Title: Technical Cooperation for the Development of Therapeutic Strategies to Control Helminth Infections of Global Importance.

Final Technical Report

Introduction

Parasitic helminths cause serious and difficult to treat diseases in humans, animals and plants. A high percentage of the earth's population, mainly in developing countries, suffers from helminth infections, most of them categorized as neglected diseases, and in Latin America almost 30% of the population is infected. Due to the scarcity of anthelmintic drugs available and the emergence of resistant parasites, the discovery of new anthelmintic drugs is mandatory. Identifying essential genes of pathogens is an important step to discover putative drug targets. With the recently available genomes of several helminth species, this can be achieved by identifying essential genes that are absent in its host (Doyle, 2010). **Proteins implicated in lipid metabolism are potential targets for anthelmintic drugs.** Helminth parasites have a restricted lipid metabolism (**Tsai, 2013**), and must acquire simple and complex lipids from their hosts for energy metabolism, membrane construction, and lipid-based signaling, the latter possibly also encompassing modifications of the host's immune and inflammatory defense systems.

Due to their poor solubility in aqueous environments, lipids must be transported between and within the cell associated with lipid binding proteins (LBP). **Helminths produce and release an unexpectedly wide range of novel LBP types structurally distinct from those of their hosts**. Some of the hypothetical roles of helminth LBPs are internal functions typical of all multicellular organisms; specialized external functions, including acquisition and distribution of lipids; and/or modulation of the host's local tissue environment, and it's innate and acquired immune systems. Lipid transporter proteins may also be important in the targeting of anthelmintic drugs; most anthelmintics are hydrophobic and may require a parasite's own carrier proteins for distribution to their site of action. Taken together, this indicates that LBPs may be good targets for chemotherapy and enhancers of drug availability.

Although mammalian LBPs have been described more than three decades ago (**Falomir, 2013**), little information is available for helminth LBPs and their biological functions (**Franchini, 2013**). Recent information has emerged from knock-down related models in *C.elegans*. Specifically, mutation and/or RNAi of some LBPs (in particular lbp-5 and npa-1) cause strong physiological disruptions such as metabolic disease, lethality and aberrant phenotypes (Xu, 2011; Curran, 2007). From the structural point of view, some helminth LBPs are known to be similar to the mammalian family of cytosolic fatty acid binding proteins (FABPs) (**Porfido, 2012**), but appear to have structural modifications that are unique to nematodes (nemFABPs) (**Ibañez-Shimabukuro, 2012; Gabrielsen, 2012)**. Other LBPs are completely unique to helminths and have no relationship with mammalian LBPs and can be potential targets for vaccines or drugs that will not be host-reactive. Examples of these LBPs are a family of nematode helix-rich LBPs, of approximately 20 kDa,

the Fatty acid and retinol-binding proteins (FAR) (**Gabrielsen, 2012; Rey-Burusco, 2012; Rey-Burusco, 2015**). Other examples are the polyprotein allergens/antigens of nematodes (NPAs) which are small, helix-rich LBPs confined to nematodes (Meenan, 2011), and Antigen B (AgB) a class of LBP found only in cestodes (**Silva Alvarez, 2015a,b)**. The availability of culture model systems for cestodes (**Prada, 2008; 2009**, Brehm, 2010; **Cucher, 2011; 2013; Cucher, 2012; Markoski, 2006; Laschuk, 2011**), and RNA interference methodology (Spiliotis, 2011; Mizukami, 2011), will enable a better understanding of these proteins functions and also of the host-parasite relationship.

The aim of this work was to contribute to the understanding of the molecular basis of LBPs functions in the parasite-host relationship and to evaluate them as potential anthelmintic drug carriers and/or targets. Knowing the structures and functions of the unusual proteins produced and released by helminths, as well as the host-parasite interactions that they may mediate, is clearly pertinent for preventing and treating helminth infections, and for the ultimate amelioration and prevention of the neglected diseases they cause. In addition, in the search for new target candidates for drug development, we have identified and analyzed the expression of microRNAs from cestodes. MicroRNAs are small regulatory non coding RNAs that exert critical functions in eukaryotic cells and are being tested as new chemotherapeutic targets against several diseases, including infectious diseases.

This project was run synergistically between groups from Argentina (Betina Córsico, Mara Rosenzvit and Marcelo Costabel), Uruguay (Ana M. Ferreira) and Brazil (Arnaldo Zaha and Henrique Ferreira) in order to integrate complementary expertise and for technical cooperation.

Specific aims

Aim 1. What are the structures of these novel proteins?
Aim 2. How do helminth LBPs interact with lipids?
Aim 3. Which are the cellular functions of helminth LBPs?
Aim 4. Can parasite LBPs be potential targets for chemotherapy and enhancers of drug assimilation?
Aim 5. Can new anthelmintic drug target candidates and inhibitory compounds be predicted and validated?

Proteins selected for analysis.
We have selected a group of different LBPs expressed by parasites of importance in our region and of global impact. The following proteins were selected:
FAR (*Necator americanus*), NPA (*Ascaris suum*), nemFABP (*A. suum*), AgB (*Echinococcus granulosus*), FABP (*E. granulosus, E. multilocularis, Taenia crassiceps*) (**Figure 1**).

For the sake of clarity, this report is divided in sections that correspond to each protein. In each section, the work performed for each protein is analyzed.

Note: cites of papers published by our groups are highlighted in bold letters.

Results

Fatty acid binding proteins (FABPs) from cestodes.

Fatty acid binding proteins (FABPs) are small intracellular proteins that reversibly bind fatty acids and other hydrophobic ligands. Usually, they are highly expressed in cells with very active lipid metabolism. In mammals, the family includes ten FABPs as well as cellular retinoid binding proteins. Many proteins of the family have later been described in nematodes, insects, molluscs and flatworms. In *Echinococcus granulosus* two FABPs had been described: EgFABP1 and EgFABP2 (Esteves, 1993, Esteves, 2003). It is worth mentioning that EgFABP1 is the only protein from cestodes whose structure has been experimentally determined, which clearly matches with the typical structure of FABPs (Jakobsson, 2003). In previous works from our laboratory, in order to gain insight into this protein´s function, we have analyzed the interaction of recombinant EgFABP1 with lipids, employing different approaches (**Porfido, 2012 and Porfido Theis, 2015**). We first found that recombinant EgFABP1 expressed in *E. coli* BL21 is loaded with fatty acids from the synthesising bacteria, and that fatty acid binding increases its resistance to proteinases, possibly due to subtle conformational changes induced on EgFABP1. By manipulating the composition of lipid vesicles and the ionic environment, we found that EgFABP1 interacts with membranes in a direct contact, collisional manner to exchange ligands, involving both ionic and hydrophobic interactions. Moreover, we observed that the protein can compete with cytochrome c for association with the surface of artificial membranes. The results suggest that this protein may be actively involved in the exchange and transport of fatty acids between different membranes and cellular compartments within the parasite. Interaction with host tissue is not excluded since members of this family of protein have been found in *E. multilocularis* and *E. granulosus* hydatid fluid (HF) (**Monteiro, 2017; dos Santos GB, 2016**). The HF that fills the hydatid cyst contains complex repertoires of parasite Excretion/Secretion (ES) products and host proteins that mediate important molecular interactions determinant for parasite survival and development, and, consequently, to the infection outcome. HF has been also extensively reported as the main source of proteins for the immunodiagnosis of echinococcosis.

What are the structures of these novel proteins?

The genomes of *E. multilocularis*, *E. granulosus* and *T. solium* (**Tsai et al, 2013**) were analyzed employing bioinformatic tools (BLASTN, BLASTP, TBLASTN and BLASTX hosted in the sequence alignment editor BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). *E. multilocularis*, *E. granulosus* and *T. solium* present at least 5 isoforms of FABPs in their genomes (**Figure 2**). At gene level, the newly described genes have certain variations in the structure, compared with previously described FABP genes in *Echinococcus* spp. In addition, one of the coding sequences encodes a FABP with a C-terminal extension unusual for this family. It is worth noticing that the coding sequences for *E. multilocularis* have been validated by cloning techniques (**Porfido, submitted and Porfido Thesis, 2015**).

Secondary structure predictions were performed on *E. multilocularis* amino acid sequence employing *PSIPRED* tool (http://bioinf.cs.ucl.ac.uk/psipred/). All the analysed sequences showed a predicted structure of ten β-strands with two α-helices between the first two strands, in good accordance to the typical arrangement of secondary structure elements in FABPs. In EmFABP4, the most striking feature is that the predicted sequence is much longer (176 amino acids) than what is expected for a FABP (around 130 amino acids). In this case, a typical FABP fold is predicted but no specific structure is assigned to the C terminus of the protein. Whether EmFABP4 is translated and conserves all the predicted amino acids is still unknown. If that was the case, it may constitute a new FABP variant in which the C terminus' fold and function should be addressed. When aligning and comparing the FABPs from *E. multilocularis* and *E. granulosus*, the closeness of these two organisms can be clearly observed (**Figure 3**). Two FABPs, FABP2 and FABP3, have exactly the same amino acid sequence in both cestodes species. Whereas FABP1, FABP4 and FABP5, have similarities that are above 97% (**Porfido Thesis, 2015; Porfido, submitted**). This situation exemplifies, once again, what has been observed in other organisms. It has been stated that FABPs from the same tissue of different species show greater amino acid similarity than FABPs isolated from different tissues of the same organism. Although that was reported for mammal FABPs, it seems likely to be the case for cestodes as well (despite the fact that FABPs' tissue distribution has not been studied in these animals yet).

Which are the cellular functions of helminth LBPs?

a) Localization studies*: Localization studies are one approach to analyze cellular functions of a protein. Immunohistochemical analysis for *E. multilocularis* FABPs was performed on sections from *in vitro* cultured metacestode vesicles. The staining showed that FABPs would be mainly localized in the innermost cells of the metacestode (**Figure 4**), in contact with the HF, where the protein could be involved in lipid exchange between the cyst cells and HF. However, as the antibody is polyclonal, it is not possible, at this point, to indicate which of the FABPs were detected. This problem will probably be solved by the development of antibodies with specificities for each FABP. Work is in progress in this sense; we are also working on the localization of FABPs in other tissue samples of different cestodes.

b) Analysis of expression of FABPs in different stages of *E. multilocularis**. Information obtained from transcriptomic data published by **Tsai et al., 2013**, as well as RNAseq material available at Huang F. et al., 2016, both suggest differential expression of different FABPs in different *E. multilocularis* stages (**Figure 5**).

Differential expression of the different FABPs in different stages of *E. multilocularis* was also analyzed employing semi-quantitative PCR. cDNA obtained from three different sources: metacestodes, activated and non-activated protoscoleces were analyzed. Elp, a constitutively expressed protein from *E. multilocularis*, was used as control (**Figure 6**). According to these results, emfabp1 and emfabp5 seem to be equally transcribed in the three analyzed stages, whereas emfabp2 seems to be more highly transcribed in metacestodes, and emfabp4 in protoscolices, both activated and non-activated.

At the protein level, our collaborators in Uruguay, have obtained evidence that indicates that, together with EgFABP1, EgFABP3 would be expressed in protoscoleces of *E. granulosus* (Personal communication, BSc. Maite Folle, UdelaR, Montevideo, Uruguay).
This differential transcription, and its subsequent translation, could suggest different functions for each FABP. The stage specific transcription pattern of some of the FABPs could, eventually, allow using them as developmental markers.
c) Silencing LBPs expression employing RNA interference methodology*. In order to perform the silencing experiments, axenic (i.e., host cell-free) primary cell cultures were carried out employing the protocols published (Spiliotis & Brehm, 2009). Samples were electroporated either with anti-gfp, anti-emfabp1 or anti-emfabp2 siRNA (α-GFP, α-FABP1, α-FABP2, respectively). A mock control was performed by electroporating cells without any siRNA, and samples without electroporation were also included in the experiment. The Western blott was developed with a polyclonal antibody raised in our laboratory against recombinant EgFABP1, which detects *E. multilocularis'* FABPs as well. (**Figure 7**). Although no clear differences were observed, it was interesting to see that FABPs expression seems to be increased after electroporation (mock sample compared to non electroporated sample). The lack of silencing could be due to many factors, being the very high expression of these proteins, as well as the many homologous genes, two of the most probable ones. Alternative strategies for silencing are considered as well.
*****These experiments were performed during a short stay of Jorge Porfido from our laboratory at our collaborator´s, **Dr. Klaus Brehm,** laboratory in Wurtzburg (Germany).

Can parasite LBPs be potential targets for chemotherapy and enhancers of drug assimilation?

a) In vitro LBP binding to inhibitors: As a first approximation to test cestodes' FABPs binding properties, a pan FABP inhibitor (HTS01037) was employed. HTS01037 is a small molecule identified by screening a chemical library to identify a small molecule that could antagonize the function of AFABP (mammalian adipocyte FABP) (Hertzel, 2009). This molecule is a pharmacologic ligand that binds to mammalian FABPs at a structurally similar position to a long-chain fatty acid. In our work, a competition assay was run where increasing amounts of the inhibitor were added to a preformed protein-ANS complex. Results show that EgFABP1 would bind the inhibitor with a lower affinity than AFABP (mammalian adipocyte FABP) (**Figure 8**).
b) Effect of FABP inhibitors on parasite viability: One of the central hypotheses of this project is that the inhibition of the lipid binding activity of proteins involved in lipid transport would affect the viability and the development of the parasite. For this set of experiments we employed *T. crassiceps*, a proper model for the study of cysticercosis caused by *T. solium*. We first confirmed the presence of FABPs in *T. crassiceps* cisticerci homogenates (**Figure 9**). Next we evaluated the effect of the pan FABP inhibitor (HTS01037) on *T. crassiceps* cisticerci. Briefly, 35 individuals were incubated in DMEM with increasing concentrations of the drug for periods of at least four days. At 24 and 72 hs individuals were collected and checked using trypan blue assay. The observed results showed that HTS01037 have a toxic effect on the cisticerci diminishing their viability and this would happen in a dose dependant manner (**Figure 10**). As a whole, we have shown that FABP inhibitors can

bind to a cestode FABP, and that this inhibitor has a toxic effect on parasite cells which express FABPs. These are very preliminary results, we are in the process of testing other inhibitors as well as classical anthelmintic drugs screening for compounds that bind specifically to parasitic but not to hosts' LBPs. We will also perform molecular docking analyses to find compounds that could bind and inhibit LBP activity.

c) Molecular docking analyses to find compounds that could bind and inhibit LBP activity: EgFABP (PDBID 1O8V) was subject of molecular docking analysis with FABP inhibitor HTS01037. Results show that the inhibitor binds with proposed target with high affinity (**Figure 11**). We also performed virtual screening analyses with HTS01037 inhibitor and with proposed natural ligands for EgFABP. Results shows over 200 possible hits. All putative compounds are being tested through ADMET software to establish their potential toxicity for rodents and humans. Once depurated, molecular docking analyses against EgFABP will be performed.

Can new anthelmintic drug target candidates and inhibitory compounds be predicted and validated? Micro RNAs as candidates for novel therapeutic interventions.

MicroRNAs (miRNAs), a class of small non-coding RNAs, are key regulators of gene expression at post-transcriptional level and play essential roles in fundamental biological processes such as metabolism and development. The particular developmental characteristics of cestode parasites highlight the importance of studying miRNA gene regulation in these organisms. In a collaborative work with Dr Henrique Ferreira (UFRGS, Brazil) we performed a comprehensive analysis of miRNAs in two developmental stages of the model cestode *Mesocestoides corti*. *M. corti* is a model for larval cestode infections (Vendelova, 2016). Like clinically relevant cestodes, *M. corti*, alteres the host immune response and releases ES products, facilitating the colonization of immunocompetent mice. In this work, using small RNAseq, we found transcriptional evidence of 42 miRNA loci in tetrathyridia larvae and strobilated worms. Tetrathyridium and strobilated worm-specific miRNAs were found, as well as differentially expressed miRNAs between these developmental stages, suggesting miRNA regulation of stage-specific features (**Figure 12**). This study confirmed that miRNAs are the main small RNA silencing molecules in cestodes being several of them absent/divergent in the host as shown in previous works with other cestodes (**Macchiaroli, 2015; Cucher, 2015, Perez, 2017**). We also showed that important pathways were predicted to be targeted by miRNAs in several cestodes (**Macchiaroli, 2017**). Altogether, these results suggest that miRNAs are candidates for novel therapeutic interventions and pave the way for further functional studies. (**Basika, 2016**).

Antigen B (AgB) from *Echinococcus granulosus*

Antigen B (AgB) is the major antigen of *Echinococcus granulosus* (EgAgB) and *Echinococcus multilocularis* (Em AgB). AgB is an oligomeric protein composed of 8 kD subunits with high alpha-helical content (**Chemale, 2005**). The heterogeneity of EgAgB apolipoprotein

component is one of its remarkable features arising from the fact that this component is encoded by a polymorphic multigene family. *E. granulosus* antigen B family comprises five isoforms named EgAgB1–EgAgB5. Recent work shows that AgB binds a wide variety of lipid classes (**Obal**, **2012**).

What are the structures of these novel proteins?

The secondary structure of EgAgB apolipoproteins have been analyzed employing different approaches. The analysis of the percentage of α-helical structure has rendered different results depending on the methods employed and whether native or recombinant subunits were analyzed (Oriol, 1975; Gonzalez Sapienza, 2003; **Monteiro, 2007**). In this work we have obtained and refined the structural models of the five subunits (AgB1 to AgB5) providing plausible tertiary structures of EgAgB apolipoproteins using homology modeling (**Silva, 2015a**). Multiple sequence alignment were obtained and used as reference for the structural prediction of the proteins by homology modeling. Based on structural templates, conformations of each apolipoprotein were then constructed using Modeller 9v3 and electrostatic profiles were predicted using Adaptive Poisson–Boltzmann Solver (APBS) program. According to the models, all apolipoproteins showed predominantly alpha helical structures in agreement with the previous data mentioned above (**Figure 13**). Moreover, the position of hydrophilic and hydrophobic amino acids defines pocket-like regions where hydrophobic ligands could interact with the proteins, and a partial charge distribution showing a plausible electrostatic profile for molecular aggregation. Another interesting feature of EgAgB apolipoproteins is their ability to form high molecular weight oligomers, which agrees with the electrostatic profile predicted by tertiary structure modeling. Demonstration that these high molecular weight components are built from the 8kDa subunits came from the analysis of the subunit composition of native EgAgB, as well as recombinant EgAgB subunits. It is worth to mention that oligomerization may represent a foot print-like feature of native *Echinococcus* antigen B, since it does not seem to be shared by other LBPs.

How do helminth LBPs interact with lipids?

EgAgB apolipoproteins self-associate into large oligomers, but the functional contribution of lipids to oligomerization is uncertain. Furthermore, binding of fatty acids to some EgAgB subunits has been reported, but their ability to bind other lipids and transfer them to acceptor membranes has not been studied. The analysis of the composition of the native lipid moiety (Obal, 2012) confirmed that EgAgB is a complex macromolecule with a high content of lipids (40% to 50% of the total mass). Analysis of EgAgB lipid component by high performance thin layer chromatography and gas chromatography revealed that this component is highly heterogeneous from highly hydrophobic compounds to phospholipids, leading to propose structural similarities with animal lipoproteins found in both invertebrate hemolymph and vertebrate plasma. The structure comprises a nucleus which contains most hydrophobic lipids surrounded by a layer of phospholipids where apolipoproteins are embedded. (**Figure 14**) (**Silva-Alvarez, 2015a**).

Lipid-free EgAgB subunits obtained by reverse-phase HPLC were used to analyze their

oligomerization, ligand binding and membrane interaction properties (**Silva Alvarez 2015b)**. Size exclusion chromatography and cross-linking experiments showed that EgAgB8/2 and EgAgB8/3 can self-associate, suggesting that lipids are not required for oligomerization (**Figure 15**). Furthermore, using fluorescent probes, both subunits were found to bind fatty acids, but not cholesterol analogues (**Figure 16**). Analysis of fatty acid transfer to phospholipid vesicles demonstrated that EgAgB8/2 and EgAgB8/3 are potentially capable of transferring fatty acids to membranes, and that the efficiency of transfer is dependent on the surface charge of the vesicles (**Figure 17**). In this work we show that EgAgB apolipoproteins can oligomerize in the absence of lipids, and can bind and transfer fatty acids to phospholipid membranes. Since imported fatty acids are essential for *E. granulosus*, these findings provide a mechanism whereby EgAgB could engage in lipid acquisition and/or transport between parasite tissues. These results may therefore indicate vulnerabilities open to targeting by new types of drugs for hydatidosis therapy.

Which are the cellular functions of helminth LBPs?.

AgB is one of the major Hydatid Fluid components synthesized in abundance by the larval stage of *Echinococcus* spp. Moreover, it constitutes the most immunogenic and specific *Echinococcus*-genus antigen for human serodiagnosis. The functions of EgAgB in parasite biology remain unclear. It may play a role in the parasite´s lipid metabolism, as it was analyzed in previous sections of this report, since it carries host lipids that *E. granulosus* is unable to synthesize. On the other hand, there is evidence supporting immunomodulating activities in EgAgB, which might involve EgAgB interactions with immune cells (Siracusano, 2008). In this work we analyzed binding of AgB subunits to monocytes and macrophages by flow cytometry (**Silva-Álvarez 2016**) (**Figure 18**). Also, involvement of the protein and phospholipid moieties in EgAgB binding to cells, as well as competition with plasma lipoproteins and ligands for lipoprotein receptors, were analyzed. The outcome of EgAgB binding on macrophage response was also studied (**Figure 19**). Our results show that EgAgB and, particularly its predominant EgAgB8/1 apolipoprotein, are potential ligands for monocyte and macrophage receptors. These receptors may also be involved in plasma lipoprotein recognition and induce an anti-inflammatory phenotype in macrophages upon recognition of EgAgB.

Fatty acid and retinol-binding proteins (FAR) from *Necator americanus* **

N. americanus FAR proteins comprise a family of unusual α-helix rich lipid-binding proteins found exclusively in nematodes. They are secreted into host tissues by parasites of plants, animals and humans. They have also proven useful for serodiagnosis, have shown promise in experimental vaccines (Fairfax, 2009) and have been proposed to facilitate infection by manipulating host lipid-mediated defences. In this work we have determined the structure and characterized in the lipid binding properties and possible biological functions a FAR protein from *N. americanus*, an intestinal blood-feeding nematode that affects around 50% of individuals belonging to aboriginal communities in the north of Argentina. This parasite and the other hookworm of humans, *Ancylostoma duodenale*, together infect

over 300 million people worldwide, causing considerable morbidity, together with adverse social and economic consequences.

What are the structures of these novel proteins?
The structure of a FAR protein from the free-living nematode *Caenorhabditis elegans* is available (Jordanova, 2009), but this protein [*C. elegans* FAR-7 (Ce-FAR-7)] is from a subfamily of FARs that does not appear to be important at the host/parasite interface. We have therefore examined *Necator americanus* FAR-1 (Na-FAR-1) from the blood-feeding intestinal parasite of humans, *N. americanus*. The 3D structure of Na-FAR-1 in its ligand-free and ligand-bound forms, were determined by NMR spectroscopy and X-ray crystallography respectively (PDB accession codes Na-FAR-1 apo 4UET and holo 4XCP). The results reveal an α-helical fold similar to Ce-FAR-7, but Na-FAR-1 possesses a larger and more complex internal ligand binding cavity and an additional C-terminal α-helix (**Rey-Burusco, 2015)** (**Figure 20**).

How do helminth LBPs interact with lipids?
Titration of *apo*-Na-FAR-1 with oleic acid, analyzed by NMR chemical shift perturbation, reveals that at least four distinct protein–ligand complexes can be formed. Na-FAR-1 and possibly other FARs may have a wider repertoire for hydrophobic ligand binding, as confirmed in the present study by our finding that a range of neutral and polar lipids co-purify with the bacterially expressed recombinant protein (**Rey-Burusco, 2015)** (**Figure 21**).

Which are the cellular functions of helminth LBPs?
Localization studies are one approach to analyze cellular functions of a protein. In this case immunohistochemistry studies employing a specific antibody raised in our laboratory we were able to show that Na-FAR-1 is present in adult worms with a tissue distribution indicative of possible roles in nutrient acquisition by the parasite and in reproduction in the male (**Rey-Burusco, 2015)** (**Figure 22**).

Nematode Polyprotein Allergenes/antigens (NPAs) of *Ascaris suum* **

NPAs represent a class of lipid binding proteins which has been described exclusively in nematodes. The term polyprotein refers to the production of NPAs as large precursors comprising tandemly repeated units that are cleaved post-translationally into multiple ~15 kDa protein units which may have similar or different functions. The nematode parasite *Ascaris* causes the most common helminth infection of humans worldwide with one quarter of the world population infected.

What are the structures of these novel proteins?
Recently, the solution structure of ABA-1A, a single unit of this family of parasite allergens from *Ascaris suum*, was determined in the presence oleic acid by protein nuclear magnetic resonance spectroscopy in Dr. Brian Smith´s laboratory. The protein adopts a novel seven-helical fold comprising a long central helix that participates in two hollow four-helical

bundles on either side (Menan, 2011). It is important to note this is the first structure of a unit of any tandemly repetitive polyprotein yet reported.
During the period of the present project we aimed at solving the solution structure of apo-ABA-1A employing NMR spectroscopy. The NMR spectra were collected and processed in AZARA. The angular restraint information will be obtained from the ^{1}H-^{15}N dipolar couplings using frequency based methods and peak positions will be determined using CCPN analysis (**Franchini, 2014**).

How do helminth LBPs interact with lipids?
a) Analysis of ligand binding site of ABA-1A employing NMR spectroscopy: Apo- and holo-ABA-1A NMR spectra were compared at different protein:ligand ratios, in order to identify ligand binding sites. Double labelled ^{13}C, ^{15}N recombinant ABA-1A was loaded with unlabelled ^{12}C oleic acid to study protein-ligand interactions. The experiment was performed at 1:1 protein:oleic acid. The double crosspeaks seen for the vinyl protons suggest two possible conformations for the fatty acid (**Figure 23**).
b) Lipidomic analysis of natural ligands bound to ABA-1A: Mass spectroscopy was applied to analyze lipids copurified with recombinant ABA-1A. Knowing the natural lipids bound by this protein could give useful information to be used in rational drug design as well as the possible functions played within the organism. As expected, most of the peaks matched with known fatty acids such as palmitoleic, palmitic and stearic acids (**Figure 24**). Surprisingly, one of the most abundant peaks matched with farnesyl monophosphate. This is the first time that this kind of ligand is suggested for NPAs, opening a broad range of possibilities to study.
c) Fluorimetruc assay: We analyzed the binding of farnesyl monophosphate (FMP) by ABA-1A by fluorimetric displacement assays. FMP showed a lower affinity than oleic acid for ABA-1A. Since neither of the ligands was able to completely displace the fluorophore, the existence of two binding sites, is suggested (**Figure 25**).
d) Titration of *apo*-ABA-1A with FMP analyzed by NMR chemical shift perturbation: Two binding events were observed, a high affinity one at lower FMP concentrations and a lower affinity binding event at ligand concentrations higher than 1:1 ratio. These results are confirmatory of the previous fluorimetric displacement assays which suggested more than one binding site for FMP (**Figure 26**).

Nematode fatty acid binding proteins (nemFABP) of *A. suum* **
Intracellular lipid binding proteins of the FABP family of animals transport mainly fatty acids or retinoids, are confined to the cytosol, and have highly similar three-dimensional structures. In contrast, nematode fatty acid binding proteins (nemFABPs) are secreted and appear in the perivitelline fluid surrounding the developing embryos of nematodes. A female *Ascaris* can produce up to 200,000 eggs a day which contaminate soil. Infective *Ascaris* eggs may survive for up to 7 years, but little is known of the biochemical basis for such long term survival. One of the most abundant components of the perivitelline fluid which surrounds the developing larva is the protein As-p18 so it may be essential to the

lipid requirements of developing infective larvae. Infection is caused by oral intake of eggs and can cause respiratory and gastrointestinal problems.

What are the structures of these novel proteins?

In this work we report the first structure of a member of the nematode-specific nemFABPs The structures of the As-p18 nemFABP with ligand bound were determined using X-ray crystallography and nuclear magnetic resonance spectroscopy. In common with FABPs, As-p18 comprises a ten β-strand barrel capped by two short α-helices, with the carboxylate head group of oleic acid tethered in the interior of the protein, but, As-p18 exhibits two distinctive longer loops between β-strands that have not been seen before in a FABP (**Figure 27**). One of these loops is adjacent to the presumed ligand entry portal, so may control ligand exchange. The second, larger loop is at the opposite end of the molecule and has no equivalent in any intracellular LBP structure yet determined. In addition to being extracellular, nemFABPs therefore represent a distinct departure from FABP structures, and may perform a crucial role in nematode reproduction, which has relevance for control of these highly pathogenic parasites of humans, animals and plants (**Ibanez-Shimabukuro, submitted)**.

How do helminth LBPs interact with lipids?

Ligand:protein interaction of As-p18 was analyzed employing NMR spectrometry and X-ray crystallography. The position and orientation of an oleate molecule was revealed, which has difference with the typical holo-FABPs. Ligands that co-purify with recombinant As-p18 were analyzed by TLC and GC analysis showing that the protein only binds fatty acids. The affinity for those ligands was analyzed by spectrofluorometry and isothermal titration calorimetry. The data provided satisfactory binding curves, consistent with a 1:1 binding stoichiometry and a micromolar constant affinity (**Figure 28**) (**Ibanez-Shimabukuro, submitted)**.

**These works were performed in collaboration with the laboratories of Dr Kennedy and Dr Smith at the University of Glasgow.

References

Chemale G, H.Ferreira, Barrett J. *Echinococcus granulosus* antigen B hydrophobic ligandbindingproperties,Biochim.Biophys.Acta. 2005. 1747:189–194.

Esteves A, Dallagiovanna B, Ehrlich R. A developmentally regulated gene of Echinococcus granulosus codes for a 15.5-kilodalton polypeptide related to fatty acid binding proteins. Mol Biochem Parasitol. 1993. 58: 215–222.

Esteves A, Portillo V, Ehrlich R. Genomic structure and expression of a gene coding for a new fatty acid binding protein from *Echinococcus granulosus*. Biochim Biophys Acta - Mol Cell Biol Lipids. (2003) 1631: 26–34.

Fairfax, K.C., Vermeire, J.J., Harrison, L.M., Bungiro, R.D., Grant, W., Husain, S.Z. and Cappello, M. Characterisation of a fatty acid and retinol binding protein orthologue from the hookworm *Ancylostoma ceylanicum*. Int. J. Parasitol. 2009. 39, 1561–157

González-Sapienza G, Cachau RE. Identification of critical residues of an immunodominant regionof *Echinococcus granulosus* antigen B. J.Biol.Chem. 2003. 278:20179–20184.

Hertzel AV, Hellberg K, Reynolds JM, Kruse AC, Juhlmann BE, Smith AJ, Sanders MA, Ohlendorf DH, Suttles J, Bernlohr DA. Identification and characterization of a small molecule inhibitor of Fatty Acid binding proteins. J Med Chem. 2009. 52(19):6024-31.

Huang F, Dang Z, Suzuki Y, Horiuchi T, Yagi K, Kouguchi H, Irie T, Kim K, Oku Y. Analysis on Gene Expression Profile in Oncospheres and Early Stage Metacestodes from *Echinococcus multilocularis*. PLoS Negl Trop Dis. 2016. 10(4):e0004634.

Jakobsson E, Alvite G, Bergfors T, Esteves A, Kleywegt GJ. The crystal structure of *Echinococcus granulosus* fatty-acid-binding protein 1. Biochim Biophys Acta. 2003 1649: 40–50.

Jordanova R, Groves MR, Kostova E, Woltersdorf C, Liebau E, Tucker PA. Fatty acid- and retinoid-binding proteins have distinct binding pockets for the two types of cargo. J Biol Chem. 2009. 284(51):35818-26

Meenan NA, Ball G, Bromek K, Uhrín D, Cooper A, Kennedy MW, Smith BO. Solution structure of a repeated unit of the ABA-1 nematode polyprotein allergen of Ascaris reveals a novel fold and two discrete lipid-binding sites. PLoS Negl Trop Dis. 2011. 5(4):e1040.

Monteiro KM , Scapin SMN, Navarro MVAS, Zanchin NIT, etal. Self-assembly and structural characterization of *Echinococcus granulosus* antigen B recombinant subunit oligomers, Biochim. Biophys.Acta. 2007. 1774:278–285.

Obal G , Ramos AL, V.Silva, A.Lima, C.Batthyany, M.I.Bessio, etal., Characterisation of the native lipid moiety of *Echinococcus granulosus* antigen B. PLoS Negl. Trop. Dis. 2012. 6:e1642.

Oriol C, Oriol R. Physiocochemical properties of alipoprotein antigen of *Echinococcus granulosus*. Am.J.Trop.Med.Hyg. 1975. 24:96–100.

Siracusano A, Margutti P, Delunardo F, Profumo E, Riganò R, Buttari B, et al. Molecular cross-talk in host‑parasite relationships: the intriguing immunomodulatory role of *Echinococcus* antigen B in cystic echinococcosis. Int J Parasitol 2008.38:1371–6.

Spiliotis M, Brehm K. Axenic in vitro cultivation of *Echinococcus multilocularis* metacestode vesicles and the generation of primary cell cultures. Methods Mol Biol. 2009;470:245-62.

Tsai IJ1, Zarowiecki M, Holroyd N, Garciarrubio A, Sanchez-Flores A, Brooks KL, Tracey A, Bobes RJ, Fragoso G, Sciutto E, Aslett M, Beasley H, Bennett HM, Cai J, Camicia F, Clark R, Cucher M, De Silva N, Day TA, Deplazes P, Estrada K, Fernández C, Holland PW, Hou J, Hu S, Huckvale T, Hung SS, Kamenetzky L, Keane JA, Kiss F, Koziol U, Lambert O, Liu K, Luo X, Luo Y, Macchiaroli N, Nichol S, Paps J, Parkinson J, Pouchkina-Stantcheva N, Riddiford N, Rosenzvit M, Salinas G, Wasmuth JD, Zamanian M, Zheng Y; Taenia solium Genome Consortium, Cai X, Soberón X, Olson PD, Laclette JP, Brehm K, Berriman M. The genomes of four tapeworm species reveal adaptations to parasitism. Nature. 2013 496(7443):57-63.

Vendelova E, Hrčková G, Lutz MB, Brehm K, Nono Komguep J. In vitro culture of *Mesocestoides corti* metacestodes and isolation of immunomodulatory excretory-secretory products. Parasite Immunol. 2016 38(7):403-13.

Publications of the group related to the project during the period of the grant

1. Franchini GR, Córsico B, Pórfido JL, Silva V, Ibañez Shimabukuro M, Rey Burusco F. Análisis Estructural y Funcional de Proteínas Solubles que Unen Lípidos de Parásitos Helmintos. *Acta Bioquímica Clínica Latinoamericana.* 47 (2): 377-84. 2013.
2. Franchini GR, Pórfido JL, Ibañez Shimabukuro M, Rey Burusco MF, Bélgamo JA, Smith BO, Kennedy MW, and Córsico B. The unusual lipid binding proteins of parasitic helminths and their potential roles in parasitism and as therapeutic targets. *Prostaglandins Prostaglandins, Leukotrienes & Essential Fatty Acids (PLEFA*). Aug 28. pii: S0952-3278(14)00141-0. doi: 10.1016/j.plefa.2014.08.003. 2014.
3. Silva-Álvarez V, Folle AM, Ramos AL, Zamarreño F, Costabel MD, García-Zepeda E, Salinas G, Córsico B, Ferreira AM *Echinococcus granulosus* antigen B: A Hydrophobic Ligand Binding Protein at the host-parasite interface. *Prostaglandins Leukot Essent Fatty Acids*. 2014 Oct 7. pii: S0952-3278(14)00152-5. doi: 10.1016/j.plefa.2014.09.008. 2014.
4. Silva-Álvarez V, Franchini GR, Pórfido JL, Kennedy MW, Ferreira AM, Córsico B. Lipid-free Antigen B Subunits from *Echinococcus granulosus*: Oligomerization, ligand binding, and membrane interaction properties. *PLoS Negl Trop Dis.* 9(3):e0003552. doi: 10.1371/journal.pntd.0003552. eCollection. 2015.
5. Rey Burusco MF, IBÁÑEZ-SHIMABUKURO M, GABRIELSEN M, ROE AJ, GRIFFITHS K, ZHAN B, COOPER A, KENNEDY MW CÓRSICO B, and SMITH BO. Diversity in the structures and ligand binding sites among the fatty acid and retinol binding proteins of nematodes revealed by Na-FAR-1 from *Necator americanus*. *Biochemical J* 471(3):403-14. 2015.
6. Silva-Álvarez V, Folle AM, Ramos AL, Kitano ES, Iwai LK, Córsico B, Ferreira AM. *Echinococcus granulosus* Antigen B binds to monocytes and macrophages: contribution of the protein and phospholipid moieties. *Parasites & Vectors*. 9(1):69. 1350-7. 2016.
7. Dos Santos GB, Monteiro KM, da Silva ED, Battistella ME, Ferreira HB, Zaha A. Excretory/secretory products in the Echinococcus granulosus metacestode: is the

intermediate host complacent with infection caused by the larval form of the parasite? *Int J Parasitol*.;46(13-14):843-856. 2016.

8. Monteiro KM, Lorenzatto KR, de Lima JC, Dos Santos GB, Förster S, Paludo GP, Carvalho PC, Brehm K, Ferreira HB. Comparative proteomics of hydatid fluids from two *Echinococcus multilocularis* isolates. *J Proteomics*. S1874-3919(17)30129-X. 2017.
9. Basika T, Macchiaroli N, Cucher M , Espínola S, Kamenetzky L, Zaha A, Rosenzvit M, Ferreira H.. Identification and profiling of microRNAs in two developmental stages of the model cestode parasite *Mesocestoides corti. Molecular and Biochemical Parasitology,* 210, 37-49. 2016.
10. Macchiaroli N, Cucher M, Zarowiecki M, Maldonado L, Kamenetzky L, Rosenzvit M.. microRNA profiling in the zoonotic parasite Echinococcus canadensis using a highthroughput approach. *Parasites & Vectors* 8:83. DOI: 10.1186/s13071-015-0686-8. 2015
11. Cucher M, Macchiaroli N, Kamenetzky L, Maldonado L, Brehm K, Rosenzvit M.C.. High-throughput characterization of Echinococcus spp. metacestodes miRNomes. International *Journal for Parasitology* 45, 253-267. 2015.
12. Pérez M, Macchiaroli N, Lichtenstein G, Conti G, Asurmendi S, Milone D, Stegmayer G, Kamenetzky L, Cucher M, Rosenzvit MC. microRNA analysis of Taenia crassiceps cysticerci under praziquantel treatment and genome-wide identification of Taenia solium miRNAs. *International Journal of Parasitology*, in press.
13. Macchiaroli N; Maldonado L; Zarowiecki M, Cucher M, Gismondi M, Kamenetzky L, Rosenzvit M. Genome-wide identification of microRNA targets in the neglected disease pathogens of the genus Echinococcus. *Molecular and Biochemical Parasitology*, in press.
14. Ibáñez-Shimabukuro M, Rey-Burusco MF, Gabrielsen M, Roe AJ, Grifiths K, Cooper A, Córsico B, Kennedy MW, Smith BO. Nematode fatty acid binding proteins exhibit unusual structural features. *Biochemical J* (submitted).
15. Pórfido JL, Kiss F, Brehm K, Rosenzvit M, Córsico B, Franchini GR. Fatty acid binding proteins in *Echinococcus* spp.: molecular cloning and in silico characterization. *PLoS Negl Trop Dis.* (submitted).

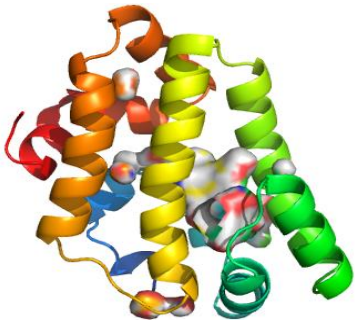
PhD Thesis related to the project defended during the period of the grant

1. **Rey-Burusco, MF.** Title: Structural and biophysical study of novel parasite proteins that bind fatty acids and retinol. Defense date: 03/**2014**. Grade: 10/10. (School of Exact Sciences, University of La Plata Argentina)
2. **Ibáñez-Shimabukuro, M**. Title: As-p18 a novel lipid binding protein of *Ascaris suum*. Defense date: 05/**2014**. Grade: 10/10. (School of Exact Sciences, University of La Plata Argentina)

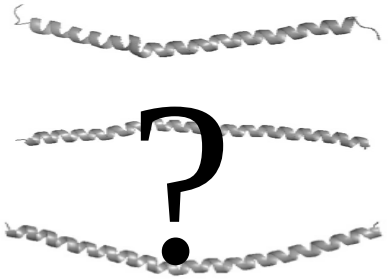
3. **Silva, MV**. Title: Structural and functional characterization of Antígeno B of *Echinococcus granulosus.* Defense date: 07/**2014**. Grade: 10/10. (School of Exact Sciences, University of La Plata Argentina)
4. **Porfido JL**. Title: Functional analysis of fatty acid binding proteins (FABPs) of *Echinococcus granulosus.* Defense date 06/**2015**. Grade: 10/10. (School of Exact Sciences, University of La Plata Argentina)

Proteins from highly pathogenic parasites in South America

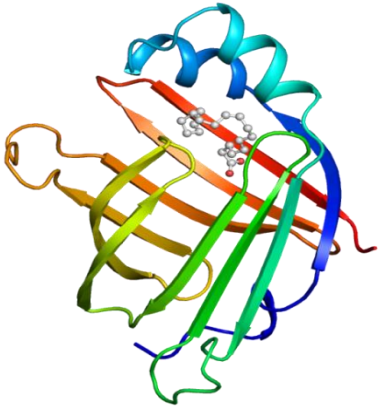
Lipid binding proteins of parasitic helminths



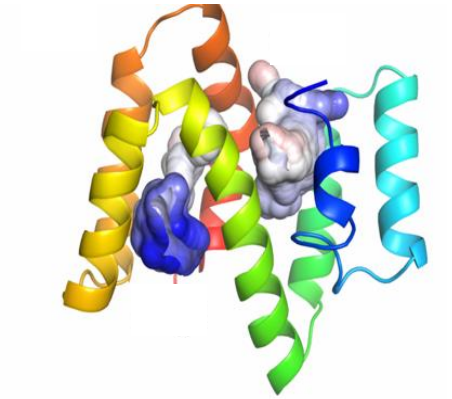
FAR
(FA and Retinoid BP)
Necator americanus



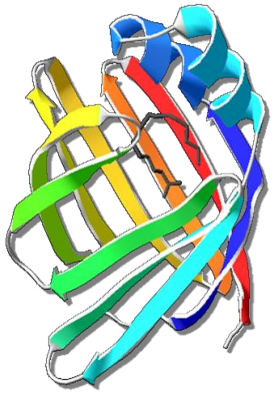
Antigen B
Echinococcus granulosus



nemFABP
(Fatty Acid BP)
Ascaris suum



NPA
(Nematode Polyprotein Allergenes)
Ascaris suum



FABP
Echinococcus granulosus

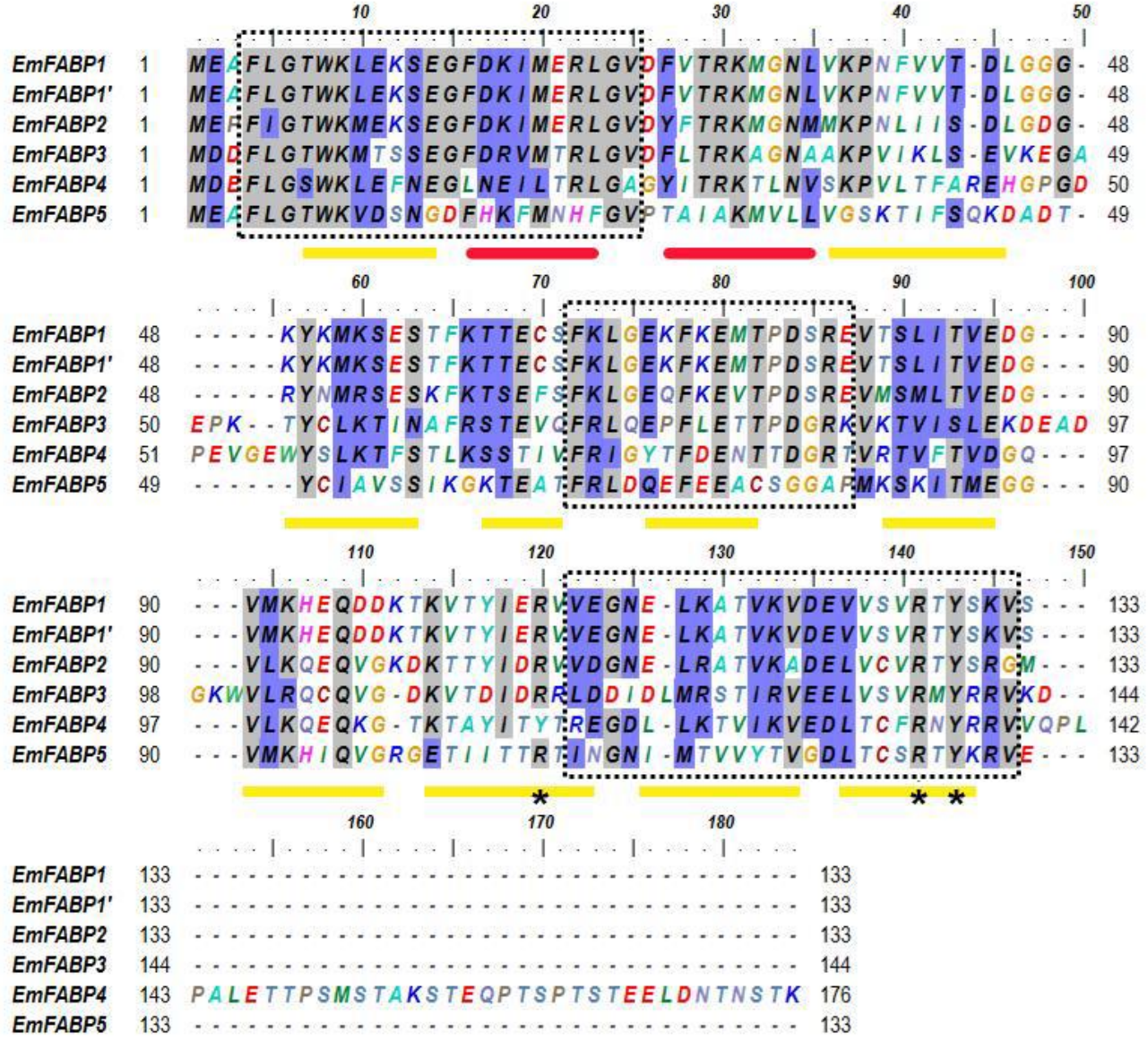


Figure 2

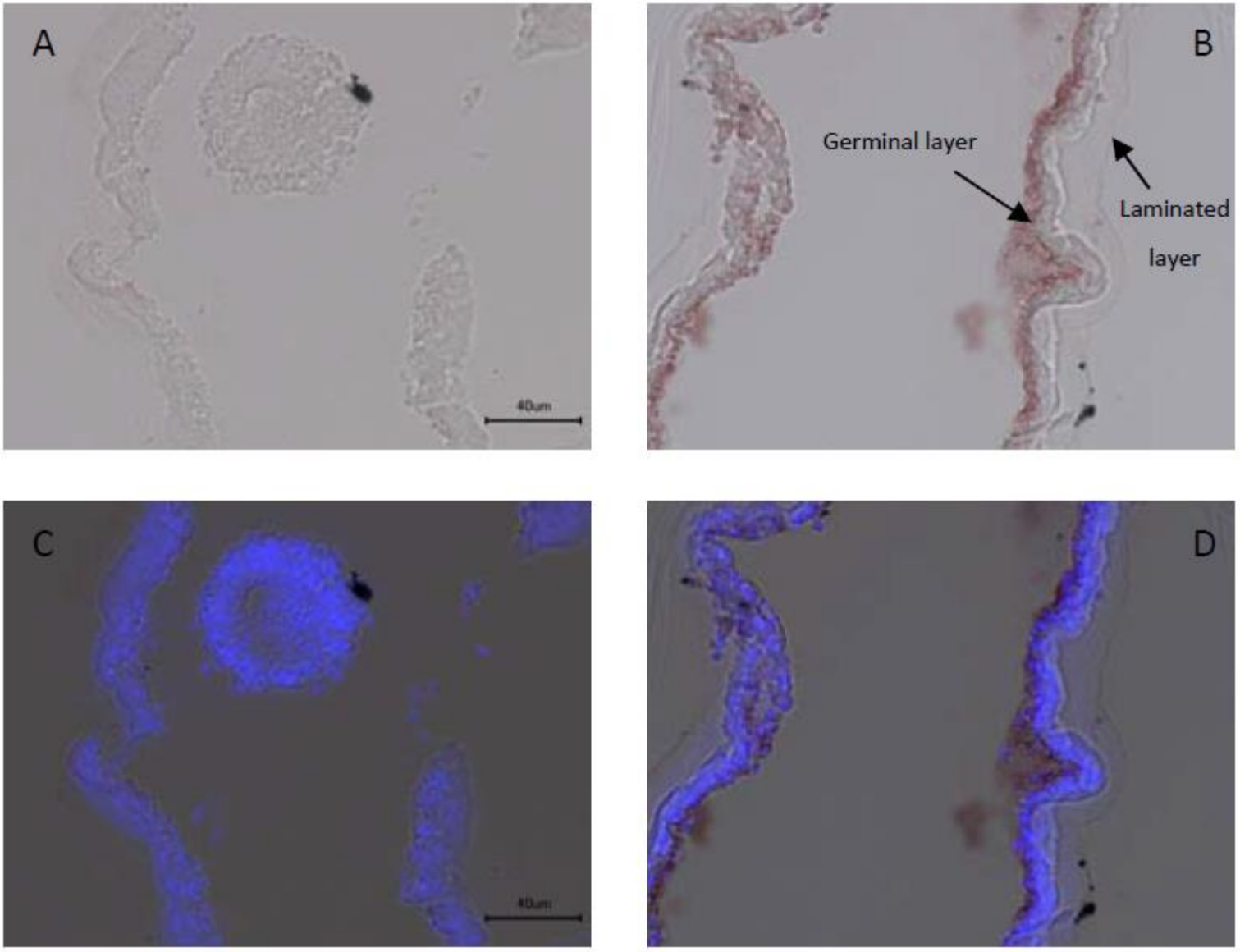
Alignment of FABP sequences found in *E. multilocularis* genome, performed with CLUSTALW software. Grey indicates identical amino acids while blue indicates similar amino acids. Asterisks show the P2 motive for ligand binding. Yellow and red bars indicate predicted secondary structure elements, β-sheets and α-helices respectively, determined with PSIPRED software. Black boxes highlights PRINTS pattern Fatty acid-binding protein signature (Accession Number: PR00178).

Figure 3

	EmFABP1	EmFABP1 [b]	EmFABP2	EmFABP3	EmFABP4	EmFABP5
EgFABP1	92,5%/97,8%	92,5%/97,8%	74,6%/88,8%	23,6%/42,4%	15,9%/27,3%	34,3%/49,3%
EgFABP1.2	62,4%/78,2%	62,4%/78,2%	61,7%/80,4%	20,1%/37,5%	13,6%/25,0%	31,6%/46,7%
EgFABP2	71,4%/87,2%	71,4%/87,2%	100%	24,3%/41,7%	14,8%/26,1%	31,6%/48,9%
EgFABP3	22,9%/41,7%	22,9%/41,7%	24,3%/41,7%	100%	14,8%/33,5%	15,3%/23,6%
EgFABP4	16,5%/27,3%	16,5%/27,3%	14,8%/26,1%	14,8%/33,0%	96,6%/97,7%	9,1%/19,9%
EgFABP5	32,8%/47,8%	32,8%/47,8%	31,3%/48,5%	14,6%/22,2%	8,5%/19,9%	95,5%/97,8%

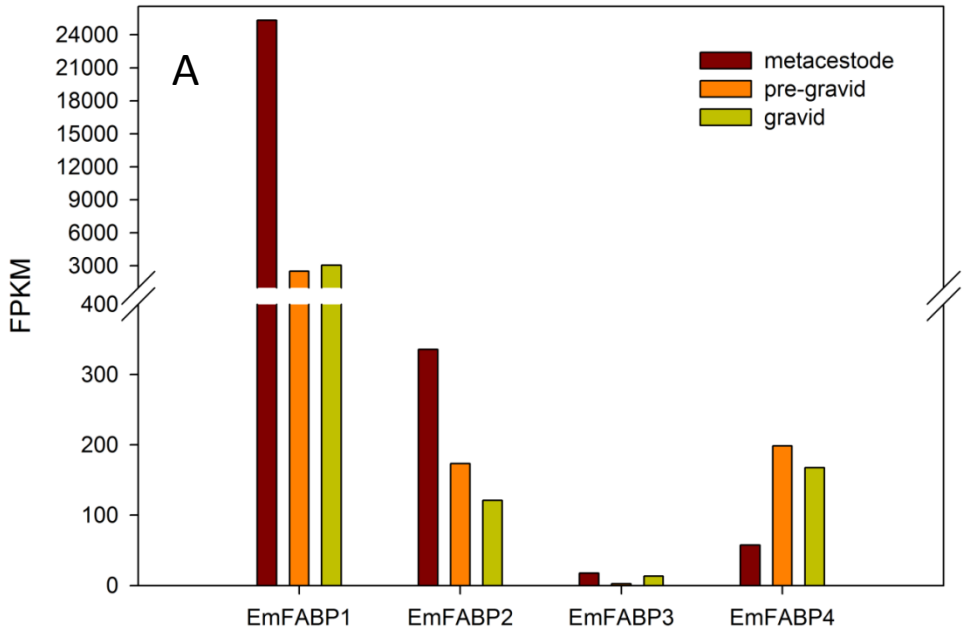
The table shows % of identity in sequences for diverse FABPs. Values shows are for amino acid identity (black) and similitude (blue).

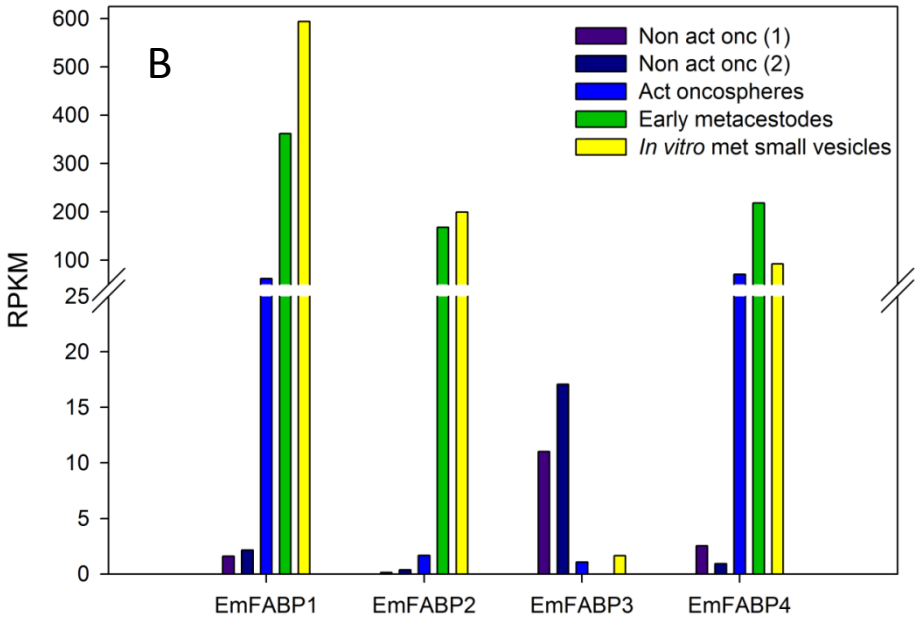
Figure 4



Localization of FABPs in sections of *in vitro* cultured *E. multilocularis* metacestode vesicles. (A) Control without anti-EgFABP1 antibody. (B) Immunohistochemical localization of FABPs employing anti-EgFABP1 antibody. (C) and (D) correspond to the same images overlayed with DAPI staining.

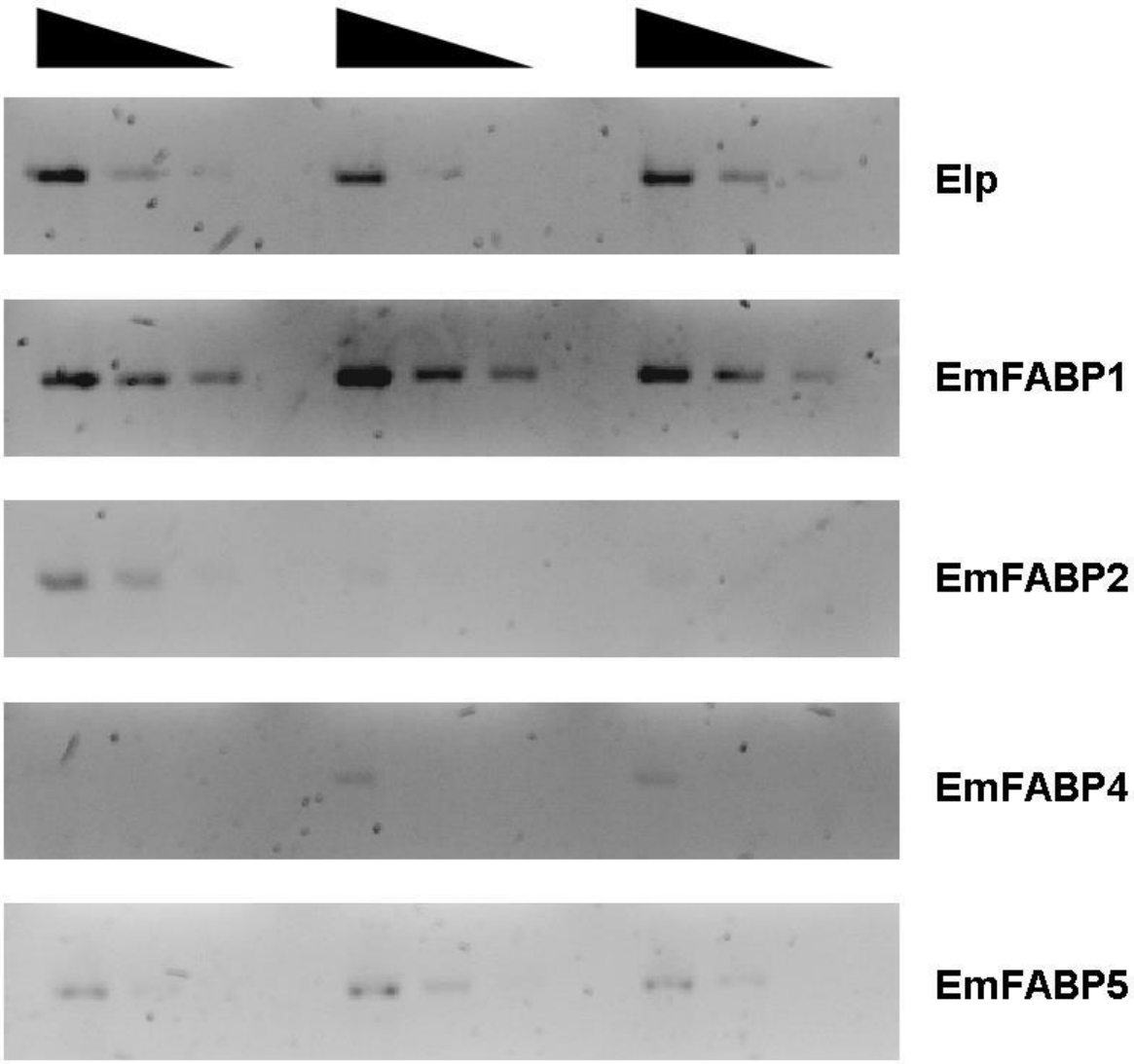
Figure 5





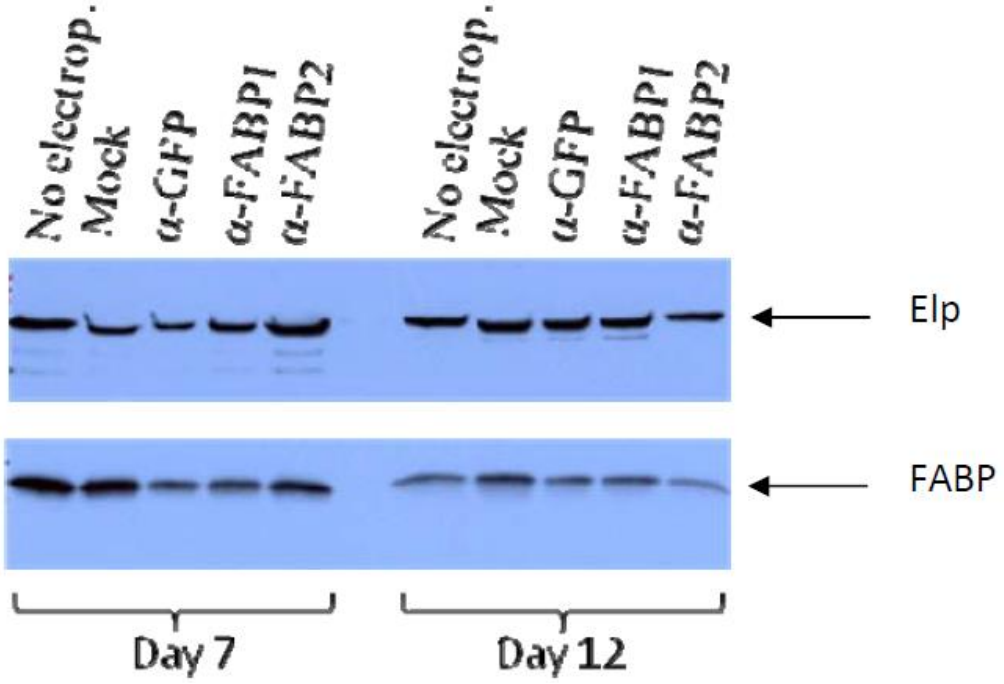
Differential expression of FABPs in different *E. multilocularis* stages. **A**. Information obtained from transcriptomic data published by Tsai et al., 2013. **B**. Analysis of differential expression of FABPs in different stages of *E. multilocularis.* Data correspond to published RNAseq material available at Huang F. et al., 2016.

Figure 6



Analysis of expression of FABPs in different stages of *E. multilocularis* using semi-quantitative RT-PCR. For each stage serial 10-fold dilutions of cDNA were employed as template. Elp, a constitutively expressed protein from *E. multilocularis,* used as control.

Figure 7

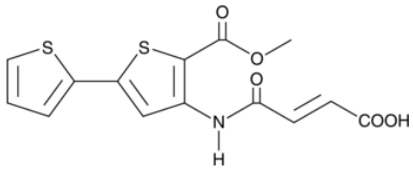


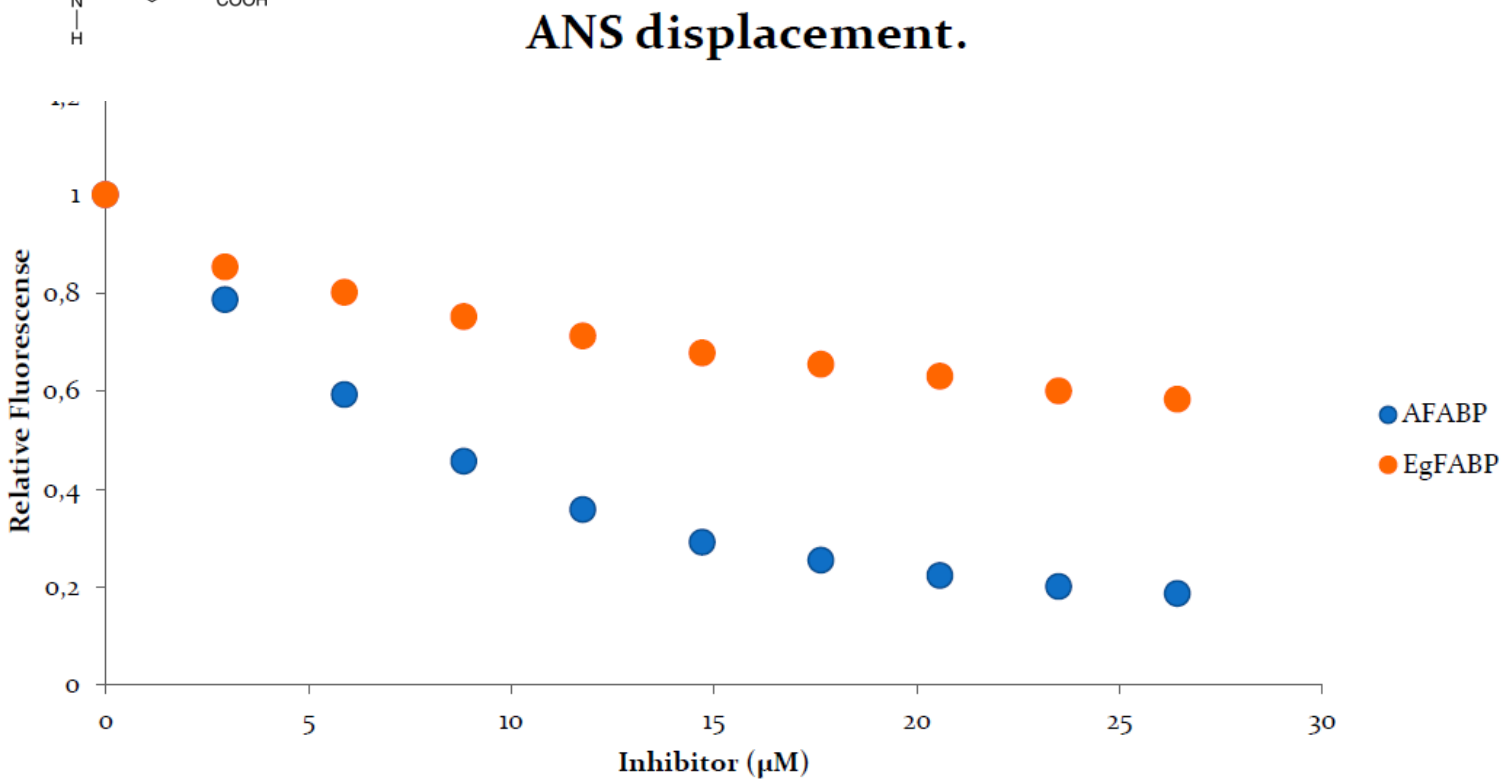
	FABP Relative Expression			
Sample	7 days after treatment		12 days after treatment	
Mock	1,00	1,00	1,00	1,00
α-GFP	1,28	0,67	0,50	0,66
α-EmFABP1	1,54	0,56	0,45	0,81
α-EmFABP2	0,96	0,44	0,31	0,52
Without electroporation	*0,71*	*0,65*	*0,73*	*0,69*

Western Blot of cell homogenates with different treatments collected on days 7 and 12, developed with anti-EgFABP1 antibody. Table bellow shows FABP relative expression. Elp expression levels was employed for normalization.

HTS 01037

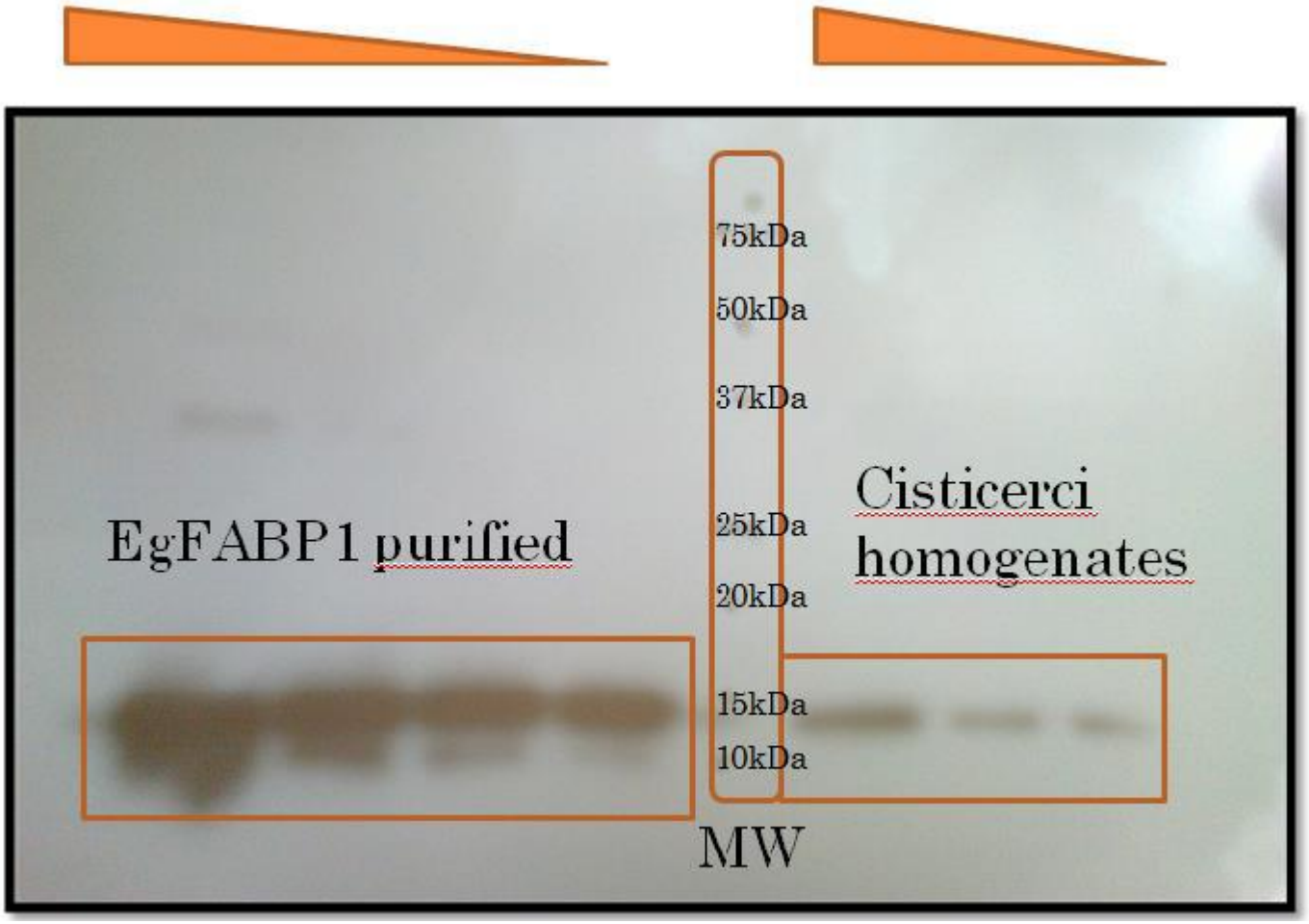
Figure 8





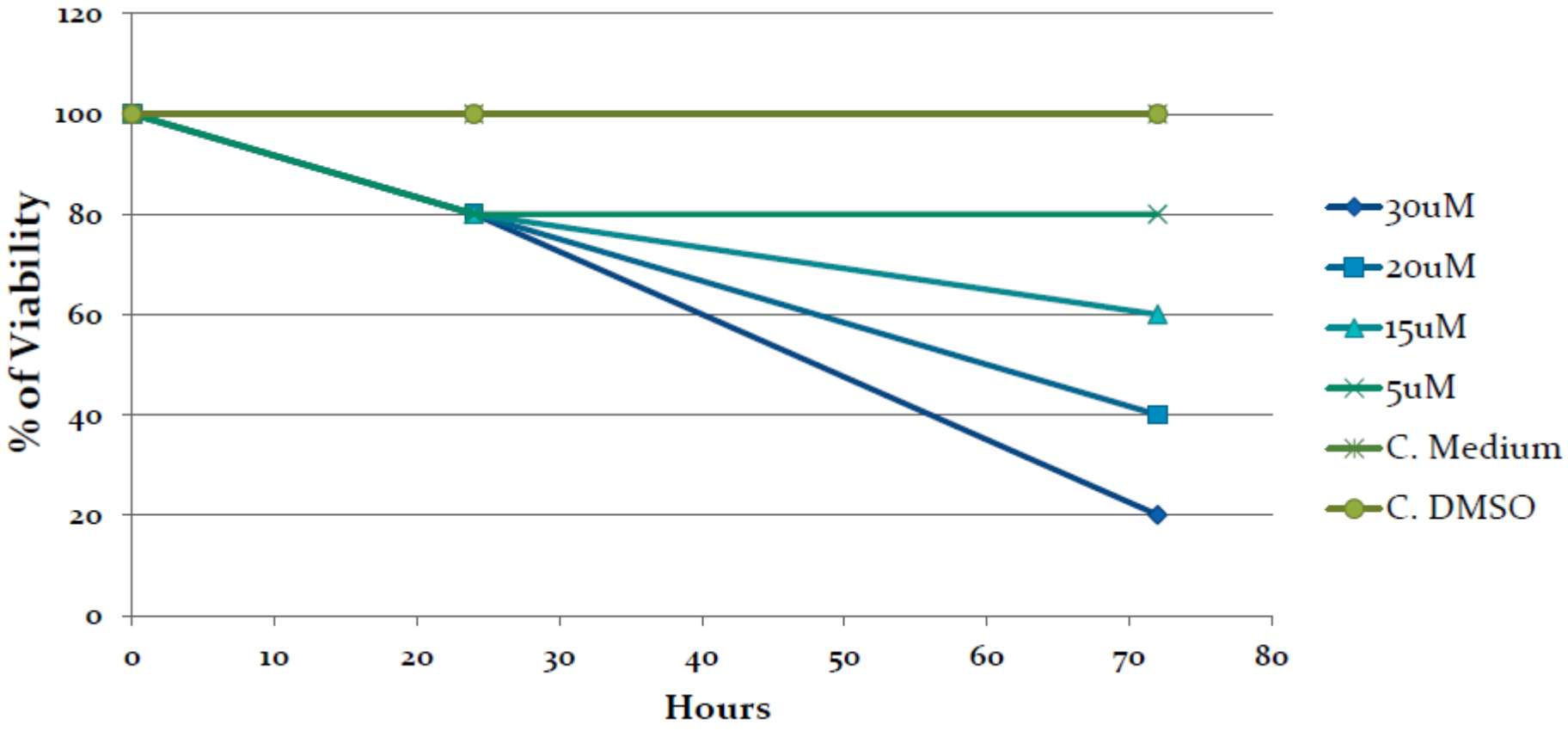
Competition assay with increasing amounts of FABP inhibitor (shown above) were added to a preformed protein-ANS complex. Mammalian AFABP was employed as a control.

Figure 9



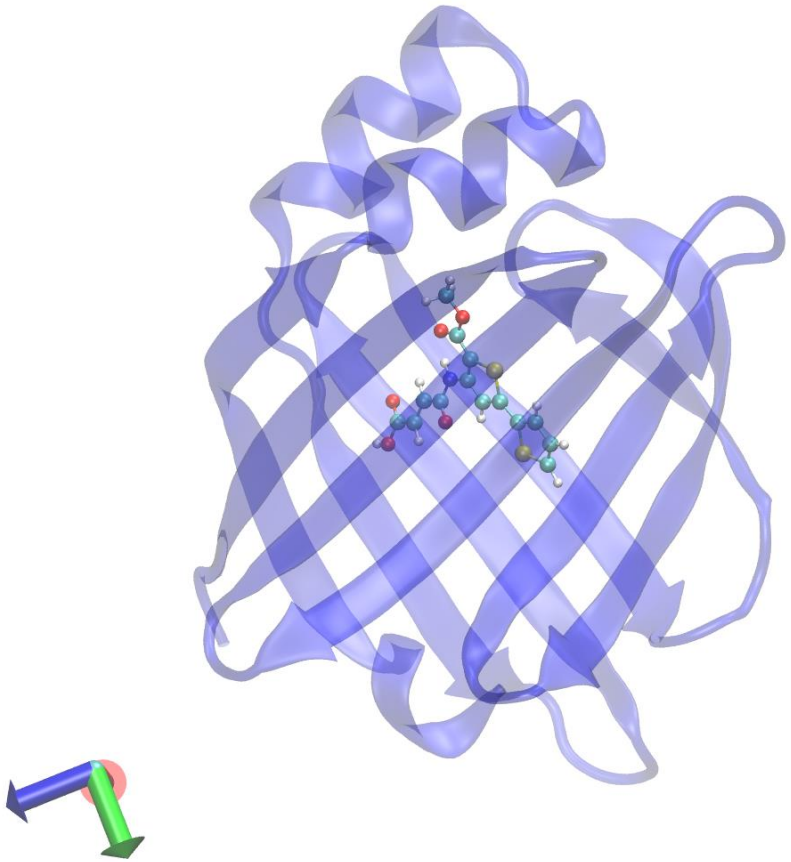
Evidence of FABP in *T. crassiceps*. Western blot of *T. crassiceps* homogenates with anti-EgFABP1 antibodies. Decreasing concentrations of purified EgFABP1 protein (15kDa) were employed as control

Figure 10



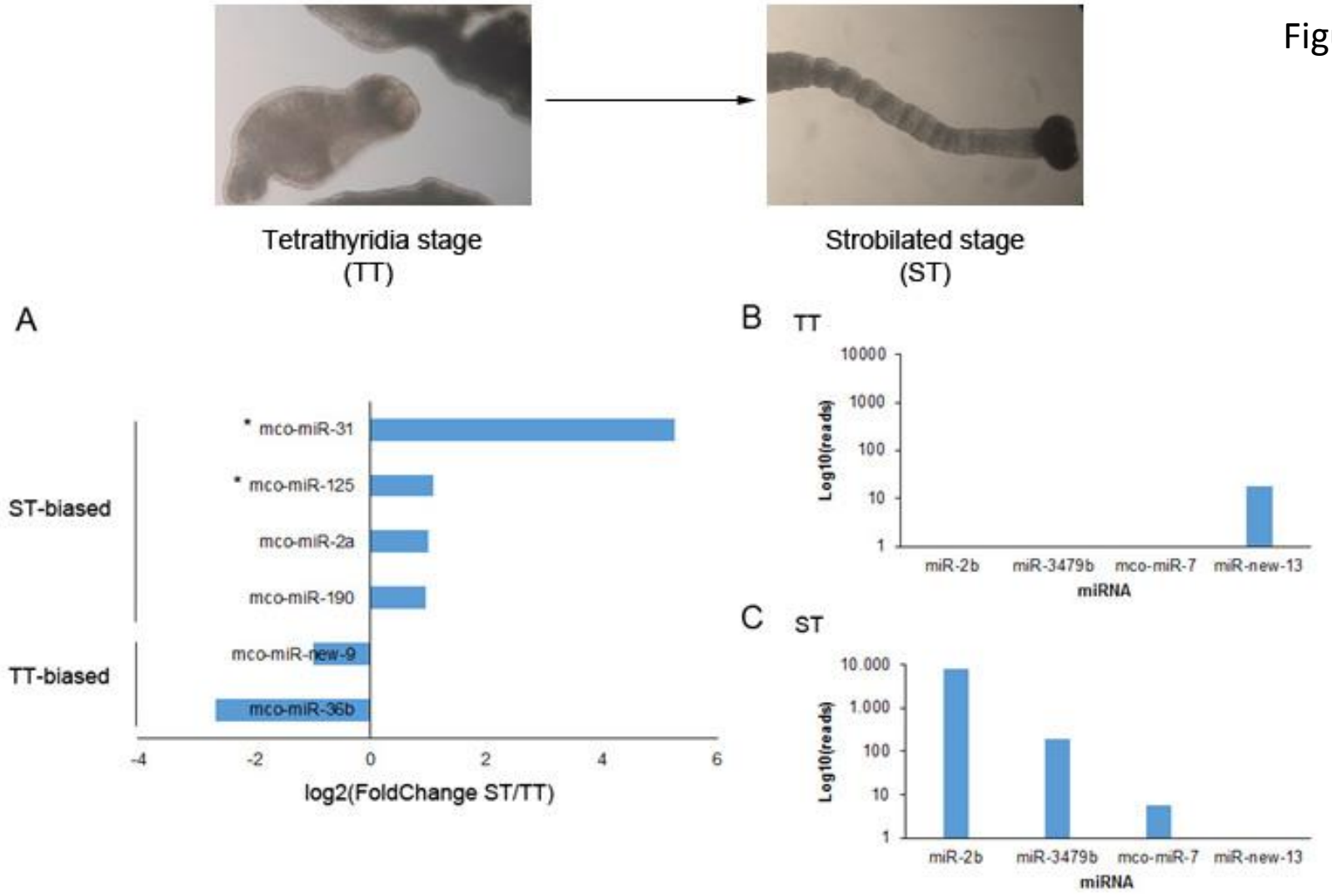
Toxic effect of FABP inhibitor HTS01037 on *T.crassiceps*. *T. crassiceps* cysticerci in DMEM medium treated with increasing concentration of HTS01037. Higher doses of inhibitor presented a stronger effect leading to death.

Figure 11



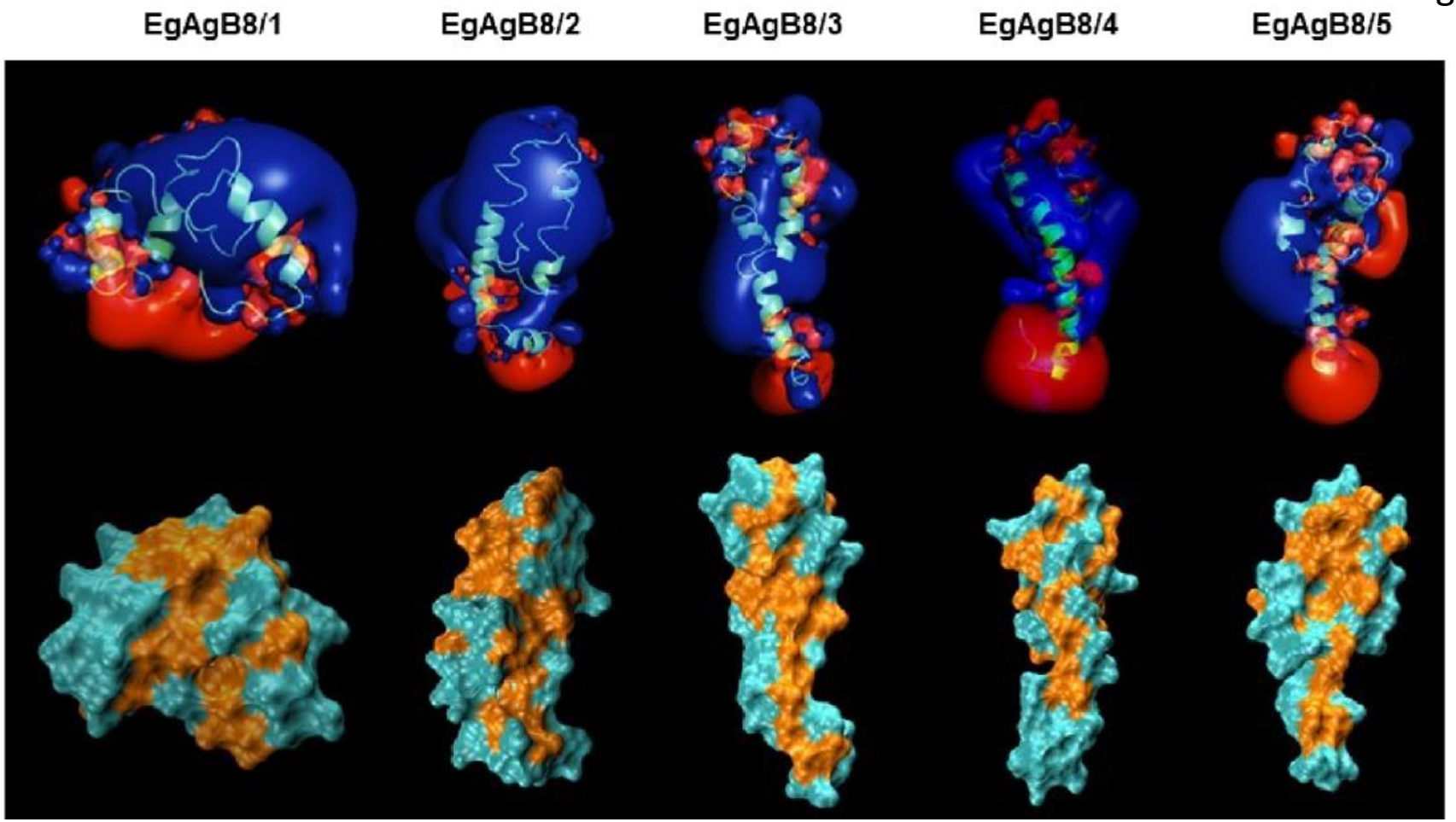
Molecular docking analyses of EgFABP (PDBID 1O8V) with FABP inhibitor HTS01037. The inhibitor locates in the typical hydrophobic ligand binding described for EgFABP1 in the chrystal structure (Jakobsson 2003).

Figure 12



Differential expression of microRNAs (miRNAs) between tetrathyridia (TT) and strobilated worm (ST) of *Mesocestoides corti*. A) Fold change analysis using DESeq algorithm. miRNA with Log2fold change ≥ ±1 and p-adjusted < 0.05 are displayed; an asterisk (*) marks stage-biased microRNAs validated by real time PCR (p-value < 0.0001, based on t-test). B and C) Normalized expression levels of miRNAs detected exclusively in TT (B) and ST samples (C); read counts of each miRNA were normalized to the total number of mature miRNA read counts in that sample.

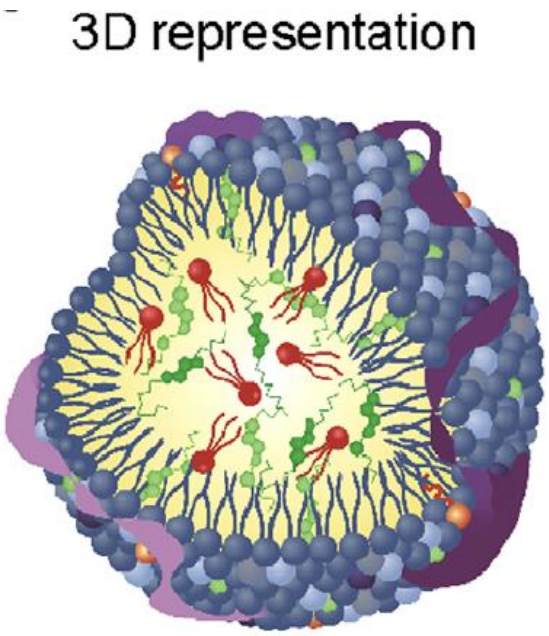
Figure 13



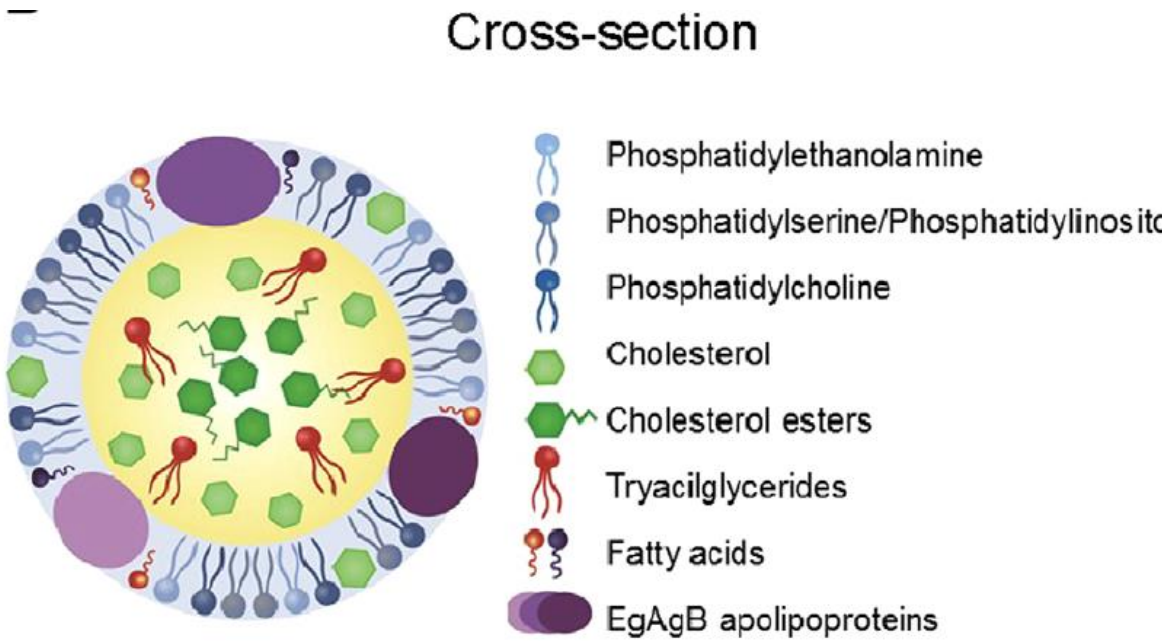
Models of AbB subunits. Models of the plausible structures of thesubunits of EgAgB8. In the first row the electrostatic profile of the apolipoproteins is shown with positive surfaces in blue and negative in red. The hydrophobic characteristics of the proteins are depicted in the second row with the non-polar amino acids in cyan and the

Hydrophilic ones inorange. According to the models, the possibility of interaction with ligands and the aggregation between monomers are evidenced, and perfectly consistent with experimental data.

A

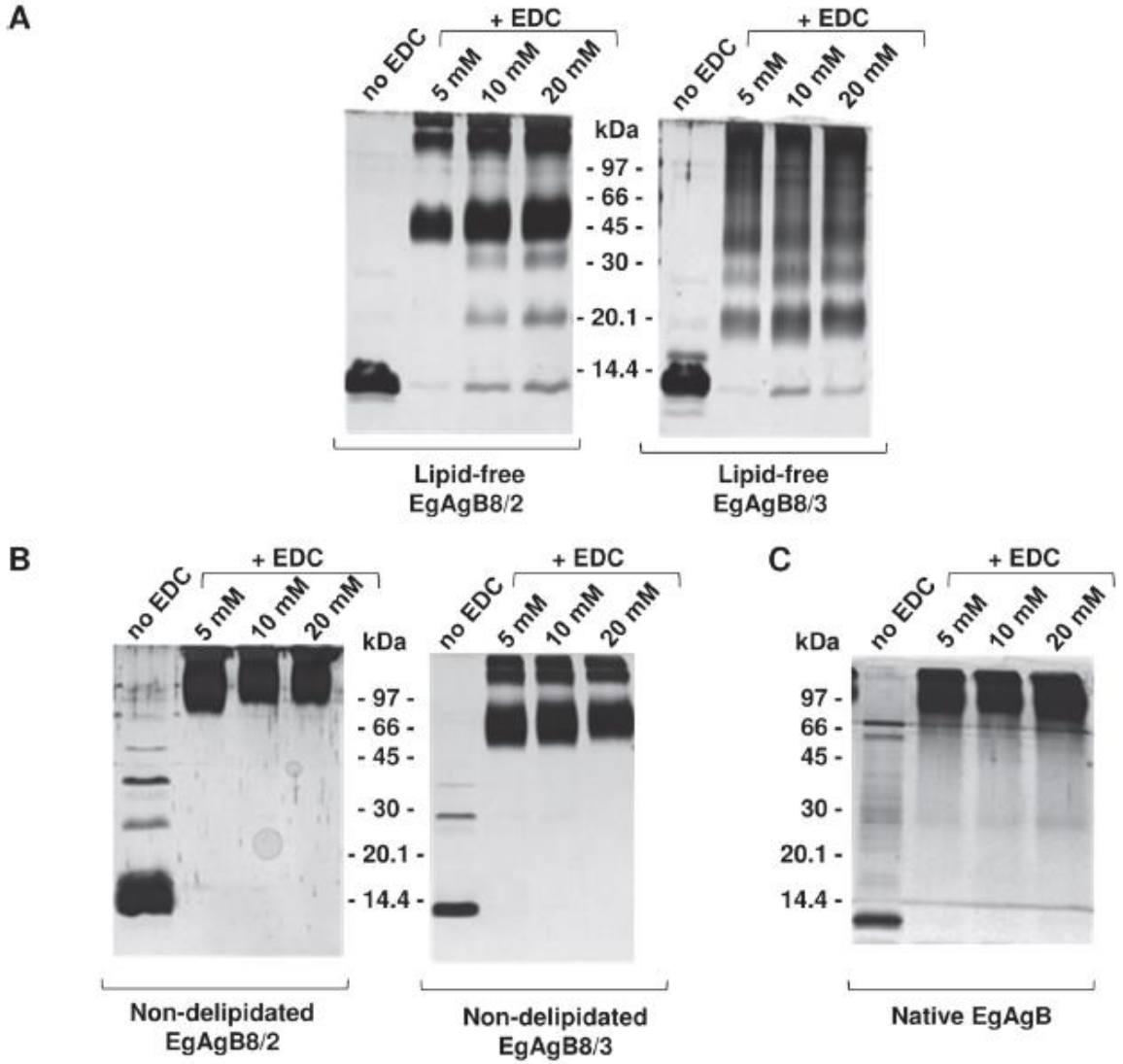


B



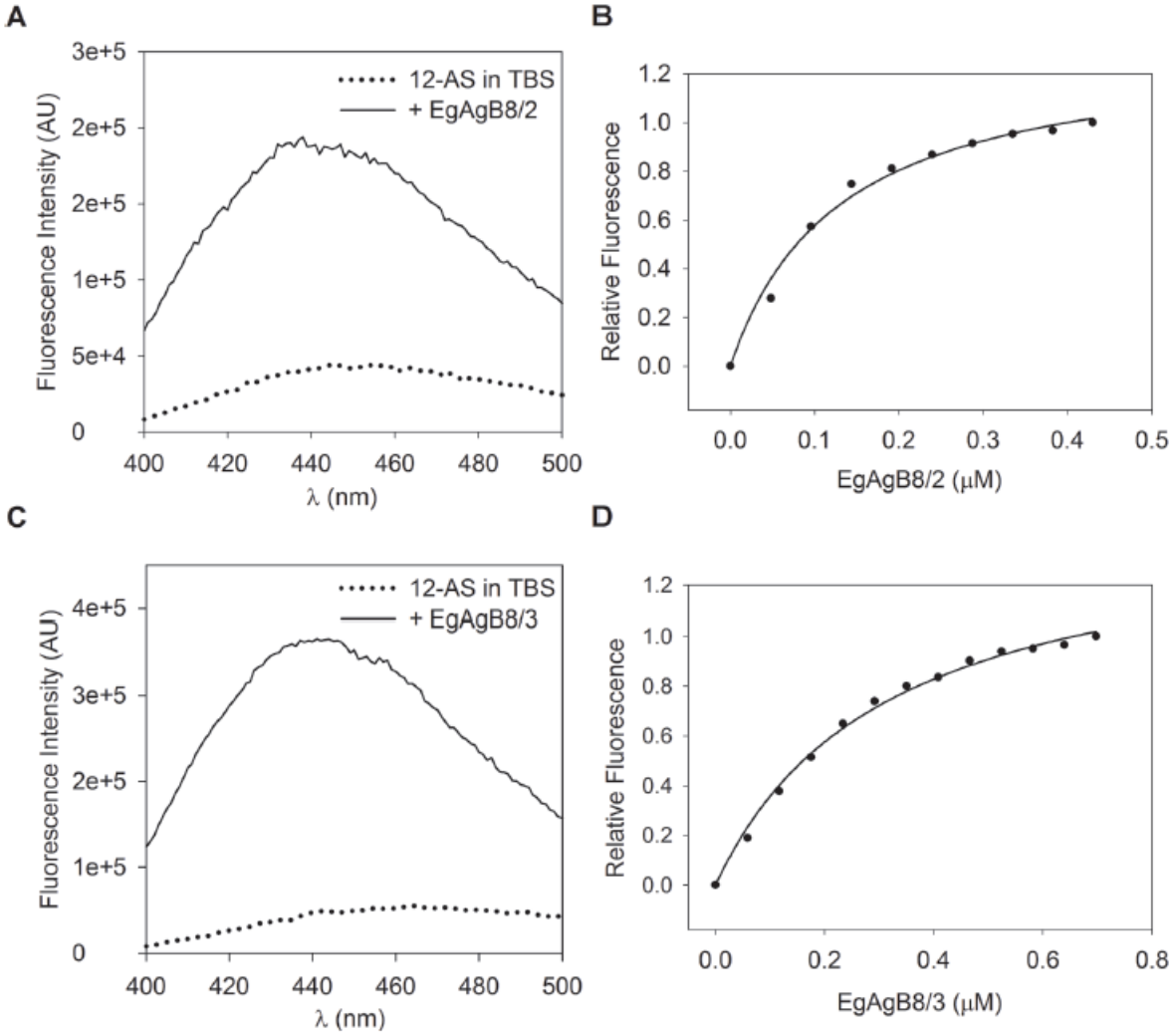
Modeling of AgB structural organization. A. 3D representation of the structural organization proposed for EgAgB. B. Schematic representation of an EgAgB lipoprotein cross-section according to the proposed model.

Figure 15



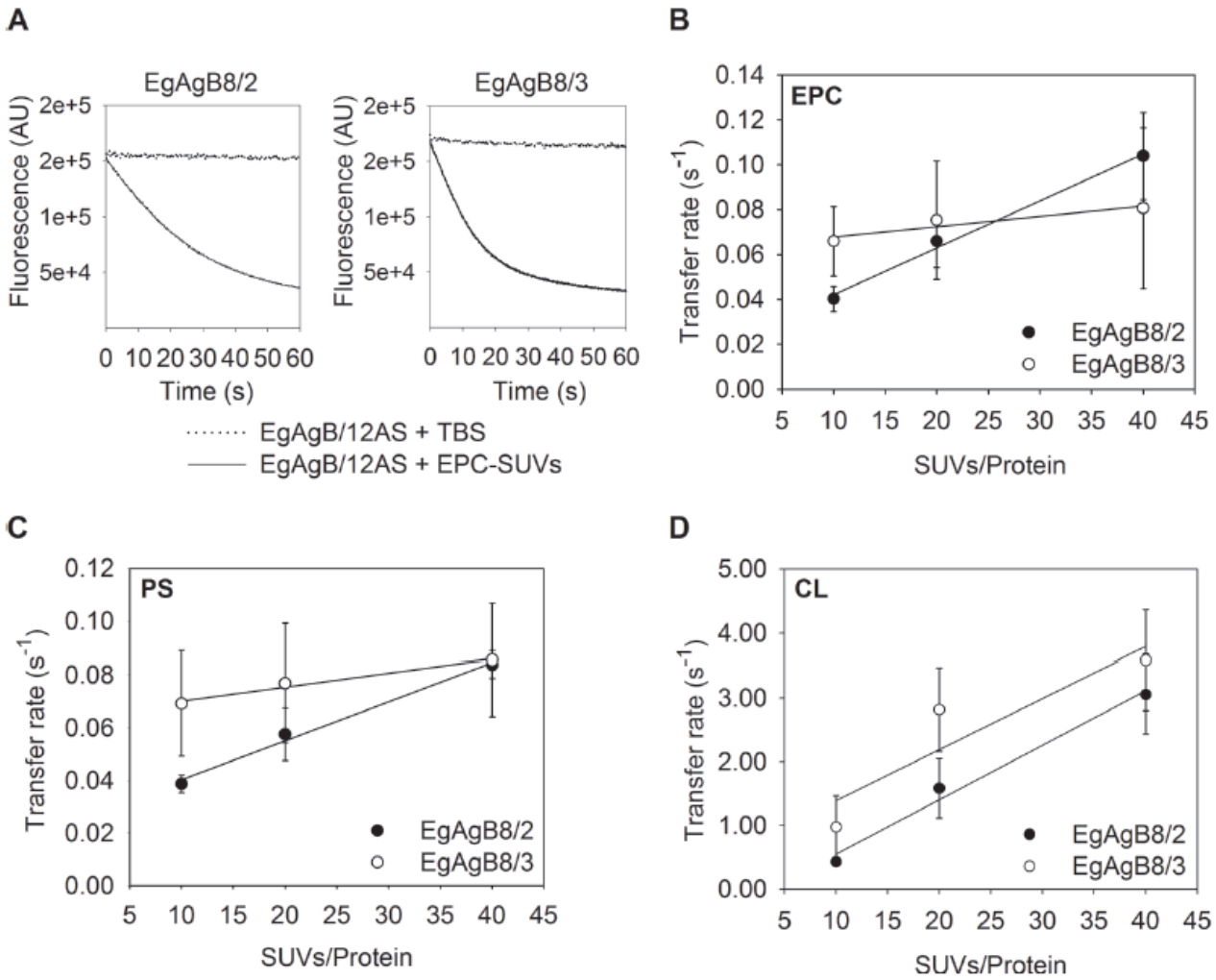
Cross-linking of rEgAgB subunits with EDC. Covalent cross-linking of proteins with EDC was carried out for 30 minutes at 25°C in PBS and separated on 15% SDS-PAGE followed by silver staining. Controls without added EDC were undertaken under the same conditions. (A) Cross-linking of lipid-free EgAgB8/2 and EgAgB8/3 subunits. (B) Cross-linking of non-delipidated EgAgB8/2 and EgAgB8/3 subunits. (C) Cross-linking of native EgAgB.

Figure 16



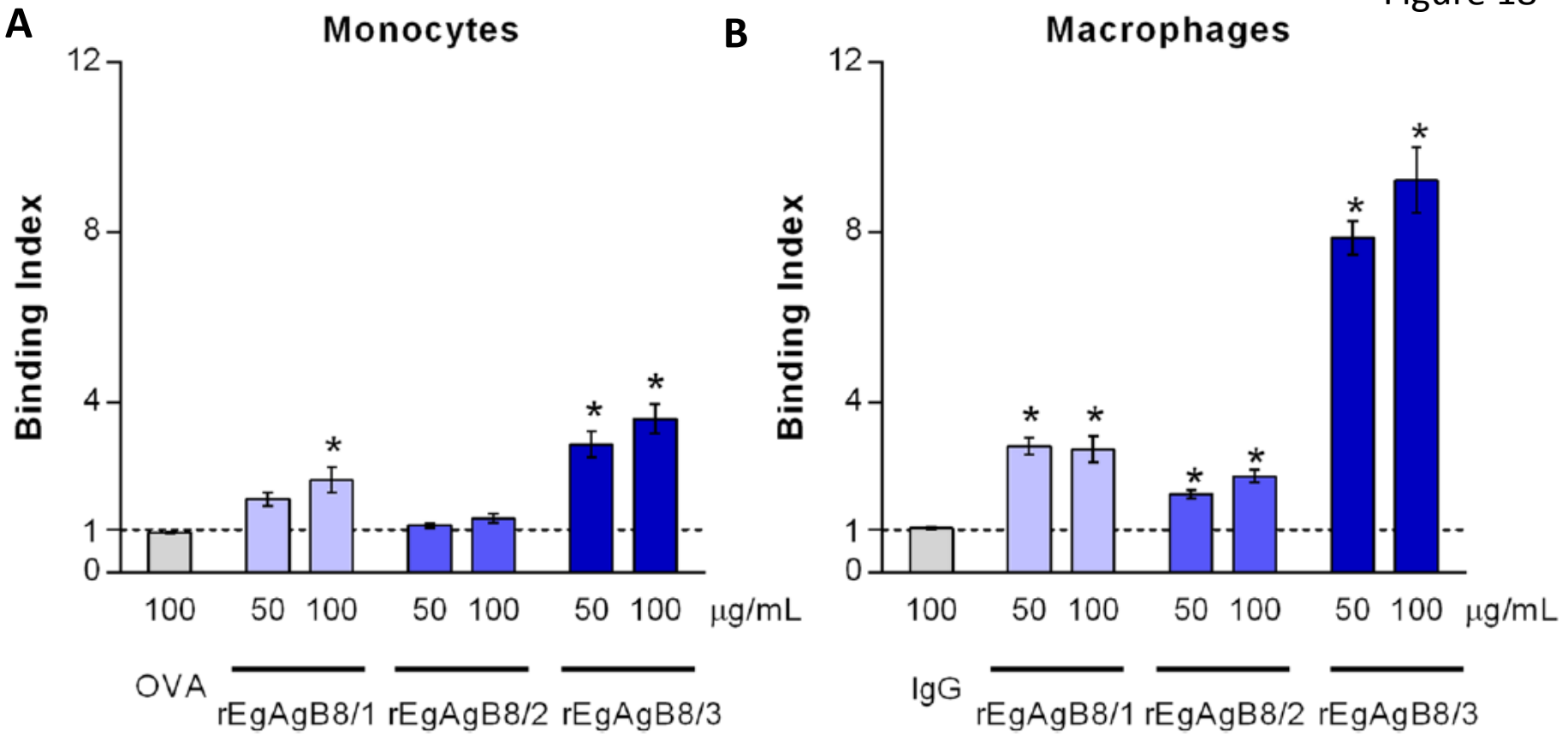
Fluorimetric titration of 12-AS with EgAgB8 subunits. Changes in relative 12-AS fluorescence were monitored from 400 to 500 nm after excitation at 383 nm upon incremental additions of EgAgB8/2 or EgAgB8/3 to a cuvette initially containing 2 mL of 0.5 μM 12-AS in TBS buffer. (A) Emission spectra of 12AS in TBS or upon adding EgAgB8/2 (0.5 μM). (B) Changes in relative 12-AS fluorescence at 440 nm were used to build the binding isotherm of the complex EgAgB8/2–12AS. (C) Emission spectra of 12AS in TBS or upon adding EgAgB8/3 (0.7 μM). (D) Changes in relative 12-AS fluorescence at 440 nm were used to build the binding isotherm of the complex EgAgB8/2–12AS. For both proteins, 12-AS spectra showed a blue shift in emission spectrum that accompanies a strong increase in fluorescence emission. The data were consistent with one binding site per monomer unit of protein and Kd values of 0.16 ± 0.09 μM for EgAgB8/2 and 0.34 ± 0.02 μM for EgAgB8/3 were obtained using SigmaPlot software. The solid line is the theoretical binding curve for complex formation. One representative experiment of three is shown for both EgAgB8 subunits.

Figure 17



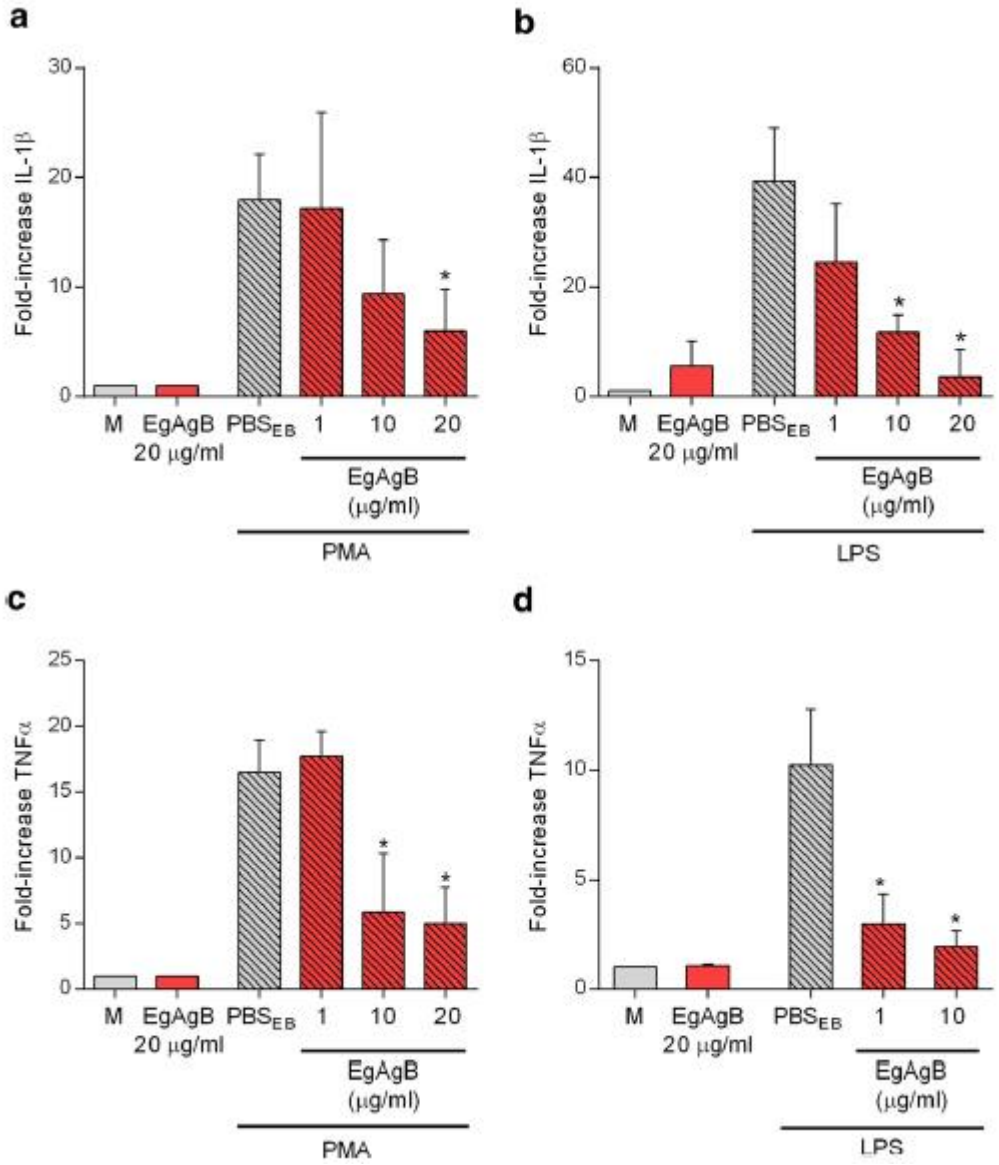
Effect of acceptor membrane concentration on 12-AS transfer from EgAgB8/2 and EgAgB8/3 to different SUVs. Transfer of 12-AS from EgAgB8/2 or EgAgB8/3 to SUVs was monitored by adding SUVs in a molar ratio of 10:1, 20:1 and 40:1 (SUVs/Protein) to the complex EgAgB8/2:12AS or EgAgB8/3:12AS (15:1 mol:mol). (A) Representative kinetic trace obtained when combining EgAgB8–12AS with NBD-PC-containing vesicles (molar ratio SUV/Protein of 10:1). Photobleaching control adding TBS instead of NBD-PC/SUVs is shown. (B) SUVs containing 100% EPC; (C) 75% EPC, 25% PS or (D) 75% EPC, 25% CL were used. For each experimental condition at least five replicates of the kinetic curves were done. All curves were well-described by a single exponential function to obtain each transfer rate value employing SigmaPlot software. Transfer rates (mean ± SD) of three independent experiments are reported. Statistical analysis of the data was carried out employing ANOVA followed by Tukey's Post Hoc Test from GraphPad Prism software.

Figure 18



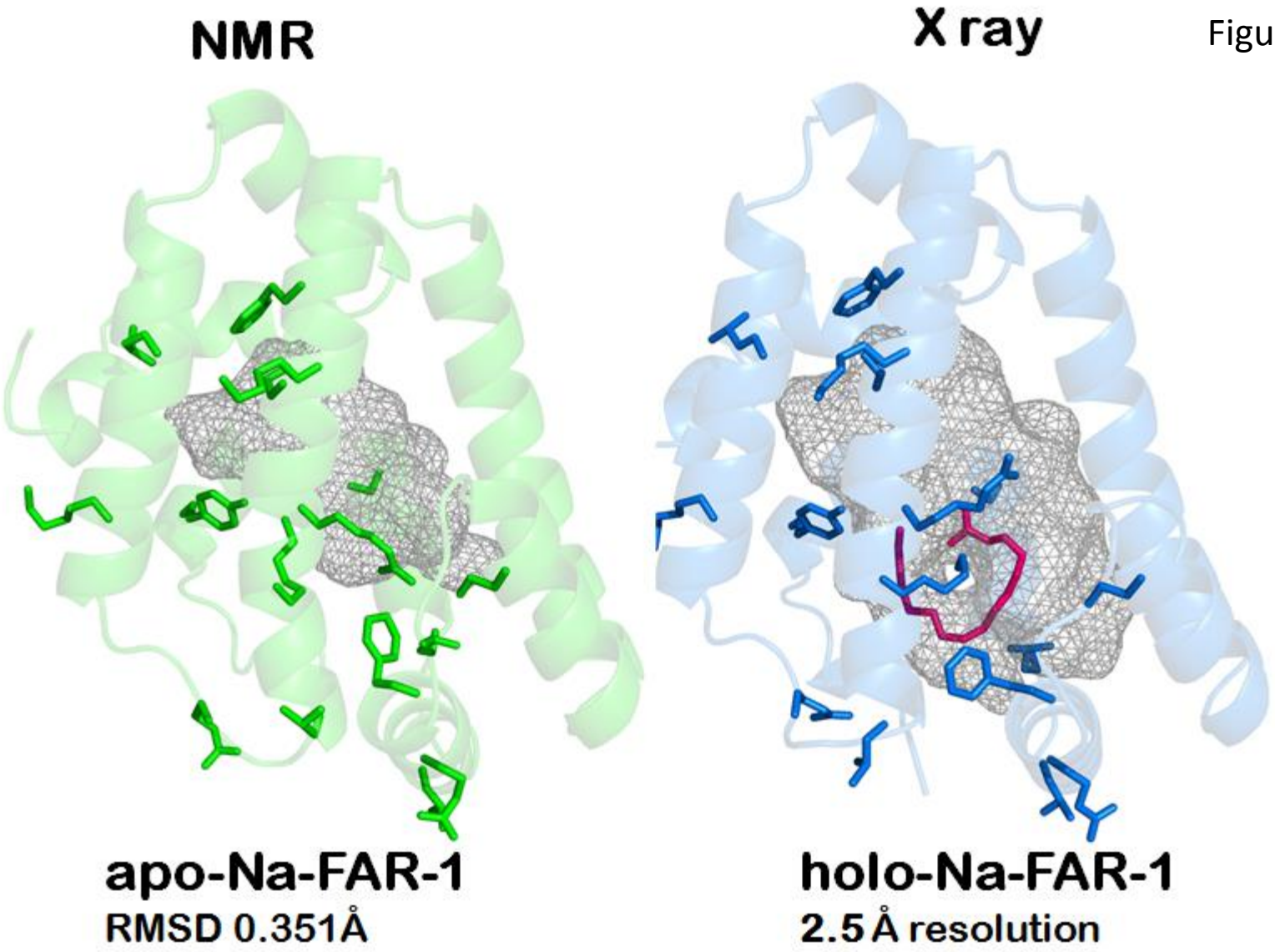
Analysis of the ability of monocytes and macrophages to bind rEgAgB8 subunits. Binding indexes of rEgAgB8 subunits to THP-1 derived monocytes and macrophages, respectively. Data are expressed as mean ± SEM of three independent experiments. Asterisks (*) indicate significant differences with respect to the control.

Figure 19



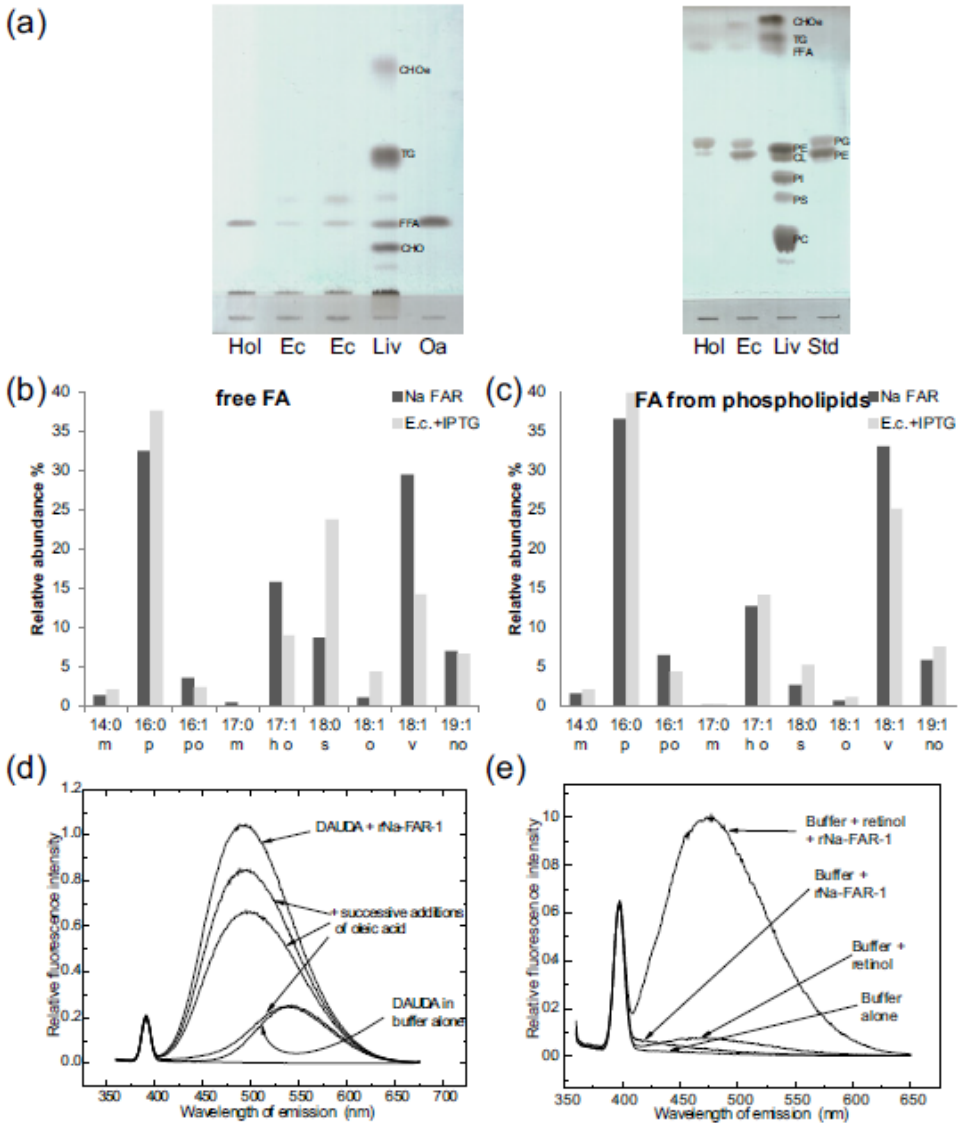
Effects of EgAgB on pro-inflammatory cytokine production by macrophages. THP-1 macrophages were stimulated with PMA (50 ng/ml) or LPS (0.1 ng/ml) for 12 h in the absence or presence of increasing concentrations of EgAgB. Secretion of IL-1β (a, b) and TNFα (c, d) were determined by ELISA in cell culture supernatant. IL-1β or TNFα levels are expressed as the fold-increase related to the basal secretion (normalised against the medium condition).

Figure 20



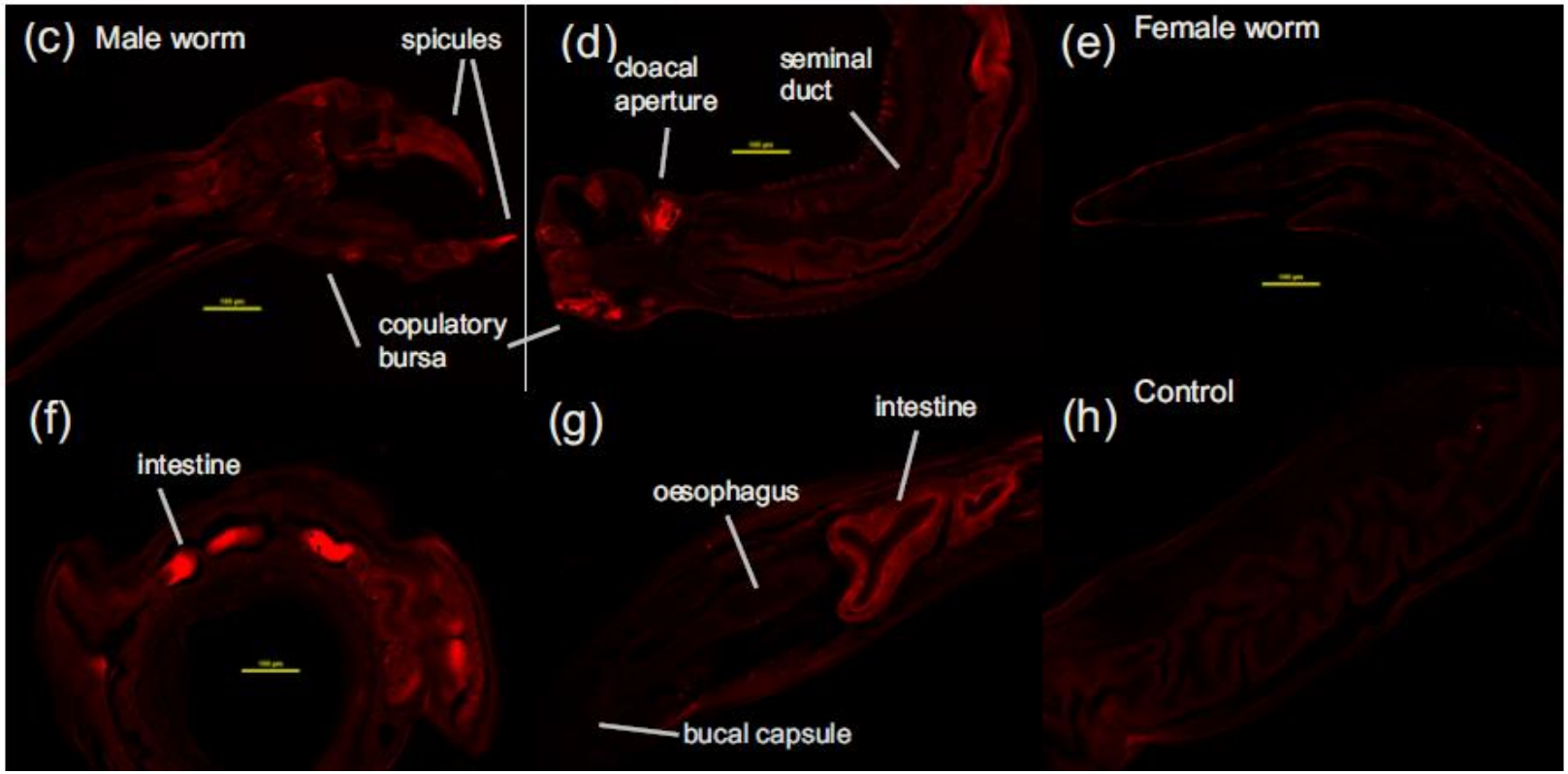
Apo- and holo-Na-FAR-1 structures shown in cartoon form. The apo structure determined by NMR, the ensemble of the 20 lowest energy structures is shown in green. The holo form determined by X-ray crystallography is shown in blue.

Figure 21



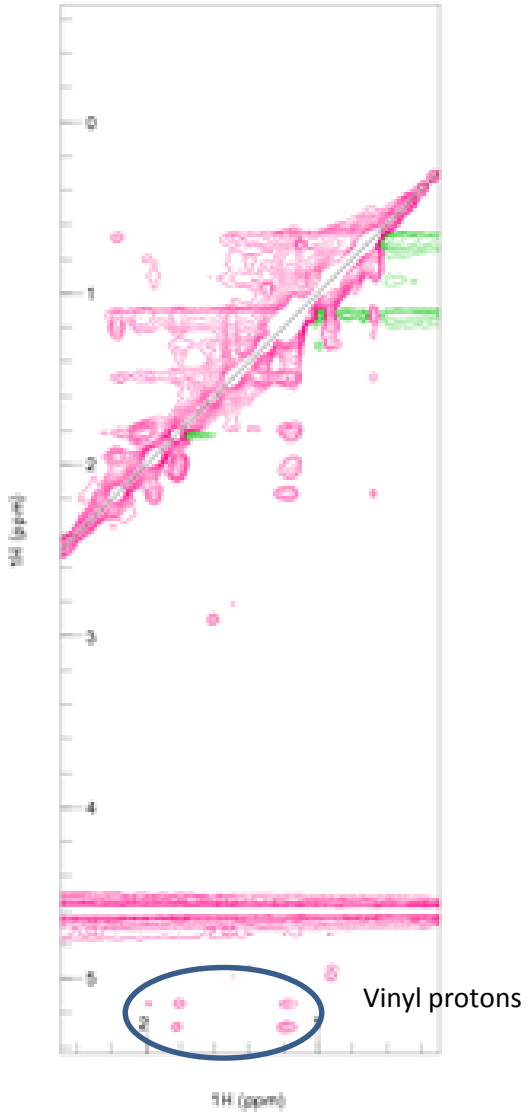
(**a**) Lipid fractions bound to bacterially-expressed Na-FAR-1 detected by TLC. Lipids were extracted from the protein and fractions were analysed by TLC in conditions for resolving separately neutral (left panel) and polar (right panel) lipid classes. Hol, Na-FAR-1 purified without an HPLC step; Ec, extract from whole E. coli cells; LIV, standard mix of lipids from rat liver homogenate; STD, E. coli whole extract. CHO, cholesterol; TG, triglycerides; CHOe, cholesterol esters; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin. (**b**) GC–MS analysis of non-esterified FAs isolated from lipids associated with Na-FAR-1 purified from E. coli (dark grey) or found in E. coli extracts (light grey). (**d** and **e**) Fluorescent ligand binding by Na-FAR-1.

Figure 22



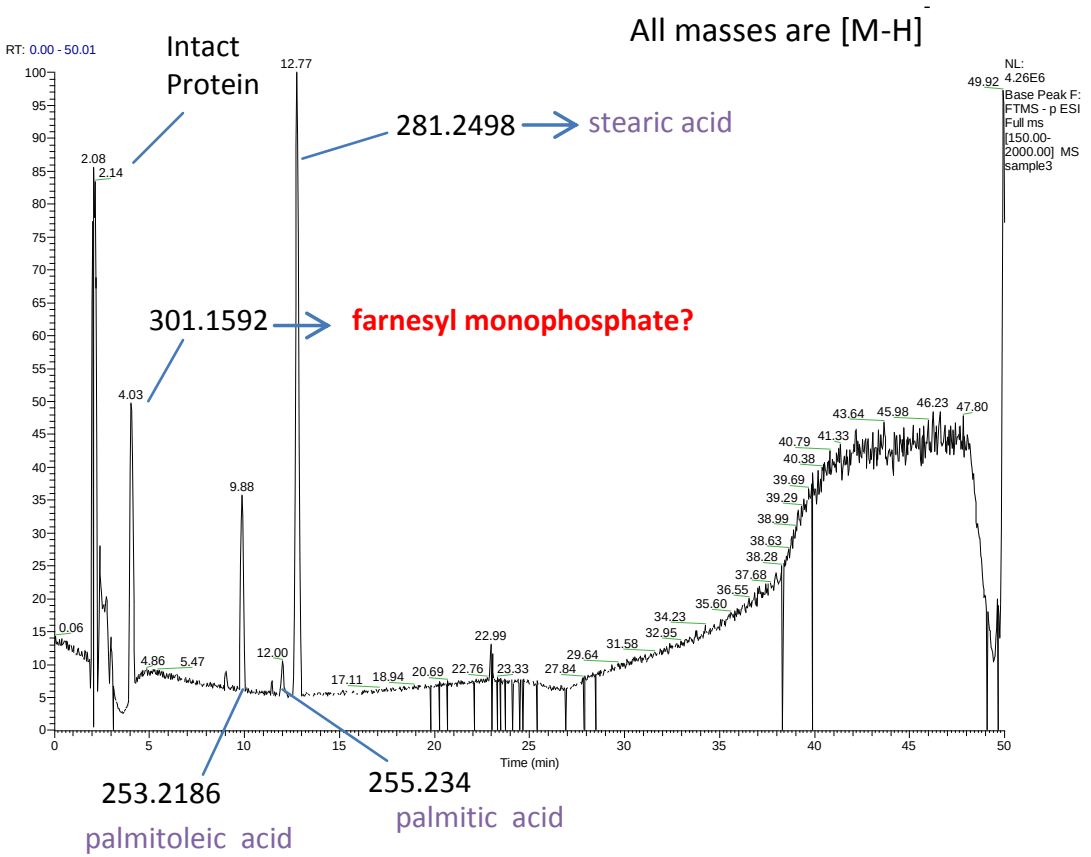
Localization of Na-FAR-1 within adult male (c and d) and female (e–g) worms. Indirect immunofluorescence localization with rabbit anti-Na-FAR-1 serum stains the intestinal cells of adult N. americanus worms. Na-FAR-1 was also detected on the copulatory bursa and cloacal aperture of male worms. (h) Control carried out using pre-immune serum. Scale bars represent 100 µm.

Figure 23



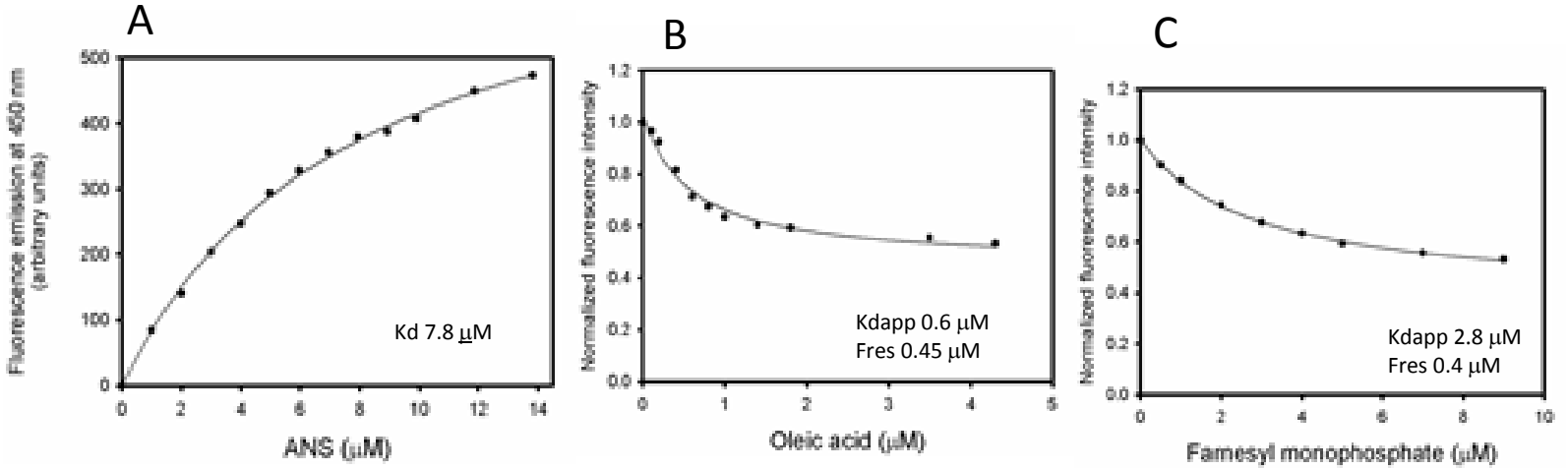
Double X-filtered NOESY spectrum (H [12C, 14N] ⍰ H [12C, 14N]) showing possible interactions or conformations of unlabeled fatty acid interacting with ABA-1A.

Figure 24



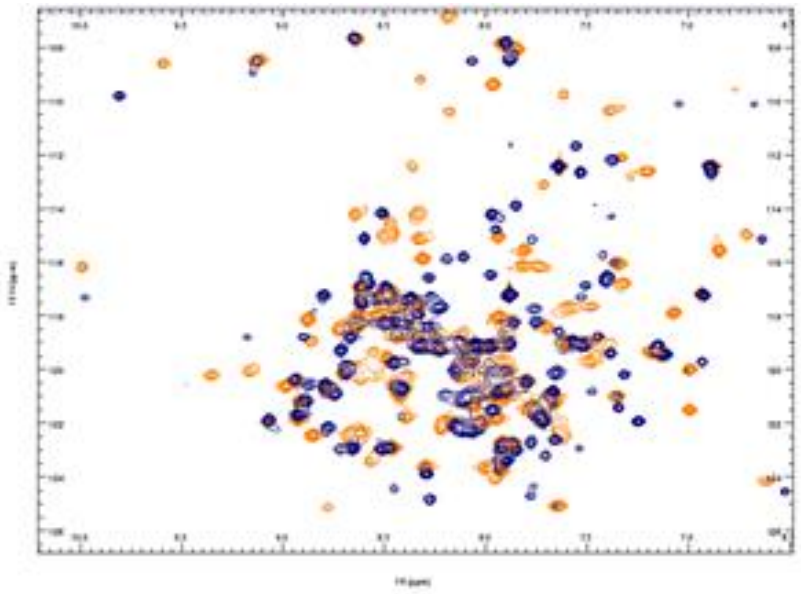
HPLC elution profile of ABA-1A bound lipids using a C 30 column followed by LTQ Orbitrap Velos MS. Lipids were eluted using a water:acetonitrile gradient. Peaks are annotated with the corresponding mass determined by negative ion mode mass spectrometry.

Figure 25



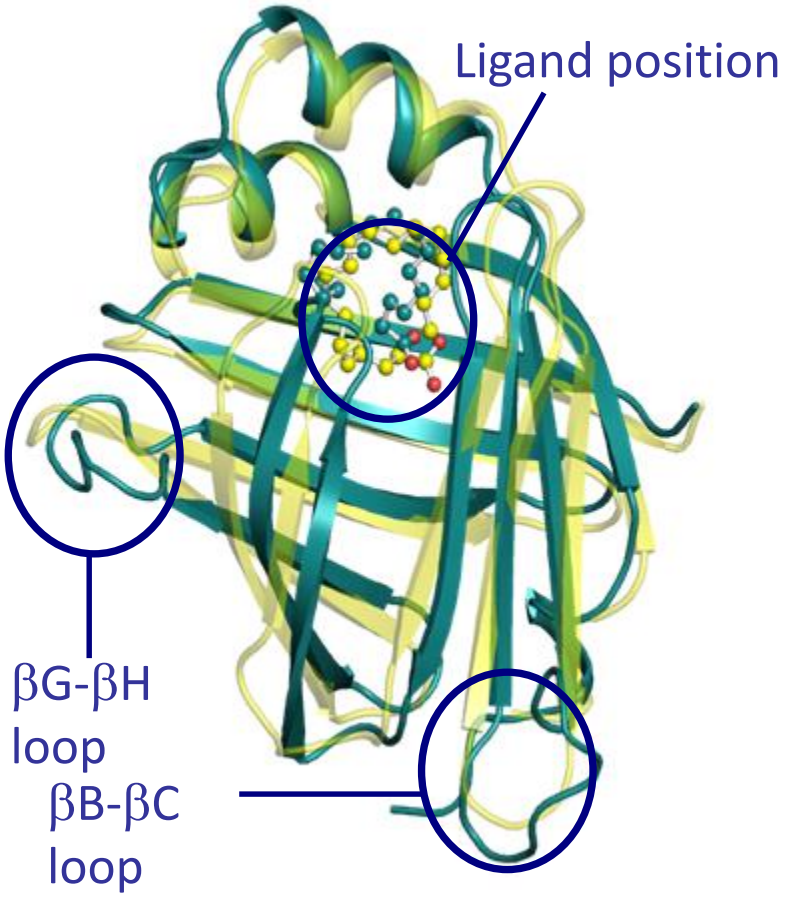
Titration of ABA-1A with the fluorescent probe 1-Anilino naphthalene-8-sulfonic acid (ANS), a one site saturation model F= Fmax1*[ANS])/(Kd1 + [ANS]) was fitted to the data. Competition of ANS bound by (B) oleic acid or (C) FMP. An initial 5:1 ANS:protein ratio was used. The continuous lines correspond to the fitting of equation F= Rres + Fmax/(1 + [ligand]/Kdapp) to the data.

Figure 26



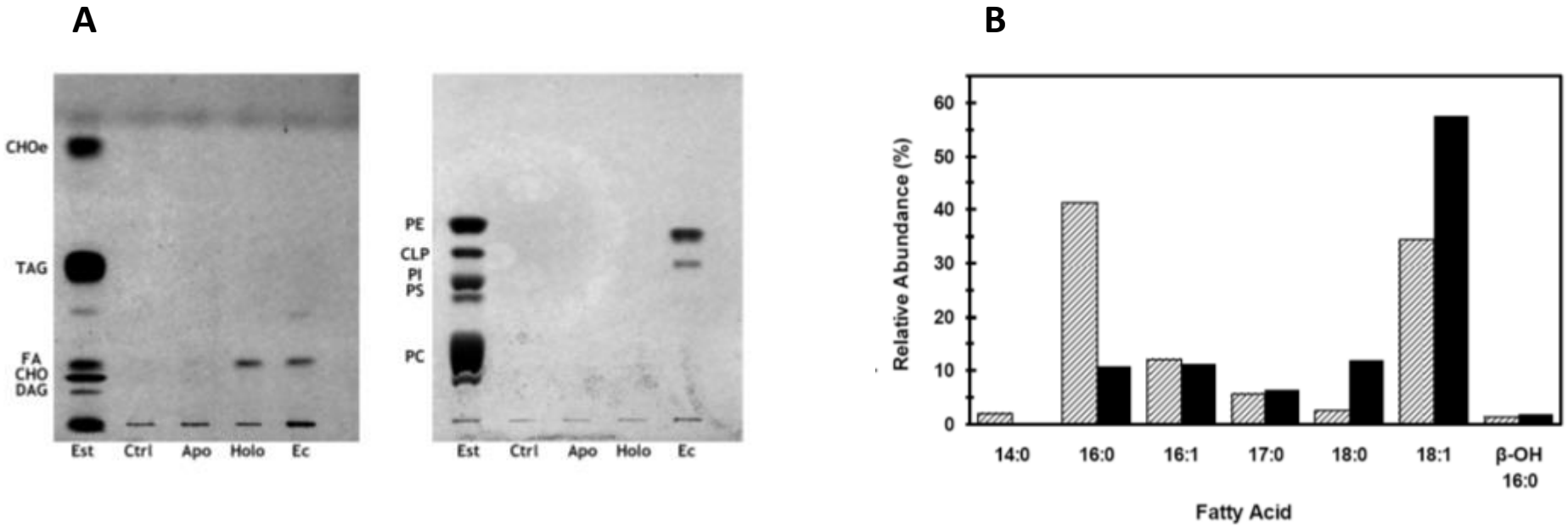
^{15}N HSQC NMR spectra of ^{15}N ^{13}C labeled ABA-1A in its apo- (blue crosspeak) and loaded with FMP (orange crosspeaks) forms. The FMP bound form was recoreded at 1:1 protein:FMP ratio. The sample was prepared in 50 mM phosphate 50 mM NaCl buffer, pH 7.4. A slow exchange mode is observed suggesting at least a first binding event of relatively high affinity.

Figure 27



Superposition of 3D structures of As-p18 (green) and mouse adipocite FABP (yellow). Overall three dimensional fold is very similar to other FABPs. However As-p18 shows prominent features highlighted in blue circles: Two extended loops between the strands bB–bC, and bG–bH.

Figure 28



(**A**)TLC plates using a mobile phase for resolving neutral **(left)** or polar **(right)** lipids. Est: Standard mix from rat liver homogenate. Ctrl 2: Control. Apo: apo As-p18 extract. Holo: non-delipidated As-p18 extract. Ec: *E. coli* extract. CHO: cholesterol; FA: fatty acids; TG: triglycerides; CHOe: cholesterol esters; PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; CLP: cardiolipin; PE: phsophatidylethanolamine. (**B**) Endogenous fatty acids (FAs) from *E coli* culture (dashed bars) and bound to As-p18 (black bars). Fatty acids were extracted, esterified, and the fatty acid methyl esters analysed by gas chromatography.