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Review

Fatty acid-binding proteins – insights from genetic manipulations

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Abstract

Fatty acid-binding proteins (FABPs) belong to the conserved multigene family of the intracellular lipidbinding proteins (iLBPs). These proteins are ubiquitously expressed in vertebrate tissues, with distinct expression patterns for the individual FABPs. Various functions have been proposed for these proteins, including the promotion of cellular uptake and transport of fatty acids, the targeting of fatty acids to specific metabolic pathways, and the participation in the regulation of gene expression and cell growth. Novel genetic tools that have become available in recent years, such as transgenic cell lines, animals, and knock-out mice, have provided the opportunity to test these concepts in physiological settings. Such studies have helped to define essential cellular functions of individual FABP-types or of combinations of several different FABPs. The deletion of particular FABP genes, however, has not led to gross phenotypical changes, most likely because of compensatory overexpression of other members of the iLBP gene family, or even of unrelated fatty acid transport proteins. This review summarizes the properties of the various FABPs expressed in mammalian tissues, and discusses the transgenic and ablation studies carried out to date in a functional context.

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1. Introduction

"Nature makes nothing in vain", said the Greek philosopher Aristotle [1]. Ever since the first discovery of fatty acid-binding proteins [2], the focus has been to elucidate the purpose of these proteins which, as we know now, belong to the family of intracellular lipid-binding proteins (iLBPs). Yet, despite the concerted effort of a generation of researchers, the scope of functions of this ubiquitous protein family and its individual members has thus far not been fully explored. Much evidence has been accumulated in support of numerous and diverse functions, although proof for any of these is difficult to establish. In recent years, new genetic tools have provided the opportunity to test proposed functions at the cellular or organismal level, for example through overexpression or targeted mutations. Especially the generation of knock-out mice, where individual FABPs have been deleted, made it possible to investigate the consequences of missing fatty acid-binding proteins. Many excellent reviews have been published on the structure, properties, gene expression, and evolution of these proteins (e.g. [3–5]), and the reader is referred to these for more detailed information. This review paper thus only briefly summarizes these aspects of iLBPs, including functions proposed. The main emphasis is a comprehensive analysis of the studies that have used genetic manipulations resulting in overexpression or deletion of FABPs in a cellular or organismal setting.

2. The family of intracellular lipid-binding proteins

2.1. Evolution and sub-families

Fatty acid-binding proteins belong to the conserved multigene family of the intracellular lipidbinding proteins having molecular masses around 15 kDa. Members of this family have been found throughout the animal kingdom, both in invertebrates and vertebrates. It is believed that individual genes of this ubiquitous gene family arose from an ancestral iLBP gene through gene duplication and diversification [6–8]. As iLBPs are not present in plants or fungi, the ancestral gene has likely evolved after the animal kingdom had separated from plants and fungi, more than 1000 million years ago. The first gene duplication may have appeared approx. 930 million years ago, well before the vertebrate/invertebrate divergence [6–8], and hence members of both branches are present in vertebrates and invertebrates. Subsequent duplications gave rise to the four subfamilies recognized today, which contain the different iLBPs found in vertebrates species [9] (Table 1). Subfamily I, which comprises proteins specific for vitamin A derivatives, can be subdivided into the cellular retinoic acid-binding proteins (CRABP-I and II) and the cellular retinol-binding proteins (CRABP-I, II, III, and IV) [10]. Subfamily II contains proteins with larger binding sites that allow binding of bulkier ligands, such as bile acids, eicosanoids, and heme. In addition to the intestinal bile acid-binding protein (I-BABP) and the liver-type (L-) FABP, this subfamily contains the basic liver-type (Lb-) FABP), the only iLBP that is not expressed in mammals. It is found in the liver of birds, fish, reptiles, and amphibians [11]. Intestinal-type (I-) FABP is the only member in subfamily III, while the remaining proteins (heart-type (H-), adipocyte-type (A-), epidermal-type (E-), myelin-type (M-), testis-type (T-), and brain-type (B-) FABP) belong to subfamily IV.

In recent years, a numerical nomenclature for the various FABP genes has been introduced [12], but the various FABPs are still named after the tissues in which they have been discovered, or are prominently expressed. It should be recognized, however, that such a classification is some-

iLBP-type	Gene	Expression	Ligands
Subfamily I CRBP I, II, III, and IV		Ubiquitous in mammalian cells	Retinol
CRABP I and II		Ubiquitous in mammalian cells	Retinoic acids
Subfamily II			
L-FABP	Fabp1	Liver, intestine, kidney, lung, and pancreas	Long-chain fatty acids, acyl-CoAs, and heme
I-BABP	Fabp6	Ileum	bile acids
Lb-FABP	Fabp10	Fish and bird liver	Long-chain fatty acids
Subfamily III			
I-FABP	Fahn?	Intestine liver	Long-chain fatty acids
1-1 / 101	1 uop2	intestine, nver	Long-chain fatty acids
Subfamily III			
H-FABP	Fabp3	Heart, skeletal muscle, brain, kidney, lung, mammary, placenta, testis,	Long-chain fatty acids
		ovary, and stomach	
A-FABP	Fabp4	Adipose tissue and macrophages liver	Long-chain fatty acids
E-FABP	Fabp5	Skin, adipose tissue, lung, brain,	Long-chain fatty acids
		heart, skeletal muscle, testis, retina, and kidney	
B-FABP	Fabp7	Brain, glia cells, and retina	Long-chain fatty acids and docosahexacenoic acid
M-FABP	Fabp8	Brain and Schwann cells	Long-chain fatty acids
T-FABP	Fabn9	Testis	Long-chain fatty acids

Table 1 FABPs as members of the intracellular lipid binding protein family

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what misleading, since no FABP is specific for a given tissue, and most tissues express various FABP-types. L-FABP, for example, is strongly expressed in the liver, and was thought to be the only FABP expressed in this tissue, at least in adult animals (recent studies have reported the presence of E-FABP, I-FABP, and A-FABP in liver as well [13,14]). But L-FABP is also found, albeit in far smaller concentrations, in the intestine, kidney, lung, and pancreas. In contrast, I-FABP and I-BABP are confined to the digestive tract and not expressed prominently in other tissues [15]. H-FABP and E-FABP are the most ubiquitously expressed iLBPs; the former is most prominent in cardiac and skeletal muscle, but also present in kidney, lung, mammary tissue, placenta, testis, stomach, ovary, and the brain [16]. The latter protein is widely expressed in skin, lung, heart and skeletal muscle, kidney, testis, and adipose tissue, as well as in the brain and the retina. In addition to H- and E-FABP, the nervous system contains two other FABPs, B-FABP that is present in brain and retina, and the M-FABP that seems to be specific for the Schwann cells forming the myelin sheath [17,18]. A-FABP was originally thought to be confined to adipocytes [19], but has been detected in macrophages as well [20]. In most cases, the expression pattern is similar in all vertebrates, with the notable exception that non-mammalian vertebrates express in their livers a basic liver-type FABP (Lb-FABP) that is different from the mammalian L-FABP, and possibly other members of the iLBP family [21].

2.2. Control of FABP gene expression

The control of tissue-specific expression of the various FABP-types is only poorly understood to date. Often, the expression of FABPs in a given tissue reflects its lipid-metabolizing capacity. In hepatocytes, adipocytes, and cardiac myocytes, where fatty acids are prominent substrates for lipid biosynthesis, storage, or breakdown, the respective FABP-types make up between 1% and 5% of all soluble cytosolic proteins; these amounts can increase further following periods of mass influx of lipids into the cell. Far smaller amounts are found in other tissues, where lipids are not essential for energy metabolism or deposit. All FABP genes contain a canonical TATA box, followed by a conserved gene structure, that is, three introns of variable length, but in identical positions separating the coding sequences. Various enhancer elements have been identified that direct tissue-specific expression, including an AP-1 site that is required for the expression of A-FABP in adjpose tissue [22], and two HNF1 α sites which control the L-FABP gene [23]. The muscle-specific expression of the H-FABP gene was mapped to a concise regulatory element that contains an atypical MEF2-binding site [24]. The promoters of the A-, I-, and L-FABP genes are regulated by C/EBP sites [25–27]. Of particular interest is the regulation by fatty acids via a peroxisome proliferator response element (PPRE); this was shown for the up-regulation of L- and A-FABP [28,29], whereas the promoters of the E- and H-FABP genes harbor non-functional PPREs [30]. It is noteworthy that genes beyond L- and A- FABP, particularly H-FABP [31], are nonetheless up-regulated by fatty acids, but the mechanism(s) by which this happens has not yet been elucidated.

2.3. Ligand affinity of FABPs

Despite a restricted cell and tissue selectivity observed for the expression of the various FABPtypes, it was and still is assumed on the basis of structural arguments that the individual members of this protein family fulfill different functions. The common denominator of all FABPs is, of course, their binding of fatty acids and/or closely related compounds. All FABPs bind long-chain fatty acids, but with differences in ligand selectivity, binding affinity, and binding mechanism [9]. These are rooted in subtle structural differences between the different subfamilies of the iLBP family. Common to all FABP-types, actually to all iLBPs, is the characteristic β -barrel structure, which is formed by two orthogonal five-stranded β -sheets [32]. The binding pocket is located inside the barrel, the opening of which is framed on one side by the N-terminal helix-turn-helix domain. Generally, one or two conserved basic amino acid side chains are required in the binding pocket to bind the carboxylate-group of the fatty acid ligand; its hydrocarbon tail is lined on one side by hydrophobic amino acid residues, on the other side by ordered water molecules [3,9], thus creating the subtle differences of the FABP-types in the enthalpic and entropic contributions to ligandbinding. The binding pocket of L-FABP (subfamily II) is considerably larger than in the other proteins, allowing the binding of two fatty acid molecules; these are bound in opposite orientation, with the carboxylate-group of the second fatty acid sticking out of the cavity and facing the solvent [33]. In I-FABP (subfamily III), the ligand is bound in a slightly bent conformation [34], while the members of subfamily IV generally bind fatty acid as a U-shaped entity [35]. As all other members of this subfamily, B-FABP binds oleic acid in this way, but its binding pocket can also accommodate very long-chain docosahexaenoic acid in a helical conformation [36].

These structural differences affect the ligand selectivity and binding affinity, as well as interactions with membranes or other proteins. Dissociation constants for long-chain fatty acids appear to be in the nano- to micromolar range. Depending on the technique used to measure these lipid–protein interactions, considerable variations for K_{ds} of a given *holo*-FABP have been reported, but the relative values for a series of *holo*-FABPs determined with one method accords quite well with those determined by the other methods. A more detailed discussion of binding affinity and constants and can be found in a recent review [9]. Studies with fluorescent fatty acid analogs and radioactively labeled fatty acids have revealed differences in the mechanism and kinetics of fatty acid-binding and release. Most FABP-types appear to be "membrane active", i.e., they exchange fatty acids with membrane structures via a collisional transfer [37]. Fatty acid transfer is facilitated through electrostatic interactions between the basic amino acid side chains in the helix–turn–helix motive that is part of the ligand portal region. L-FABP, however, does not interact with membranes and exchanges fatty acids via a diffusional transfer [38].

2.4. Functions of FABPs

As mentioned before, numerous functions have been proposed for FABPs, but supporting evidence in many cases remains circumstantial. The presence of intracellular-binding proteins certainly increases the solubility of fatty acids and may thus protect cellular structures from damage by an excess of these amphipathic molecules. By the same token FABPs create a larger cytosolic fatty acid pool that enhances substrate availability to fatty acid metabolizing enzymes. FABPs enhance uptake of fatty acids into the cell by increasing their concentration gradient, due to minimizing unbound fatty acid in the cell [39]. Consistent with these general roles in fatty acid transport and metabolism, the FABP content in most cells is generally proportional to the cells' rates of fatty acid metabolism. As alluded to in Section 2.2, hepatocytes, adipocytes, and cardiomyocytes have the highest content of their respective FABP-types, while cells that me-

tabolize small fatty acid pools contain much less of these proteins [40]. Moreover, increased fatty acid exposure leads to a marked increase in FABP expression. Endurance training or pathological nutrient changes, as seen in diabetes, result in up to 30% higher levels of FABP in skeletal muscle cells [41], and similar effects have been seen in hepatocytes and adipocytes after exposure to chronically elevated extracellular lipid levels [40]. FABPs are directly involved in this up-regulation; as small intracellular proteins they appear to have ready access to the nucleus and target fatty acids to transcription factors in the nuclear lumen, such as the subtypes α , β , and γ of the peroxisome proliferator-activated receptor (PPAR). L- [29] and A-FABP [28] have been demonstrated to be controlled by these transcription factors, which are activated by fatty acids or other hydrophobic agonists [42]. A physical contact between L-FABP and PPAR α occurs during this activation, and therefore it has been suggested that L-FABP is considered a co-activator in PPAR-mediated gene control [43]. Moreover, the interaction between the fatty acid transporter FABP and the fatty acid-activated nuclear receptor PPAR constitutes a mechanism on how fatty acids, once taken up by the cell, become signaling molecules for conveying messages to the nucleus [43]. By binding to its response element (PPRE) in the promoter of a target gene, the fatty acidactivated nuclear receptor thus links intracellular fatty acid levels to gene expression. In a similar way, E-FABP interacts with PPAR β and A-FABP with PPAR γ [44]. Finally, certain FABPs also seem to be involved in the control of growth and differentiation, either through their binding of mitogens or carcinogens [45,46], or directly through protein-protein interactions with other cellular components. Two proteins that have been shown to reduce the growth of breast cancer cells, mammary derived growth inhibitor (MDGI) [47] and a related protein that is encoded by the MDGI-related inhibitor gene (MRG) [48] were later identified as H-FABP [49] and B-FABP [50], respectively. There is little doubt that FABPs influence all of the above-mentioned pathways and events in vitro; however, in vitro studies cannot reveal whether the proteins are functionally relevant in living cells and organisms. Those answers can be generated by looking at consequences of genetically altered FABP expression in intact cells or animals. As shown below, such studies have dramatically enhanced our understanding of FABP function, but also opened new questions about their roles in vivo.

3. Functional analysis of FABPs by genetic models

3.1. L-FABP (Fabp1)

Early experiments measured the influence of enhanced L-FABP expression in mouse fibroblasts. Transfection of fibroblasts with L-FABP cDNA led to a ~50% increase in fatty acid uptake, and rapid incorporation into triacylglycerols and phospholipids [51]. The total phospholipid mass was 70% higher than in non-transfected control cells, with particularly strong increases in phosphatidylserins and -inositols. Concomitant with elevated phospholipids levels, the cholesterol:phospholipid ratio decreased dramatically [52]. In transfected cells, the fatty acid composition shifted to a higher proportion of polyunsaturated fatty acids, suggesting that L-FABP not only mediated fatty acid uptake, but also altered the cellular lipid environment. In this respect it is noteworthy that L-FABP, apart from binding two fatty acid molecules, also binds two molecules of their CoA-esters with comparable affinities for the first binding site [53]. To assess the importance of L-FABP in vivo, the knock-out mice for the *Fabp1* gene recently were generated by two laboratories [54,55]. Given the importance of the liver in the overall lipid metabolism, it is perhaps surprising that no changes in the appearance, gross morphology, and viability were observed in L-FABP null mice. These mice, as well as their livers, were of normal weight, and the serum levels for triacylglycerols and fatty acids were unchanged. While the fatty acid-binding capacity of liver cytosol was drastically reduced, due to the absence of L-FABP, the overall levels of total lipids, non-esterified fatty acids, and triacylglycerols remained normal [54,55]. Both studies found markedly reduced fatty acid uptake from the plasma, and a clear reduction of intracellular triacylglycerol depots. In L-FABP null mice, hepatic cholesterol, cholesterol esters, and phospholipids were found to be increased in one study [54], but essentially unchanged in the other [55].

The fatty acid-binding capacity of the 15 kDa fraction of the liver cytosol, which should comprise all members of the iLBP family, was reduced by 80% in L-FABP null mice, thus eliminating the possibility that the ablation of the L-FABP gene led to significant compensatory increase in expression of another FABP, as has been observed for A- and E-FABP (see below). However, the cellular sterol carrier protein (SCP)-2 was dramatically increased, perhaps causing the observed accumulation of cholesterol. This compensation may be a post-translational event, as the amounts of the precursor protein SCP-X was proportionally reduced. As SCP-2 binds not only cholesterol but also, although less strongly, fatty acids and their CoA-esters [56], it appears that the overexpression of the protein is a partial remedy for the lack of cytosolic binding capacity for these ligands. Indeed, cytosolic levels of acyl-CoA-binding protein (ACBP), another binding protein for long-chain acyl-CoAs, was also increased in L-FABP null mice [57]. Nevertheless, while the cytosolic binding capacity for both fatty acids and long-chain acyl-CoAs remained lower in null mice, their respective pools in the livers of null mice were normal. The cytosolic levels of L-FABP and SCP-2 are interdependent as, vice versa, the ablation of the SCP-X gene resulted in increased expression of L-FABP [58]. The mechanism by which this compensatory up-regulation occurs has been elucidated. Because of the lack of SCP-X/SCP-2 branched-chain phytanic acid is no longer degraded and accumulates in drastically enhanced concentrations in tissues and serum. Wolfrum et al. [59] demonstrated that the rise in phytanic acid is responsible for the induction of L-FABP expression via the transcription factor PPARa, for which phytanic acid is a strong activator. Yet, the metabolic consequences of the ablation of L-FABP and SCP-X genes are not necessarily complementary; lack of SCP-X/SCP-2 and the concomitant rise in L-FABP led to lower liver concentrations of cholesterol esters, but also of triacylyglycerols [58].

Newberry et al. [55] investigated the response to fasting-induced changes in hepatic fatty acid flux in L-FABP null mice. After 48 h of fasting, the increases in fatty acid uptake, fatty acid oxidation, and triacylglycerol levels were much lower in L-FABP null mice than in control animals. Similar results were obtained by Erol et al. [60] in L-FABP null mice fed on standard, ketogenic, or diabetogenic diets: in all cases fatty acid oxidation rates were much lower than in identically treated wild-type animals. Isolated liver cells from L-FABP null mice, when incubated with palmitate, oxidized this substrate more slowly than cells from control animals. However, this is not the consequence of changes in the β -oxidation pathway: no differences were seen in the oxidation of albumin-bound fatty acids by liver homogenates. There were no changes in the expression of enzymes of the β -oxidation pathway (MCAD, ACO) [60] or of membrane fatty acid transporters (CD36, FATP) [55], which are under the control of PPAR α . Obviously, fatty

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acids reach this nuclear receptor via a mechanism not involving L-FABP. It appears that the lower fatty acid oxidation rates observed after starvation of the knock-out mice were directly caused by the lack of L-FABP and the resulting limits in intracellular fatty acid storage capacity. This led to an early saturation of the pathways of fatty acid oxidation and storage in fasting conditions.

Other knock-out models that have contributed to our understanding of L-FABP include $HNF1\alpha^{-/-}$ mice as well as $PPAR\alpha^{-/-}$ mice. $HNF1\alpha^{-/-}$ mice showed very fatty, enlarged livers, probably primarily a consequence of the increased transcription of enzymes involved in fatty acid synthesis and uptake. In spite of the elevated lipid metabolism seen in these mice, the expression of L-FABP expression was greatly reduced [23]. Since levels of PPAR α , which mediate the induction of L-FABP by fatty acids, are not altered, it appears that HNF1 α is directly involved in the expression of the L-FABP gene. Indeed, the L-FABP promoter contains two HNF1 α -binding sites, and the authors demonstrated in transfection studies that HNF1 α is required for the expression of L-FABP in the liver.

Ablation of the PPAR α gene abolished the induction of L-FABP expression by PPAR α agonist [61], confirming that the up-regulation in liver is mediated by this transcription factor. Consequently, overexpression of PPAR α in human hepatoma cells increased L-FABP expression and stimulated oleate uptake. Blocking of L-FABP translation by antisense strategies eliminated this effect and reduced FABP expression (-84%) and oleate uptake (-66%) [62]. Interestingly, the regulation of L-FABP in intestinal cells is controlled in a different manner. Its up-regulation by fatty acid is mediated by PPAR β , and PPAR α knock-out mice did not alter L-FABP inducibility or expression in the intestine [63].

3.2. I-FABP (Fabp2)

Cellular studies on I-FABP function are not conclusive. Following I-FABP overexpression in mouse fibroblasts, no changes in uptake of fatty acids from the medium were observed, but increased esterification and formation of triacylglycerols and cholesterol esters [51,64]. Transfection of embryonic stem cells with I-FABP resulted in a >1.7-fold increase in fatty acid uptake and intracellular diffusion [65]. On the other hand, the addition of I-FABP message to an I-FABP negative line of intestinal epithelial cells did not alter esterification rates [66]. To this end it will be interesting to see which physiological consequences, if any, the ablation of the L-FABP gene has for the intestine. In this tissue, three different members of the iLBP family are expressed, namely L-FABP, I-FABP, and I-BABP, although in different segments [15]. L-FABP is expressed mostly in the proximal region, while I-BABP is restricted to the distal part. I-FABP is expressed throughout the intestine, but most strongly in the distal part. The individual contributions of these proteins to lipid absorption and metabolism are difficult to assess.

A study with I-FABP knock-out mice, which were viable and fertile, clearly demonstrated that I-FABP is not essential for the absorption of dietary fat; in fact, fat absorption was not at all affected by the loss of I-FABP [67]. A compensatory up-regulation of co-expressed L-FABP or I-BABP was not observed. Both genders exhibited elevated plasma levels of insulin, but normal levels of glucose. Male animals weighted more and had elevated levels of plasma triacylglycerols, regardless of their diet, while females were of normal weight. On high fat diet, however, females gained less weight than their wild-type littermates. Correspondingly, livers of male I-FABP null

mice were significantly larger, but no such changes were seen in females. Reasons for these gender differences remain unresolved at present. It thus appears that fatty acid uptake can be mediated by the remaining iLBPs as they are normally expressed, without the need for increased amounts of either L-FABP or I-BABP.

3.3. H-FABP (Fabp3)

This protein has been found in a great variety of mammalian tissue cells (see Section 2.1), but it is generally co-expressed with other FABP-types, at least temporarily. A case in point is the identification and characterization in mammary epithelial cells of growth-inhibiting MDGI [68] (see Section 2.4), which later turned out to be an isolation mixture of H- and A-FABP [43]. A notable exception to co-expression are heart and skeletal muscle cells where H-FABP concentrations are subject to diurnal variations [69] as well as the physiological state of the cells due to stress and nutritional manipulations. In muscle cells, H-FABP is believed to be involved in fatty acid uptake and subsequent transport toward mitochondrial β -oxidation systems; thus increased fatty acid exposure in vitro and in vivo has been shown to result in elevated H-FABP expression, both at mRNA and protein levels [31,70,71]. Conditions in which plasma lipids are elevated, such as obesity and diabetes [71,72], result in elevated H-FABP levels in myocytes, as does endurance training in many organisms, including insects [73], birds [74], rodents [70], and humans [75].

From the foregoing it appears plausible that higher FABP levels are required for enhanced fatty acid uptake and metabolism. However, in vitro studies in intact cells have not always supported this conclusion. In human breast cancer cells, overexpression of H-FABP resulted in a moderate increase in fatty acid uptake [76], whereas transfection of Madin–Darby canine kidney cells with H-FABP cDNA constructs did result in 8-fold increase in H-FABP content, yet uptake rates for palmitate remained unchanged [77]. Similarly, fatty acid uptake rates were not altered by the overexpression of H-FABP in L6 myoblasts [78]. One possible explanation for these results could be that all these cells already contain an excess of H-FABP, which would not be rate-limiting for uptake. Consequently, experiments with cells having reduced H-FABP levels or none at all should be more conclusive.

In 1999 Binas et al. [79] created a H-FABP knock-out mouse. While these animals appeared to be of normal phenotype with no obvious differences in viability and fecundity, physiological differences in fatty acid metabolism of heart and skeletal muscle were readily detected. In isolated cardiomyocytes palmitate uptake was reduced by 45% [80]. Inspection of the heart itself revealed that the uptake of fatty acids was severely inhibited, while plasma concentrations of free fatty acids were increased. Due to this inability of the heart to obtain sufficient amounts of fatty acids, cardiac metabolism switched towards glucose oxidation. Physiological consequences of these metabolic switches included a reduced tolerance to exercise, in which the H-FABP null mice fatigued quickly, and localized cardiac hypertrophy in older animals [79]. Interestingly, fatty acid metabolism in skeletal muscle was somewhat less affected as revealed by a 30% reduction of fatty acid uptake in H-FABP^{-/-} mice. Skeletal muscle cells from these mice at rest showed reduced rates for fatty acid oxidation and esterification as well as for glucose oxidation. Cells from soleus muscle, when electrically stimulated to contract, retained the capacity to substantially increase fatty acid metabolism. This indicated that lack of H-FABP does not preclude efficient fatty acid metabolism in these cells. It is possible that other proteins compensated for the lack of H-FABP in the knock-out mice, but these apparently were not members of the iLBP family, as the 15 kDa fraction of cardiac cytosol did not contain any fatty acidbinding proteins [81,82].

Mammary gland is another organ in which H-FABP is prominently expressed in the course of cell differentiation and formation of ductuli followed by lactation [83]. Initially characterized as mammary derived growth inhibitor (Section 2.4), H-FABP as well as an 11 amino acid C-terminal peptide of the protein was shown to inhibit the growth of human breast cancer cells in vitro [68]. Human MCF-7 breast cancer cells which do not express H-FABP were transfected with bovine H-FABP [84], and the effects on fatty acid metabolism and cell proliferation were studied. While fatty acid uptake was modestly increased, no changes in fatty acid metabolism were observed [76]. These cells, however, exhibited lower tumorgenicity in nude mice [84]. Wang and Kurtz [85] transfected three different human breast cancer cell lines (MCF-7, MDA-MB468, and MDA-MB231) with a novel peptide production vector, so that they expressed and secreted the C-terminal peptide of H-FABP. Xenografts of each of these cell lines onto athymic nude mice suppressed tumor growth; in addition, co-injection of transfected MCF-7 cells and wild-type MDA-MN486 cells resulted in suppressed tumor growth from both cell lines, indicating a paracrine action of the secreted peptide. Thus, growth inhibition appeared to be unrelated to the ligand-binding capacity of this FABP-type. On the other hand, overexpression of H-FABP in transgenic mice had no effect on cell proliferation or differentiation in the mammary gland [86]. Similarly, the ablation of the H-FABP gene showed no discernable effects on the mammary gland, and it was concluded that the protein does not play a role in regulating the development or function of this tissue [87]. Thus, the biological function of H-FABP in the mammary gland remains unclear. Similarly, little is known about the effect of the lack of H-FABP in tissues other than heart or mammary gland. It would be interesting to see which, if any, changes occur in the brain, a tissue that contains significant levels of H-FABP together with E-FABP in its neurons but which, by common knowledge, does not use fatty acid as energy source.

3.4. A-FABP (Fabp4)

Substantial A-FABP expression is limited to adipocytes and macrophages [20,88]. Treatment of myoblasts with fatty acids can result in the *trans*-differentiation of these cells to adipocytes [89], with a concomitant rapid increase in A-FABP expression [90]; the *trans*-differentiation and the induction of A-FABP expression have been shown to be triggered by a rise in PPAR β [91]. In contrast, differentiation to myotubes is accompanied by an increase in H-FABP expression [31,92] and a reduction in both A-FABP and E-FABP [93]. Early cell transfections by Prinsen and Veerkamp [78] have shown that transfection of rat myoblasts with either A- or H-FABP did not alter fatty acid uptake rates, thus providing further evidence that these FABP-types are not rate-limiting in cellular fatty acid uptake. However, overexpression of A-FABP resulted in a 2-fold increase in cell proliferation rates; these myoblasts could not be induced to differentiate into myotubes. While some concomitant changes were seen in the phospholipid composition of these A-FABP transfected rat myoblasts, insights into the biological functions of A-FABP could not be delineated from these cell transfection studies.

Targeted disruption of the A-FABP gene did not lead to any gross morphological changes [94]. The mice appeared normal and healthy, and their adipocytes developed normally. When fed a high lipid diet, normal and A-FABP null mice gained weight similarly. Unlike their wild-type littermates, however, obese A-FABP^{-/-} mice did not show increased plasma triacylglycerol levels, and serum levels for insulin, fasting glucose, and cholesterol were also markedly lower. While the obese wild-type mice became insulin resistant and diabetic, the knock-out mice remained glucose and insulin tolerant. Thus, the ablation of the A-FABP gene generated an interesting model in which obesity and insulin resistance became uncoupled [95]. Within the adipocyte the loss of A-FABP was compensated by overexpression of E-FABP, which is present in the normal adipocyte only in very small amounts. Although total FABP levels were still lower than in wild-type mice, the intracellular concentration of free fatty acids was actually increased. This finding has been interpreted as supporting the role of A-FABP as a fatty acid transporter between intracellular compartments: if the efficient transport of fatty acids towards lipid storage or export is disrupted, free fatty acids accumulate in the cytosol. The observed reduction in lipolysis (-40%) would also support the notion that the lack of A-FABP leads to a down-regulation of lipid export from adjpocytes [96]. This had been initially attributed to the ability of A-FABP to bind and activate hormone sensitive lipase [97], but a recent study by the same authors demonstrated that E-FABP also interacts with hormone sensitive lipase [98]. Moreover, it appears that the activation of the enzyme activity is mostly the result of the binding of released fatty acids to FABP, which thus removes the product of the lipolytic reaction. While I- and L-FABP do not physically interact with the enzyme, their presence in solution activated hormone sensitive lipase in a similar manner as the A- and E-paralogs. Apparently, stimulation of the lipase depends on efficient sequestration of the fatty acid liberated. Consequently, the rate of lipolysis was markedly increased in adipocytes from transgenic animals that overexpress E-FABP [99]. Such mice also developed insulin resistance [100], a fact that underlines the role E-FABP in obesity and diabetes (see below).

A closely related pathological event is the development of atherosclerosis in diabetic and/or obese animals. In a recent study Makowski et al. [101] took advantage of two genetic models, the A-FABP^{-/-} strain and the Apo-E^{-/-} strain, where the latter is characterized by spontaneous development of atherosclerotic lesions independent of obesity or elevated plasma levels of insulin and glucose. No significant differences were seen in serum cholesterol, triacylglycerols, and glucose levels between crossbreds of A-FABP^{+/+}/Apo-E^{-/-} knock-out and A-FABP^{-/-}/Apo-E^{-/-} double knock-out strains, which also did not differ strongly in their insulin sensitivities. Compared to the A-FABP expressing strain, atherosclerotic lesions in the proximal aorta were reduced by 2/3 in the double knock-outs lacking both A-FABP and Apo-E, indicating that the absence of A-FABP protected these animals from the development of atherosclerosis. Using a bone marrow transplant model in which A-FABP^{-/-}/Apo-E^{-/-} mice were donors to recipient A-FABP^{+/+/} Apo- $E^{-/-}$ strains, the investigators created animals in which A-FABP is expressed in adjocytes but not in the bone marrow derived macrophages. The lack of A-FABP in macrophages did not alter the serum glucose and lipid levels, but resulted in a marked decrease ($\sim 50\%$) in the area of atherosclerotic lesions [101]. Similar differences were seen in animals with severe hypercholesteremia [102]. A-FABP thus appears to play a crucial role in various manifestations of the metabolic syndrome, influencing both metabolic changes through its expression in adjocytes, and vascular damage through its expression in macrophages.

3.5. E-FABP (Fabp5)

E-FABP is expressed most strongly in epidermal cells, but also in many other tissues, including mammary gland, brain, liver, kidney, lung, and adipose tissue. Since all these cells express additional members of the iLBP family, the function of E-FABP is especially difficult to elucidate. One study [103] that has focused on rat mammary epithelial cells found that E-FABP overexpression may, under suitable conditions, induce metastasis in some human cancers: a benign, nonmetastatic rat mammary epithelial cell line was transfected with E-FABP. Following inoculation into syngeneic rats, almost one-third of these animals developed metastases, while all animals treated with non-transfected cells remained free of metastases [103]. Thus, E-FABP seems to a have the opposite effect of both H-FABP and B-FABP (see below), which inhibit tumor growth in mammary cells. Changes in H- or B-FABP expression were not analyzed in these cells, but in light of more recent studies that suggest that the expression of the various FABP members is interdependent [99,96], it is tempting to speculate that overexpression of E-FABP results in lower expression rates for the tumor-suppressing members of this gene family.

In nervous tissues, E-FABP seems to be required for neuronal development. E-FABP is expressed in astrocytes and glia of pre- and perinatal brain, and, unlike B-FABP, also in neurons [18]. E-FABP expression is induced following peripheral nerve injury, suggesting a role in the regeneration of neurons as well [104]. To examine whether E-FABP plays a role during neurite outgrowth, PC12 cells (which are a useful model for the study of neuronal differentiation) were transfected with an antisense E-FABP vector to create an E-FABP-deficient cell line. Cells lacking E-FABP responded less strongly to nerve growth factor treatment than control cells as revealed in a visibly decreased neurite expression [105].

Cell culture models have proven very useful for mechanistic studies. As it was demonstrated in HepG2 cells and in 3T3-L1 adipocytes that L-FABP interacted with PPAR α [43] and A-FABP with PPAR γ [106], respectively, it has been shown that in transfected COS-7 cells E-FABP served as co-activator in the nucleus as well, in this case for PPAR β -mediated gene control [44]. In elegant studies these authors constructed a mutant E-FABP with an added nuclear export signal, thus depleting the nucleus of transfected cells of E-FABP. In these COS-7 cells, PPAR-mediated reporter gene expression was significantly reduced over cells transfected with the wild-type protein. Transfection of cultured mouse keratinocytes with E-FABP antisense plasmids resulted in decreased levels of E-FABP, and these cells did not display proper PPAR β -mediated cell differentiation [44].

The interdependency of expression levels for the various FABP-types is clearly visible for E-FABP. In transgenic mice overexpressing the E-FABP gene the adipocyte levels of E-FABP were reduced by 50% [99]; added together, the total FABP content in these adipocytes was \sim 50% higher than in wild-type cells. Between the transgenic and wild-type mice, there were no detectable differences in gross morphology, and the body weight and serum concentrations of free fatty acids were also unchanged. However, both basal and hormone stimulated lipolysis was markedly enhanced in the transgenic animals [99]. Thus, it appears that the rate of lipolysis in adipocytes depends on the total FABP concentration, but is independent of the particular type of FABP.

The expression pattern was reversed in E-FABP knock-out mice, where the lack of E-FABP in adipocytes was compensated by the overexpression of A-FABP [14]. Adipocytes showed higher

capacity for insulin-dependent glucose transport, while E-FABP overexpression in the above mentioned transgenic animals reduced sensitivity to insulin [99].

The complete loss of E-FABP in the epidermis did not alter the fatty acid composition of the epidermal membrane, where fatty acids are essential components of the water permeability barrier of the skin [14]. However, functional analysis revealed a lower transepidermal water loss in the knock-out mice than in wild-type animals, and the water permeability barrier recovered more slowly following its damage by acetone treatment [107]. Thus, it appears that E-FABP plays an important role in maintaining the water permeability of the skin that cannot be easily compensated by other proteins, but more detailed analysis is still needed to fully understand the mechanism by which the protein exerts this action.

Interestingly, the lack of E-FABP in the liver of both E-FABP^{-/-} and E-FABP^{+/-} mice during the perinatal development was compensated by an overexpression of H-FABP. In the liver of wild-type animals, H-FABP levels are undetectable, but data from EST-libraries indicate that H-FABP is expressed during embryonal development [108]. Otherwise, there were no apparent changes in morphology and histology between wild-type and E-FABP^{-/-} mice [14].

The effects of the deletion of both the E-FABP and H-FABP genes was studied in lung type II alveolar cells [109]. These cells produce and secrete lung surfactant, composed mainly of dipalmitovl-phosphatidylcholine (DPPC). It has been suggested that E-FABP, due to its high affinity for palmitate, is instrumental in the formation of DPPC [110]. While the double knock-out mice were phenotypically normal, the uptake of exogenous palmitate was significantly reduced. Inside the cell, palmitate was incorporated preferentially into DPPC, and only at reduced levels into other phospholipids or neutral acylglycerides. It was demonstrated that the de novo formation of DPPC was reduced as well, but overcompensated by higher rates of re-acylation of other phospholipids. Interestingly, the loss of intracellular fatty acid-binding capacity due to the lack of both FABP genes normally expressed in alveolar cells was compensated by an unrelated protein capable of transporting fatty acids. Following the suggestion of Stremmel et al. [111] that fatty acids are transferred in the plasma membrane from translocators to calveolin-1, Guthmann et al. [109] demonstrated that the expression of this protein is increased in the double knock-out mice. Together with similar increases in the expression of the membrane transporter fatty acid translocase (FAT), which mediates fatty acid import, and the nuclear receptor PPAR, the overexpression of calveolin-1 appears to partially restore fatty acid signaling to the nucleus. Indeed, treatment of these cells with the selective PPAR γ agonist pioglitazone further increased the expression of calveolin-1, FAT, PPAR γ , and RXR α , which acts as heterodimer with PPAR in fatty acid-dependent gene regulation. The pioglitazone-treated cells from E-FABP^{-/-}/H-FABP^{-/-} mice were, in fact, phenotypically identical to wild-type TII cells, with similar palmitate uptake, oxidation, and incorporation into phospholipids [109]. Thus, it is apparent that various other components of fatty acid transport and signaling pathways are available to compensate for the loss of the FABPs in these cells.

3.6. B-FABP (Fabp7)

The occurrence of B-FABP in nervous tissue has been mentioned in earlier sections of this review. More specifically, B-FABP is expressed in various regions of the murine brain in the mid-

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term embryonic stage, but with progressing differentiation, B-FABP expression decreases. The protein is strongly expressed in radial glia cells of the developing brain, especially in the preperinatal stadium, but only weakly in mature glia of the white matter. Neurons of the gray matter do not express B-FABP, but H-FABP and E-FABP. B-FABP is distinguished from other members of the iLBP family by its strong affinity for n - 3 polyunsaturated fatty acids, in particular docosahexaenoic acid (DHA). As this very long-chain fatty acid is an important nutrient for nervous tissues, whose absence has been linked to severe disorders, it has been considered a natural ligand for B-FABP [112]. Pathologically, B-FABP is overexpressed in patients with Downs syndrome, and it has been suggested that this overexpression contributes to the associated neurological disorders [113]. Since B-FABP is found in nuclei of cultured radial glia [114], it appears likely that it mediates the action of its ligand(s) on gene expression, either indirectly or through interaction with a nuclear receptor; the latter has been reported for other members (see Sections 3.1, 3.4 and 3.5) of this protein family, but not yet for B-FABP.

Based on these observations, it appears that the ligand-binding properties of B-FABP are important for neurogenesis. On the other hand, addition of anti B-FABP antibodies to mixed cultures of glia and cerebellar neurons inhibited neuron migration, and B-FABP was found in the culture media [114]. These findings suggest that a growth inhibitory activity of B-FABP similar to that found before for H-FABP in the mammary gland influences the correct migration of developing neurons into cortical layers.

B-FABP (= MRG) is also prominently expressed in mammary tissue [48,50]. Overexpression of B-FABP in MDA-MB-231 human breast cancer cells resulted in growth inhibition [115] and significant morphological changes. Treatment with DHA resulted in a stronger growth inhibition for B-FABP overexpressing cells than for non-transfected control cells. Interestingly, DHA-induced growth inhibition was also observed when non-transfected cells were treated with B-FABP, indicating an extracellular role for this protein as well. To determine whether B-FABP can modulate the growth of breast cancer cells in vivo, Shi and co-workers [48] transfected human breast cancer cells with B-FABP cDNA and studied tumor proliferation in athymic nude mice. As previously observed for H-FABP, the overexpression of B-FABP clearly inhibited tumor growth in this model system.

These studies may point to a protective function of B-FABP against breast cancer proliferation, but other experiments also suggest a role in mammary gland differentiation. Overexpression of B-FABP (placed under the control of a strong promoter) in transgenic mice did not affect ductal development, but resulted in some developmental changes normally observed in early pregnancy, both morphologically and biochemically, as evident by the increased expression of the milk protein casein [115]. Thus, it appears that B-FABP may mediate the differentiating effects of pregnancy.

To date, no B-FABP knock-out model has been reported. However, given the similar growth inhibitory actions of B-FABP and H-FABP, it would not be surprising if the ablating of B-FABP alone has no overt effects on mammary growth and differentiation. It is likely that the expression of both proteins is interdependent, just as it was observed for L- and I-FABP in the intestine, and A-and E-FABP in adipocytes. Unfortunately, the expression of the other FABP-types was not measured in the transgenic mice overexpressing H-FABP or B-FABP, respectively. Definite insights into the biological roles of these two proteins in the differentiation and growth of brain structures or the mammary gland may only be possible from knock-out mice that lack both the

H-FABP and the B-FABP gene, and perhaps even E-FABP, the third FABP-type thus far found in these tissues.

4. Outlook

The genetic manipulations of FABP expression described in this paper and summarized in Table 2 helped to pinpoint certain essential cellular functions either to a specific FABP-type, or to an ensemble of FABP-types. Pertinent examples are the promotion of atherogenesis by A-FABP expressed in macrophages and the stimulation of lipolysis by A-FABP and other FABP-types in adipocytes, respectively. Thus, the extraordinary conservation and the maintenance of multiple types of FABPs with subtle differences in binding selectivities for fatty acids implies critical im-

Table 2 Transgenic and knock-out mice models used in FABP research

Mouse strain	Phenotype	Biochem./physiol. differences	Gene expression
L-FABP			
L-FABP ^{-/-}	Normal	Liver: FA uptake↓, TAG↓, cholesterol↑; fasting response↓	SCP-2↑, ACBP↑
SCP-X/SCP-2 ^{-/-}	Normal	TAG↓, cholesterol↓	L-FABP↑
HNF4 $\alpha^{-/-}$	Fatty livers		L-FABP↓
$PPAR\alpha^{-/-}$			L-FABP induction by PPAR agonists↓
I-FABP			
I-FABP ^{-/-}	Normal, enlarged liver (male)	Plasma insulin↑ TAG↑ (male)	None detected
H-FABP			
H-FABP ^{-/-}	Normal	Exercise intolerance, heart, skeletal muscle: fatty acid uptake↓	None detected
H-FABP ^{BLG-promoter}	Normal	No effects on mammary development	None detected
A-FABP			
A-FABP ^{-/-}	Normal	Obesity does not cause diabetes, adipocytes: lipolysis↓	E-FABP↑
		macrophages: cholesterol uptake↓	E-FABP unchanged
A-FABP ^{-/-} /Apo-E ^{-/-}	Normal	Cholesterol uptake, atherosclerotic lesions↓	E-FABP unchanged
E-FABP			
E-FABP ^{fabp4-promoter}	Normal	Adipocytes: lipolysis↑	A-FABP
E-FABP ^{-/-}	Normal	Adipocytes: insulin sensitivity↑	A-FABP
		transepidermal water loss↓ liver:	None detected H-FABP↑
E-FABP ^{-/-} /H-FABP ^{-/-}	Normal	Alveolar type II cells: palmitate uptake \downarrow , β -oxidation \downarrow , surfactant synthesis impaired	Calveolin-1↑, FAT↑, PPARγ↑

portance for cellular function. The studies in knock-out and transgenic animals have clearly demonstrated that the expression of the various FABP-types is in many cases interdependent. It can thus be assumed that grave functional consequences of the loss of a particular FABP are often prevented by the compensatory overexpression of another member of this gene family, or even of unrelated transport proteins. Although on first sight that may be interpreted as an argument against essential roles of this gene family, it is the conviction of the authors of this article that the opposite is true: FABPs as part of the iLBP family are so important that their loss cannot be tolerated; the numerous, at first glance 'redundant' members of this gene family provide not only a means for fine-tuning the expression of FABP-types in individual cells, but also present a safety mechanism in case any of the genes becomes non-functional. This concept is supported by observations in non-mammalian species. In teleost fish, where a whole-genome duplication has appeared approx. 200 million years ago, most, if not all members of the iLBP family appear to be still present in two isoforms. In the zebrafish, Danio rerio, Wright and co-workers have identified two isoforms each for H-FABP [116], B-FABP [117], CRABPI [118], and CRBPI and II [119], with slightly different expression patterns [120]. In contrast, no un-expressed pseudogene for any iLBP was found in the zebrafish genome. Evolutionary theory expects that duplicated genes are expressed and remain functional only if there is selection advantage. In the absence of differing binding characteristics or major changes in expression, it must be assumed that the presence of redundant copies of these genes serves as a safety mechanism against the loss of these vital proteins. On the other hand, very few members of the iLBP family have evolved in invertebrates. The Drosophila melanogaster [121] and Anopheles gambiae [122] genomes contains only one FABP-type, which is similar to that of the subfamilies I and IV seen in mammals (i.e., H-FABP and cellular retinoid-binding proteins) [5]. In all genetic screens reported on Flybase [123] only five alleles of this FABP gene were detected, and none of these affected the coding sequence or promoter region of the gene. Thus, it is very likely that any mutation of the protein itself would be lethal.

How, then, can contemporary genetic tools be used to get further insights into essential functions of the iLBPs? It seems to be necessary to remove all FABP-types expressed in a given tissue by cross-breeding individual knock-out strains, to assess the true consequence of the lack of intracellular fatty acid transporters. The creation of such multiple knock-out models, currently in progress in several laboratories, may achieve this.

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