of L-FABP in human liver or other L-FABP expressing tissues (e.g. intestine, kidney, etc.). Further, it is not known whether the Thr^{94}/Ala substitution alters the structure and thereby function of L-FABP. In the T94A mutation of L-FABP (FABP1), at codon 94 there is a replacement substitution of an alanine for the threonine [184-187]. Examination of the crystal structure of L-FABP shows that there is a pair of threonines (T93 and T94) which are located near the high affinity ligand binding pocket [78]. While the hydroxyl group in the T93 side chain is oriented into the binding cavity, its nearest neighbor T94 has its hydroxyl group oriented outward into the solvated environment [184]. Although binding affinities could be affected as in the case of the intestinal fatty acid binding protein I-FABP (also called FABP2) [188], it would be more likely that effects on L-FABP interactions with other proteins (e.g. CPT1, PPARα) and thus mitochondrial oxidative activity (e.g. via L-FABP/CPT1 interaction) or expression regulation (e.g. via L-FABP/PPARα interaction) would be more pronounced.

Lessons learned from an analogous I-FABP SNP mutation in humans are instructive. In I-FABP (FABP2), an A54T mutation first reported in Pima Indians and subsequently shown to occurr in most populations with an allelic frequency near 30% [189]. The A54T mutation enhances ligand binding affinity approximately two-fold, increases intestinal fatty acid absorption, and is associated with increased fatty acid oxidation and insulin resistance [188, 190,191]. It is important to note that there are only two promoter alleles associated with the 54 codon whether coded for the alanine or the variant threonine [192]. *In vitro* studies using luciferase activity showed that the promoter carried on the Thr54 allele resulted in a threefold decrease in expression as compared to the promoter carried on the Ala54 allele [192]. Taken together, the studies with the I-FABP Ala54/Thr mutation indicate that further work needs to be done to clarify the functional consequence of the L-FABP Thr94/Ala mutation, especially the effect on expression of this protein in the liver. Furthermore, while analyses of L-FABP structural models suggest that the Thr^{94}/Ala substitution should influence, but not likely abolish, function of L-FABP, there are as yet no reported data directly examining the effect of the Thr^{94}/Ala substitution on L-FABP structure, ligand specificity, ligand affinity, interaction with other proteins (e.g. PPARα, CPT1, etc.), function *in vitro* or function in cultured cells.

It is important to note that the alterations in L-FABP phenotype due to Thr^{94}/Ala substitution in Caucasians may not necessarily extend to other human populations, as has been the case for the Ala54/Thr mutation in I-FABP [188,190,191,193]. The Ala^{54}/Thr substitution in I-FABP is associated with increased BMI, body fat, and fasting plasma triglycerides in aboriginal Canadians and with higher fasting lipid oxidation rate, higher fasting plasma HDL and LDL

triglycerols, and increased postprandial lipemic response in Finns (rev. in [193]. In contrast, in healthy young Europeans, the Ala^{54}/Thr substitution is not associated with postprandial responses to fat and glucose tolerance tests-suggesting that in addition to the differences in promoter makeup there may be environmental factors, other genetic factors, and selection of study population that may also explain the difference between this and earlier studies of the I-FABP Ala^{54}/Thr polymorphism [193].

In summary, while the studies with SNPs resulting on single amino acid substitutions in L-FABP (or I-FABP) in some human populations are informative, they may not necessarily extend to other human populations, and may be difficult to directly correlate at this point with the effects of total ablation of L-FABP in mice—a much more homogenous inbred population than humans.

11. Conclusions regarding the role of L-FABP in obesity

While some discrepancies exist, the overall experimental data obtained *in vitro*, with cultured cells, and L-FABP gene-ablated mice strongly support the hypothesis that L-FABP may play an important role in LCFA utilization by liver versus adipose tissue. L-FABP enhances LCFA uptake, intracellular LCFA transport, and targets bound LCFA and/or LCFA-CoA to intracellular organelles esterification (endoplasmic reticulum), storage (lipid droplets), secretion (VLDL), or most importantly, oxidation (mitochondria, peroxisomes) (rev. in [6, 139,142,146,148,151,152]. Thus loss of L-FABP was predicted to redirect LCFAs towards storage in adipose tissue and induce weight gain/obesity—especially in response to high fat diet. This possibility was supported by studies with L-FABP null mice generated by complete deletion of all 4 exons of the L-FABP gene, backcrossing to the C57BL/6N substrain (less susceptible to diet induced obesity). Increased weight gain and obesity was observed in L-FABP null mice fed control low fat (4.3 %) chow for >6 mo, pair-fed defined control (phytol-free and phytoestrogen-free) chow for shorter time, or pair-fed defined isocaloric high fat diet. Under all of these conditions, L-FABP gene ablation inhibited LCFA oxidation and promoted weight gain and/or obesity, especially in L-FABP null females and less so males fed high dietary fat. Thus, loss of L-FABP did not protect mice from the deleterious effects of fat in the diet. A similar phenotypic pattern was also exhibited by adipocyte fatty acid binding protein (A-FABP) null mice, which showed increased weight gain as compared with their wild-type counterparts when fed a high-fat diet [194]. I-FABP null mice also exhibit a higher weight gain than their wild-type counterparts when fed either a low-fat or a high-fat diet [195].

The physiological significance of studies with L-FABP null mice compared to humans is underscored by the fact that L-FABP accounts for as much as 7-11% of cytosolic protein in normal human liver [196], nearly 2-fold more than in normal mouse liver wherein L-FABP comprises 3-5% of cytosolic protein (rev. in [4,6]. L-FABP levels are very responsive to high fat diet (rev. in [15,24,26-35] and to peroxisome proliferators and lipid lowering drugs such as fibrates (rev. in [26,34,36-39].

The pathological significance of studies with L-FABP null mice in humans is indicated by the finding that human genetic variations in the L-FABP gene impact blood lipoprotein/lipid levels, response to lipid-lowering therapy with fenofibrate (a cholesterol synthesis inhibitor) and glycogenolysis [216-219]. Decreased expression of L-FABP (as well as I-FABP) occurs in the fat-laden enterocytes of the proximal intestine of humans with genetic lipid malabsorption syndromes such as abetalipoproteinemia and Anderson's disease [228]. Conversely, hepatic L-FABP levels are increased nearly 2-fold to 20% of cytosolic protein in patients with Reyes syndrome—a condition characterized in part by visceral fatty degeneration, block of fatty acid oxidation, and peroxisomal proliferation [227]. Thus, while direct comparisons between the

human mutations and L-FABP null mouse cannot yet be accomplished, the impact of further research to understand the role of L-FABP in obesity is becoming increasingly evident.

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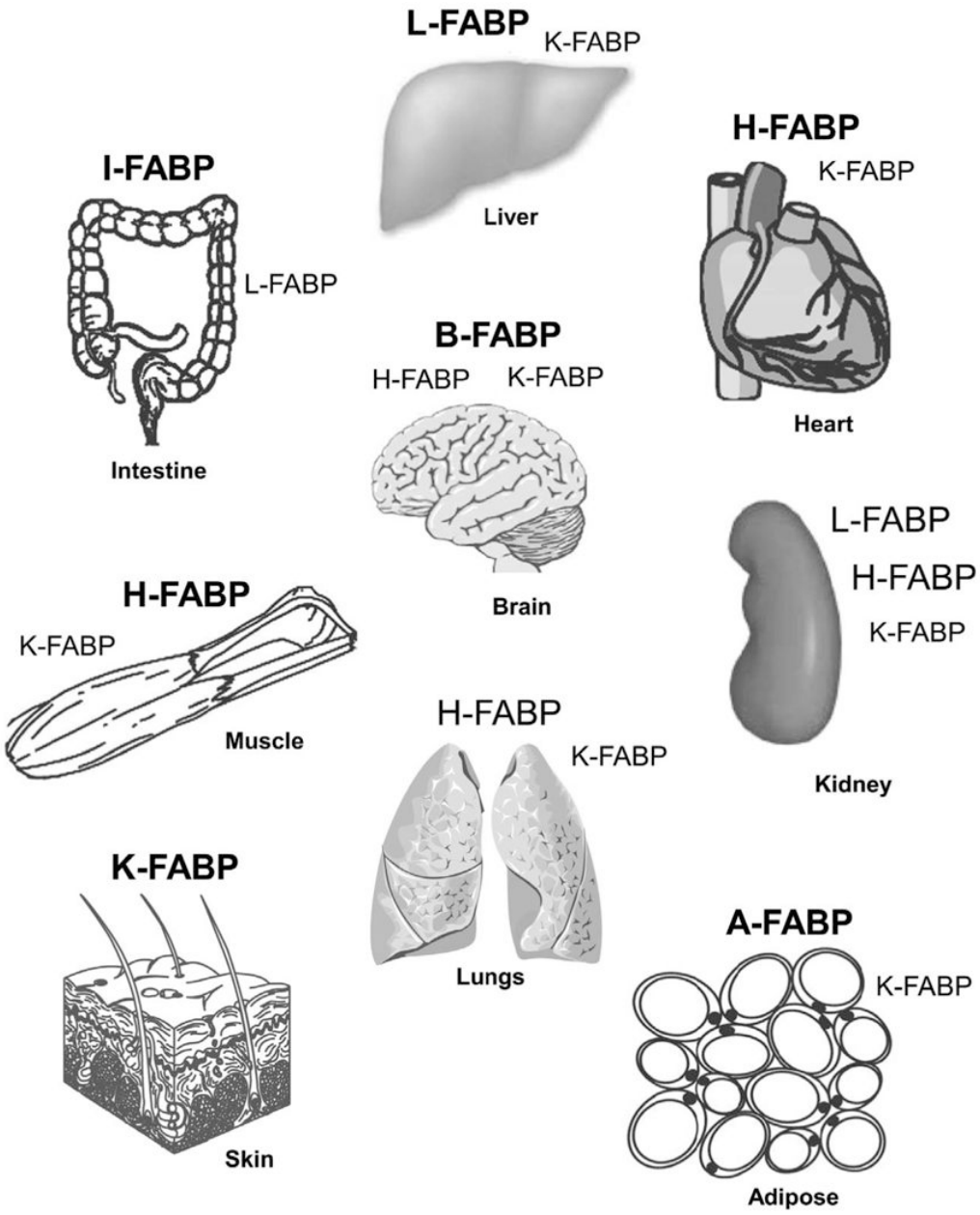

Figure 1. Distribution of fatty acid binding proteins (FABPs) in tissues important for long chain fatty acid (LCFA) metabolism

FABPs present in tissues at highest concentration are shown in large bold letters. FABPS present at lower concentration are shown in large unbold letters. Low expression is shown with small bold letters. The nomenclature of the long chain fatty acid binding protein family has been described [21]: L-FABP, liver type fatty acid binding protein (Fabp1 gene); I-FABP, intestinal type fatty acid binding protein (Fabp2 gene); H-FABP, heart type fatty acid binding protein (Fabp3 gene); A-FABP, adipocyte type fatty acid binding protein (Fabp4 gene); K-FABP, keratinocyte type fatty acid binding protein (also called epidermal fatty acid binding protein, E-FABP, Fabp5 gene); B-FABP, brain type fatty acid binding protein (Fabp7 gene). Additional members of the FABP family (not shown) that bind other types of ligands include: M-FABP, myelin (peripheral) type fatty acid binding protein (Fabp8 gene); T-FABP, testis

type fatty acid binding protein; ILBP, ileal bile acid binding protein (Fabp6 gene); CRBP I and II, cellular retinol binding proteins I and II; and CRABP I and II, cellular retinoic acid binding proteins I and II.

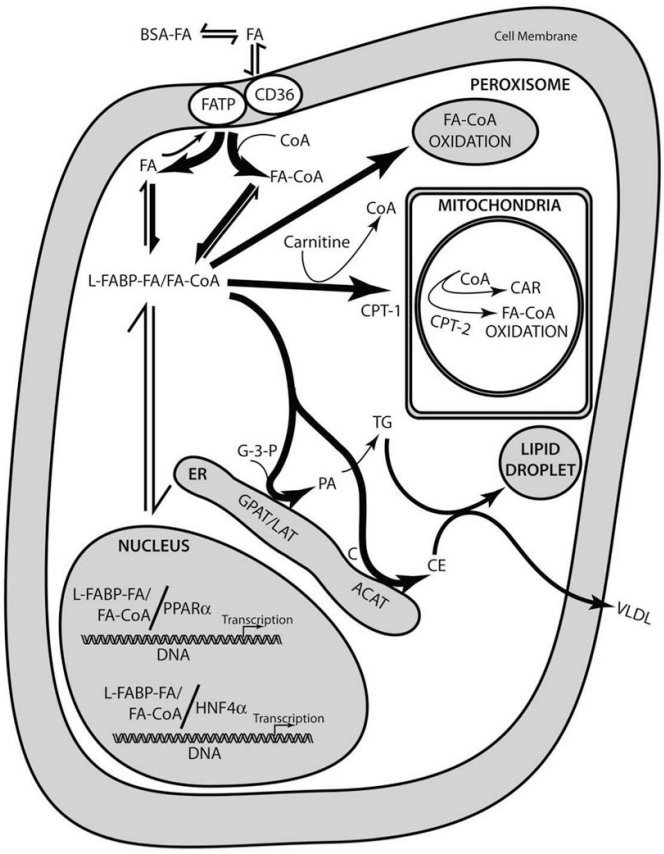

Figure 2. Model of L-FABP functions in living cells

Bold arrows refer to reactions most greatly enhanced by L-FABP. Abbreviations are as follows: BSA, serum albumin; FA, long chain fatty acid; FATP, plasma membrane fatty acid transport protein; CD36, plasma membrane fatty acid translocase protein; CoA, coenzyme A; L-FABP, liver fatty acid binding protein; CPT-1, carnitine palmitoyl transferase I (outer mitochondrial membrane); CPT-2, carnitine palmitoyl transferase II (inner mitochondrial membrante); CAR, carnitine; G-3-P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; TG, triacylglyceride; C, cholesterol; ACAT, acyl CoA cholesterol acyl transferase; CE, cholesteryl ester; VLDL very low density lipoprotein (applies only to the liver); ER, endoplasmic reticulum; PPARα, peroxisome proliferator activated receptor α; HNF4α, hepatocyte nuclear factor 4α.

A.

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           . ...... .  ... . ... . . .   .. .......... ..  ..      .. . ..
Rat        MNFSGKYQVQ SQENFEPFMK AMGLPEDLIQ KGKDIKGVSE IVHEGKKVKL TITYGSKVIH 60
Mouse      MNFSGKYQLQ SQENFEPFMK AIGLPEDLIQ KGKDIKGVSE IVHEGKKIKL TITYGPKVVR 60
Human      MSFSGKYQLQ SQENFEAFMK AIGLPEELIQ KGKDIKGVSE IVQNGKHFKF TITAGSKVIQ 60
Bovine     MNFSGKYQVQ TQENYEAFMK AVGMPDDIIQ KGKDIKGVSE IVQNGKHFKF IITAGSKVIQ 60
           .... .....  . ..... .  ..  .....  ..... ... ... ...  .  ... .. .
Rat        NEFTLGEECE LETMTGEKVK AVVKMEGDNK MVTTFKGIKS VTEFNGDTIT NTMTLGDIVY 120
Mouse      NEFTLGEECE LETMTGEKVK AVVKLEGDNK MVTTFKGIKS VTELNGDTIT NTMTLGDIVY 120
Human      NEFTVGEECE LETMTGEKVK TVVQLEGDNK LVTTFKNIKS VTELNGDIIT NTMTLGDIVF 120
Bovine     NEFTLGEECE MEFMTGEKIK AVVQQEGDNK LVTTFKGIKS VTEFNGDTVT STMTKGDVVF 120
           .. ....
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Mouse      KRVSKRI 127
Human      KRISKRI 127
Bovine     KRVSKRI 127
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B.

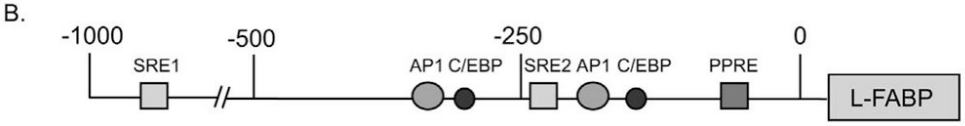

Figure 3. Sequence homology in the liver fatty acid binding proteins (L-FABPs) from different species

(A) Amino acid sequence alignment for L-FABP derived from rat, mouse, human, and bovine. Identical amino acids are indicated by dots. **(B)** Promoter region of the human L-FABP gene. Response elements within the promoter for two putative sterol response elements (SRE 1 and 2), activator protein (AP1), CCAAT/enhancer binding protein (C/EBP), and the peroxisomal proliferator response element (PPRE) are indicated.

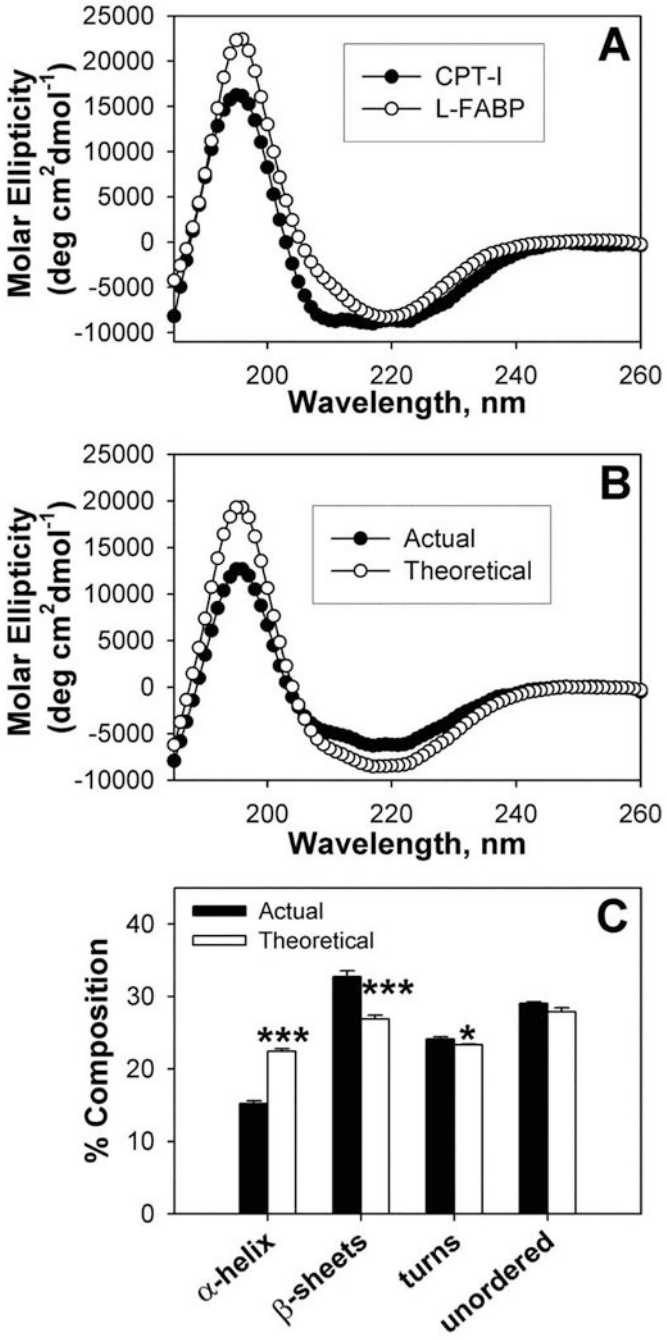

Figure 4. Interaction of L-FABP with carnitine palmitoyl transferase I (CPT1) determined by circular dichroism

(A) Individual far-UV circular dichroic (CD) spectra of WT CPTI C-terminal 89-residue peptide (*filled circles*) and an equal amino acid molarity of L-FABP (*open circles*). **(B)** Comparison of the far-UV CD spectra of an equal amino acid molarity mixture of WT CPT peptide and L-FABP obtained experimentally (actual, *filled circles*) and the theoretically expected spectrum (theoretical, *open circles*) if no conformational change occurred (i.e. the average of the two proteins). **(C)** Proportion of secondary structures (e.g. α–helix, β-sheet, turn, unordered) in equal molarity mixtures of CPT peptide and L-FABP obtained experimentally (actual, *filled bars*) and the theoretically expected (theoretical, *open bars*). Asterisks represent

significant differences between the actual and theoretical for each compositional component; * $P < 0.05$; *** $P < 0.001$.

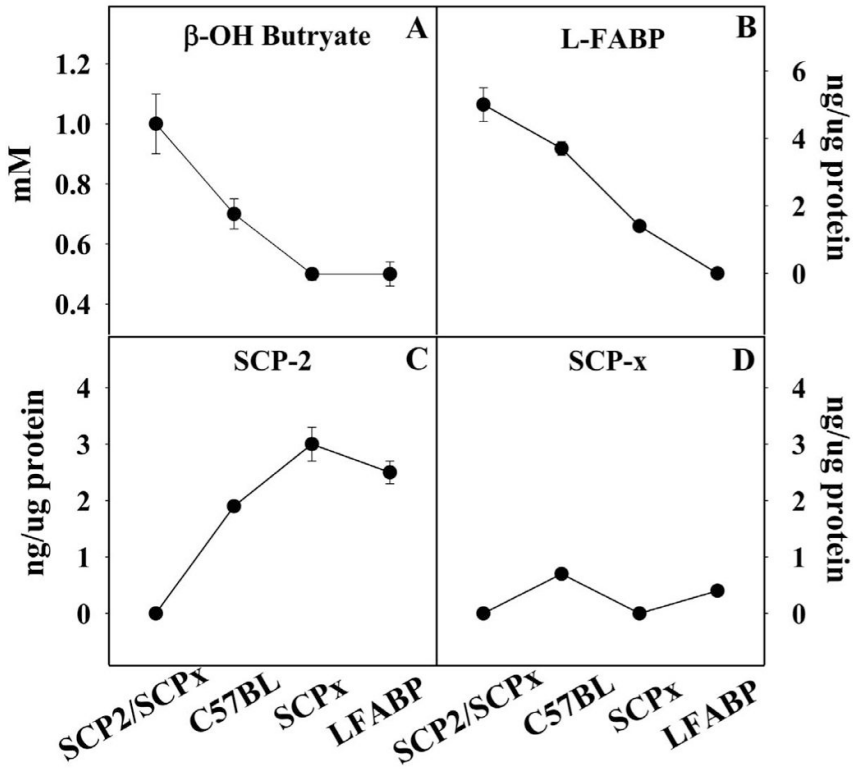

Figure 5. Correlation of LCFA oxidation with hepatic L-FABP levels *in vivo*

Levels of serum β-hydroxybutyrate **(A)** as well as L-FABP **(B)**, SCP-2 **(C)**, and SCP-x **(D)** in liver homogenates were determined in male SCP2/SCP-x null, C57BL6N wild-type, SCP-x null, and L-FABP null mice fed control standard rodent chow diet fed *ad libitum*.

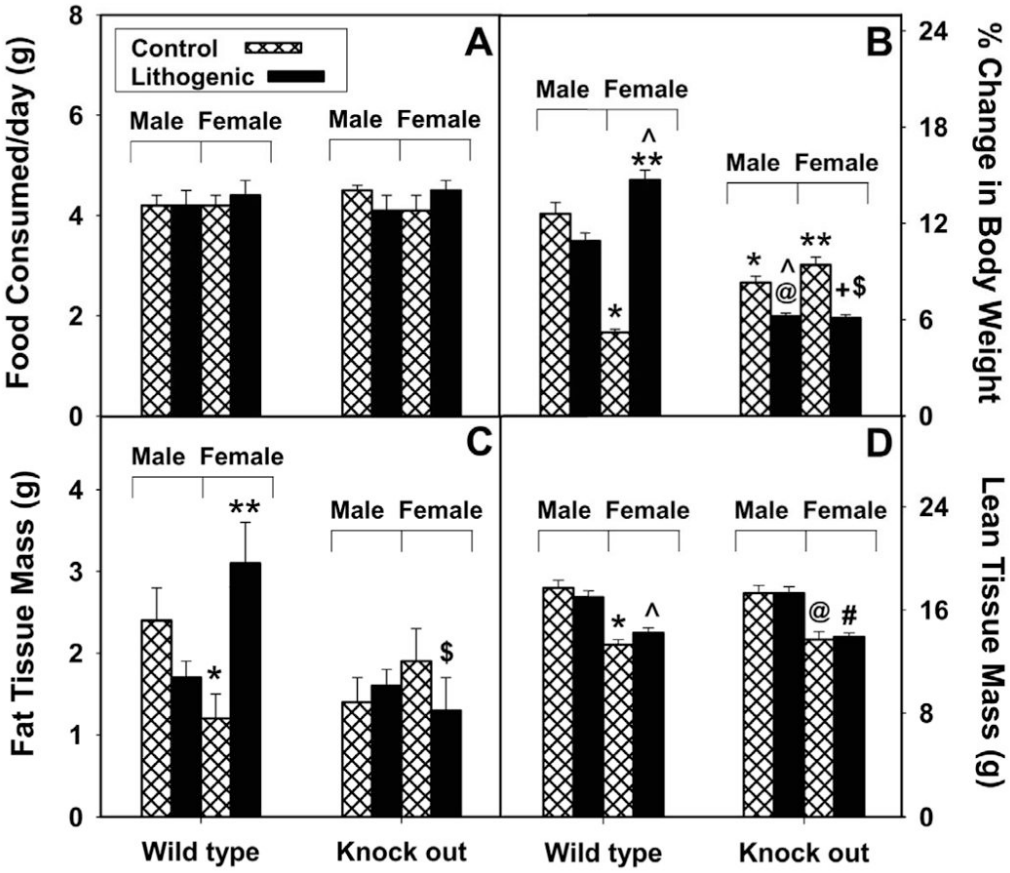

Figure 6. Effect of lithogenic diet on body weight, fat tissue mass, and lean tissue mass

Average daily food consumption **(A)**, percent change in body weight **(B)**, fat tissue mass **(C)**, and lean tissue mass **(D)** was determined in male and female mice fed a control (hatched bar) and lithogenic (solid bar) diet for 4 weeks. Values represent the mean ± SEM, n=5-7. Statistical analysis was as follows: * $p \leq 0.04$ vs male L-FABP +/+ on control-diet; * * $p \leq 0.03$ vs female L-FABP +/+ on control-diet; @ $p \leq 0.004$ vs male L-FABP -/- on control-diet; + $p \leq 0.001$ vs female L-FABP -/- on control-diet; ˆ $p \leq 0.005$ vs male L-FABP +/+ on lithogenic diet; # $p \leq 0.004$ vs male L-FABP -/- on lithogenic diet; $ $p \leq 0.02$ vs female L-FABP +/+ on lithogenic diet.

Table 1

Effect of L-FABP gene ablation on weight gain in mice fed control chow *ad libitum* or pair-fed defined (phytol-free, phytoestrogen-free) control chow diets.[a]

Type of control chow diet	Feeding	Age *(mo)*	Sex	Body Weight (g)	
				Rate of gain	*Final Weight*
Standard rodent chow	*ad libitum*	< 6 mo	M, F	No change	No change
Standard rodent chow	*ad libitum*	> 6 mo	M, F	Increase	Increase
Defined rodent chow	pair-fed	2.5 mo	M, F	No change	No change
Defined rodent chow	pair-fed	3.5 mo	F	Increase	Increase
Defined rodent chow	pair-fed	3.5 mo	M	No change	No change

[a]L-FABP null mice, age- and sex-matched (up to N10) to wild-type littermates by heterozygote/heterozygote breeding, were fed continuously on control (Rodent Diet 8604, standard low fat, 5% of energy from fat) chow from Harlan Teklad, Madison, WI. Alternately, mice were fed on this control chow for 2 mo and then pair-fed for 18 or ≥42 days on defined control (AIN-76A phytol-free, phytoestrogen-free, 5% calories from fat) chow from Research Diets, New Brunswick, NJ. Food consumption and mouse weight were determined every other day. There were no differences in food consumption in response to L-FABP gene ablation.

Table 2

Effect of L-FABP gene ablation on whole body fat tissue mass (FTM) and lean tissue mass (LTM) in mice fed control chow *ad libitum* or pair-fed defined (phytol-free, phytoestrogen-free, 5% fat) control chow diets.[a]

Type of control rodent chow diet	Feeding	Age *(mo)*	Sex	Change in Tissue Mass			
				FTM (g)	LTM (g)	FTM (%)	LTM (%)
Standard	*ad libitum*	< 6	M, F	0	0	0	0
Standard	*ad libitum*	≥ 6	F	+++	0	+++	- -
Standard	*ad libitum*	≥ 6	M	++	+	++	-
Defined	pair-fed	2.5	M, F	0	0	0	0
Defined	pair-fed	3.5	F	+++	++	+++	+
Defined	pair-fed	3.5	M	+/0	0/-	+/0	0/-

[a] L-FABP null mice, age- and sex-matched (up to N10) to wild-type littermates by heterozygote/heterozygote breeding, were fed continuously on control (Rodent Diet 8604, standard low fat, 5% of energy from fat) chow from Harlan Teklad, Madison, WI. Alternately, mice were fed on this control chow for 2 mo and then pair-fed for 18 or ≥42 days on defined control (AIN-76A phytol-free, phytoestrogen-free, 5% calories from fat) chow from Research Diets, New Brunswick, NJ. Whole body fat tissue mass (FTM) and lean tissue mass (LTM) were determined by dual emission x-ray absorptiometry. 0, +, and − refer to no difference, increase, or decrease versus wild-type mice.

Table 3

Effect of L-FABP gene ablation and high fat diet on mouse whole body phenotype.[a]

Sex	Change in Body Weight (g)	Change in Tissue Mass	
		FTM (%)	LTM (%)
M	0/+	+	0
F	+	+ +	+

[a] L-FABP null mice, age- and sex-matched to wild-type littermates by heterozygote/heterozygote breeding, (N6 back-cross generation) were fed for 7 wks standard (4.5 gm% fat) standard Rodent Diet 8604 chow from Harlan Teklad, Madison, WI. Thereafter, the mice were individually housed and switched to a defined low fat (4.3% fat) control diet (Diet D12450, phytol-free, phytoestrogen-free) from Research Diets, New Brunswick, NJ. After 1 week on this diet, half of the mice in each group continued on the defined low fat control diet while the other half were pair-fed a high fat (24% fat) isocaloric diet based on the same defined control diet. Food consumption and mouse weight were measured every 2 days for 12 weeks. Food consumption (g or kcal) did not differ in response to L-FABP gene ablation. Whole body fat tissue mass (FTM) and lean tissue mass (LTM) were determined by dual emission x-ray absorptiometry at the beginning and end of the dietary study. 0, +, and − refer to no difference, increase, or decrease vs age-and sex-matched littermate wild-type L-FABP +/+ mice.

Table 4

Potential factors contributing to observation of somewhat different obese phenotype noted with "L-FABP null/GFP overexpressor" mice

Differences were derived by comparisons of methodology reported for "L-FABP null/GFP overexpressor mice" [143,163-165,182,197] with those for the original "L-FABP null mice" mice [45,56,59,71,[98],[142],[152],[153],162,167,176,177]. Additional references in the table refer to other publications demonstrating such differences in factors/parameters can affect mouse phenotype/obesity/lipid metabolism.

Factor	Parameter	Affects Mouse Phenotype/Lipid Metabolism/Obesity	Additional References
Construct	Lipid phenotype	Yes	[198-200]
	Peptide fragments?	Yes	[201-208,208,209,209-215]
	GFP overexpression	Yes	[216-221]
Backcrossing	C57BL/J substrain more obesity susceptible	Yes	[222], http://jaxmice.jax.org/jaxnotes/511/511n.html
	Backcross generation number of L-FABP null	No	[98]
Control mice	Vendor supplied vs wild-type littermates	Yes	
	Age	Yes	[223-225]
	Sex	Yes	[58,169]
Diets	Vendor source, non-defined, not phytol free, not phytoestrogen free	Yes	[58,168,171,172]
	Ad libitum feeding, length of time on high fat diet	Yes	
Intestinal microflora	Obesity	Yes	[226-231]