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1 Historical overview of studies on fatty acid binding proteins

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Abstract

Fatty acid binding proteins (FABPs) were first described in the 1970's by diverse groups whose studies ranged from cellular binding-uptake of hydrophobic ligands to aspects of neurological diseases. Soon roles for fatty acid (FA) solubilisation, protection from chemical damage and FA delivery to the correct destination became apparent. Each FABP was suggested to have structural features enabling them to specifically collect and deposit their ligands interacting directly with cellular components. The search for these structural features involved the examination of cellular structures with appropriate

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'sticky fingers' that could provide the keys for specificity. In this sense seminal contributions were the determination of FABP amino acid sequences together with the use of recombinant DNA technologies. Identification of genes encoding adipocyte and hepatocyte FABPs, aP2 and L-FABP, revealed numerous putative cis-acting sequences indicating an intricate regulatory system for expression that continues to be studied intensively. Methods for measuring binding constants were standardized for fluorescent-labelled fatty acids and analogues. Two distinct mechanisms for fatty acid uptake/localization by FABPs were proposed. Binding properties, uptake mechanisms and gene expression studies supported the view that different members of a gene family had cell/tissue specific functions. Putative functions were then proposed for the most members of the family under study. A role for dietary fatty acid absorption was proposed for the intestine FABP, I-FABP, and functions related to cardiac β-oxidation and peroxisome b-oxidation were found for heart FABP, H-FABP and liver FABP, L-FABP, respectively. An important role in cell differentiation was attributed to aP2, underscoring the central roles FABPs can play in cell physiology. As we entered the 21st century, a major role for these proteins to facilitate entry and subsequent intracellular transport and compartmentalization of cell lipids was firmly established. Despite the long road on the paths of FABPs the precise role of the members of this protein family is still an open question. The aim of this chapter is to provide an overview of the initial studies that led to a now extensive network of research in which FABPs are involved. A summary of the early findings by investigators who first initiated the study of these proteins is provided, some of whom continue to carry the flag, highlighting when significant leaps in understanding were made.

Introduction

Fatty acid binding proteins (FABPs) constitute an important protein family, with its members exhibiting great diversity in physicochemical properties as well as at the level of primary structure. Remarkably, in spite of the diversity of protein sequences a highly conserved tertiary structure is found suggesting that FABPs have shared common ancestry (see chapter 2). The FABP family is included in the Calycins superfamily [1], with a minimum of nine members found as small intracellular proteins in a wide variety of tissues in both vertebrate [2] and invertebrate animals [3]. Although a FABP-like protein has been found in bacteria [4], proteins related to FABPs have not been reported in plants. Family members are named by the predominant cell type in which they were first identified and or are predominantly expressed, *i.e.*, ones expressed in the liver are named liver

fatty acid binding proteins (L-FABP), those expressed in intestine are named intestinal fatty acid binding proteins (I-LFAB), *etc*. The corresponding genes are named with a numerical nomenclature, *i.e*, *fabp1*, *fabp2*, *etc.* [2]. They are characterized by high-affinity (non-covalent) binding of hydrophobic ligands, have a mass of 14-15 kDa and form a characteristic β-barrel structure. A typical FABP consists of 127-134 amino acid residues with 10 β-strands folded into a barrel capped by two α helices. The ligand binding site, *e.g.*, for a fatty acid (FA), is within the large water-filled interior with a large number of polar and charged amino acid *R* groups on the inner surface [5]. FABPs share between 20 - 70% sequence identity between family members within a species, whereas the same FABP type may share greater than 95% identity from different vertebrate species [3]. Many proteins in the family have been shown to be of importance in clinical medicine (see chapter 4).

The objective of the present chapter is to walk through the beginnings of the history of the study of FABPs, and to render homage to those researchers who devoted their careers to unravelling the intricacies of FABP structure-function. With this objective, I will highlight historically relevant findings that changed the course of investigation of FABP structure-function. The current state of knowledge of the physiological and clinical aspects of vertebrate FABPs, and a discussion of their evolutionary history will be reviewed in other chapters of this book. Finally, chapters on insect FABPs and a discussion of on going efforts to develop FABP-based vaccines to control helmith diseases are also included.

The discovery

FABPs were first introduced to the scientific community almost 50 years ago by different research groups who sought an explanation for various fundamental questions, ultimately then to converge on the properties and functions of the FABP family. One group studied hepatic uptake, storage, conjugation, and excretion of bilirubin. They observed that after the injection of physiologic amounts of tritium-labeled unconjugated bilirubin, radioactivity was found principally in the hepatic cell cytosol at rates and concentrations that were unexpectedly high for a mechanism involving albumin-linked transfer [6-9]. The involvement of protein in hepatic cell bilirubin localization, with immunologic and electrophoretic properties different from albumin, had been suggested previously. Levi *et al*. (1969) [8] suggested that the hepatic localization of injected bilirubin was dependent on cytoplasmic protein fractions isolated from hepatocyte homogenates, designated as fractions “Y” and “Z”. These protein fractions were also able to

bind various organic anions including: bilirubin, sulfobromophtalein, indocyanine green and Evans blue.

Three years later, the identification of cytoplasmic protein with fatty acid binding affinity, and designated FABP, was reported thus providing a basic model to explain translocation of fatty acids from cell surface to other intracellular sites [10-11]. The protein fraction reported as FABP by these workers had properties that were virtually identical to the protein fraction designated “Z” by Levi and co-workers. Similar proteins were subsequently isolated from jejunum, intestinal mucosa, liver, myocardium, adipose tissue and rat kidney. The estimated apparent molecular weight was 12,000 kDa according to elution characteristics by gel filtration using cytochrome C as reference. These studies indicated the presence of FABP in the cytoplasm, and raised the possibility that they might also be associated with the plasma membrane to function in fatty acid uptake. In addition, although precise measurements for determining FA binding affinity/stoichiometry were needed, it was clear from these early studies that these parameters would be greatly affected by FA length and degree of saturation thereby indicating steric and hydrophobic constraints on FA-FABP interactions. It was further suggested that changes either in the availability of fatty acids or in fatty acid requirements of specific tissues might be among the determining factors in which FABPs play a role. These early workers also considered that FABPs would fulfil functions other than cytosolic transport, *e.g.*, the regulation of intracellular metabolism of fatty acids. Also in the 1970’s, studies on allergic neuritis resulted in the focus of research on another member of the FABP family. The protein was first isolated from both peripheral nerve myelin and spinal cord myelin from rabbit and bovine sources, and referred as the “P2 protein” [12-15].

At present, members of the protein family are named according to the tissue from which they were first identified and found to be predominantly expressed (liver, intestine, heart/muscle, adipocyte, epidermis, ileum, brain, testis and myelin): L-FABP; I-FABP, H-FABP, A-FABP, E-FABP, Il-FABP, B-FABP, T-FABP, and My-FABP. Many FABPs are found expressed in several tissues. FABPs have also been identified in such diverse organisms as chicken, fishes, fruit fly and worms [16-24]. A FABP-like was also reported in bacteria as noted above [4].

From sequence to structure

Important contributions for the advances in FABP research came in the 1980s. During these years, the amino acid sequence of one member of this protein family, P2 myelin, was determined [25], and the first reports on

FABP structural properties were published [26-28]. The amino acid sequence of the P2 protein of peripheral myelin was analyzed for regions of probable α-helix, β-structure, β-turn, and unordered conformation using several algorithms commonly used to predict secondary structure in proteins. A high β-sheet content and virtual absence of α-helix in P2 was shown by circular dichroism spectroscopy. The general model of the P2 protein that was first reported was that of a "Greek key" β-barrel, consisting of eight anti-parallel β-strands with a two-stranded ribbon of antiparallel β-structure emerging from one end. This model had an uncharged, hydrophobic core and a highly hydrophilic surface [28].

Additional FABP sequence data from other species [29] and from new FABP family members were subsequently obtained [30-31]. Significant advances in the area were achieved using molecular cloning/engineering methods. Gordon and co-workers were first able to clone a full-length cDNA from processed *fabp* mRNA in 1983 [32]. The availability of full-length cloned FABP cDNAs, introduced into an appropriate expression vector, provided a powerful tool for analysing the molecular mechanism of fatty acid-protein interaction in providing the ability to specifically alter the coding region. Recombinant rat L-FABP expression and purification was first reported in 1984, by Lowe and co-workers [33].

Subsequently, in a short time accurate structural data was obtained and used to further refine our understanding of the FA-FABP interaction in relation to the specific roles FABPs play in intracellular lipid transport and compartmentalization. European and U.S. research groups had been trying to obtain crystals of FABPs from different sources for subsequent x-ray structure analysis. Alter obtaining crystals of FABPs from either recombinant I-FABP or bovine L-FABP, preliminary X-ray data was collected [34-35], but only low resolution spectra were obtained. After clarification of difficulties, however, a cartoon diagram was soon published, showing the remarkable structural properties of FABPs: ten anti-paralell β-strands joined by Gly and Asn containing sharp turns, and two α-helices between the first and second strand. The FA binding site was located in a large water-filled cavity whose surface was composed of a large number of polar and charged amino acid *R* groups. This conformation was defined as a “clam shell-like” structure [36]. At the same time, the structure of the other known member of the family, P2 myelin, was also solved [37]. This structure was obtained using the native protein, isolated from bovine intradural spinal root, and fully supported the structure obtained with the recombinant protein derived from I-FABP. Refined structures later published, confirmed these results and indicated that this structure was basically conserved between all identified

FABPs [36-44]. Since that time, structural data relevant to FABPs has increased enormously, particularly with the sequencing of several vertebrate and invertebrate genomes. In addition, the structure of the FABP-like protein of bacterial origin was recently solved indicating a long evolutionary history for the FABP family. At this time, hundreds of sequences and structures of FABPs from different family members and diverse animals are deposited in public databases.

Gene structure and regulation of expression

L-FABP and aP2 were the first two members of this family whose genomic organization was defined [45-46]. The L-FABP transcription unit spans 3790 nucleotides and contains four exons interrupted by three introns [45]. This organization is the same for all vertebrate *fabp* genes examined [47]. A different organization was found in invertebrates, here the position of the intron is conserved but not the number [3]. Similarity of the exon structures was found in terms of size and terminal amino acids, however, it should be recalled that these proteins share only 23% overall amino acid identity [46]. It should be noted that the amino acid sequences present in the first exon of the L-FABP gene (*fabp1*) were found to be highly similar to the same region in other FABPs, however, it not currently known what potential role these conserved exonic sequences may have in FABP structure or function.

Investigation of the regulation of FABP gene expression was a primary focus for understanding FABP function. A number of conserved DNA sequences that might serve as *cis* regulatory elements for FABP gene expression were identified in genomic sequences adjacent to FABP genes. Sequences have been found in *cis* to FABP genes predicted to interact with transcriptional regulatory factors responsive to, *e.g.*, fatty acids and dexamethasone [48-50], glucocorticoids and as well cAMP for regulation of the aP2 gene (*fabp4*) [51-52]. In addition, putative binding sites for c-jun/c-fos, C/EBP [53] and Ap-1 transcription factors were found that likely regulate the mouse myelin P2 gene (*MP2*) [54]. A putative regulatory *cis*-acting sequence termed ARF6 [55-56], was later shown to be the target of a heterodimeric complex of mPPARγ2 and RXR [57], for regulation of *fabp1* gene [58]. Peroxisome-activated proliferators and hepatocyte nuclear factor, 1 α(HNF1alfa), were shown to act in *fabp1* promoter activation [59-60]. Finally, the *in vitro* induction of the *fabp4* by insulin-like growth factor I [61] was shown to involve the AP-2 like transcription factor in the regulation of the chicken R-FABP gene [62] during development. A *cis*-acting region for myocyte enhancer-binding factor, CarG-like of H-FABP [63], was found

more recently. Currently there is evidence supporting the hypothesis that transfer and channelling of long chain fatty acids to PPARα in the nucleus is mediated by L-FABP [64].

Towards understanding FABP function

The functional roles of FABPs have intrigued scientists since their discovery, and despite the advances made, a comprehensive understanding of FABP function is still far from complete. First reports concerning putative function of liver and intestinal FABPs were developed by Arias and co-workers [7, 9, 65-66] and Ockner and co-workers [10-11, 67-69]. Papers collectively discussing diverse functional aspects were then published, some of which will be mentioned below.

Ligand binding properties

Stoichiometry and binding kinetics were determined for different purified FABPs with presumptive *in vivo* ligands, binding sites were first characterized and comparisons were made of binding affinities for different ligands in an effort to better understand function. A group of quantitative methods were developed to accomplish this, the most important of which was for the accurate determination of concentrations of bound and unbound FA. Measurements of equilibrium binding constants were first done using methods that physically separated free FA from FABP-bound FA. In 1972, Ockner and co-workers examined, *e.g.*, binding affinities of radio-labelled FAs of different lengths and degrees of saturation, as well as their affinities for neutral lipids. Partially purified FABP from rat jejunum was added to each of various radioactive ligands and percent binding was determined after gel filtration using Sephadex G-75. These initial measurements showed that binding affinity was related to both length and degree of saturation of the FA to suggest that binding interactions were controlled by hydrophobic and steric properties of the ligand. Other experiments suggested that negative charges in the binding pocket were neither necessary nor sufficient for binding [10]. A radiochemical assay for FABP:FA binding was subsequently developed that made use of a long-chain hydrophobic alkyl ether matrix of Sephadex by Glatz and Veerkamp in 1983 [70]. In this assay, the FA ligand-bound protein and ligand-free protein were easily separated by selective binding of the latter to the matrix. The matrix, commercially known as Lipidex-1000, was initially developed by Ellingboe *et al.*, for the separation of hydrophobic compounds in the 1970's [71].

Consideration was given to the possibility that the equilibrium measurements themselves might influence the measurement, since

separation of the components should influence equilibrium. To address this problem in a general way, spectroscopic methods that distinguish between the bound and free states in solution were developed, and in which a fractionation or separation step was omitted. Various fluorescent probes were then assayed for use and compatibility with these equilibrium binding measurements. One of the most useful of these fluorescent probes was developed by Richieri and co-workers in 1992. They developed a method to directly measure FABP:FA using fluorescent-modified I-FABP that was labelled with the polarity-sensitive acrylodan, a fluorophore unusual sensitive to small changes in polarity [72]. This compound was selected because of its unusual sensitivity to small changes in polarity. The probe was licensed as ADIFAB by Molecular Probes Inc. (US 4.952.496 and US 5.470.714). This probe provided quantitave monitoring of free fatty acids without resorting to separationve biochemical methods. Anthroyloxy-fatty acids [73-75] and dansyl undecanoic acid (DAUDA) [76-77] were also used to measure FABPs binding properties. DAUDA was introduced by Wilkinson and Wilton as a useful fluorescent probe for the detection and estimation of bound FABPs in cell-free extracts prepared from fresh tissue. The probe binds FABPs with high-affinity and has a considerable fluorescence enhancement allowing for detection at the level of 1 μg/ml. Anthroyloxy-palmitic acid was also used by Spener and co-workers to locate and identify FABPs intracellularly as well as for determination of oleic acid/FABP binding ratios [73]. Using this method, they reported a single binding site for fatty acids for a single FABP molecule. The binding affinities of different types of FABPs and fluorescent anthroyloxy-fatty acids were also studied by the Veerkamp group [78-79].

To avoid the influence of the bulky fluorescent side-chains on the binding affinity of fatty acids by FABPs, Nemecz and co-workers used the naturally occurring polyunsaturated fatty acid, parinaric acid [80]. Parinaric acid was initially used as a membrane probe by Hudson and co-workers [81]. Cis-parinaric acid was found to be the closest structural analog of any known intrinsic membrane lipid and in addition had several experimentally useful optical properties. These properties included a very large fluorescent Strokes shift, a long fluorescence lifetime enabling very detailed measurements to be made and an almost complete lack of fluorescence when in water [82-83]. Both fluorescent anthroyloxy-fatty acids and *cis*-parinaric acid have been used extensively for FABP-FA binding and kinetics determinations.

Fatty acids uptake and transfer

Despite much evidence that cellular FA uptake occurs by a passive mechanism, active transport of FAs was also suggested from the early

experiments. Looking back in perspective at the numerous studies indicating that uptake is most likely a passive process [65, 83-84], the postulated existence of specific receptor sites for fatty acids in the plasma membrane was not discarded from the beginning [67]. The fact that after uptake, FA rapidly leaves the plasma membrane to enter the cytosol, suggested the existence of some unknown facilitating mechanism. FAs strongly bound to plasma membrane fractions were found to readily dissociate into the aqueous cytosol, inferring the presence of a soluble acceptor with high-affinity for fatty acids. FABPs which were already known at this time, remained as the most likely candidates for the function of facilitating the removal of FA from the cell membrane inner surface, for passage of the FA to the appropriate intracellular destination [67].

In 1984 a simple experimental system was developed by Brecher *et al.* for studying the movement of long-chain FAs between multi-lamellar liposomes and soluble proteins capable of binding fatty acids. This system served to verify the rapid and selective transfer of FA from liposome to hepatic FABP, and further suggested that FA could be transported quite readily between and across phospholipid bilayers [86]. The movement of free FAs between small uni-lamellar vesicles and the mechanism by which FABPs might be involved in intracellular FAs traffic, was studied by Storch and co-workers. Their approach was to specifically focus on the transfer mechanism itself [87-90] by measuring the transfer of FAs using some of fluorescent probes discussed previously (*e.g.*, n-9-anthroyloxy-labeled fatty acid analogues). Storch and coworkers proposed two distinct mechanisms for free FA transfer from FABPs to artificial membranes: the “collisional” [91] and the “diffusional” mechanisms [92]. The collisional transfer mechanism proposed a direct interaction between a FABP and a membrane to in turn transfer the FA from the protein directly to the membrane. The diffusional transfer mechanism, on the other hand makes an assumption that the aqueous phase serves as part of the route of transfer of FA from FABP to the acceptor membrane [91]. It was shown that the H-FABPs and I-FABPs use a mechanism consistent with the collisional transfer model, while L-FABPs use the alternative diffusional mechanism. The careful development of these studies allowed for establishing, for the first time, an important distinction between different FABPs at a functional level.

Tissue expression

Tissue expression levels rat H-FABP, I-FABP and L-FABP were examined as another strategy for developing a better understanding of the functional role of FABPs. Distinctive patterns of expression for these three

FABP types were found, further supporting the idea that individual types are responsible for distinct, specific functions in FA transport and metabolism. Gene expression of the heart type was found to be widely distributed within an individual, as expression was found localized in heart, stomach, muscle, testis, ovary, kidney, brain, and adipose tissue of the vertebrates studied. Additional distinct patterns of expression were found for both the liver type, in liver, small and large intestinal epithelium and adipose tissue, and for the intestinal type whose expression was localized to the small and large intestinal epithelium and the stomach [92]. Similar results were found when examining FABPs expression patterns in other vertebrate organisms as discussed by Paulussen *et al*.[93].

The intestinal and liver FABPs were models for the study of the functional roles FABPs might play in lipid metabolism. Evidence for a physiological function for I-FABP in the intestine was first documented by Ockner and Manning, in 1974 [64]. Their study showed that diet influences I-FABP levels, and as well that anatomical localization of *fabp2* expression was consistent with a role for maximal lipid absorption in the grastro-intestinal tract. *In vivo* binding of FA to FABP was also found localized in association with intestinal *villi* consistent with a proposed absorptive function. These studies indicated a role in FA transport through the cytosol to sites of metabolic processing as well as in compartmentalization following adsorption [67]. Subsequent work using immuno-cytochemical localization methods for intestinal mucosa supported this since the I-FABP was found concentrated in apical portions of the mature *villus* cells, thereby unifying a consistent model proposed from a number of studies [94].

The first demonstration that I-FABP interacts or binds with dietary long-chain fatty acids as part of the mechanism for in their intracellular transport was reported by the Sacchettini group, who employed a useful *in vitro* model for analysis of lipid trafficking, *i.e.*, the Caco-2 cell line [95]. These cells act to mimic small intestinal epithelium, do not express I-FABP endogenously and are capable of absorbing long-chain fatty acids as well as having the ability to secrete chylomicrons, [96-97]. The endocytes of the Caco-2 cell line, now widely used, are also useful for studying FA traffic involving FABPs, since lipid metabolism is highly localized in this cell type.

The liver FABP, first known as Z protein (discussed above), was the object of numerous studies on its functional role, during the decade following its discovery [98-101]. Studies with L-FABP soon revealed dramatic differences in FABP function when compared with those of other FABP types. L-FABP was unique in having two distinct ligand-binding sites within its cavity and in being able to bind different kinds of ligands simultaneously [102]. L-FABP also exhibits both cytoplasmic and nuclear localization

[103-104]. *Fapb1* was found to be expressed in both liver and intestine, and with the complexities of hepatic lipid metabolism, L-FABP is currently believed to be the most functionally diverse FABP family member.

A proposal was made in which the non-mitochondrial β-oxidation fatty acid oxidation pathway (*i.e.*, peroxisome β-oxidation) involved *L-FABP* since the expression of the gene was localized to peroxisomes. There was an observed increase in *L-FABP* levels inside peroxisomes after clofibrate (an hypo-lipidemic drug that induces peroxisomes proliferation) induction [101, 105-107]. These initial observations were subsequently extended with additional inducers including several peroxisome proliferators. Taken together these results a mechanism of action of hypo-lipidaemic drugs was additionally proposed to be mediated, in part, by interaction of these agents with L-FABP [59]. Soon a link between L-FABP and peroxisomal β-oxidation was clarified with the observation that FABPs may bind ligands that were quite reminiscent of peroxisome proliferator-activated receptors (PPARs) agonists and were in fact induced by peroxisome proliferatiors. It is worth noting that PPARs are members of the orphan family of nuclear receptors, and were well-known to be involved in fatty acid-dependent regulation of gene expression. Issemann *et al*., postulated a model in which L-FABP binds FA in the cytosol for specific targeting of the FA to the nucleus, where it interacts with PPAR to affect transcription [108].

A quite different functional role was proposed for heart FABP in consideration of typically low levels of FA synthesis by the heart (about 0.1% of the total body synthesis). A highly effective FA transport (uptake) system was thought to function for supply of this nutrient in that translocation of FA from the circulatory system to a myocyte mitochondrial matrix might require passage across eight successive hydrophobic-hydrophilic barriers. H-FABP was proposed to act as an FA-carrier in this multi-step translocation system, and the idea was supported by myocyte mitochondrial localization of the protein. The authors of this model proposed that H-FABP acts as the carrier controlling the flux of acylcarnitine entering heart mitochondria and accordingly controlling, indirectly but very efficiently, the catabolic system which transports and then utilizes FA by β-oxidation [109-111].

Involvement of FABPs in cell differentiation processes were found for the adipocyte binding protein, also known as aP2 or LBP. Studies on the role of aP2 in adipocyte differentiation were initiated by Spiegelman and co-workers who investigated 3T3-L1 cells, which resemble adipocytes during development. As a useful model for study of the mechanisms of cell differentiation at molecular level, they proposed that aP2 expression might be specifically associated with adipocyte differentiation. RNA blotting

experiments showed an increased level of gene expression linked with cell development [112]. Transfection experiments with the entire gene also showed an up-regulation of *fabp4* during this process [46].

FABPS at the end of XX century

Upon entering the 1990's, it was clear based on DNA and protein sequence relationships that the FABPs of mammalian origin were divided into at least the three easily distinguished types: namely the hepatic, intestinal and cardiac FABPs, and the then new adipocyte form. Evidence supported the concept that the major role of these proteins was to facilitate the entry of lipids into cells and/or their subsequent intracellular transport and compartmentalization in which specific functions were suggested of each type. Much work has been done since that time for defining specific as well as general structure-function relationships for FABPs. Dissociation constants for FABP:FA complexes were found to be in the range of 1 microM to 1:1 stoichiometries, with the exception of the hepatic FABP that binds 2 fatty acids. Concomitantly, ligand specificities were found to vary widely for the different FABPs types. At the subcellular level, hepatic- and cardiac-type FABPs were found differentially distributed. The intracellular sites at which FABPs were found in some cases involved the use of immunoelectron microscopy together with a gel chromatography-immunofluorescence assay. In such studies an association of hepatic FABP was demonstrated with microsomal fractions, mitochondrial outer membranes and nuclei, while cardiac FABP was localized/confined to the mitochondrial matrix and to nuclei [113]. Tissue specific and specific intracellular localization of FABPs provided important basic information on studies related to their own function, and as well in dissection of the molecular organization of cell. Emerging areas involve the use of transgenic mice as tools to extend our understanding of lipid metabolism at the cellular level to the systems level *in vivo* [114-116]. Numerous recent studies continue to reveal functional roles for FABPs that are both diverse, very specific and of central importance for processes ranging from metabolic homeostasis to cell development. Our current understanding of the functional roles of FABPs remain incomplete, which may only further underscore a highly complex evolutionary history in the interaction of protein with lipid as biological molecules.

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