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Table of contents

1. Introduction	9
1.1 The chicken as a model organism.....	9
1.2 The Lipid droplet as a complex organelle	11
1.3 Lipid trafficking: a brief overview	14
1.4 Energy Homeostasis: Lipogenesis and Lipolysis	17
1.4.2 Patatin-like Phospholipases	21
1.4.2.1 ATGL (Adipocyte Triglyceride Lipase; PNPLA2)	25
1.4.3 Arylacetamide Deacetylase (AADA)	28
1.5 Aims of the Thesis	30
2. Materials and Methods	31
2.1 Chemicals and Enzymes	31
2.2 Bacterial Strains and Vector Systems.....	31
2.3 Oligonucleotide Primer.....	36
2.4 Antisera and Antibodies	38
2.5 Animals	38
2.6 Molecular Biological Methods: DNA	38
2.6.1 cDNA Synthesis	38
2.6.2 Polymerase Chain Reaction (PCR)	39
2.6.3 DNA Gel Electrophoresis	39
2.6.4 DNA Gel Extraction	39
2.6.5 Vector Preparation	40
2.6.6 DNA Ligation	40
2.6.7 Transformation of competent E.coli	41
2.6.8 Mini preparation of Plasmid DNA	41
2.6.9 Midi preparation of Plasmid DNA	42
2.6.10 Quantitative Real Time PCR (RT-qPCR)	42
2.7 Molecular Biological Methods: RNA	43
2.7.1 Isolation of total RNA	43
2.8 Biochemical Methods: Protein.....	43
2.8.1 Preparation of Triton X-100 Total Protein Extracts	43
2.8.2 Preparation of Triton X-100 Membrane Protein Extracts	44
2.8.3 Determination of Protein Concentration (Bradford).....	44
2.8.4 SDS-Polyacrylamide Gel Electrophoresis	45
2.8.6 Western Blot Analysis	46
2.8.8 Antibody Generation.....	48
2.8.9 Chromogenic Esterase Assay using pNitrophenyl-Esters	48
2.8.10 Fluorogenic Lipase assay using 4-methyl umbelliferyl heptanoate (MUH)	49
2.9 Immunohistochemistry.....	50
2.9.1 Isolation and Cultivation of chicken primary hepatocytes	50
2.9.2 Fixation and Fluorescence Staining of chicken primary hepatocytes	50
3. Results	51
3.2 Monitoring of PNPLA3 expression in different tissues	54
3.3. Influence of fasting and refeeding on PNPLA3	55

3.4 Influence of hormonal treatment on PNPLA3.....	58
3.5 Influence of treatment with TO901317 on PNPLA3 mRNA expression	60
3.5 Immunohistochemistry of PNPLA3 in primary hepatocytes.....	61
3.6 Effect of fasting and refeeding on AADA	62
3.6 Analysis of tissue specific expression of AADA isoforms	64
3.8 Cloning, expression and purification of ggStAR	65
3.9 Search for evidence that AADA substitutes for HSL in the chicken	66
3.8.1 AADA activity against pNP-esters	66
3.8.2 AADA activity against MUH	69
4. Discussion	71
5. References.....	75
6. Abbreviations	81
Danksagung	85
Curriculum Vitae	87

Abstract

Although fat generally has a bad reputation, it is essential for many functions of the human body. Cholesterol, for example, is necessary for assembly of cell membranes; triglycerides form large energy stores for times of nutritional scarcity and are also precursors in many other metabolic pathways that give rise to substances needed by the body. Whether lipids are stored or re-mobilised to gain energy is a tightly regulated process. This regulation is important, as de-regulated lipid metabolism may result in diseases such as arteriosclerosis, heart attacks, diabetes, and/or the associated metabolic syndrome.

Lipases are important enzymes involved in the metabolism of lipids. A relatively newly defined family of lipases are the patatin-like phospholipases. These proteins all have a common domain derived from patatin, a protein first identified in potato tubers. In potatoes, patatin serves not only as a hydrolase, but also as a storage protein. It has been shown that some members of the patatin-like phospholipase family fulfil their function rather as lipase, whereas others don't show lipase activity at all. One family member with unclear function is PNPLA3. PNPLA3 shares remarkable homology with ATGL, which was shown to be a very important lipase in adipose tissue. However, PNPLA3 does not exhibit lipase activity *in vivo*, and upon changes in nutrition, PNPLA3 is regulated exactly opposite to ATGL. One part of this work was to find out more about the function of PNPLA3 in our model organism, the chicken (*Gallus gallus*). At the mRNA level, it was already known which chicken tissues express PNPLA3. Here, to monitor PNPLA3 at the protein level, an α -PNPLA3 antibody was raised. Then, the expression of PNPLA3 under different conditions (e.g. fasting, hormonal treatment) was quantitated at the mRNA level using qPCR, and at the protein level using Western Blotting. Employing these techniques, it was shown that PNPLA3, as suggested before, acts *in vivo* as a protein that aids lipid storage rather than as a lipase. Indications for this are that PNPLA3 expression decreases upon fasting and increases upon refeeding, likely to store lipids and replenish energy stores. Also the fact that PNPLA3 is higher in adipose and liver of a mutant hyperlipidemic chicken strain than in normal laying hen tissues indicates a role in lipid storage. Unfortunately, the entire cDNA sequence of PNPLA3 in the chicken is not known. Therefore, it is not yet possible to produce PNPLA3 recombinantly and in order to further test its lipase activity.

The breakdown of triglycerides is achieved in several steps that depend on various enzymes. The first key enzyme is ATGL, which breaks down triglycerides into diglycerides and fatty acids. The diglycerides have to be cleaved into monoglycerides, a step that in humans is catalyzed by HSL (hormone sensitive lipase). In chickens, the enzyme that breaks down diglycerides has not been identified so far; however, the breakdown occurs.

It was shown that AADA (arylacetamide deacetylase) in rodents and humans shares significant homologies with HSL and also exhibits esterase activity. Therefore we hypothesized that AADA may fulfil the function of HSL in the chicken. Changes in AADA expression upon different conditions (fasting, refeeding, etc.) were monitored at mRNA and protein levels.

I tested whether there is an AADA-like protein that is predominantly present in fat tissue (HSL is abundant in fat tissue, AADA rather in the liver) and finally, performed assays to test (i) for esterase and lipase activity of AADA and (ii) if known interaction partners of mammalian HSL would also interact with chicken AADA.

The obtained results were contradicting: no interaction between AADA and FABP4, a known binding partner of HSL was detected, but apparently StAR, another HSL interaction partner, was able to modulate AADA activity against certain substrates in esterase and lipase assays. Interestingly, StAR itself showed detectable endogenous activity against 4-methylumbelliferyl hepanotate, a substrate used in lipase assays; however, StAR does not share common lipase motifs nor any other common lipase feature. Further investigations are required to elucidate if StAR has other functions apart from its known role as cholesterol transporter, and to definitely answer the question whether AADA indeed can substitute for HSL in the chicken.

Zusammenfassung

Fette sind essentiell für viele Funktionen unseres Körpers. Cholesterin ist ein wichtiger Bestandteil jeder Zellmembran, Triglyzeride stellen große Energiespeicher dar und sind oft Ausgangsmaterial für den Aufbau anderer vom Körper benötigter Stoffe. Ob Fette eher zur Speicherung eingelagert oder abgebaut werden, um Energie zu mobilisieren, wird vom Körper mithilfe von Enzymen reguliert. Diese Regulierung ist wichtig, da ein falsch regulierter Fettstoffwechsel die Ursache bzw. ein Faktor für verschiedenste Erkrankungen, wie Arteriosklerose, Herzinfarkt, Diabetes, oder das häufig damit assoziierte Metabolische Syndrom sein kann.

Lipasen sind wichtige Enzyme, die am Abbau von Fetten beteiligt sind. Eine jüngst definierte Familie von Lipasen ist jene der Patatin-like Phospholipases (PNPLAs). Diese Lipase-Familie lässt sich strukturell auf ein Protein zurückführen, das Patatin aus der Kartoffelknolle. Patatin hat in der Kartoffel einerseits Hydrolaseaktivität, dient aber andererseits auch als Speicherprotein. Für die Proteine der Patatin-like Phospholipases hat sich gezeigt, dass nicht alle Familienmitglieder wirklich eine Aufgabe als Lipase erfüllen. Ein Familienmitglied, dessen Rolle derzeit eher unklar ist, ist PNPLA3. Einerseits zeigt PNPLA3 große strukturelle Gemeinsamkeit mit ATGL, einem Protein, das eindeutig eine Lipase ist, hat aber andererseits *in vivo* selbst keine detektierbare Lipase-Aktivität, und wird auch genau umgekehrt zu ATGL reguliert. Teil dieser Arbeit war es, die Funktion des PNPLA3 in unserem Modell-Organismus, dem Huhn (*Gallus gallus*), weiter zu klären. Da bisher nur auf dem mRNA Niveau bekannt war, in welchen Geweben des Huhnes PNPLA3 exprimiert wird, wurde PNPLA3 nun auch auf dem Protein-Niveau betrachtet. Dafür wurde ein anti-PNPLA3 Antikörper hergestellt. Die Expression von PNPLA3 wurde unter verschiedenen Bedingungen (Fasten, Hormonelle Behandlung, etc.) nicht nur auf dem mRNA sondern auch auf dem Protein-Niveau erforscht. Daraus ergab sich, dass PNPLA3, wie schon häufiger vermutet, im Körper offenbar eher die Funktion eines Aufbauproteins als die einer Lipase übernimmt. Hinweise dafür sind etwa, dass die PNPLA3 Expression beim Fasten eher abnimmt und stark ansteigt wenn das Fasten abgebrochen wird. Außerdem ist PNPLA3 in Fett und Leber von mutanten, hyperlipidämischen RO Hühnern, welche mehr Lipide einlagern, stärker exprimiert als in normalen Hühnern. Da im Huhn nicht die gesamte cDNA-Sequenz des PNPLA3 Gens bekannt ist, ist es leider nicht möglich, PNPLA3 rekombinant herzustellen, um dann z.B. Lipase-Assays durchzuführen, und die Funktion von PNPLA3 als Lipase so weiter zu widerlegen – dies ist für künftige Arbeiten geplant.

Der Abbau von Triglyzeriden durch die sogenannte Lipolyse erfolgt in mehreren Schritten und ist von mehreren Enzymen abhängig. Im Menschen erfolgt der Abbau von Triglyzeriden zu Diglyzeriden und einer Fettsäure mithilfe von ATGL (adipocyte triglyceride lipase), das Diglyzerid wird dann von HSL (hormone sensitive lipase) weiter abgebaut.

Im Huhn ist kein Enzym bekannt, das in der Lage ist Diglyzeride abzubauen, dennoch findet dieser Schritt statt. Da gezeigt wurde, dass AADA (Arylacetamid Deacetylase) nennenswerte Homologie mit HSL sowie Esterase-Aktivität besitzt, wollten wir herausfinden, ob AADA im Huhn möglicherweise die Aufgabe von HSL übernimmt.

Dafür wurde auf mRNA- und Proteinebene beobachtet, wie sich die AADA Expression unter verschiedenen Ernährungsbedingungen ändert, und ob es eine AADA Isoform gibt, die vorwiegend im Fett vorkommt (HSL ist in Fettgeweben reichlich vorhanden, AADA kommt eher in der Leber vor). Es wurden Versuche gemacht, um zu bestimmen ob AADA Esterase- bzw. Lipaseaktivität besitzt, und ob Interaktionspartner von HSL (FABP4, StAR) auch mit AADA interagieren können. Hier gab es widersprüchliche Ergebnisse: einerseits konnte Interaktion zwischen AADA und FABP4 festgestellt werden. Andererseits konnte StAR die Aktivität von AADA - zumindest gegenüber gewissen Substraten, die in Lipase bzw. Esterase assays verwendet werden - modulieren. Interessanterweise zeigte StAR auch selbst Aktivität gegenüber einem dieser Substrate, 4-Methylumbelliferylheptanoat (MUH), obwohl nicht bekannt war, dass StAR Lipaseaktivität besitzt und auch strukturell keine Gemeinsamkeiten mit Lipasen zeigt. Weitere Forschungsarbeit wird nötig sein, erstens um zu klären, ob StAR weitere bisher unbekannte Funktionen im Fettstoffwechsel besitzt, und zweitens, ob AADA wirklich HSL im Huhn ersetzen kann, da diese Frage noch nicht eindeutig beantwortet werden kann.

1. Introduction

1.1 The chicken as a model organism

The domesticated chicken (*Gallus gallus domesticus*) is the premier non-mammalian model organism in disciplines such as developmental biology or medical research. It has been already used as a model in the pre-genomic era and served William Harvey, when he performed experiments which revealed the morphology and function of the blood circulation system (Harvey, 1628).

Now that the chicken genome is almost entirely deciphered, there are even more implications for the chicken as a model than before. The chicken genome is composed of approximately 1.2 billion base pairs and approximately 16.000-20.000 genes organized in 39 chromosomes, of which 9 are so-called macro- and 30 are so-called micro-chromosomes. There are indications that the micro-chromosomes are by far richer in genes than macro-chromosomes (McQueen et.al. 1996). Sex chromosomes are designated W and Z, hens carrying WZ and roosters carrying ZZ. In the chicken genome, there is only a very small amount of repetitive DNA sequences compared to mammalian DNA, and there is also only a very low number of pseudogenes. Also, a wide range of single nucleotide polymorphisms has been found in the chicken (Burt, 2007).

Unlike the viviparous mammals, the chicken is an oviparous (egg-laying) organism. Because of the fact that embryos grow in eggs outside the mothers' body, the embryos' energy supply must be ensured before the egg is laid. This happens by incorporation of energy-rich molecules into a special compartment of the egg, the yolk. Yolk consists mainly of Vitellogenin and VLDLs (very low density lipoproteins). These molecules are transported into the yolk during follicle development via receptor-mediated endocytosis. The receptor necessary for the internalization of VLDLs and vitellogenin into the yolk is a member of the LDL receptor family, and is termed LR8, as it contains eight binding motifs rich in cystein residues.

In the laying hen, each day one oocyte enters a developmental cycle, gets enriched in yolk components and finally ovulates. The major developmental stages in follicle development are the small white and large white follicle, the small and large yellow follicle and follicles F5, F4, F3, F2 and F1, with F1 being the next follicle to ovulate.

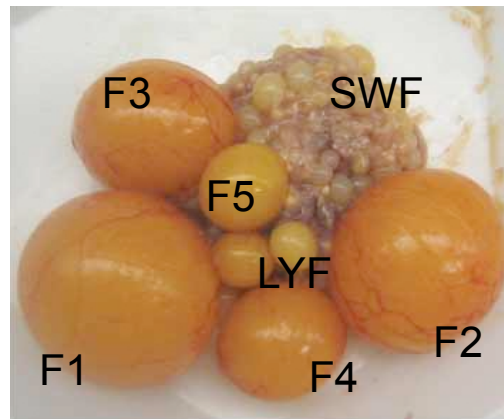


Figure 1.1 Stages of chicken follicle development, SWF = small white follicle; LYF = large yellow follicle

In the Restricted Ovulator (R/O) chicken strain, a naturally occurring infertile mutant, LR8, a 95kDa receptor needed for the transport of yolk components into the yolk, is unfunctional because of a mutation in an EGF repeat, where a cysteine is substituted for a serine residue (Bujo et.al.1995). Because of this, yolk precursors are not transported into the developing oocytes, the oocytes show an incomplete development and become necrotic before they can ovulate. Because the transport of yolk precursors into the oocytes does not work, R/O hens accumulate fat in their bodies, resulting in a hyperlipidaemic and potentially atherosclerotic phenotype. The accumulation of fat is especially drastic in the liver which becomes ryellowish because of the high fat content. Roosters of the R/O chicken strain have a normal phenotype, although the gene defect is located on the Z chromosome (Bujo et.al. 1994). In fact, one would expect a severe phenotype in roosters who possess two Z chromosomes; however, the LR8 gene is not expressed much in other cells than oocytes, and therefore roosters are not affected by the mutation in the LR8 gene.

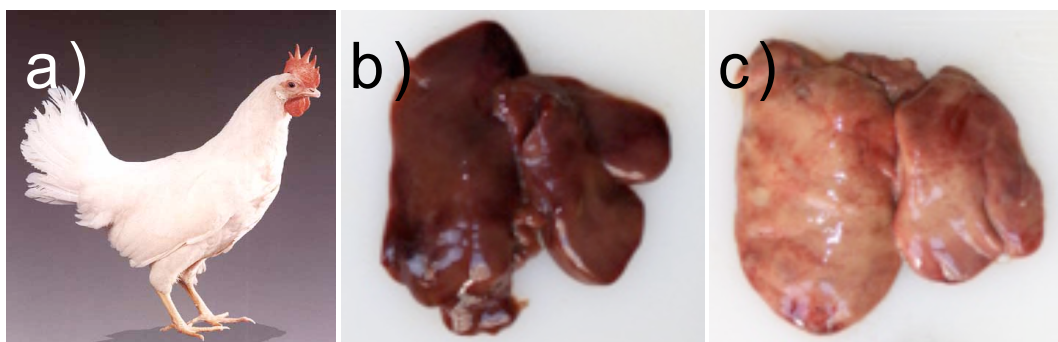


Figure 1.2 White leghorn hen (a) Healthy chicken liver (b) and chicken liver with fat accumulation (c)
(a) from <http://www.moorlandspoultry.co.uk/white-star.html>
(b) and (c) adapted from <http://www.seriouscats.com/recipes/images/liver%20on%20board%201.jpg>

The hyperlipidaemic phenotype of the R/O hens offers a range of possibilities to investigate differential gene expression in various tissues, and so the chicken serves as an appropriate model organism for the aims of my diploma thesis.

1.2 The Lipid droplet as a complex organelle

Organisms have the ability to store excess nutritional energy that is not needed at the moment in special storage forms in certain tissues. Triacylglycerols (TG) are the main storage form of fat in adipose tissue. But apart from the storage of fat in adipocytes, eukaryotic cells are also capable of storing a limited amount of neutral fat in so-called lipid droplets within the cytosol. In animal cells lipid droplets are sometimes also called adiposomes, and in plant cells they are referred to as oil bodies. In former times lipid droplets weren't paid too much attention to, but in the meantime they are regarded as dynamic organelles resembling lipoproteins.

Like lipoproteins, lipid droplets have a core that contains TG and cholesterol esters (CE) and are – in contrast to vesicles - surrounded by a phospholipids monolayer that is coated with specific proteins.

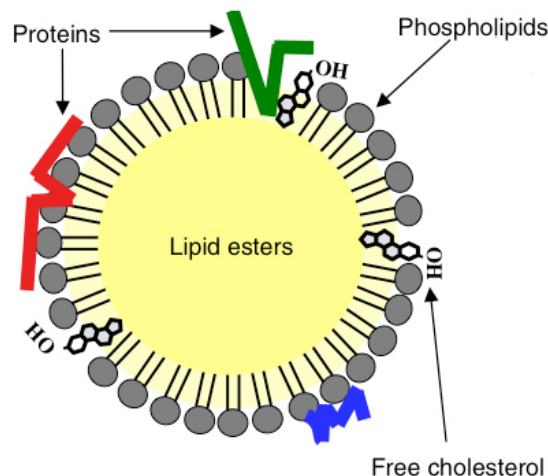


Figure 1.3 Lipid droplet, PAT proteins associated to the phospholipid monolayer are shown in pink, green and blue. (adapted from Fujiimoto et al. 2008).

Although the energy store provided by lipid droplets is small, it is nevertheless important: the stored lipids can serve as substrates for the biosynthesis of membrane phospholipids, the products of TAG hydrolysis may affect cell signalling or gene expression by acting as ligands for certain receptors, on the cell surface or the nucleus, respectively.

As already mentioned, like lipoproteins, lipid droplets are coated, and thereby also regulated, by a certain family of proteins, the perilipin family of proteins, also called PAT-proteins (the abbreviation PAT is derived from the first identified members of the perilipin family, Perilipin A, Adipophilin and IIP47).

Some perilipin family members are always found on the surface of lipid droplets, others only under lipogenic/lipolytic conditions, so it appears likely that they regulate the packing and the transport of lipid droplets and their metabolites.

To date, there are five important known members of the perilipin family: Perilipin A (and its shorter isoforms B and C), Adipophilin, TIP47, OXPAT, and S3-12. Although they belong to the same protein family they do not share many structural features.

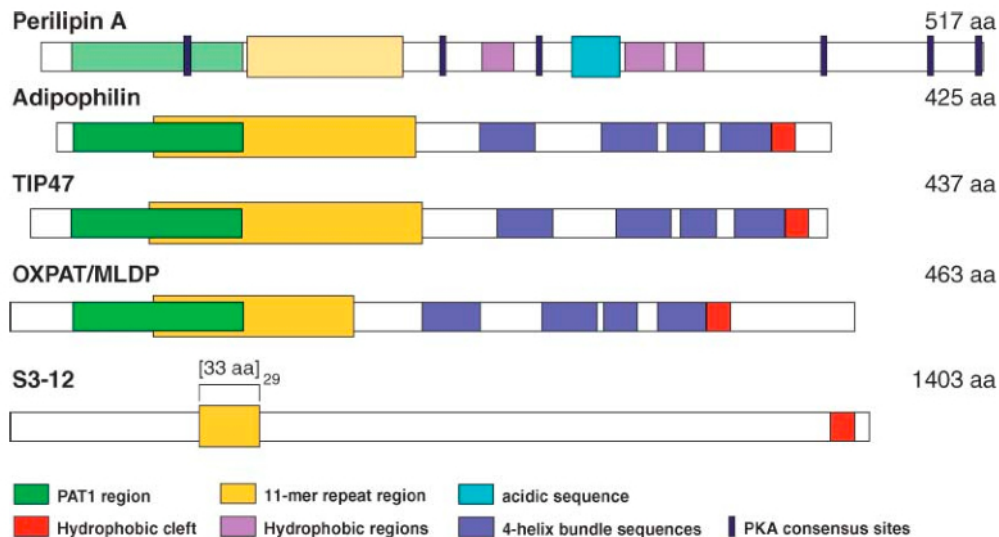


Figure 1.4 Perilipin family members and structure The mouse perilipin family members are depicted. A faint colour represents less sequence similarity and stronger colour higher similarity of sequences. (from: Brasaemle, 2007)

In Figure 1.4 one can see that adipophilin, TIP47 and OXPAT are closer related to one another than to perilipin. S3-12 is missing a lot of features that are provided in the other family members. Except S3-12, all family members contain a PAT domain (green) at the N-terminus, consisting of about 100 amino acids that are highly conserved. The function of the PAT domain is not known.

An 11-mer repeat region (yellow) of variable size overlaps with the PAT domain and is also present in S3-12. The 11-mer repeat region is predicted to fold into amphipathic helices.

There is contradicting data on whether or not the 11-mer repeat sequences are necessary for targeting the protein to the nascent lipid droplet (Yamaguchi et al. 2006; Ohsaki et al. 2006, Garcia et al. 2003). Anyhow the amphipathic helices seem to be necessary to place the protein on the lipid droplet in a distinct manner so that it performs its function optimally.

Following the 11-mer repeat region in TIP47, there is a 4-helix bundle region (blue) that also forms amphipathic helices. This region in TIP47 obviously provides stability to the protein both in the cytosol and when bound to the lipid droplet (Wolins et al. 2001); furthermore the region also resembles the 4-helix bundle region on the N terminus of ApoE that is also exchangeable between lipoproteins and the blood (Hatters et al. 2006). Although adipophilin shares high sequence similarity in this 4-helix bundle region, it doesn't share the ambiguous state of TIP47 as adipophilin was shown to be highly unstable in the cytoplasm (Xu et al. 2005).

OXPAT, that is also closely related to TIP47 and adipophilin, also has the 4-helix bundle region, appears to be stable in the cytoplasm, but becomes rapidly recruited to nascent lipid droplets (Wolins et al. 2006). Adipophilin, TIP47, OXPAT, and S3-12 share one region of 14 amino acids that is not found in the sequence of perilipin A, which folds into an hydrophobic cleft (red).

Perilipin A has some unique structural features among the family members: instead of the 4-helix bundle region and the hydrophobic cleft region, it contains three hydrophobic regions (purple) interspaced by a strongly acidic region (turquoise). Additionally, Perilipin A is the only family member that contains consensus sites for phosphorylation by Protein Kinase A (PKA).

	Perilipin A	Adipophilin	TIP47	OXPAT	S3-12
Localized to Lipid droplet	constitutive	constitutive	transient	transient	transient
Cytoplasmic stability	instable	instable	stable	stable	instable
PKA sites	6	none	none	none	none
Tissue distribution	White and brown adipose tissue	All tissues <i>except</i> adipocytes	All tissues	heart	White adipocytes

Table 1.1 Summary of characteristics of Perilipin family members

It is unclear how exactly lipid droplets are formed within the cell. The generally proposed model is that they emerge from the endoplasmic reticulum (ER) by “budding” from the ER membrane to form a droplet. This is quite probable, as ER proteins have been found to be present in lipid droplets isolated from various cell types, e.g. 3T3-L1 adipocytes (Wolins et. al. 2005).

It is tempting to suggest that the lipid droplet formation takes place at the ER, because this is also the place where lipases that mobilize the energy from the lipid droplets are found. But it was also shown by cryo-electron microscopy that the fatty acid composition of the lipid droplet membrane differed from that of the phospholipids of the rough ER (Tauchi-Sato et. al. 2002). These data suggest that lipid droplets probably are synthesized in a different manner; nevertheless, the model of emergence from the ER today is the prevailing one.

Lipid droplets in the cell usually aren’t uniform, but a heterogeneous pool in respect to their different size, localization, and protein coating. For example, under resting conditions, most droplets are coated with perilipin A and centrally located in the cell. When there is an influx of fatty acids into the cell, at the periphery a new pool of smaller droplets forms that are coated with S3-12 and TIP47. Over time, they “mature”, become larger and acquire adipophilin on their surface (Wolins et. al. 2006).

Whether differently coated lipid droplets also serve for different specialized functions in energy storage and metabolism remains to be elucidated. However, perilipin A - the best characterized family member of perilipin proteins - was found to play an important role in lipolysis, as later described in 1.4.4.

1.3 Lipid trafficking: a brief overview

Dietary lipids and cholesterol that are taken up have to be distributed from the gastro-intestinal tract to different peripheral tissues where they are needed for energy supply or biosynthesis of, e.g., steroid hormones or membrane lipids. Excess lipids are stored in the adipose tissue for times of energy scarcity. Lipids are stored as triacylglycerols (TG) in the body.

There is one major problem that the body has to overcome to transport and store TG in the adipose tissue, namely the hydrophobicity of the TG and the polarity of the blood. Blood is an aqueous liquid, whereas TG are lipophil, so TG can't be transported as such within the blood stream. Instead, TG have to be packed in some sort of transport vehicle, the so-called lipoproteins. Lipoproteins are made up of a core containing TG and cholesterol esters (CE) and a monolayer of phospholipids that associates with apolipoproteins. Apolipoproteins have a structure containing amphiphilic helices that can integrate between phospholipids, thereby making the lipoproteins more soluble in the aqueous milieu. So apoproteins and phospholipids form a kind of amphiphilic interface, whereas the core with the TG and CE is lipophilic. The amphiphilic interface enables the particle to be transported within the blood.

There are four types of lipoprotein particles: Chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). There are also different forms of apolipoproteins, namely ApoA1 and ApoA2, ApoB100 and ApoB48 (a truncated form of ApoB100), ApoC and ApoE. The major characteristics of lipoprotein particles are listed in Table 1.2.

	Chylomicrones	VLDL	LDL	HDL
Density [g/ml]	<0,95	0,95-1,006	1,019-1,063	1,063-1,21
Size [nm]	100-1000	50	20	10nm
Content	85% TG 15% Protein	50% TG 20%Phospholipid 10% CE 10% Protein	40% CE 20% Protein 20%Phospholipid 10%TG	50%Protein 25%Phospholipid 15% CE
Proteins	ApoB48; E; C	ApoC; B100; E	ApoB100	ApoC; A;E
Built in	Colon	Liver	Liver	Liver
Function	Transport of TG and Cholesterol to the periphery	Transport of TG to peripheral tissue	Transport of CE to the liver and periphery	reverse cholesterol transport
Half life	5min	3hours	2,5 days	3,5 days

Table 1.2 Characteristics of Apolipoproteins (from Horn et al. 2005; Löffler 2001)

How does the packing into lipoproteins work?

Dietary TG are metabolised in the gastro-intestinal tract. In the gut they are broken up into monoacyl-glycerol and two fatty acids. These single components are lipophilic and small enough to be taken up via diffusion by the enterocytes. In the enterocytes they are rebuilt to TG and then together with CE, phospholipids and apoproteins they become assembled as chylomicrons. Chylomicrons are the largest lipoprotein particles, as they contain a huge amount of TG and CE. The chylomicrons are released into the lymph to avoid the storage of their TG and CE in the liver, enter the blood stream at the ductus thoracicus, and supply peripheral tissues with their load. .

The chylomicrons then reach the liver as chylomicron remnants containing little TG. In the liver, in the postprandial period, VLDLs are synthesized. VLDLs are smaller than chylomicrons because they contain less TG and have a composition of apoproteins different from chylomicrons. VLDLs travel to the periphery and supply tissues, especially the adipose tissue, with TG and CE. As in the case for the conversion of chylomicrons to chylomicron remnants, a special extracellular lipase is needed, i.e. lipoprotein lipase (LPL). This lipase is attached to endothelial cells of blood vessels, and has a high affinity for both chylomicrons and VLDLs and needs ApoC-II on lipoprotein particles as a co-factor. In the presence of insulin (after food consumption) LPL becomes active and starts to act on lipoproteins in the circulation. LPL cleaves TG into monoacylglycerol and free fatty acids that can be taken up into cells where they are then re-built to TG. By giving off TG to peripheral tissues, VLDLs diminish in size and become LDLs, which contain mainly cholesterylesters in their core.

LDL particles are taken up by cells by receptor-mediated endocytosis via LDL receptors. When cholesterol enters the cell, a feedback loop stops the cells' own cholesterol production and instead the cell uses the cholesterol delivered from the LDL particle.

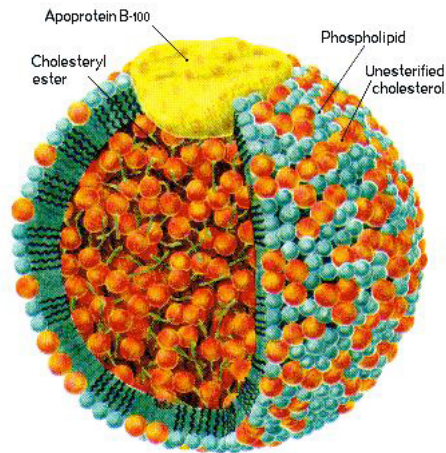


Figure 1.5 The LDL particle, The relatively big ApoB100 protein (yellow) is associated with the surface consisting of phospholipids (turquoise) and unesterified cholesterol (orange). The core consists of cholesteryl esters and a small amount of TG. The phospholipid layer is only a monolayer. From: <http://foodspace.files.wordpress.com/2008/05/ldl.jpg>

The LDL concentration in the blood is associated with an increased risk for arteriosclerosis. If the LDL concentration in the blood is increased due to limited clearance of LDL from the blood, the LDL particles become oxidized and attach to the intima of blood vessels. Macrophages recognize oxidized LDL but not via the LDL receptor.

Instead they recognize LDL with (a) so-called scavenger receptor(s). Because of this, there is no feedback loop to stop the macrophages' cholesterol production and the cells accumulate cholesterol, becoming so-called "foam cells". Foam cells form plaques within the blood vessel and clog the vessel, thereby blocking the blood flow and increasing the risk of a heart attack.

Although every cell is capable of synthesizing cholesterol, all cells lack mechanisms to degrade cholesterol. There are only two ways to eliminate cholesterol from the organism: by scaling off mucosa cells containing cholesterol from the colon and export them with the feces or by forming bile acid from cholesterol. HDL particles are in permanent contact with other lipoproteins and can take up TG, phospholipids, and cholesterol esters from chylomicrons and VLDL and either redistribute them to other lipoprotein particles or to tissues in need of cholesterol for biosynthetic processes. They are also able to eliminate cholesterol from macrophagic plaques, which is why HDL is commonly referred to as "the good cholesterol" (Horn et al. 2005).

1.4 Energy Homoestasis: Lipogenesis and Lipolysis

Although most people would say that fat is not healthy for the body, a certain amount of fat is essential for our survival. Lipids supply our bodies with energy, and they are especially important, as the major component of every cell membrane are (phospho-)lipids which surround the cells in bilayers. Adipose tissue also serves as an endocrine organ and is able to produce and secrete endo- and paracrine factors, the so-called adipokines (e.g. leptin), which modulate e.g. insulin sensitivity.

Our body has the ability to synthesize, break down, and store fatty acids. The body stores fat in times of plenty in the adipose tissue and breaks it down in times of fasting to gain energy in the form of ATP for the body. TG are the major storage form of fat in the body. They are made up of four single components, a glycerol molecule and three fatty acid molecules. TG are more energy-rich molecules than carbohydrates, and so they form a perfect energy depot.

The biosynthesis of TG is called lipogenesis, the degradation of TG to gain energy is generally called lipolysis. Whether the body performs lipogenesis or lipolysis is dependent on the supply with energy, i.e. food, and on hormones. Insulin, the hormone emitted after a meal to lower the blood glucose level again, activates lipogenesis so that fats are stored in the adipose tissue. Glucagon, the antagonist of insulin, and adrenalin, emitted under stress when more energy is needed, activate lipolysis so that energy is produced by the breakdown of TG (β -Oxidation of fatty acids).

As metabolic diseases related to aberrations in energy homeostasis, such as obesity, diabetes, the metabolic syndrome, arteriosclerosis, and heart attacks are a growing problem in the Western world, it is crucial to understand the underlying regulating mechanisms and to investigate the interactions between molecules involved in lipid metabolism for a better understanding of lipid metabolism forwards new therapeutic possibilities.

One group of enzymes that is crucial to regulate lipolysis are the lipases. Key lipases are discussed in the following sections.

1.4.1 Hormone sensitive Lipase (HSL)

The fasting body has to mobilize its energy stores for necessary metabolic processes. To gain energy the body fat stores can be broken down by lipases. Among the lipases, those that cleave TG, DG, or MG depending on their affinities for these substrates are an important group. In different tissues, different lipases are present. In adipose tissue, it was thought for a long time that the hormone-sensitive lipase (HSL) was the most important lipase catalyzing lipolytic reactions. Apart from its high expression in adipose tissue, HSL is also present in ovary, testis, and adrenal, reflecting the fact that adipose tissue and its proteins also serve a role as endocrine organ.

Surprisingly, HSL seems not to be related to any other mammalian lipase known, but shares noticeable sequence homology with a lipase of an antarctic bacterium, *Moraxella TA144* (Feller et al 1991, Langin et al. 1993).

HSL is a serine hydrolase with the catalytic Serine residue found in the characteristic lipase motif G-X-S-X-G (Holm et al. 1994). The Serine residue together with Aspartic Acid and Histidine builds a classic Ser-Asp-His catalytic triad (Osterlund, et al. 1997). The multi-domain structure that is proposed for HSL due to sequence and molecular modeling analysis (Osterlund et al. 1997; Smith et al. 1996) is depicted in Figure 1.6. The N-terminal site of HSL is now proposed as a binding domain for HSL interaction partners and comprises about 300 amino acid residues that are variable. The C-terminal domain is the catalytically active site of about 400 amino acid residues, making up 48kDa. The C-terminal domain also contains a regulatory module necessary for activation of HSL by Protein Kinase A. In its functional form HSL acts as a dimer, although HSL monomers also exhibit enzymatic activity (Shen et al. 2000).

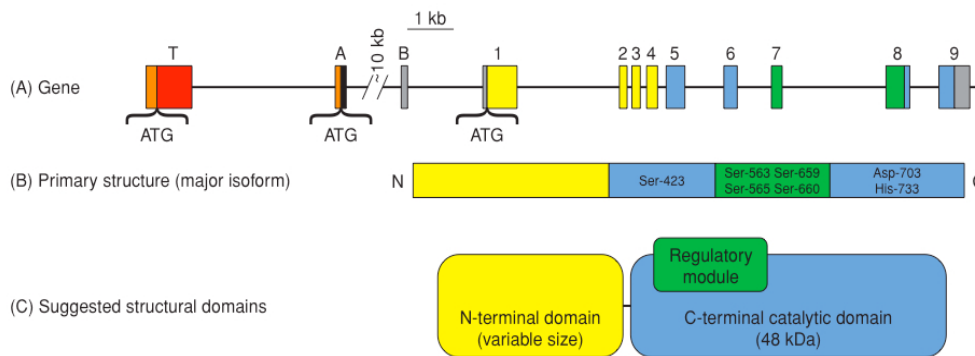


Figure 1.6 HSL multi-domain structure (from Yeaman 2004), in (A) the gene structure (intron/exon organization) is depicted, three different ATG (AUG, respectively, in the mRNA) start codons result in three possible isoforms of HSL that have different size. (B) represents the primary protein structure, yellow being the N-terminal domain, the C-terminal domain is coloured in blue with the regulatory domain for PKA in green. (C) shows the proposed domain-structure, the colour code is the same as in (B).

As one can see in Figure 1.6, there are three ATG (AUG, respectively, in the mRNA) start codons, resulting in three different isoforms of HSL, that range in their size from 84kDa to 130kDa (Holm et al. 1988).

As already mentioned, HSL action is controlled by phosphorylation by PKA. If a lipolytic signal is received by the cell, such as docking of catecholamine (or ACTH, glucagon, noradrenalin or adrenalin) to a β -adrenergic receptor, conversion of ATP to cAMP by adenylate cyclase occurs. Increased cAMP levels in the cell activate Protein Kinase A (PKA) which in turn phosphorylates HSL to activate it. The phosphorylation of HSL results in a 2-fold increase of HSL activity - however, lipolytic rates increase by 100-fold in the adipose tissue in response to lipolytic stimuli (Nilsson et al. 1980, Londos et al. 1985). Therefore, it was supposed that the phosphorylation of HSL could not be the only step necessary for effective lipolysis.

The discovery that perilipin, a lipid droplet associated protein (Greenberg et al. 1991; also see 1.2), is also regulated in its action by phosphorylation by PKA, finally answered the question why HSL activity after phosphorylation only increased slightly, while lipolytic rates increased massively. It was found that perilipin associates with lipid droplets only under unstimulated basal conditions, but dissociates from the lipid droplet upon hormonal stimulation, thereby providing free access for HSL to the lipid droplet surface. Concluding, the phosphorylation of HSL and perilipin by PKA results not only in activation of HSL but also in translocation of HSL from the cytosol to the lipid droplet (Su et al. 2003; Sztalryd et al. 2003). This is schematically shown in Figure 1.7.

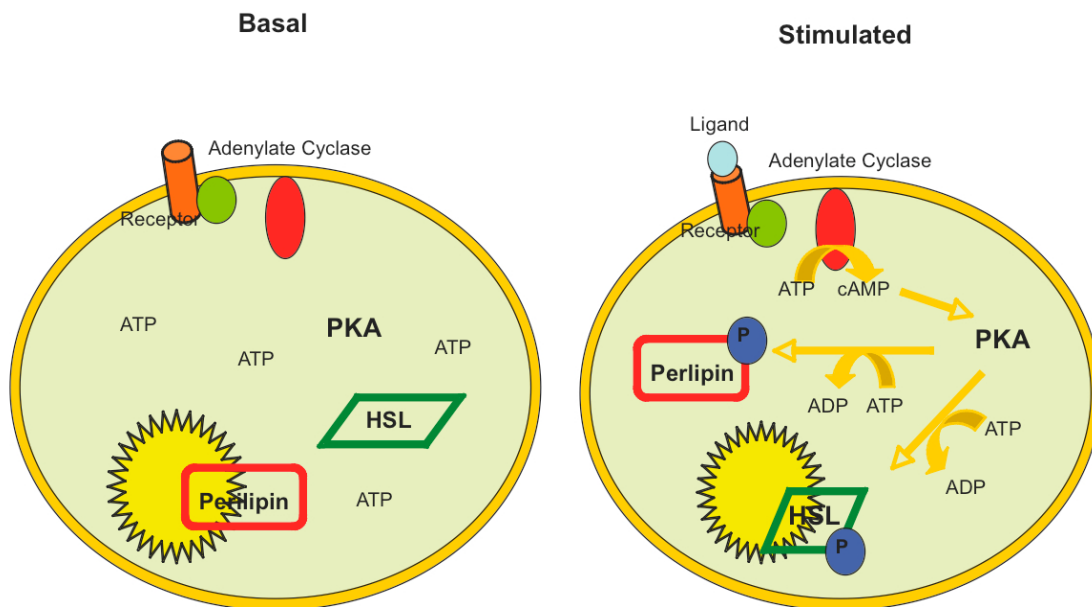


Figure 1.7 Activation of HSL and perilipin by PKA, under basal conditions, HSL is located in the cytoplasm and perilipin is bound to the lipid droplet, both proteins are dephosphorylated. Upon a lipolytic stimulus, PKA becomes activated by an increase of the intracellular cAMP concentration, and phosphorylates both HSL and perilipin – perilipin dissociates from the lipid droplet, giving way for HSL action on the lipid droplet surface. (© Carolin Besenböck)

Furthermore HSL may be activated by the MAPK (mitogen activated proteins kinase) pathway by the MAPK ERK. There are 4 phosphorylation sites for ERK present in the HSL molecule and ERK, as PKA, is able to increase the activity of HSL about 2-fold (Greenberg et al. 2001). However, the physiological relevance of ERK signaling on HSL activity is not yet well understood.

At the beginning of the new millennium, some groups succeeded in generating HSL knock- out mice. These mice showed a surprising phenotype. It was expected that mice lacking HSL would present massive TG accumulation in the plasma and organs, and would be obese. In fact HSL^{-/-} mice were quite lean and did not show TG but DG accumulation in adipose tissue, muscle, and testis (Wang et al. 2001; Haemmerle et al 2002). Male HSL^{-/-} mice were sterile due to oligospermia (Osuga et al. 2000), but the molecular mechanism underlying the development of sterility in HSL knock-out mice is not understood.

The observation that HSL⁻ deficient mice did not accumulate TG but DG led to the proposal that not HSL was in fact the prime lipase of adipose tissue, but a DG lipase that would have to act in concert with a TG lipase for effective lipid breakdown. This TG lipase was demonstrated to be ATGL and is further discussed in 1.4.2.

HSL has a number of interaction partners, two of which are of interest for this work and shall be further discussed here. As the exact function of the HSL N-terminus was not yet characterized, it was supposed that it may serve as binding site for interaction partners of HSL. In 1999, Shen and colleagues could show that HSL is interacting with FABP4 (fatty acid binding protein 4; also adipocyte lipid binding protein, ALBP) and that this interaction is dependent on an intact N-terminal region (Shen et al. 1999).

Fatty acid binding proteins are single-chain polypeptides of low molecular weight (approx. 15kDa). They usually form complexes with fatty acids in a 1:1 stoichiometry (Bernlohr et al. 1997) and facilitate their diffusion between different cell compartments (Shen et al. 2001).

It has been shown that lipid binding proteins form barrels and sequester fatty acids from the cytosol by taking them up into the cavity of this barrel. From their observations, Shen and colleagues proposed a model of interaction for FABP4 and HSL, where FABP4 is bound to the HSL N-terminal region in the basal state and dissociates from it when HSL actively cleaves its substrates to produce fatty acids that are then sequestered by FABP4, possibly resulting an facilitated efflux of fatty acids from the cell (Shen et al. 1999). It was also proposed, that the binding of fatty acids by FABP4 prevents HSL from self-inhibition via feedback mechanisms. Furthermore, the group showed that co-expression of FABP4 and HSL in CHO cells increases lipolysis by 50%.

A second protein that was shown to interact with HSL is StAR (steroidogenic acute regulatory protein). StAR actually serves as a mitochondrial cholesterol transporter which transduces cytosolic cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) where the cholesterol is converted into pregnenolone by P450_{sc}, the cholesterol side chain cleavage enzyme – which is the first and rate limiting step in steroid hormone biosynthesis. StAR is produced as a 37kDa precursor protein that is then cleaved to a 30kDa mature protein (Stocco et al. 1991). It is well conserved among different species, such as mammals, birds and amphibians (Bauer et al. 2000). It is mostly expressed in the adrenal cortex, in steroidogenic cells of ovary and testis, and to some extent also in the brain (Sugawara et al 1997).

The StAR protein has a quite unique sequence; its structure contains 2 long α - helices at the N- and the C-terminus, and two α -helices that together with nine antiparallel β -sheets form an α -/ β fold. Most strikingly there is a hydrophobic pocket that provides exactly the dimensions needed to take up a single cholesterol molecule (Murcia et al. 2006).

As the protein is also stable and fully active in the cytoplasm and its half-life of 15-60min is much longer than needed for an acute steroidogenic response, it was proposed that the protein probably serves more functions than known to date.

HSL is a lipase that is abundant not only in adipose tissue, but also in steroidogenic tissues. HSL and StAR are both expressed in rodent adrenals. Interestingly, both proteins are also activated in the same manner: as described previously, HSL is activated by PKA as a result of ligand binding to a G protein coupled receptor and the subsequent increase of cAMP levels. The same is true for StAR: it is activated by PKA, and PKA was shown to be essential for StAR steady state levels (Manna et al. 2005).

Shen and colleagues performed GST-pull down experiments with HSL and StAR from adrenals isolated after StAR expression was induced with ACTH (Shen et al. 2003). The group also performed mutational studies and found that the sites needed for HSL and StAR interaction, which are the first 62 n-terminal amino acids and a region between amino acid residue 180 and 221 of StAR.

Co-expression of StAR and HSL in hamster ovary cells resulted in an approximately 75% increased level of hydrolysis of cholesteryl ester substrates, indicating that StAR is able to modulate HSL activity.

Unlike FABP4, StAR has no lipid binding properties – StAR may affect HSL action by altering HSL conformation to allow better substrate access or may prevent free fatty acids from inhibiting HSL. The interaction of StAR and HSL also has a positive effect on intracellular trafficking of cholesterol between compartments. The interaction of HSL and StAR was, however, only shown in adrenal cells and only for the StAR family member present there (StAR – also referred to as StarD1).

However, there are other family members like StarD4 and StarD5 that are ubiquitously expressed in all tissues and may also interact with HSL. Therefore, further investigations of the StAR/HSL interaction are necessary.

1.4.2 Patatin-like Phospholipases

The so-called family of patatin-like phospholipases (PNPLAs) is a protein family that has quite recently been described to function in lipid metabolism. PNPLAs are derived from a common ancestor, the Patatin protein which was first identified in potato tubers. Patatin acts as a storage protein in potato tubers and makes up about 40% of the potatoes' soluble proteins (Vancanneyt et al., 1989). It also contains the consensus serine lipase motif G-X-S-X-G and acts as an acyl-hydrolase.

After the identification of Patatin, Patatin-like phospholipase family members have been found and investigated, for example in yeast (Kurat et al. 2006) reflecting that the Patatin-like phospholipase family may play an important role in lipid metabolism and is therefore conserved from yeast over avian species to mammals.

Characteristically all Patatin family member proteins share the so-called Patatin domain (Pfam 01734). All family members contain the G-X-S-X-G active site motif of lipases, but instead of general hydrolase activity some of the family members exhibit phospholipase activity.

The Patatin-like phospholipase domain shows some characteristic features, such as a three-layer α - β - α architecture, and instead of the typical catalytic triad, in Patatin proteins there is a catalytic dyad consisting of Serine and Aspartic Acid (see Fig. 1.8).

Protein folding produces a 3D arrangement that differs from other hydrolases, by placing the catalytic Serine into a nucleophilic bow and producing an oxyanion-hole that is thought to be able to stabilize the transition state of the enzyme-substrate complex (Rydel et al. 2003).

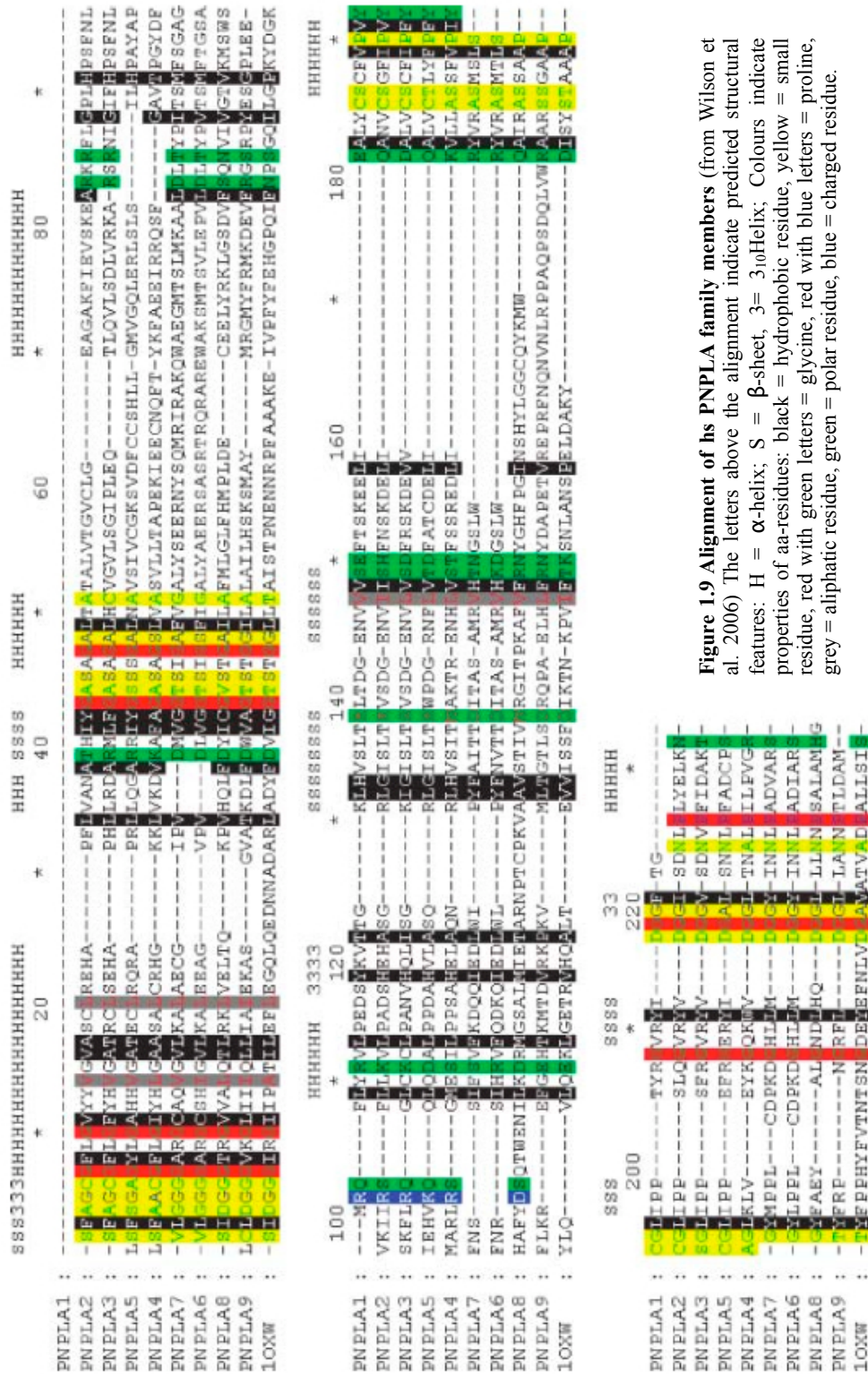


Figure 1.8 Patatin domain structure (PAT17); helices are shown in blue, β -sheets are numbered and drawn in pink, coils are depicted in yellow. The active site Serine S77 and Aspartic Acid D215 are shown in green (from Rydel, 2003).

Apart from the highly conserved Patatin domain and certain hydrophobic residues, the actual sequences do not share high sequence similarity within a species or between different species (Wilson et al. 2006; see Fig. 1.9).

Also, the tissue expression patterns vary much between different species - e.g. PNPLA3 in humans and the chicken is highly expressed in the liver, whereas in mice it has highest expression levels in adipose tissue.

In humans, nine Patatin-like phospholipase family members have been identified, which are designated PNPLA1-9; in our model organism, the chicken, there are known homologs to all human PNPLAs except for PNPLA5; in mice no homolog to human PNPLA4 is known. Characteristics of human PNPLA family members are listed in Table 1.3.



	Enzymatic Activity	Function	Expressed in	Co-activators	Cellular localization
PNPLA1	unknown	unknown	unknown	unknown	unknown
PNPLA2	Lipase, Transacylase	TG break-down	white adipose tissue	CGI-58	Lipid droplets
PNPLA3	Hydrolase, Transacylase	unclear	adipocytes, liver	unknown	Adipocyte membranes
PNPLA4	Retinol ester hydrolase	Epidermal morphogenesis?	muscle, liver, skin	unknown	unknown
PNPLA5	TG hydrolase	Adipocyte differentiation?	lung, adipocytes	unknown	unknown
PNPLA6	Lysophospholipase	Maintenance of axon integrity	nervous system	unknown	ER and Golgi
PNPLA7	Lysophospholipase	Adipocyte differentiation?	Prostate, pancreas	unknown	ER Lipid droplets
PNPLA8	Phospholipase	Importance in mitochondria?	heart	unknown	Mitochondria peroxisomes
PNPLA9	Phospholipase	Lipid remodelling Cell signalling	ubiquitous	unknown	Cytoplasm

Table 1.3 Some characteristics of mammalian PNPLA family members (Kienesberger et al. 2008)

As one can see from Table 1.3, little is known about Patatin-like phospholipases, especially the biochemical functions of the various family members are unknown, although functions are suggested. It is also problematic that the enzymatic activities of most family members could only be shown *in vitro*, but *in vivo* often no such activities could be detected. It is known that many family members, such as PNPLA2, PNPLA3, PNPLA5 and PNPLA7 are regulated in accordance with the nutritional status (fasting, insulin signaling).

The two best characterized patatin-like phospholipase family members, PNPLA2 and PNPLA3, are further described in the following sections.

1.4.2.1 ATGL (Adipocyte Triglyceride Lipase; PNPLA2)

To mobilize energy stored as TG in the adipose tissue in times of nutritional scarcity, the body is dependent on lipases that are able to break down TG to DG and further to MG, FFA and glycerol. For a long time it was believed that hormone-sensitive Lipase (HSL, see 1.4.1) was the only lipase present in adipose tissue to fulfill this function. However, when HSL knock-out mice were generated, they did not show the expected obese phenotype and/or TG accumulation in the plasma at all (Wang et al. 2001, Zimmermann et al. 2004, Okazaki et al. 2002). Instead they showed massive accumulation of DG, thereby suggesting that there must be at least one additional lipase present in the adipose tissue. Subsequently, ATGL was identified to be this additional lipase in the adipose tissue (Zimmermann et al. 2004; Villena et al. 2004; Jenkins et al. 2004).

ATGL/PNPLA2 (also called Desnutrin) shares the classic features common among all Patatin-like phospholipase (PNPLA) family members, such as the Patatin domain and the G-X-S-X-G lipase motif. The murine ATGL gene is highly expressed in white and brown adipose tissue and codes for a protein of 54kDa. Zimmermann et al. showed by transfection of COS-7 cells that ATGL is in fact a triglyceride lipase, which is, unlike HSL, unable to cleave cholesteryl- or retinyl esters. Finally ATGL knock-out mice showed the phenotype that was expected for HSL knock out mice, with massive accumulation of TG in white adipose tissue but also in non-adipose tissues (e.g the heart), giving rise to the conclusion that loss of ATGL function cannot be compensated by HSL function (Haemmerle et al. 2006). ATGL obviously has especially high affinity for TG substrates, and while HSL has TG lipase activity, its affinity for DG substrates is 10-fold higher than for TG substrates (Frederikson et al. 1981). Therefore Zimmermann and colleagues proposed a model in which ATGL is responsible for the first step of TG breakdown, the cleavage of TG to DG and one FFA, and HSL subsequently breaks down the resulting DG to MG and a second FFA (Zimmermann et al. 2004).

Whereas HSL is regulated by phosphorylation and de-phosphorylation in response to cAMP levels by Protein Kinase A (PKA), no activation of ATGL was observed upon phosphorylation by PKA. However, there is a connection between PKA signaling and ATGL activation – soon after ATGL had been identified, a regulatory protein interacting with ATGL, CGI-58 (comparative gene identification 58) was. To date, the exact mechanism of regulation of ATGL by CGI-58 is still not completely clear. However, it is known that in hormonally unstimulated cells, CGI-58 binds to perilipin, a major lipid droplet protein (see 1.2).

When cells were stimulated by a lipolytic stimulus, such as catecholamine binding to an β -adrenergic receptor on the cell, cAMP levels rise, activating PKA, which in turn phosphorylates perilipin. This phosphorylation results in the dissociation of CGI-58 from perilipin and its co-localization with ATGL (Grannemann et al 2007). Mutations within the ATGL gene and within the CGI-58 gene have been discovered and shown to cause certain pathologies, so-called neutral lipid storage diseases, which indicates that these two proteins are crucial for normal lipid metabolism.

1.4.2.2 PNPLA3 (Adiponutrin)

PNPLA3, also called Adiponutrin, is another well-investigated member of the Patatin-like phospholipase family. The human PNPLA3 gene, located on chromosome 22, codes for a 52kDa transmembrane protein. It is highly expressed in adipose tissue and in liver. The precise function of adiponutrin is unclear and there are contradicting data whether it acts as lipogenic or lipolytic factor –although to some extent PNPLA3 has lipase activity *in vitro*, there are data showing that *in vivo* PNPLA3 does not exhibit lipase activity at all (Zechner et al. 2005, Kershaw et al. 2006).

Within the patatin-like phospholipase family, PNPLA3 shares highest sequence identity with ATGL (PNPLA2). However, the expression pattern of PNPLA3 upon changes in the nutritional status of the cell is the exact opposite of ATGL: the expression level of PNPLA3 is known to decrease upon fasting and to increase upon refeeding (Baulande et al. 2001), suggesting that PNPLA3 rather acts as an anabolic enzyme. In humans, the expression of PNPLA3 mRNA is positively linked to obesity (Johannson et al. 2006) and is reduced if body weight is lost (Liu et al. 2004). It was also shown that, in humans, PNPLA3 expression is regulated by insulin and blood glucose levels, and that PNPLA3 expression differs in healthy humans and individuals suffering from Type 1 or Type 2 diabetes (Moldes et al. 2006).

It is known that many genes involved in lipid metabolism are regulated by synergistic action of the LXR (liver X receptor) and SREBP1-c (sterol regulatory element binding protein 1-c): LXR agonists enhance the synthesis of fatty acids mainly by activating the gene for SREBP1-c, which then turns on many genes involved in lipogenic actions (Liang et al. 2002, Chen et al. 2004). Recently it was shown that also the PNPLA3 gene is, at the molecular level, regulated by a LXR/SREBP1-c synergy (Huang et al. 2010). The group of Huang showed that mice overexpressing SREBP1-c had a 12-fold increase of PNPLA3 mRNA levels in the liver, suggesting a regulation of PNPLA3 by SREBP1-c.

Treatment with an LXR agonist, T0901317, only had a positive effect on PNPLA3 mRNA levels if SREBP1-c was expressed, thus in *srebp1-c*^{-/-} knock-out mice the LXR agonist had no effect, thereby indicating that the increased expression of PNPLA3 is not a result of a direct action of LXR, but is dependent both on LXR and SREBP1-c.

The investigations of the PNPLA3 gene gained even more significance when a single nucleotide polymorphism within the PNPLA3 gene associated with non-alcoholic fatty liver disease, was identified (Romeo et al. 2008, Kotronen et al. 2009). Non-alcoholic fatty liver disease (NAFLD) is the most common pathologic condition of the liver in the Western World. NAFLD can occur in different grades of severity, ranging from an increased excessive storage of fat in the liver (hepatic steatosis), liver inflammation, or cirrhosis that can lead to hepatic cancer (Browning et al. 2004) – these symptoms are, however, not caused by alcohol abuse or other life style-related factors. Also the high liver fat content is not related to a high BMI or general obesity. It is estimated that about 10% of all liver transplantations performed are due to cirrhosis as a result of NAFLD (de Alwis et al. 2008).

It had been observed that in the United States, different ethnics group such as Hispanics, African-Americans and Caucasians have a different predisposition to develop non-alcoholic fatty liver disease. Based on this observation, Romeo et al. performed a genome-wide association scan of nonsynonymous sequence variations and found that an allele of PNPLA3 that carried a SNP was strongly associated with liver fat content (Romeo et al. 2008).

The SNP substitutes a cytosine for a guanine in codon 148 resulting in a change from isoleucine to methionine. The isoleucine residue at position 148 is very well conserved among different species (see Figure 1.10) and therefore may be crucial for PNPLA3 function.

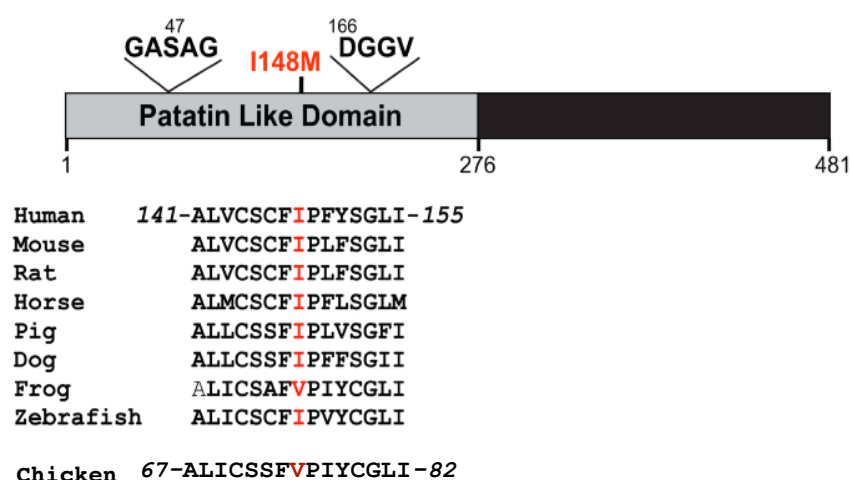


Figure 1.10 Conservation of Isoleucine 148 in PNPLA3 among different species (adapted from Kotronen et al. 2009). Chicken protein sequence was obtained from NCBI Gen Bank (EU419875.1) and aligned with the human residues 141-155 shown here using ClustalW2. The N-Terminus of ggPNPLA3 is still unknown, therefore residues are numbered 67-82. Like in the Frog, the important residue here is a Valine instead of Isoleucine.

Romeo and colleagues showed that the I148M allele of the PNPLA3 gene is very common in Hispanics, the ethnic group having the greatest risk to develop NAFLD and the highest liver fat content. In African-Americans, the group with the least prevalence for developing NAFLD, another allele – S453I – was found which seems to be associated with especially low liver fat content. Moreover, in people carrying the I148M allele the plasma levels of two liver enzymes (ALT and AST) which are responsible for liver inflammation were elevated, thereby suggesting that PNPLA3 plays a role in liver inflammation. Interestingly, there was no association between the SNP and BMI, insulin resistance, or plasma TG and LDL levels. A European group later showed that the observations of Romeo et al. were also true for European populations (Kotronen et al. 2009).

Although the PNPLA3 gene is well investigated and it is now known how PNPLA3 is regulated and that there are pathologies associated with PNPLA3 mutations, the exact function of PNPLA3 remains unclear and further research is required.

1.4.3 Arylacetamide Deacteylase (AADA)

Arylamines and heterocyclic amines are carcinogens that need to be eliminated from the cell. For this the cell employs certain enzymes. The principal detoxifying enzyme is arylamine N-transferase (NAT) that competes with CYP1A2 (a member of the cytochrome P450 family) for the primary arylamine substrate. Arylacetamide deacetylases are capable of converting the arylacetamide back into a primary arylamide, thereby making it available for NAT. Therefore, arylacetamide deacteylases are proposed to be detoxifying enzymes necessary to degrade carcinogens (Sato, 1987). Human AADA is a 45kDa protein present in high amounts in liver, small intestine, and kidney, tissues susceptible to cancerogenesis, which need high amounts of detoxifying enzymes.

While lipases of adipose tissue are well known and characterized, it is still unclear if there are hepatic lipases promoting β -oxidation and thereby energy generation from TG stores. However, in the 1990's Probst et al. identified liver arylacetamide deacteylase also as an esterase in addition to its detoxifying activity (Probst et al. 1991 and 1994).

When the AADA amino acid sequence is aligned with different eukaryotic and prokaryotic esterases, it shows highest identity with Moraxella TA 144 lipase 2 and hormone-sensitive lipase. HSL is thought to be related to Moraxella lipase 2, but AADA shows even higher sequence similarity to this enzyme than HSL itself.

Moraxella TA144	G	A	S	P	S	R	I	V	L	S	G	D	S	A	G	G	C	L	A	A	L	V	A	Q	Q
Hs AADA	G	V	N	P	E	R	I	G	I	S	G	D	S	A	G	G	N	L	A	A	A	V	T	Q	Q
Hs HSL	G	S	T	G	E	R	I	C	L	A	G	D	S	A	G	G	N	L	C	F	T	V	A	L	R

Figure 1.12 Alignment of the AADA active site motif with Moraxella lipase 2 and HSL; all lipases show the same active site motif G-D-S-A-G.

Apart from the aligned sequences of the active site motifs shown in Figure 1.12, AADA has another interesting region that appears to be closely related to HSL (42%): it starts at Arg104, contains the active site serine, and ends at Tyr216. This stretch contains the dipeptide His-Gly112 which is thought to be necessary for the binding of lipids to HSL (van Oort et al. 1989). Another region that shows 40% identity is located on the C-terminal part of AADA (Val317 to Leu381). In human HSL this region is required for hormonal control, and converts HSL into its active form when cAMP signalling occurs (Langin et al. 1993). However, in AADA this hormonal control region is not found in this stretch, raising the possibility that AADA is not hormonally controlled.

The sequence similarity in important regions required for lipolytic activity suggests that AADA has more functions than known. In fact, in lipase and esterase assays, AADA could be shown to exhibit esterase activity: pNitrophenylacetate (pNP) and 4-Methylumbelliferylheptanoate (4-MUH) were used as substrates to monitor hydrolytic activity of AADA, which exhibited activity against these lipid esters (Lo et al. 2010).

Cells stably expressing AADA contained significantly less TG than control cells. These differences were abolished when E600, a lipase inhibitor, was added to the cells. If E600 was removed again, the preformed TG was turned-over rapidly again in both AADA and control cells, indicating that AADA does not take part in TG hydrolysis (Lo et al. 2010). There was also no activity against cholesterol esters. As AADA was shown to be located in the ER, Lo and colleagues proposed a model for AADA activity in which AADA exhibits activity against endogenous DG synthesized in the ER compartment, whereas TG stores in lipid droplets are not accessible for AADA.

Many genes in lipid metabolism are regulated diurnally according to the food intake over a 24 hour cycle. AADA was shown to be expressed in such a diurnal pattern, with the highest expression in the post-absorptive phase. This pattern resembles the pattern of VLDL secretion. As VLDL secretion is dependent on a cycle of lipolysis and re-esterification of TG, AADA might support hepatic fuel homeostasis (Trickett et al., 2001). This is also consistent with the model proposed by Lo et al., as fatty acid release in the ER could result in re-esterification of lipolytic products by ER resident acyltransferases such as AADA, thereby supporting VLDL production.

1.5 Aims of the Thesis

In my diploma thesis I had two more or less independent projects: one was the characterization of PNPLA3 in the chicken, the other was the quest for a chicken analog of HSL.

In our model organism, the domesticated chicken *Gallus gallus domesticus*, the tissue distribution and expression patterns of the different Patatin-like Phospholipase family members have already been investigated (Saarela et al. 2008) when I started my diploma thesis. PNPLA3 expression was shown to be especially high in skeletal muscle, liver, and testis, resembling the human expression pattern with high expression in liver, muscle, and adipose tissue rather than, for example, that of mice (Lake et al. 2005). Therefore, and for reasons mentioned in 1.1, the chicken appeared to be a good model to study PNPLA3 function. I wanted to investigate changes in PNPLA3 expression under a variety of conditions in wild type and mutant animals not only at the mRNA level, as it had been done before, but also at the protein level. For this I needed to raise an antibody against ggPNPLA3. Cloning and generating this antibody was a major part of my work. With the antibody, I was then also able to monitor the translational state of PNPLA3 in various tissues and under different conditions, obtaining insight into its regulation and physiological role(s).

Interestingly, although it is known that there is DG breakdown in the chicken, the enzyme performing this step is unknown. In mammals the enzyme necessary for this is HSL; in chicken, however, a homologue of HSL has not been identified. Since AADA was shown to share sequence homology with HSL in rodents and mammals, we hypothesized that AADA may take over the function of HSL in the chicken. It was shown that HSL interacts at least in vitro with StAR (see 1.4.1), a protein involved in steroidogenesis; therefore I cloned ggStAR and expressed it recombinantly to test whether StAR would also interact with AADA. I also tried to determine if there were AADA isoforms that are mainly expressed in fat tissue, because an analogue of HSL should be present predominantly in adipose tissue. Furthermore, I tried to show that AADA has not only esterase, but also lipase activity.

2. Materials and Methods

2.1 Chemicals and Enzymes

Chemicals that were used for the production of solutions and buffers were purchased from Amersham Bioscience, Amresco, AppliChem, Fluka, Merck, Roth, Sigma-Aldrich and Star Lab.

Enzymes used for restriction digests were obtained from Fermentas or Promega.

Polymerases used were High Fidelity PCR Mix by Fermentas and High Velocity DNA Polymerase by Boline. Additionally for RT-PCR either LightCycler® 480 SYBR Green I Master by Roche or KAPA SYBR FAST qPCR LightCycler480 by PeqLab were used.

Furthermore DNase I and Superscript® II Reverse Transcriptase, both by Invitrogen, were used for cDNA production.

Protein markers were obtained from Bio-Rad, DNA markers were bought from Fermentas.

2.2 Bacterial Strains and Vector Systems

Strain	One Shot TOP10 Chemically Competent E.Coli
Genotype	F- <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80/lacZ\Delta M15$ $\Delta lacX74$ <i>recA1</i> <i>araD139</i> $\Delta(ara-leu)7697$ <i>galU galK rpsL (StrR) endA1 nupG</i>
Reference/Source	Invitrogen

Table 2.1 Bacterial Strain used for transformation

Strain	One Shot BL21 (DE3)
Genotype	F- <i>ompT hsdSB (rB-mB-) gal dcm (DE3)</i>
Reference/Source	Invitrogen

Table 2.2 Bacterial Strain used for expression of recombinant proteins

Vector	TOPO TA Vector
Size	3931bp
Genotype	LacZ α , M13 reverse priming site, MCS, T7 promotor/primer, M13 forward priming site, f1 origin, Kan ^R ORF, Amp ^R ORF, pUC origin
Reference/Source	Invitrogen, TOPO TA Kloning Kit

Table 2.3 Cloning and sequencing vector

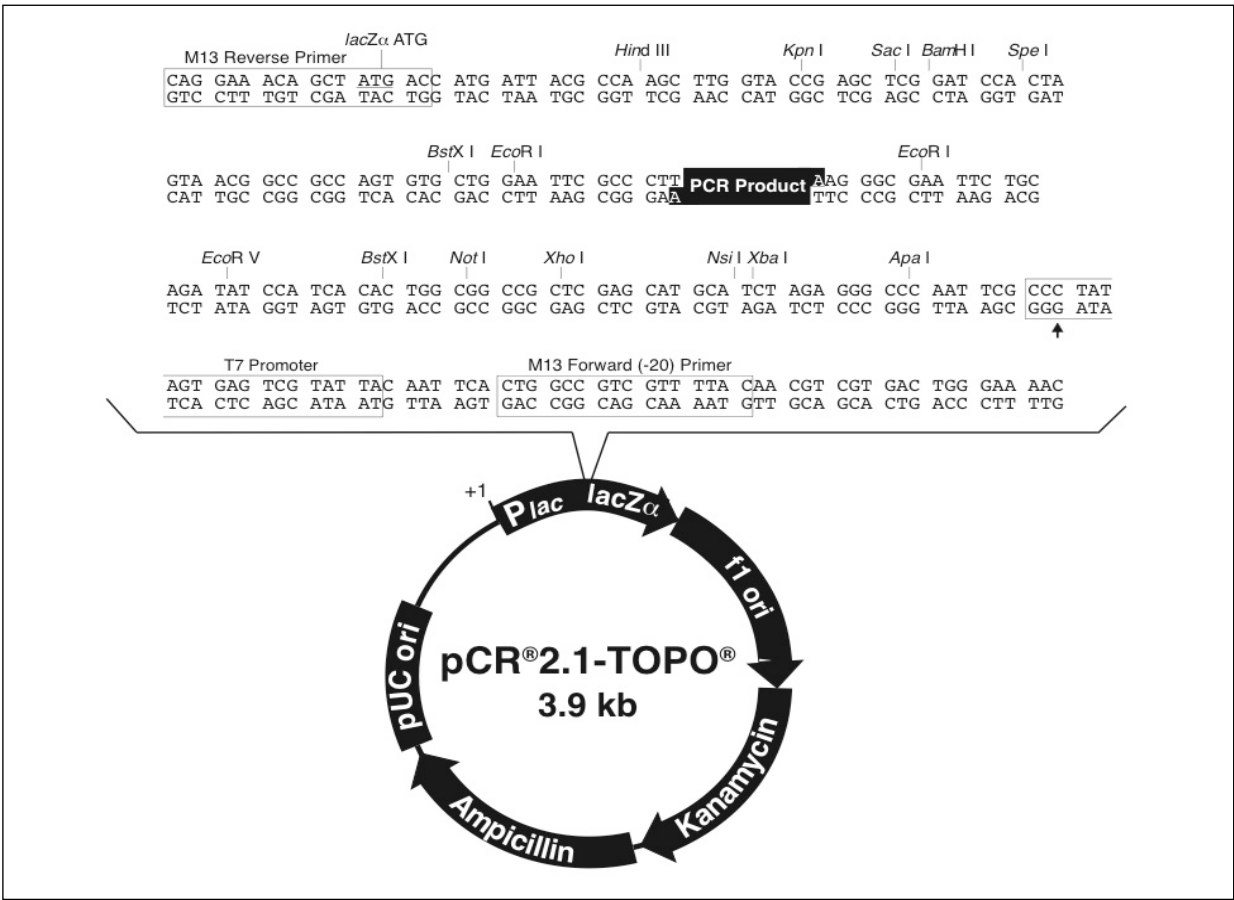


Figure 2.1 TOPO vector map

Vector	pBluescript SK+
Size	3kb
Genotype	f1 origin, β -Galactosidase fragment, MCS, M13 forward priming site, T7 promotor/primer, KS primer, SK primer, T3 promotor, Lac promotor, pUC origin, Amp ^R
Reference/Source	Invitrogen

Table 2.3 pBluescript Cloning vector

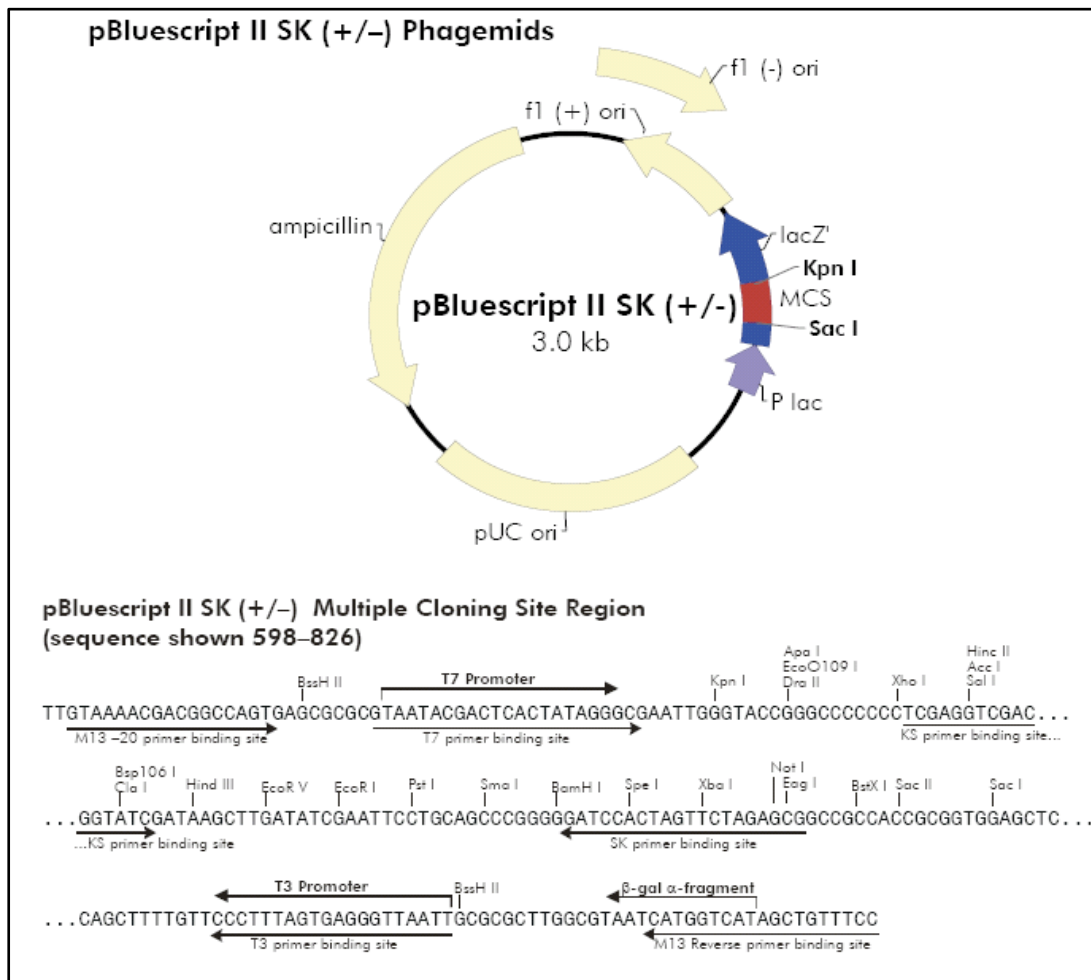


Figure 2.2 pBluescriptSK+ vector map

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Vector	pET 25b+
Size	5547bp
Genotype	T7 promotor, MCS, 6xHis tag coding sequence, T7 terminator, BR322 origin, f1 origin
Reference/Source	Novogene/Merck

Table 2.5 Expression vector for 6xHis tagged proteins

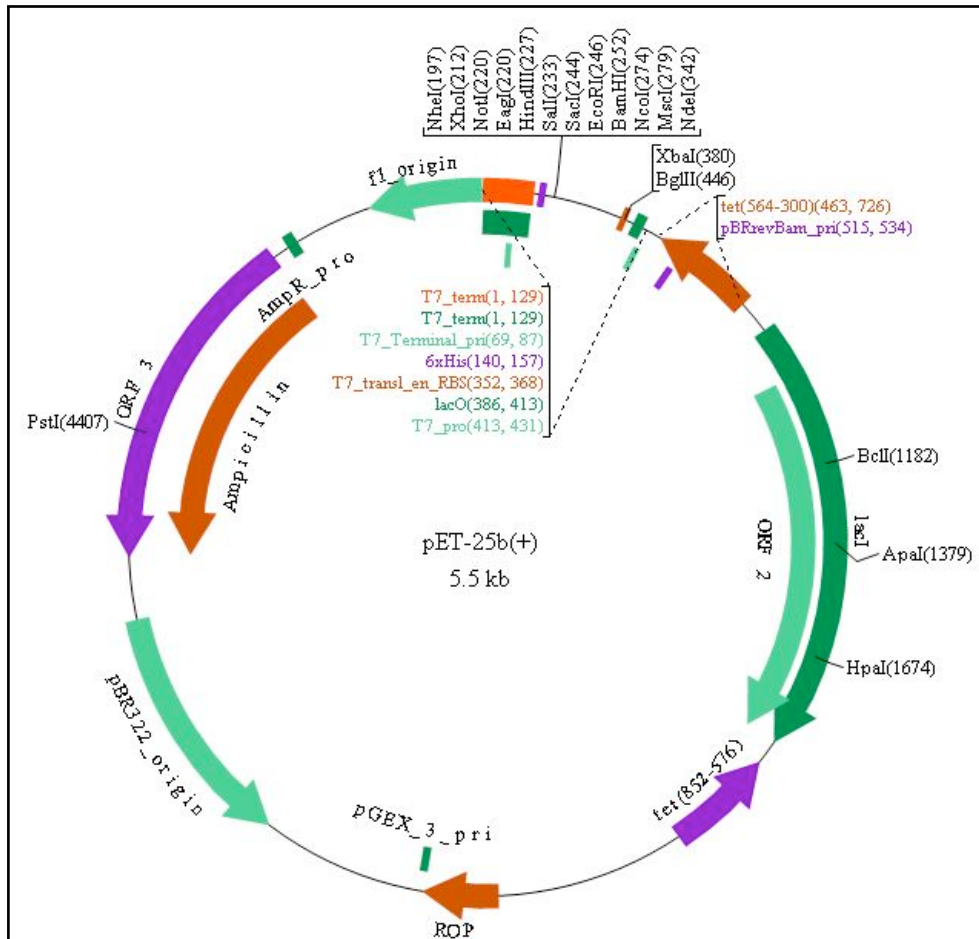


Figure 2.4 pET25b+ vector map

2.3 Oligonucleotide Primer

All oligonucleotide primers used for cloning or RT-qPCR were synthesised at MWG Eurofins.

M 13 Sequencing primer

forward 5' GTAAAACGACGGCCAGT 3'
reverse 5' AACAGCTATGACCATG 3'

T7 primer

Promotor 5' TAATACGACTCACTATAGGG 3'
Terminator 5' GCTAGTTATTGCTCAGCGG 3'

PNPLA3 qPCR Primers

Forward 5' GTTGTCCAGGCATTGATCTG 3'
Reverse 5' ATCACACTCCCCAGCAAAAG 3'

StAR qPCR Primer

Forward 5' CGTGGATCAGCCAGGTCCGCA 3'
Reverse 5' TCACTCAGCATCCTCGGGGAC 3'

StAR full length cloning primers (GST tagged)

Forward 5' AAGGATCCATGCTGCCCGCCACCTTCAAGC 3'
Reverse 5' CGGGCAGCGGGCTGCTGAGAATTCAA 3'

AADA qPCR Primer

Forward 5' GGCAGTGCTCTACTTCCAC 3'
Reverse 5' TCCAGTGCAAGTTGAAGACG 3'

A [LOC768489, Gallus gallus](#) similar to LOC495387 protein

Forward 5' GCTGTTAGCCAGACCCTGTC 3'
Reverse 5' CACAAGCAAGACGTCCTGAA 3'

B arylacetamide deacetylase-like 4, gallus gallus LOC768457

Forward 5' AGGTCCTGAAGGGCTCTCAT 3'
Reverse 5' ATGAGACGGTGAAACGGTTC 3'

C [LOC429936, Gallus gallus](#) similar to LOC495387 protein

Forward 5' CGAAGTCCATGAAGCAGTCA 3'
Reverse 5' ACAACGGCATTGAGTAACC 3'

D [LOC768597, *Gallus gallus*](#) similar to LOC495387 protein

Forward 5' GCCCTCCTATCAGCAGAATG 3'
Reverse 5' ACAACGGCATTTCGAGTAACC 3'

E [LOC768647, *Gallus gallus*](#) similar to LOC495387 protein

Forward 5' AACCTGCCGCTATCTTGCTA 3'
Reverse 5' TATTGTCTGTGGGGACAGCA 3'

F arylacetamide deacetylase-like 4, *gallus gallus* LOC495387

Forward 5' CAAGGGGATACAACCCACAC 3'
Reverse 5' AGGACAATGGAGTCCCAGTG 3'

G [LOC768580, *Gallus gallus*](#) similar to LOC495387 protein

Forward 5' CGTGCTGCGTTCTATGTGTT 3'
Reverse 5' ATGAGACGGTGAAACGGTTC 3'

H arylacetamide deacetylase-like 3, *gallus gallus*

Forward 5' CTGTGTCAATGCCACCATTC 3'
Reverse 5' CCAAAGATACGTGCCCAGAT 3'

I arylacetamide deacetylase-like 1, *gallus gallus*

Forward 5' TGCTGTTTGTATGCCACCAT 3'
Reverse 5' CTGCAAAGCAAGATGAACCA 3'

J arylacetamide deacetylase *gallus gallus*

Forward 5' GGCAGTGCTCTACTTCCAC 3'
Reverse 5' TCCAGTGCAGTTTGAAGACG 3'

 β -Actin qPCR primers

Forward 5' AGCTATGAACTCCCTGATGG 3'
Reverse 5' ATCTCCTTCTGCATCCTGTC 3'

2.4 Antisera and Antibodies

Antibody	Dilution
Rabbit α chicken AADA	1:500
Mouse α chicken FABP4	1: 20000
Mouse α chicken PNPLA3	1: 1000
Mouse α GAPDH	1:15000
Mouse α 6x His	1:1500
Goat α rabbit IgG	1:50000
Goat α mouse IgG	1:1500
Goat α rabbit IgG Alexa 488	1:1000
Biotinylated goat α rabbit IgG	1:800
Streptavidin HRP	1:500
DAPI nuclei stain	1:4000

Table 2.6 Antisera and antibodies used for Western Blots and Immunostainings

2.5 Animals

White Leghorn laying hens (30-40 weeks old) were purchased from Heindl Co. (Vienna, Austria) and maintained on layer's mash with free access to water and feed under a daily light period of 16 hr.

2.6 Molecular Biological Methods: DNA

2.6.1 cDNA Synthesis

cDNA Synthesis was carried out using Superscript II Reverse Transcriptase (Invitrogen) after DNase I (Invitrogen) digest. Superscript Reverse Transcriptase II produces first strand cDNA.

To get 1 μ g of cDNA, 1 μ g of RNA (obtained as described in 2.6.1) was used. To 1 μ g RNA, 1 μ l 10x Buffer and 1 μ l DNase I were added, adjusted with ddH₂O to a final volume of 10 μ l and then left on room temperature for 10min. 1 μ l of 25mM EDTA was added and the reaction was inactivated by heating at 65°C for 15min.

Then 1 μ l of 10mM dNTPs and 1 μ l of Oligo dT Primer were added, the mixture was incubated on 65°C for 5min. It was then briefly chilled on ice before adding 4 μ l of First Strand Buffer and 2 μ l of 1mM DTT. The mixture was incubated at 42°C for 1min. Thereafter 1 μ l of Reverse Transcriptase (200U) was added and the solution was incubated at 42°C for 50min. The reaction was inactivated by heating at 70°C for 15min.

The cDNA was stored at -20°C until further use as template in PCR and RT-qPCR.

2.6.2 Polymerase Chain Reaction (PCR)

All PCR reactions to amplify specific parts of DNA were performed using a T3000 Thermocycler by Biometra. Polymerases used are listed in 2.1. The set up of the PCR reaction mix and of the PCR program is described in Table 2.7 and Table 2.8, respectively.

DEPC H ₂ O	18,75µl
Buffer/MgCl ₂	2,5µl
DMSO	1,25µl
Primer fwd	0,5µl
Primer rev	0,5µl
dNTPs	0,5µl
cDNA	0,5µl
Polymerase	0,5µl

Table 2.7 PCR reaction mix

Initial Denaturation		94°C 5min
Denaturation	40 Cycles	94°C 30sec
Annealing		xx°C 30sec
Elongation		72°C xxsec
Final Elongation		72°C 10min
Cooling		4°C forever

Table 2.8 Example of a PCR program set-up

Specific primers for the desired fragment were used. The annealing temperature for each PCR program was adjusted for the melting points of those primers. The elongation temperature of each program was chosen depending on the length of the desired fragment.

2.6.3 DNA Gel Electrophoresis

DNA gel electrophoresis was performed to separate different DNA fragments or PCR products by their size. DNA electrophoresis gels were prepared from 1% (w/v) StarPure Agarose (Starlab) and TAE buffer, containing ethidium bromide. Ethidium bromide intercalates with DNA and makes DNA visual under UV light. As a size marker, the 100bp ladder by Fermentas was used. Samples were mixed with DNA loading buffer containing glycerol to increase the density of the sample, and insure that it stays within the gel slots, and a stain (Bromphenol Blue or Xylen Cyanol) as a front marker. The gel was run in TAE buffer at 100V for 30min.

<u>TAE Buffer</u>	<u>5x DNA loading buffer</u>
2M Tris pH 8,0	50 % Glycerol
5,7% Acetic Acid	0.1 M EDTA 50x TAE Bromophenol blue or Xylen Cyanol FF
0,5M EDTA	

2.6.4 DNA Gel Extraction

The gel extraction was performed using Quiagens QiaQuick Gel Extraction Kit. Bands were cut out of the agarose gel using a scalpel. 300µl of Buffer QC were added to the gel piece and the gel piece was molten at 50°C shaking. 100µl isopropanol were added and the solution was mixed by inverting. It was then loaded onto a column and centrifuged for 30sec at full speed.

The flow through was discarded, 500µl Buffer QC were added to the column, and again centrifuged for 30sec at full speed. The flow through was again discarded, 700µl of Wash Buffer were added and the sample was centrifuged again. The flow through was again discarded and another brief centrifugation was done to remove all ethanol from the Wash Buffer. The column was then placed into a new collection tube and the sample was eluted from the column using 40µl of ddH₂O.

Eluted DNA was stored at -20°C.

2.6.5 Vector Preparation

2.5.5.2 pBuescript SK+

1µg of pBluescript SK+ was mixed with 0,2µl of Acetyl-BSA, 2µl of EcoRV-Buffer and 1µl of EcoRV and then brought to a final volume of 20µl. The vector was digested for 1,5h at 37°C. The digest was then inactivated by heating at 65°C for 10min.

The now linearised plasmid was then ready to use for ligation.

2.6.5.2 pGEX 6P.2

1µg of pGEX6P.2 vector DNA was mixed with 14µl of dH₂O, 2µl Tango Buffer (Fermentas) and 1µl each of EcoRI and BamHI (both Fermentas) and then digested for 1,5h at 37°C.

The vector DNA was then loaded onto an agarose gel and the vector band was cut out and gel extraction was performed as described in 2.4.

2.6.5.3 pET 25b+

1µg of pET 25b+ vector DNA was added to 14µl dH₂O, 2µl Tango Buffer (Fermentas) and 1µl of NcoI and EcoRI (both Fermentas) each. The mixture was incubated for 1,5h at 37°C.

Then 13,5µl of 1M sodiumacetate, 27µl of isopropanol and 7µl dH₂O were added. The vector DNA was precipitated either over night at -20°C or for 2h at -80°C.

After that, a DNA pellet was obtained by centrifugation for 30min at 13000rpm at 4°C.

The pellet was washed once with 70% ethanol and then dried for 5min at 37°C.

2.6.6 DNA Ligation

2.6.6.1 Ligation into pBluescript SK+

16µl of gel extracted PCR product were added to 1µl of EcoRV-digested and heat inactivated pBluescript SK+, 2µl of ligation buffer and 1µl of T4 ligase (both Promega) were added and ligation was performed over night at 18°C.

2.6.6.2 Ligation into pGEX6P.2

After Midi-Preparation of the desired DNA sequence in pBluescript SK+, the DNA was digested using 5µg of Midi-DNA, 2µl Tango buffer, 1µl EcoRI, 1µl BamHI and water up to 20µl, to produce sticky ends for ligation into pGEX6P.2. After digestion for 1,5h at 37°C, the DNA was loaded onto an agarose gel and the insert band was cut and gel extraction was done as described in 2.4.

Then 14µl of gel extracted insert DNA and 3µl of gel extracted pGEX6P.2 vector DNA were mixed with 2µl ligation buffer and 1µl T4 ligase and ligation was performed over night at 18°C.

2.6.6.3 Ligation into pET 25b+

The desired DNA piece was obtained by digestion of 5µg of Midi-DNA with 2µl Tango Buffer, 1µl NcoI, 1µl EcoRI and water up to 20µl. Digestion was done for 1,5h at 37°C and afterwards the DNA was loaded onto an agarose gel, after which the insert band was cut out and gel extraction was performed as described in 2.4.

17µl of gel extracted insert DNA were pipetted directly onto the dried pET 25b+ vector pellet, the pellet was resuspended and 2µl of ligation buffer and 1µl of T4 ligase were added. Ligation was performed over night at 18°C.

2.6.7 Transformation of competent E.coli

A 50µl aliquot of One Shot TOP10 chemically competent E.coli (Invitrogen) was thawed. Per 25µl of cells, 5µl of ligation reaction were added and the cells were incubated on ice for 30min.

Cells were then heat-shocked at 42°C for 30sec to take up plasmid DNA. They were then briefly chilled on ice, 250µl of SOC medium were added and the cells were grown at 37°C for 1,5 hour.

Cells were then spread onto LB plates containing either kanamycin (100mg/ml) for TOPO TA plasmids, ampicillin (100mg/ml) for pGEX6P.2 and pET25b+ or ampicillin and X-Gal for blue/white-selection of pBluescript SK+. Plates were put into a 37°C incubator over night.

2.6.8 Mini preparation of Plasmid DNA

For small scale preparation of DNA, the Promega Pure Yield Plasmid Mini Kit was used, for all centrifugation steps an Eppendorf table top centrifuge was used. 600µl of bacterial over night culture was mixed with 100µl of Lysis Buffer and subsequently 200µl of Neutralization Buffer were added to stop the lysis reaction. The samples were centrifuged for 5min at 13000rpm to pellet the precipitate.

The cleared lysate was then put onto mini columns and the columns were centrifuged for 1min at 13000rpm to allow binding of DNA. DNA was then washed first with 200µl of Endonuclease Removal Buffer and then with 400µl of Wash Buffer containing ethanol. DNA was then eluted into a fresh tube using 40µl of pure water. The obtained DNA was stored at -20°C.

2.6.9 Midi preparation of Plasmid DNA

For medium scale DNA preparations, the Promega Pure Yield Plasmid Midi Kit was used, all centrifugation steps were performed using the Sorvall GSA and HB-6 rotors. 100ml bacterial overnight culture were harvested by centrifugation for 15min at 3000rpm. The pellet was then resuspended in 3ml Resuspension Buffer, 3ml of Lysis Buffer were added and cells were left to lyse for 3min. 5ml of Neutralization Solution were added and the solution was incubated on RT for 2min to allow a precipitate to form. The Lysate was then poured onto clearing columns and centrifuged for 5min at 2000rpm to separate the liquid containing the DNA and the precipitate. The cleared lysate was then transferred onto a DNA binding column and DNA was bound by centrifuging for 5min at 2000rpm. The DNA on the column was then washed first by adding 5ml of Endotoxin Removal Wash and centrifugation for 5min at 2000rpm and then by adding 20ml of Wash Buffer and centrifugation for 10min at 2000rpm. The column was then placed into a new falcon tube and 500µl of pure water were put onto the column resin to elute the DNA by centrifugation for 3min at 2000rpm.

DNA concentration was measured using a NanoDrop 2.0 Spectrophotometer.

2.6.10 Quantitative Real Time PCR (RT-qPCR)

Quantitative Real Time PCR serves to detect the level of transcription of a certain gene e.g. in various tissues or at different time points. It is based on the classic PCR technique to amplify DNA, and simultaneously it allows the detection of the actual amount of amplified transcript by fluorescence measurement. Fluorescing dyes that intercalate with dsDNA such as SYBR Green are added to the PCR reaction mix, and with each cycle of PCR the fluorescence signal becomes more intensive, as there is more dsDNA with which the dye intercalates. The DNA amount in the samples can be quantified using standard curves and the actual level of transcription can be calculated by normalizing the results of RT-qPCR to a house-keeping gene (β -Actin or GAPDH), also quantified with RT-qPCR.

All RT-qPCR experiments were done with a Roche LightCycler® 480. As SYBR Green mixes, either LightCycler® 480 SYBR Green I Master by Roche or KAPA SYBR FAST qPCR LightCycler480 by PeqLab were used. Each PCR reaction was set up as in Table 2.9

Ultra pure water	4,5µl
Primer fwd. 25pmol	0,75µl
Primer rev. 25pmol	0,75µl
SYBR Mix	7,5µl

Table 2.9 qPCR Reaction Mix

To the 13,5µl of Master Mix, 1,5µl of cDNA were added, either undiluted or diluted 1:10 in ultra pure water. The qPCR was then run using an appropriate program, adjusted to the melting temperatures of the primers used.

2.7 Molecular Biological Methods: RNA

2.7.1 Isolation of total RNA

Total RNA from tissues was isolated using TRIzol Reagent (Applied Biosystems Inc.). 5ml TRI reagent was used per gram wet weight tissue (4-5g for fat tissues). The tissue sample was homogenized with the TRI reagent using an Ultra-Turrax homogenizer. Samples were incubated at RT for 5min, then 200µl of chloroform per ml of TRI were added, vortexed and incubated at RT for 15min.

The samples were then centrifuged at 12000g, 4°C for 15min. The clear upper phase containing the RNA was taken up and transferred into a new tube. 500µl isopropanol per 1ml of TRI were added, the samples were mixed by vortexing and then incubated at RT for 15min.

Precipitated RNA was obtained as a pellet by centrifugation at 12000g, 4°C for 30min. The RNA pellet was washed once with 75% ethanol and then briefly air-dried. Depending on the tissue and on the size of the pellet, the pellet was resuspended in an appropriate volume of ultra pure water.

RNA concentrations were quantified using a NanoDrop 2.0 spectrophotometer.

2.8 Biochemical Methods: Protein

2.8.1 Preparation of Triton X-100 Total Protein Extracts

1g of fresh or frozen tissue was homogenized in 4ml of homogenization buffer using an Ultra-Turrax T25 homogenizer. The samples were then centrifuged at 620x g at 4°C for 10min. The supernatant was transferred into a new tube and 1/20 volume of 20% Triton X-100 was added. The solutions were briefly mixed and then left to incubate on ice for 30min. The solution was then centrifuged in an Beckman ultra-centrifuge for 1h at 300.000 x g at 4°C. After centrifugation the supernatant was transferred in aliquots to new tubes and quick-frozen in liquid nitrogen. The protein extracts were stored at -80°C. Protein concentration was measured using the Bradford assay.

Homogenization buffer

20mM HEPES, pH 7.4

300mM sucrose

150mM NaCl

0,5mM PMSF

2,5µM Leupeptin

2.8.2 Preparation of Triton X-100 Membrane Protein Extracts

5ml Buffer A were added to 1g of fresh or frozen tissue and the tissue was homogenized within the buffer using an Ultra-Turrax homogenizer until no more chunks were visible. The samples were centrifuged for 10min at 3000x g. The supernatant was then transferred into a new tube appropriate for ultra-centrifugation and was again centrifuged in an Beckman Ultra-Centrifuge at 100.000x g for 1h at 4°C. The supernatant of this centrifugation was discarded and the pellet was resuspended using 3ml of Buffer A flushing it through a 18gge needle several times, followed by a few flushes with a 22gge needle. The samples were then centrifuged for 1h at 4°C at 100.000 x g.

The supernatant was again discarded and the pellet was resuspended in 1ml of Buffer B, flushing it through 18gge and 22gge needles as before. Then 4% of the total volume of 4M NaCl were added and the sample was sonicated for 30sec. 26% of the total volume of dH₂O and 20% of the total volume of 5% v/v Triton X-100 were added and the samples were carefully mixed avoiding foaming.

The samples were again centrifuged for 1h at 4°C, 100.000 x g. The supernatant representing the protein extract was stored in appropriate aliquots at -80°C after quick-freezing in liquid nitrogen.

Protein concentration was determined using Bradford assay.

Buffer A

20 mM Tris/HCl

1mM CaCl₂

150 mM NaCl

Proteinaseinhibitor

Buffer B

250mM Tris/Maleat, pH 6

2mM CaCl₂

Proteinaseinhibitor

2.8.3 Determination of Protein Concentration (Bradford)

For Bradford assays, Quick Start Bradford Dye and BSA (both from Biorad) were used. 1ml of Bradford reagent was mixed with 5µl of BSA, resulting in a final concentration of 10µg/µl BSA. 1ml of reagent was mixed with 1µl of protein extract or purified recombinant protein. Absorption of light was measured at 595nm wavelength, using 1ml of Bradford as a blank, before measuring the samples.

The protein concentration was calculated according to the formula below,.

$$\frac{c(\text{BSA}) \mu\text{g}}{\text{OD595nm}} \times \frac{\text{OD595nm}}{\mu\text{l Protein}} = c(\text{Protein}) \mu\text{g}/\mu\text{l}$$

2.8.4 SDS-Polyacrylamide Gel Electrophoresis

In a molecular filter, such as an electrophoresis gel, molecules are supposed to be separated exclusively by their size. Under physiological conditions proteins are charged, and therefore won't migrate through a gel simply according to their size. The charge of SDS masks the actual charge of the protein by binding to the protein. SDS is added to the rest of the gel components and also to the gel running buffer, so that the electrophoresis occurs under denaturing conditions.

Gels were prepared as in Table 2.10

	Stacking Gel	10%	12%	14%
	4%			
dH2O	1525μl	2025μl	1675μl	745μl
1,5M Tris HCl pH 8,8	-	1250μl	1250μl	1250μl
0,5M Tris HCl pH 6,8	625μl	-	-	-
Bis-PAA	325μl	1650μl	2325μl	3255μl
10% SDS	25μl	50μl	50μl	50μl
10% APS	12.5μl	25μl	25μl	25μl
TEMED	5μl	5μl	5μl	5μl

Table 2.10 Preparation of Polyacrylamide Gels

Recombinant proteins and protein extracts were separated on 10%, 12% and 14% gels with a thickness of 0,75 mm using the BioRad Mini gel system. First, the components of the separation gels were mixed, the gel was poured, overlayed with isopropanol and polymerized for about 20min. The isopropanol was then rinsed off and the stacking gel components were mixed and poured onto the separation gel. A comb was placed into the liquid stacking gel to produce slots, and the gel was polymerized for 20min. Then the comb was removed, slots were rinsed with running buffer and the electrophoresis chamber was assembled and filled with running buffer.

Samples were prepared using 4x Laemmli Buffer and 1M DTT to produce reducing conditions, samples were heated at 95°C for 10 minutes before being loaded onto the SDS-PAA gel.

Gels were run at 100V for 10 minutes and then at 140V until the front marker ran out of the gel. After the gel run, either Coomassie Staining or Western Blotting was performed.

<u>Running Buffer</u>	<u>4x Laemmli buffer</u>
250mM Tris	8ml 78% glycerol
1,92M Glycine	6ml 20% SDS
1% SDS	0.4ml 1M Tris-HCl pH 7.4 - 7.5
	ddH ₂ O to a final volume of 20ml
	+25mM Dithiothreitol (DTT) for reducing conditions

2.8.5 Protein Detection by Coomassie Staining

After the gel run, SDS polyacrylamide gels were carefully detached from the glass slide and then put into Coomassie Blue Staining Solution for one hour. The gel is stained by Coomassie and destained in Destain Solution, leaving only the proteins stained. The gel was vacuum dried at 70°C.

Coomassie Blue solution

10% Acetic acid
25% Isopropanol
0.287g Coomassie Brilliant Blue R250 in ddH₂O.

Destain

10% Acetic Acid
30% MeOH in ddH₂O

2.8.6 Western Blot Analysis

After the gel run, proteins were transferred from the gel onto a Hybond-C membrane (Amersham) using the wet blot technique. A so-called "sandwich" was prepared: onto each side of a clamp two sponges soaked in transfer buffer were placed, three Whatman papers soaked with transfer buffer were placed onto each sponge. The gel was placed onto one part of the sandwich and an Hybond C nylon membrane was put onto it. Air bubbles were removed and the sandwich was clapped together. The sandwich in its clamp was placed into a transfer chamber (Biorad) filled with transfer buffer. Transfer was performed for 1h at 100V.

The membrane then was reversibly stained with Ponceau Solution to mark lanes and the marker proteins. The Ponceau was then removed with distilled water and the membrane was blocked with 5% dry milk in TBS-T for 1h at room temperature. Afterwards the membrane was incubated with the first antibody in an appropriate dilution over night at 4°C. Then, the membrane was washed three times with 1x TBS-T and incubated for one hour at room temperature with the second antibody, anti mouse IgG or anti rabbit IgG, respectively. After incubation with the second antibody, the membrane was washed as before, and then incubated with 1ml of Enhanced Chemiluminescence (ECL) Reagent. Then a film was exposed to the membrane in the dark room and developed using Developer and Rapid Fixer by AGFA.

<u>Ponceau S</u>	<u>Transfer Buffer</u>	<u>TBS-T</u>
0.2% Ponceau S	250mM Tris	250mM Tris
1x PBS	1,92M Glycine	1,4M NaCl
3% Trichloroacetic acid		25mM KCl
		1% Tween 20

2.8.7 Expression and Purification of recombinant Proteins

Recombinant proteins were obtained from bacterial expression cells, namely BL21 by Invitrogen which had previously obtained the DNA sequence coding for the protein. Cells were grown at 37°C in LB amp medium until an optical density OD₆₀₀ of 0,6-0,7 was reached. Induction was achieved using Isopropyl β -D-1- thiogalactopyranoside (IPTG) at a final concentration of 1mM.

Cells were grown for 3 hours and then harvested by centrifugation at 4°C for 10min at 3000 x g in a Sorvall centrifuge using a GS-3 rotor. The cell pellet was resuspended in 8M Urea containing protease inhibitors (Roche), and the samples were sonicated 12 times for 30 seconds. The lysate was then centrifuged at 2300 x g for 10 minutes at 4°C. The cleared supernatant was transferred into a new tube and incubated over night in an appropriate binding buffer at 4°C, with either 500 μ l Ni-NTA agarose (Quiagen) for His-tagged proteins or Glutathione sepharose B4 beads (Sigma) for GST-tagged proteins, respectively.

On the next day, the beads were sedimented at 2300 x g for 5min at 4°C. The supernatant was transferred to a new tube as a control of protein binding to the beads. The beads were then washed three times in an appropriate washing buffer, and wash fractions were also stored as controls. The protein was finally eluted from the beads buffers containing either Imidazol for Ni-NTA agarose or L-Glutathione for Glutathione sepharose B4 beads, respectively. For elution the beads were incubated three times with 500-700 μ l of elution buffer for 30min at 4°C rotating. Protease inhibitor was added to the eluted protein samples.

The extent of purification was tested on SDS-PAA gels using Coomassie Staining.

<u>Binding Buffer A</u>	<u>Binding Buffer B</u>
8M Urea pH8,5	TBS-T
 <u>Washing Buffer A</u>	 <u>Washing Buffer B</u>
8M Urea pH6,3	TBS-T
 <u>Elution Buffer A</u>	 <u>Elution Buffer B</u>
8M Urea	TBS-T
100mM Imidazol	100mM Glutathion

2.8.8 Antibody Generation

As insoluble proteins have to be eluted in 8M Urea, the urea concentration of the eluted peptide used for antibody generation is typically too high and could cause renal failure in the animal used for antibody production. Thus, the solution has to be dialysed to remove the urea almost completely.

Dialysis buffers containing different concentrations of urea were prepared. Elutions were pooled in dialysis tubing and the sample was dialysed against each buffer for a minimum of three hours. The protein concentration was then measured using Bradford. The purified protein, a fragment of chicken PNPLA3 made up of 50 amino acids, located in the middle of ggPNPLA3, was then injected intra-dermally into either white Balb C mice or into a white New Zealand rabbit. Mice were given the peptide three times in intervals of three weeks with 100µg of peptide at each injection. Rabbits were also injected three times with the peptide in intervals of three weeks, receiving 250µg of peptide with each injection. For all injections the purified protein was mixed with GEBRO complete adjuvant.

One week after the last injection, the antisera were obtained. The blood was incubated at RT for 1 hr and afterwards stored o.n. to let coagulation proceed, then the samples were centrifuged at 4000 rpm for 15 min. The supernatant representing the antiserum was aliquoted and stored at -20°C. The obtained serum was tested by Western blot analyses. Preimmune serum from the same rabbit served as control.

Dialysis Buffer

25mM Tris HCl pH 7,4

100mM NaCl

5% Glycerol

Urea added up to 6M, 4M, 2M or 0M respectively

2.8.9 Chromogenic Esterase Assay using pNitrophenyl-Esters

A commonly used assay used to determine lipase and/or esterase activity employs p-nitrophenyl (pNP) esters as substrates for a putative enzyme (Gilham and Lehner, 2005). When these esters are cleaved by an enzyme, p-nitrophenol accumulates and makes a chromogenic reaction, turning the buffer solution yellow. The change in colour can be quantified photometrically at a wavelength of 410nm. There are various types of esters available commercially, short-chain acyl esters such as p-nitrophenyl acetate, or longer chain acyl esters e.g. p-nitrophenyl dodecanoate (also called p-nitrophenyl laurate). If an enzyme is capable of cleaving short-chain acyl esters, this is an indication for esterase activity; cleavage of long-chain acyl esters indicates lipase activity.

For the assay, the putative esterase/lipase was freshly expressed and purified, at maximum 2 days before the assay. A basic buffer was prepared from Tris-HCl pH8, NaCl and Triton X-100. Stock solutions of p-nitrophenyl acetate and p-nitrophenyl laurate in CH₂Cl₂ were prepared. The freshly purified substrate was incubated with possible co-factors/activators for 1h at room temperature before starting the assay. 20-50µl of p-nitrophenyl ester stock solution was added to 10ml of basic buffer under constant vortexing to mix the organic phase with the aqueous solution. Then 200µl of buffer containing substrate were added to each well of a 96-well plate and the enzyme preparations (different dilutions, co-incubated with co-factors, etc.) were added to the substrate solution. The chromogenic reaction was then observed by measuring the absorbance of the solution at 410nm each 5min over 40min.

<u>Basic Buffer</u>	<u>Stock 1</u>	<u>Stock 2</u>
20mM Tris-HCl pH8	200mM p-Nitrophenyl acetate	200mM p-Nitrophenyl laurate
150mM NaCl	in CH ₂ Cl ₂	in CH ₂ Cl ₂
0,01% Triton X-100		

Note: In assays of the activity against long-chain acyl esters, emulsifying agents, such as gum arabicum (0,01%) or Triton (up to 2%) should be added to the basic buffer.

2.8.10 Fluorogenic Lipase assay using 4-methyl umbelliferyl heptanoate (MUH)

Another assay to test for lipase activity of a protein employs 4-methylumbelliferyl heptanoate as substrate. The substrate is water-soluble and the methyl-umbelliferone compound is strongly fluorogenic after hydrolysis of the ester bond. Fluorescence is measured at 355nm (excitation) and 460nm (emission). However it is critical, that MUH resembles rather a monoacylglycerol than triacylglycerol (Gilham and Lehner 2003).

For the assay, freshly expressed and purified enzyme was used. Different dilutions of the enzyme were prepared in Buffer B, and co-incubations of the enzyme and putative co-factors were performed in Buffer B for 1h at room temperature – Buffer B was added to a total volume of 180µl. 10µl of a 100mM stock solution of MUH in Tetrahydrofuran were added to 10ml of Buffer A just before the assay. The 180µl Buffer B were mixed with 20µl of MUH in Buffer A in a 96 well plate, resulting in a total volume of 200µl. The samples' fluorescence was then measured each minute over 10 minutes using a VICTOR V2 fluorimeter.

<u>Buffer A pH 8</u>	<u>Buffer B pH8</u>	<u>MUH Stock</u>
20mM Tris HCl	137mM NaCl	100mM MUH
1mM EDTA	2,7mM KCl	in Tetrahydrofuran
300µM Sodiumtaurodeoxycholate	10mM Na ₂ HPO ₄	
in PBS	2mM KH ₂ PO ₄	
	in PBS	

2.9 Immunohistochemistry

2.9.1 Isolation and Cultivation of chicken primary hepatocytes

Chicken primary hepatocytes were obtained from chicks either one day before or after hatching by decapitation and isolation of the liver. The liver was then rinsed with PBS and minced with a scalpel into very small pieces. These pieces were incubated with Collagenase and DNase for 30min. The samples were centrifuged for 1min at 200 x g. The supernatant was aspirated and the cell pellet containing hepatocytes and erythrocytes was treated for 5min with Ammonium-Chloride Buffer to swell and eradicate the erythrocytes.

The reaction was stopped by adding DMEM medium and the sample was again centrifuged. The isolated hepatocytes were then suspended in DMEM medium and cells were seeded at different concentrations onto coated 8well microscope slides. Cells were allowed to attach to the slide over-night at 37°C, 5% CO₂.

<u>Collagenase Buffer</u>	<u>Ammonium-Chloride Buffer</u>
1,1ml Collagenase (150U/ml)	0,14M NH ₄ Cl
0,15ml DNase	0,17M Tris.HCl pH 7,2
2,25ml 400mM Glucose	
up to 20ml with PBS	

2.9.2 Fixation and Fluorescence Staining of chicken primary hepatocytes

Cells were removed from sterile conditions and the medium was sucked off, the cells were washed with PBS carefully so as to not detach them. A 4% paraformaldehyde solution was used to fix the cells, cells were incubated for 15min shaking at room temperature with PFA. Cells were again carefully washed twice with PBS and then permeabilized with 0,2% Tween-20 for 5min at room temperature. Cells were washed two more times with PBS and then incubated for 1h in Blocking Solution. Then, the cells were incubated over-night with different dilutions of the first antibody in blocking solution. Subsequently, cells were washed three times with PBS and incubated with the second antibody, (either goat- α -rabbit IgG-Alexa 488 or goat- α -mouse IgG-Texas Red), and counter-stained with DAPI for 1h at room temperature. After incubation with the second antibody, cells were washed three more times with PBS and then mounted using Fluorescence Mounting Medium by Dako and covered with coverslips. The processed samples were analyzed under the fluorescence microscope (Zeiss).

<u>PBS Buffer pH 7.3</u>	<u>PFA solution</u>	<u>Blocking solution</u>
1,4M NaCl	4% (w/v) PFA	1% BSA
25mM KCl	1ml 5M NaOH	3% inactivated goat serum
81mM Na ₂ HPO ₄	in PBS	in PBS
15mM KH ₂ PO ₄		

3. Results

In general, my diploma thesis had two aims:

- 1) To further examine chicken PNPLA3 expression at the mRNA and protein levels under different conditions, such as in the fasted and refed state and after treatment with various reagents such as hormones, LXR agonists etc. As an antibody against chicken PNPLA3 did not exist, a polyclonal antibody was raised by me.
- 2) To investigate whether arylacetamide deacetylase (AADA) might take over the role of HSL in the chicken. Therefore, esterase and lipase assays were carried out, using AADA and interaction partners of HSL.

3.1 Antibody Generation

To be able to observe the expression of PNPLA3 not only at the mRNA but also at the protein level, an antibody against chicken PNPLA3 (ggPNPLA3) was raised. Therefore, a 150bp stretch of the chicken PNPLA3 cDNA, yielding a 50 amino acid polypeptide, was chosen for antibody production. The sequence of the ggPNPLA3 gene was obtained from the Ensemble Genome database (see. Fig. 3.1).

```

5' - GCTGAAGCATGTGCAGATGTTCTGGCATTGGCCAAAGAAGCTAGAAAGCGGAATCTTGGT 60
      CCTCTTCATCCTTCATTTAATGTGATAAAGATAATAAGAGATGGGTTGATGAGAAATCTG 120
      CCAGAAAACACTCACCTGTTGTCATCGGGCAGGCTGTGCATTTCACTGACTAGAGTCTCA 180
      GATGGTAAAAACACACTGATATCTAATTTTAACTCTAAAGAAGAAGTTGTCCAGGCATG 240
      ATCTGTAGCTCATTTGTCCCAATTTATTTGTGGCCTCATTCGCCATCATTTAGAGGTGTG 300
      CGTACGTGGATGGAGGAATCAGTGACAACCTGCCTCAGTATGAATCTAAGAATACCATC 360
      ACCGTTTCACCTTTTGTCTGGGGAGTGTGATATCTGTCCAAAGGGGAATTCTGCCAACTT 420
      CATGAAATGAATGTGACCAACACCAGCATTTCAGCTCAGTTTGGGGAACCTTTATCGTTTA 480
      ACACAAGCACTCTTTCTCCAGAACCTAAGGTACTAGGTGAGATCTGTGAGCAAGGATAT 540
      TCAGATGCTTTTAAATTCCTGAAAGAGAATGGTATTCTGAATGACTCAATTTATGTCAGC 600
      TTGTCCTTCACCAAAACAAATCCTCATGAAGCTGCACAACACATTGACTATATGAAGAAA 660
      AGAAATAAGTCTGAAAACAGTAGAATGAAACTTCACAAGTGGAAGTACTCGGTGAGCAG 720
      ATGAAGCAAAATCCGTGGCCTCTGGAGAAGAGTATATTTGAGAGTCTACCTCCTAGGCTT 780
      CGTAAAGCGCTTCAGGAAGCTTGTAAAGAACCAATGGATTCTATGCTCAGTTCTCTAAA 840
      CTCTTCCCAGTGCGGGTGATATCCTATCTGATGCTACCATATACACTACCTGTGGAGTCT 900
      GCTTATTCTGCTGCTTTACGGTTAGTAAACTGGTTTCCTGACATGCCTGCTGATGTTTCA 960
      TGGATGCAAGAACAACCTTCTTCAGATTGCTGGTACAGTTTATTCCCAGGCAAAAAAGAGG 1020
      CTGTTCTGTGACATCCAGGAAGACAATTACACAGCACTAAGGAAATGTCAGACTGCCCGG 1080
      GCTACTGTGGAATTTTCATTCTTCGTACTGCCATCTTAAATGCCTCATTCTTCTGCGGAT 1140
      ATTGAAACCTGGCTCTGGGAATCTTCGTGCTACATGGACCTCTGTTATGAAACATACTTCT 1200
      CCTAAGACAAAGGAGCAGTCAGACTTAAACTCCTATCCTAATTTTCTCCCAATTCTGAT 1260
      GAATCTGGTTTGGAAATAGATTTTGACTCCTCCTCAGAGACAAGTTTCCATAAACAGTGT 1320
      CTTTCTGGAAATGAGTTT - 3' 1138
  
```

Figure 3.1 The chicken PNPLA3 cDNA sequence. The 5' ATG start codon as well as the 3' Stop codon can not be shown, as the PNPLA3 gene sequence in the chicken is not completely known. The region marked in light grey was chosen for antibody generation; letters in red signify primer binding sites (green arrow = forward primer with additional NcoI restriction site [AACCATGGTCCATCCAGGAAAGACAATTAC]; blue arrow = reverse primer with additional EcoRI restriction site [TTGAATTCGGGTCCATGTAGCACGAAGATTCT])

To introduce restriction sites that would facilitate the cloning into different vectors, nucleotides corresponding to certain restriction sites were added to the primers. A list of primers can be found in part 2.3.

In Figure 3.2 the protein sequence, deduced from the known PNPLA3 DNA sequence is shown. The peptide used for antibody generation is depicted bold red. A part of the PNPLA3 sequence is still unknown, because of this the protein sequence does not start with the obligatory methionine residue.

```

AEACADV LALAKEARKRNLGPLHPSFNVIKIIRDGLMRNLPENTHLLSSGRLCISLTRVS 60
DGKNTLISNFNSKEEVVQALICSSFVPIYCGLIPPSFRGVRYVDGGISDNLPQYESKNTI 120
TVSPFAGECDICPKGNSANFHEMNVTNTSIQLSLGNLYRLTQALFPPEPKVLGEICEQGY 180
SDAFKFLKENGILNDSIYVSLSFTKTNPHEAAQHIDYMKKRNKSENSRMETSQVEVLGEQ 240
MKQNPWPLEKSIFESLPPRLRKALQEACEKPNGFYAQFSKLFVPRVISYLMPLPYTLPVES 300
AYSAALRLVNWFPDMPADV RWMQEQLLQIAGTVYSQAKKRLFCTSRKDNYTALRKQTAP 360
ATVEFHSSYCHLKMPHSSAD IETWLWESSCYMD SVMKHTSPKTKEQSDLNSYPNFLPNSD 420
ESGLEIDFDSSSETSFHKQCLSGNEF 446

```

Figure 3.2 PNPLA3 protein sequence. the C-terminally located peptide chosen for antibody generation is shown in bold red letters; in the recombinant protein a 6x His-tag is attached to the peptide.

The selected DNA fragment was amplified in a PCR reaction using LH liver cDNA (produced from tissue extracted total RNA) as a template. A polymerase with proof-reading function to prevent deletion or insertion of bases was used. The product of the PCR reaction was the desired 150bp PNPLA3 fragment with additional restriction sites (NcoI and EcoRI). The PCR product was visualized on a 1% agarose gel containing ethidiumbromide (see Fig. 3.3).

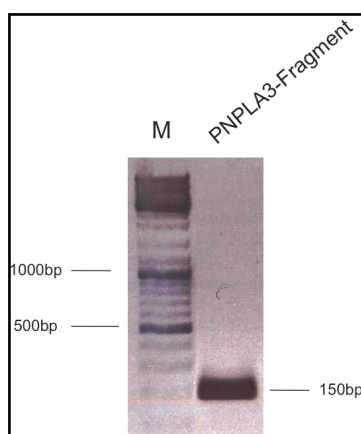


Figure 3.3 PCR for cloning of a PNPLA3 fragment used for antibody generation. M designates the marker (100bp DNA ladder by Biorad), the PNPLA3 fragment is well amplified and appears as a thick band.

The PCR product was then cloned first in to a pBluescript and then into an pET25b+ vector. The pET25b+ vector is supposed to add a C-terminal 6xHis tag to the peptide. After the correctness of the fragments' sequence was confirmed, the fragment was transformed into E. coli BL21 expression cells and the protein expression was induced using 100mM IPTG (see Fig. 3.4).

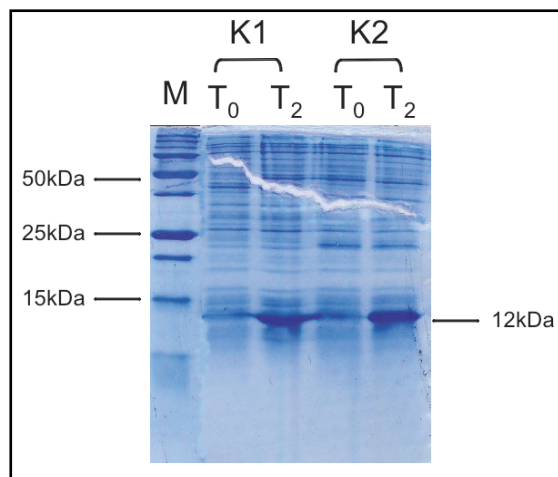


Figure 3.4 Induction of the expression of a his-tagged PNPLA3 fragment in E.coli BL21. Bacterial cells were grown to OD600 0,6 and protein expression was induced using 100mM IPTG. After induction cells were allowed to grow for 2 hours. Cells were harvested by centrifugation and resuspended in PBS. Samples were prepared for SDS-PAGE by adding reducing Laemmli Buffer. PAA-Gels were Coomassie stained after electrophoresis. T0 = before induction; T2 = 2 hours after induction. The protein of 6kDa together with the 6kDa 6x His tag yielded a recombinant protein of 12kDa.

There was efficient expression of the desired tagged peptide but as the peptide was quite insoluble, purification was difficult. The protein was purified using a lysis buffer containing 8M urea. The protein was bound to Ni-NTA agarose beads and eluted from the beads employing a pH gradient from pH8 to pH4,5. Urea was eliminated using step-wise dialysis to decrease the urea concentration without precipitating the protein.

After three injections of 100µg purified protein each every three weeks, the mice used for antibody generation were sacrificed and the antibody was tested for its function and specificity. A Western Blot was performed to test whether the antibody worked (see Fig. 3.5). The recombinant His-tagged PNPLA3 fragment was used as a control for the function of the antibody.

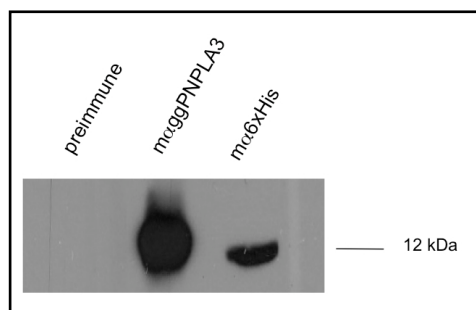


Figure 3.5 Western Blot to test for function of the polyclonal maggPNPLA3 antibody. Purified recombinant protein also used for injection was used as control, 2µg of recombinant protein were loaded in each lane of a 14% PAA gel and subjected to SDS-PAGE. Proteins were subsequently transferred onto a nitrocellulose membrane. The recombinant protein of 12kDa size was detected with the maggPNPLA3 antibody via the PNPLA3 fragment and also with the mα6xHis antibody via the His tag attached to the recombinant protein. No band is visible when the recombinant protein is incubated with preimmune serum. The Western Blot was performed under reducing conditions.

As expected, the protein is recognized by the $\alpha 6xHis$ antibody via the His-tag attached to the protein. Preimmune serum did not give an immunoreactive signal, whereas the immune serum contains antibody against chicken PNPLA3. The antibody was shown to work in Western Blots both under non-reducing and, slightly better, under reducing conditions (data not shown). Therefore, all further Western Blot experiments were performed under reducing conditions.

3.2 Monitoring of PNPLA3 expression in different tissues

In our model organism, the chicken, the expression patterns of all patatin-like phospholipase family members in different tissues had been previously elucidated - at the mRNA level - in our laboratory (Saarela et al. 2008). Thus, it was known that in the chicken the expression of PNPLA3 is highest in liver, muscle and testis (see Figure 3.6).

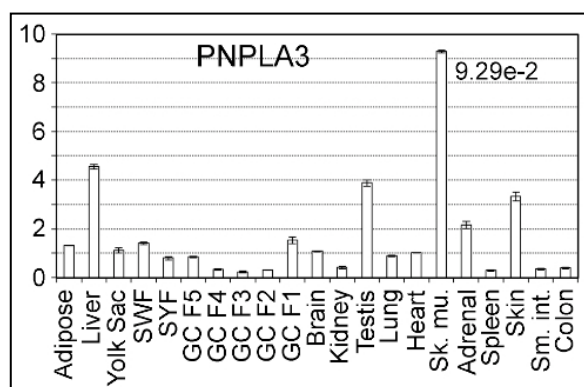


Figure 3.6 Expression of PNPLA3 mRNA in various chicken tissues (from Saarela et al., 2008)

In additional qPCR experiments, I also observed that expression was highest in liver, muscle and testis (see Figure 3.7). Furthermore, it was found that there are higher PNPLA3 expression levels in the liver of the R/O chicken compared to wild type, and that PNPLA3 expression is higher in hen liver than in rooster liver.

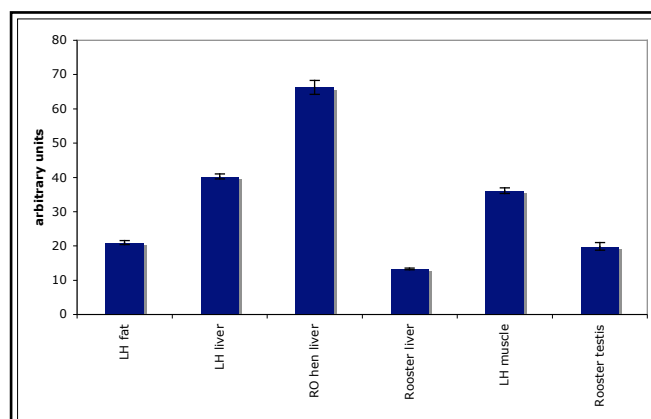


Figure 3.7 Expression of PNPLA3 mRNA. cDNA was prepared from 1 μ g/ μ l RNA and used diluted 1:10 in qPCR. Gene specific primers for the ggPNPLA3 and β -actin genes were designed; PNPLA3 expression was normalized to β -actin expression. Each chicken gene was analyzed three times using the Roche Lightcycler 480 and two replicates of each tissue were employed in each analysis. Error bars indicate the standard deviation. Statistics were done using MS Excel.

After raising an antibody against chicken PNPLA3, Western Blot was performed to monitor the expression of PNPLA3 in various tissues also at the protein level (see Figure 3.3).

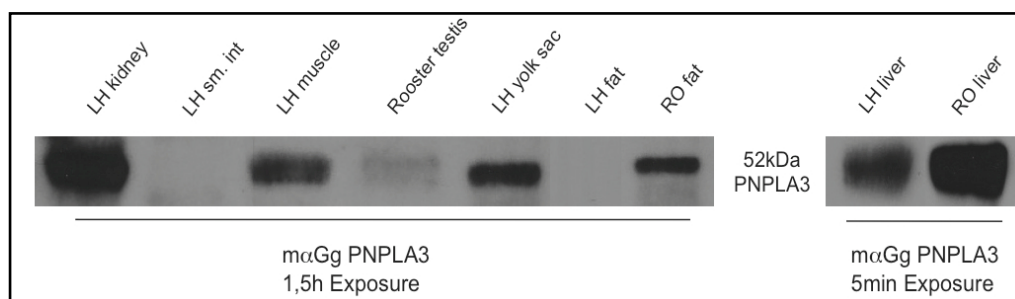


Figure 3.8 Western Blot analysis of PNPLA3 expression in various tissues; 50μg of membrane protein extract of each tissue were loaded onto a 10% PAA gel and subjected to electrophoresis. Proteins were transferred to a nitrocellulose membrane, PNPLA3 was detected using a m-α-ggPNPLA3 polyclonal antibody diluted in 5% BSA-TBST.

Monitoring PNPLA3 at the protein level, some surprising results were found. Because in qPCR experiments mRNA expression was high in liver, muscle and testis, it was expected that in the Western Blot strong bands would appear in these tissues. In liver, and even stronger in the liver of R/O chicken, solid bands appeared as expected. But in e.g. muscle only a faint band appeared, and an even fainter one in testis, although mRNA levels in these tissues were especially high. Maybe this is due to poor stability of the mRNA or due to low translation rates.

3.3. Influence of fasting and refeeding on PNPLA3

It has been reported that PNPLA3 expression levels in humans and mice decrease upon fasting and increase upon refeeding (Liu et al. 2004, Moldes et al. 2006, Kershaw et al. 2006). Therefore an experiment to monitor the influence of fasting and refeeding on chicken PNPLA3 was designed. Two hens were fasted for 18 hours, one of them was then sacrificed, the other one was refed for 24 hours and sacrificed thereafter. Additionally, one hen was put on a high-energy fat diet for three weeks and sacrificed afterwards. Liver tissue was taken from these animals, mRNA was isolated from the tissues and transcribed to cDNA, and also liver membrane protein extracts were prepared. The cDNA was used in quantitative PCR, the protein extracts were used for Western Blot experiments.

As published for humans and rodents, also in the chicken, PNPLA3 levels decrease upon fasting and increase upon refeeding (see Fig. 3.9). Interestingly, after a period of fasting and subsequent refeeding the PNPLA3 expression rises dramatically, meanwhile it only increases slightly in chickens after a three week fat diet.

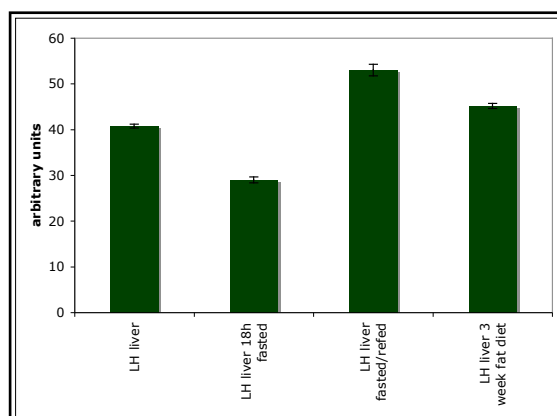


Figure 3.9 qPCR analysis of PNPLA3 expression upon different nutritional conditions. Gene specific primers for the ggPNPLA3 and β -actin genes were designed; PNPLA3 expression was normalized to β -actin expression. cDNA was prepared from $1\mu\text{g}/\mu\text{l}$ RNA and used diluted 1:10 in qPCR. Each chicken gene was analyzed three times using the Roche Lightcycler 480 and two replicates of each tissue were employed in each analysis. Error bars indicate the standard deviation. Statistics were done using MS Excel.

Next, it was tested whether the expression observed at the mRNA level was similar to that at the protein level. Therefore, a Western Blot with the protein extracts obtained from liver tissue of fasted, refed and hens on high fat diet was performed. For detection of the PNPLA3 protein the polyclonal m α ggPNPLA3 antibody, was used and to detect GAPDH a m α GAPDH antibody was used as control.

As at the mRNA level, also at the protein level, PNPLA3 decreases upon fasting and increases upon refeeding (see Fig. 3.10): compared to the “normal/basal” LH liver sample, the band visible in the 18h fasted sample is weaker, whereas the band after refeeding appears much stronger. The signal in the three week fat diet liver sample is only slightly stronger than that in the basal sample, but weaker than the band in the refed sample, showing the same pattern as at the mRNA level. The housekeeping gene GAPDH was used as control, the strength of the GAPDH signal should appear quite similar in all tissues to ensure that differences in the intensity of PNPLA3 bands are not due to uneven loading but to differential expression.

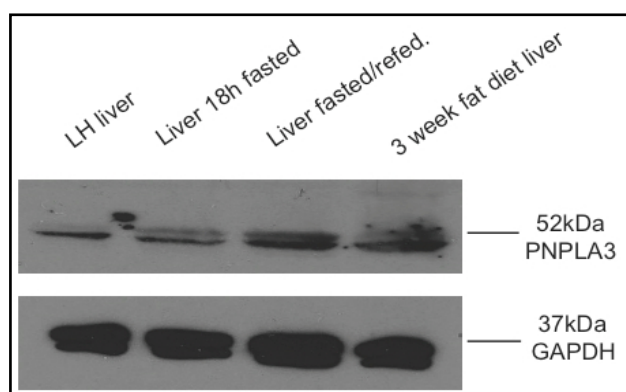


Figure 3.10 PNPLA3 protein levels upon different nutritional conditions. 50 μg membrane protein extract per lane were subjected to SDS PAGE and blotted onto a nitrocellulose membrane, the membrane was cut between the 50 and 37kDa markers, PNPLA3 was detected on the upper part using m- α -ggPNPLA3 antibody, GAPDH was detected on the lower part using a mouse- α -GAPDH antibody. Antibodies were diluted in 5% BSA-TBST.

The same experiment was performed with samples obtained from restricted ovulator (R/O) chicken: two R/O hens were fasted for 18 hours, one was sacrificed after that, the other one was refed for 24 hours before sacrificing it. Livers were isolated and protein extracts as well as RNA were prepared from them. RNA was then used to generate cDNA. qPCR and Western Blot were performed, using cDNA and protein extracts, respectively.

Basal PNPLA3 expression levels in R/O hen liver are higher than in the wild type laying hen. In accordance to the wild type expression pattern, PNPLA3 expression in the liver of fasted R/O chicken decreases - but upon refeeding there is no sudden increase in PNPLA3 expression as was observed in the wild type chickens (see Fig. 3.11). A possible explanation for this might be that the R/O chicken generally has more energy stores than the wild type chicken and therefore induction of PNPLA3 expression is attenuated following refeeding.

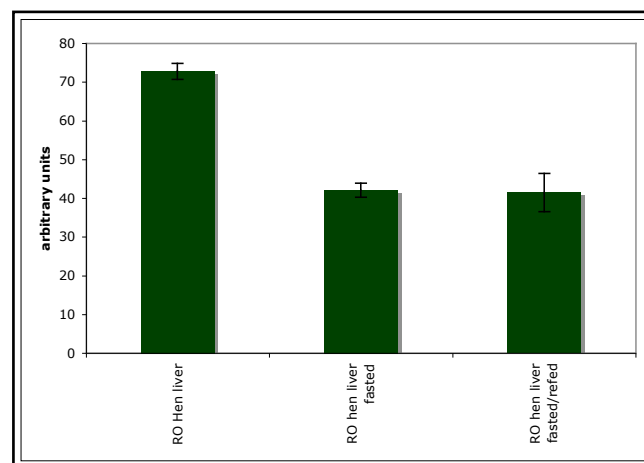


Figure 3.11 qPCR of PNPLA3 mRNA expression in fasted/refed R/O chicken. Gene specific primers for the ggPNPLA3 and β -actin genes were designed; PNPLA3 expression was normalized to β -actin expression. cDNA was prepared from 1 μ g/ μ l RNA and used diluted 1:10 in qPCR. Each chicken gene was analyzed three times using the Roche Lightcycler 480 and two replicates of each tissue were employed in each analysis. Error bars indicate the standard deviation. Statistics were done using MS Excel.

A Western Blot was performed with samples from liver tissue of fasted and refed R/O chickens. This blot is shown in Figure 3.12. As before, GAPDH was used as a control for even loading. Here the band in the fasted R/O liver sample is clearly weaker than the other bands. Untreated R/O liver and the fasted/refed liver sample appear unchanged. Generally the bands are more intense than in the corresponding blot with wild type chicken tissues (Fig. 3.10) in agreement with PNPLA3 expression in the R/O chicken being higher.

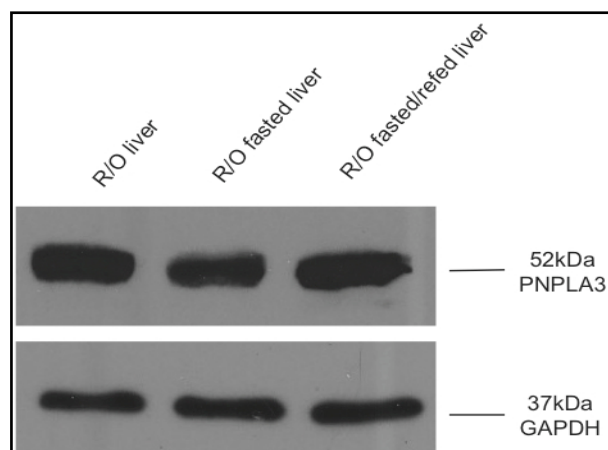


Figure 3.12 Expression of PNPLA3 protein upon fasting and refeeding in R/O chicken. 40 μ g of membrane protein extract from liver tissue were loaded per lane onto a 10% PAA gel, proteins were blotted onto a nitrocellulose membrane and the membrane was cut in two pieces to be able to detect both GAPDH and PNPLA3. PNPLA3 was detected on the upper part using m- α -ggPNPLA3 antibody, GAPDH was detected on the lower part using a mouse- α -GAPDH antibody. Antibodies were diluted in 5% BSA-TBST.

Taken together, qPCR and Western Blot results suggest that PNPLA3 may play a role in the generation and storage of energy-rich molecules such as dietary lipids: in the R/O chicken, where lipid metabolism is quite abnormal, PNPLA3 expression is generally higher than in wild type chicken. Moreover, PNPLA3 expression decreases at both the protein and the mRNA level when animals are fasted; the exactly opposite expression pattern would be expected for a lipase.

3.4 Influence of hormonal treatment on PNPLA3

Many genes in lipid metabolism have different expression patterns in the females compared to males. It was observed that this is also true for PNPLA3 which showed in qPCR significantly higher expression of mRNA in the hens' liver than in the roosters' liver. Therefore, the influence of oestrogen treatment on the expression of PNPLA3 in rooster liver was tested.

Roosters received treatment with oestrogen (one dose every 24h) over 48 or 72 hours, and were then sacrificed. The liver tissue was isolated and RNA and protein isolation was performed. RNA was transcribed to cDNA and cDNA and protein extracts were used in qPCR and Western Blot, respectively.

In qPCR experiments, in the liver of control roosters, PNPLA3 expression at the mRNA level was lower than in the liver of laying hens. When roosters were treated with oestrogen for 48 hours, the levels of PNPLA3 expression in the liver increased and even exceeded the expression level in laying hen livers. In livers of roosters treated for 72 hours with oestrogen, the level of PNPLA3 expression was even higher than after 48 hours of oestrogen treatment (see Fig. 3.13).

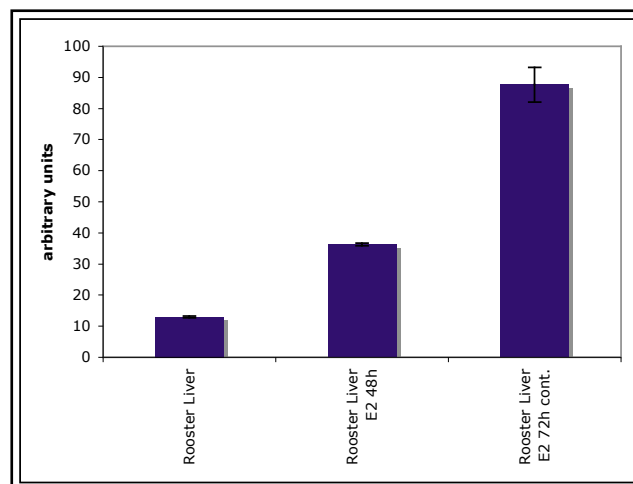


Figure 3.13 qPCR analysis for PNPLA3 mRNA expression in rooster liver after a hormonal treatment with oestrogen. Gene specific primers for the ggPNPLA3 and β -actin genes were designed; PNPLA3 expression was normalized to β -actin expression. cDNA was prepared from 1 μ g/ μ l RNA and used diluted 1:10 in qPCR. Each chicken gene was analyzed three times using the Roche Lightcycler 480 and two replicates of each tissue were employed in each analysis. Error bars indicate the standard deviation. Statistics were done using MS Excel.

Again, the protein expression does not exactly mirror the mRNA expression pattern. As expected, in rooster liver there is less PNPLA3 protein than in laying hen liver. After 48 hours of oestrogen treatment, the rooster liver band appears as strong as that in the laying hen. But, although mRNA levels of PNPLA3 were high after 72 hours of oestrogen treatment, in the liver protein sample after 72 hours of oestrogen treatment, PNPLA3 protein expression appeared weaker than before (see Fig. 3.14).

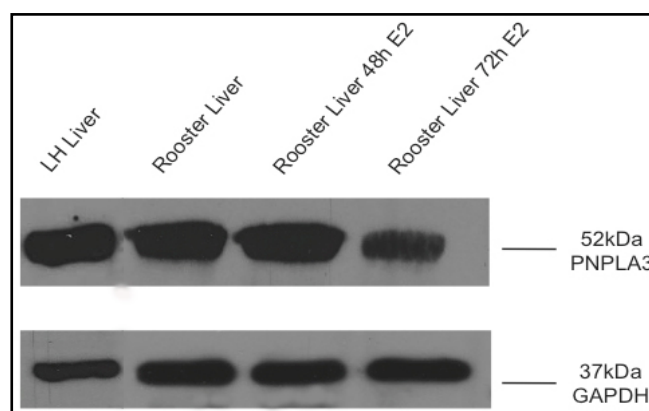


Figure 3.14 PNPLA3 protein levels after estrogen treatment of roosters (Western Blot under reducing conditions), 40 μ g of liver membrane protein extract per lane were subjected to SDS PAGE and blotted onto a nitrocellulose membrane, the membrane was cut between the 50 and 37kDa marker bands, PNPLA3 was detected on the upper part using m- α -ggPNPLA3 antibody, GAPDH was detected on the lower part using a mouse- α -GAPDH antibody. Antibodies were diluted in 5% BSA-TBST.

In conclusion, it was shown that treatment with oestrogen could abolish the sex-specific differences in PNPLA3 expression at both the mRNA and the protein level.

3.5 Influence of treatment with TO901317 on PNPLA3 mRNA expression

Recently it was shown in a mouse model that, as many other genes in lipid metabolism, also PNPLA3 expression seems to be regulated by LXR/SREBP signalling (Huang et al. 2010). Treatment of cells or animals with the LXR agonist TO901317 appears to increase the expression of PNPLA3 – at least in the liver. Therefore, it was tested whether TO901317 would also affect PNPLA3 expression in the chicken and, if so, in which tissues. Chicken swere treated with either DMSO or TO901317 (which was dissolved in DMSO) for 72 hours and then sacrificed. RNA was isolated from various tissues and cDNA was prepared from RNA. Quantitative PCR was carried out using these cDNAs. Results were normalized to β -Actin (Fig. 3.15).

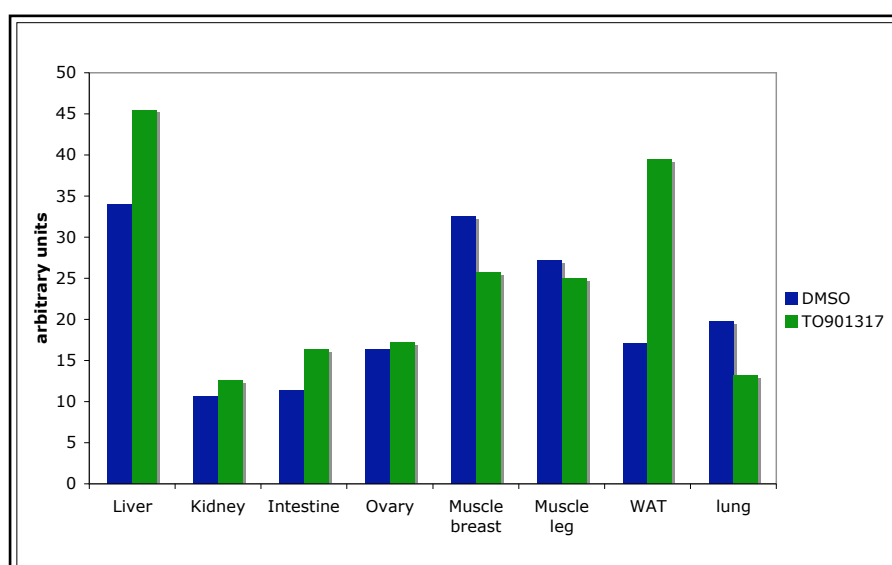


Figure 3.15, PNPLA3 mRNA expression after treatment with TO901317 for 72h, blue bars indicate DMSO treated chicken (control), green bars stand for chicken treated with the LXR agonist TO901317. Gene specific primers for the ggPNPLA3 and β -actin genes were designed; PNPLA3 expression was normalized to β -actin expression. cDNA was prepared from 1 μ g/ μ l RNA and diluted 1:10 before use in qPCR experiment. Each chicken gene was analyzed three times using the Roche Lightcycler 480 and two replicates of each tissue were employed in each analysis.

As expected, TO901317 treatment elevated clearly the level of PNPLA3 expression in liver and white adipose tissue (WAT). We can only speculate that in the chicken, the effect of TO901317 on PNPLA3 expression is not a direct one, but also depends on SREPB as shown previously in rodents (Huang et al. 2010).

3.5 Immunohistochemistry of PNPLA3 in primary hepatocytes

As the α -ggPNPLA3 antibody worked under reducing and also under non-reducing conditions, I attempted immuno-histochemical experiments to localize PNPLA3 in the cell.

Primary chick hepatocytes were isolated and cultured. They were then fixed to a microscope slide and stained with the m- α -PNPLA3 antibody, followed by incubation with a fluorophor-conjugated α -mouse IgG antibody. Samples were allowed to dry over night.

Samples were first monitored with a non-confocal fluorescence microscope to check if controls (samples incubated only with blocking solution or pre-immune serum) did not show fluorescence, and if immune samples incubated with the α -ggPNPLA3 antibody showed a fluorescing signal. This was true (data not shown due to poor image quality), thus the immune samples were also used in confocal microscopy (Fig. 3.16). Cell nuclei are stained with DAPI (blue), the PNPLA3 staining appears red. At least to some extent PNPLA3 appears to localize to lipid droplets (circular structures).

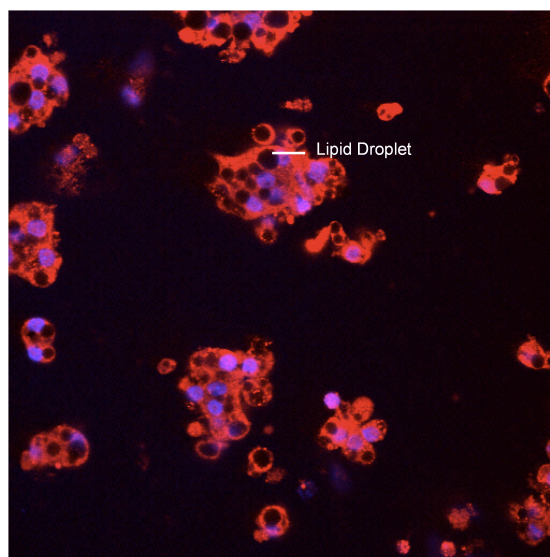


Figure 3.16 Confocal Fluorescence images of PNPLA3 stainings in chicken primary hepatocytes. Hepatocytes seeded onto microscope slides were cultivated for 24h before fixation and staining. Fixation was performed using 4% PFA. Cells were either incubated with pre-immune mouse serum, immune serum containing α -ggPNPLA3 antibody or neither of them. Sera were diluted in 2%BSA-PBS. The fluorophor conjugated antibody Texas Red was used as secondary antibody and nuclei were counter stained with DAPI. Pictures were taken on a Zeiss fluorescence microscope using AxioVision software. Lipid droplets appear as unstained circular structures.

While these results are promising despite their preliminary nature, further experiments clearly are required.

3.6 Effect of fasting and refeeding on AADA

Arylacetamide deacetylase (AADA), an enzyme whose reported primary function is the detoxification of arylamides, was shown to have esterase activity as well (Probst et al. 1994). Many esterases also have lipase activity (in fact it is difficult to exactly distinguish esterase and lipase activity). Therefore, the working hypothesis was that AADA in the chicken may act as a lipase which would substitute for HSL.

If AADA were a lipase, one would expect that its expression would increase upon fasting so that energy stores would be mobilized, and decrease again after refeeding. This was tested by qPCR at the mRNA level and by Western Blot at the protein level. To obtain the tissue samples needed for the experiment, chicken were fasted and refed as described for the experiment in 3.3.

qPCR experiments revealed that, as expected for a lipase, the mRNA levels of AADA in fact increased upon fasting and decreased again after refeeding (see Fig. 3.17).

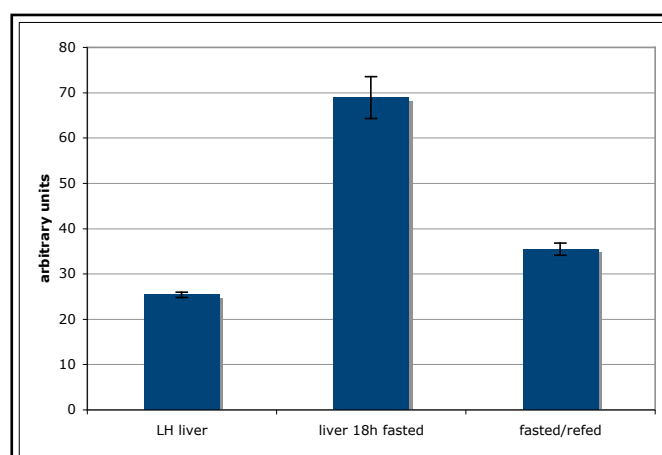


Figure 3.17 qPCR analysis of AADA expression upon fasting and refeeding. Gene specific primers for the ggAADA and β -actin genes were designed; PNPLA3 expression was normalized to β -actin expression. cDNA was prepared from 1 μ g/ μ l RNA and used undiluted in qPCR experiment. Each chicken gene was analyzed three times using the Roche Lightcycler 480 and two replicates of each tissue were employed in each analysis. Error bars indicate the standard deviation. Statistics were done using MS Excel.

To monitor AADA expression at the protein level, a Western Blot using protein extracts from fasted and refed chicken liver tissue was performed. Samples were normalized using GAPDH as loading control. A polyclonal rabbit- α -ggAADA antibody was used to detect AADA. The Western Blot is shown in Figure 3.18: as expected from qPCR analysis, AADA protein was elevated in the fasted liver sample when compared to untreated liver and liver from hens refed after fasting.

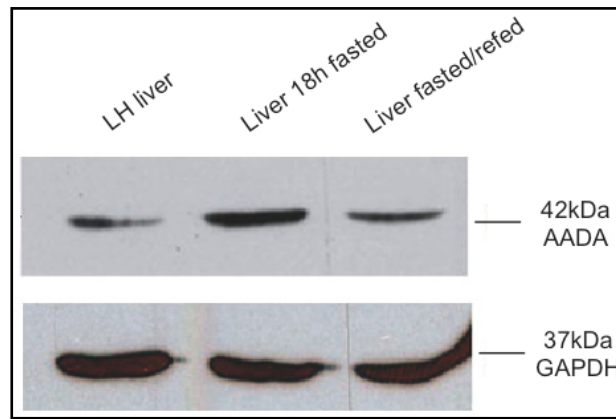


Figure 3.18 Expression of AADA protein on fasting and refeeding. 50 μ g of liver membrane protein extract per lane were subjected to SDS PAGE and blotted onto a nitrocellulose membrane. Each sample was loaded twice so that AADA and GAPDH could be detected independently. AADA was dedected using rabbit- α -ggAADA antibody and GAPDH was detected using m- α -GAPDH antibody. Both antibodies were diluted in 5% Milk-TBST.

Summarizing the results of qPCR and Western Blots, AADA could in fact serve as a lipase in the chicken. However, AADA is only in liver present to high extent, but as true substitute for HSL its presence in adipose tissue would be important. Therefore, further elucidation of the role of AADA in lipid metabolism is required.

3.6 Analysis of tissue specific expression of AADA isoforms

If our hypothesis that AADA takes over the function of HSL in the chicken (or avian species in general) were true, AADA should be highly expressed in adipose tissue, as is HSL. AADA expression is highest in the liver - however, there are proteins that share high similarity with AADA. Therefore, primers specific for these „isoforms“ were designed, and in qPCR analysis it was tested, whether there was an isoform highly expressed in adipose tissue. Sequences of different AADA-like proteins were obtained from the NCBI nucleotide database. The genes and their corresponding primer pairs were designated with letters from A to J (full names and primer sequences are listed in 2.3).

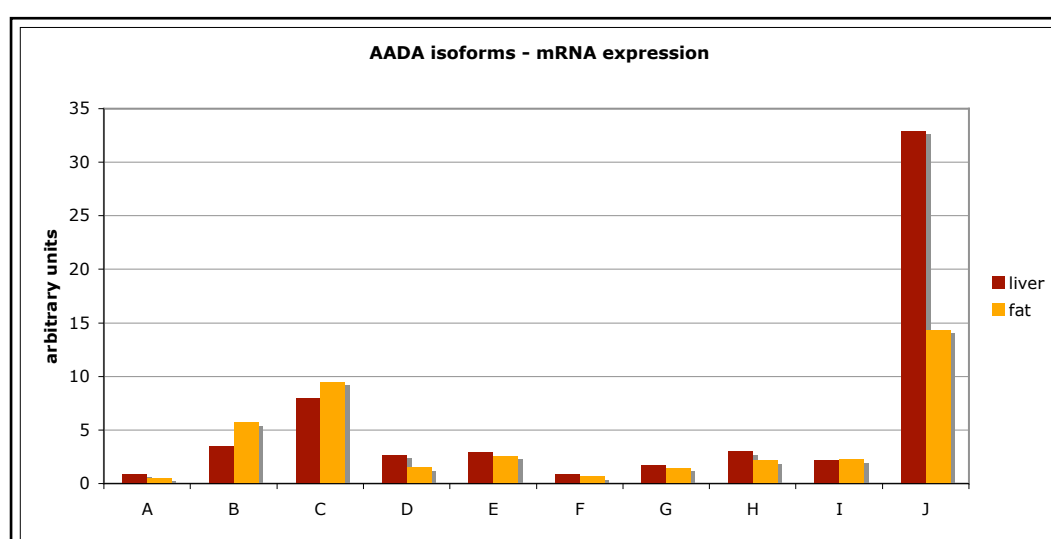


Figure 3.19 qPCR analysis of tissue specific expression of AADA isoforms. Gene specific primers for the AADA-like genes designated with letters A to J and the β -actin genes were designed; expression of AADA-like genes was normalized to β -actin expression. cDNA was prepared from 1 μ g/ μ l RNA and used undiluted in qPCR experiments. Each chicken gene was analyzed two times using the Roche Lightcycler 480 and two replicates of each tissue were employed in each analysis. Red bars = liver tissue; yellow bars = adipose tissue.

All AADA like genes (designated A-I) have much lower expression levels than AADA (J) itself (see Fig. 3.19). However, genes B and C showed higher expression than the other AADA-like genes. And surprisingly, these two genes also showed higher expression in the adipose than in the liver tissue. However, expression in the adipose tissue was still low compared to classic *Gallus gallus* AADA (J). Further investigation of these two genes appears promising and is required to further examine the role of AADA in lipid metabolism.

3.8 Cloning, expression and purification of ggStAR

As mentioned in 1.4.1 the steroidogenic acute regulatory protein (StAR) was shown to interact with HSL (Shen et al. 2003). I wanted to test if StAR would also interact with AADA the putative HSL analogue in the chicken. Therefore I first cloned chicken StAR as full length protein.

Primers for ggStAR 5' and 3' ends were designed (see 2.3, the G.g. StAR sequence was obtained from the Ensemble genome browser) and the sequence coding for StAR was amplified in a PCR reaction, yielding a DNA fragment with the expected length of 900bp (Fig. 3.20).

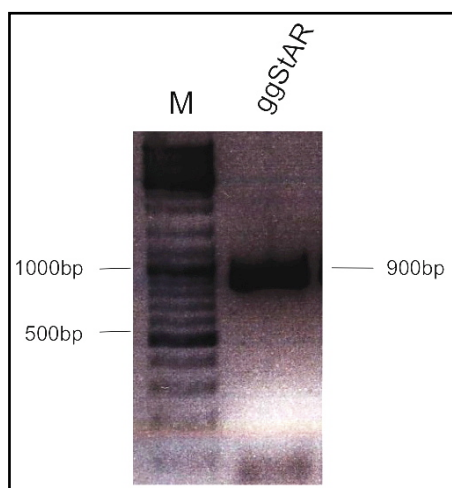


Figure 3.20 PCR amplification of the ggStAR gene; the 100bp DNA ladder (Biorad) was used as marker (M).

The PCR product was cloned into the pGEX6.2 vector and, after correctness of the sequence was confirmed, transformed into E.coli BL21 expression cells. By induction with IPTG, the expression of StAR was achieved (Fig 3.21). The amount of StAR increases after induction (T0) over three hours (T1-3). StAR has a size of 37kDa, the pGEX vector adds a GST tag of 26kDa to the protein which therefore appears as a band of 63 kDa.

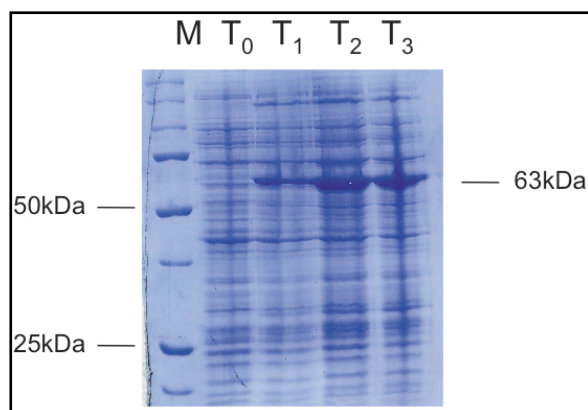


Figure 3.21 Expression of ggStAR protein. BL21 cells were grown to OD600 0,7 and protein expression was induced using 100mM IPTG. Cells were grown for three hours; each hour a sample was taken. Samples were centrifuged to harvest cells, cell pellets were resuspended in PBS and reducing Laemmli Buffer was added. Cell lysates were loaded onto a 10% PAA gel and subjected to SDS-PAGE. After electrophoresis, proteins on the gel were Coomassie stained.

The protein was then purified using glutathione Sepharose 4B beads by binding and subsequent elution using 100mM glutathione. Protein concentrations were determined using Bradford assay and appropriate aliquots were used in Esterase Assays (see. 3.9).

3.9 Search for evidence that AADA substitutes for HSL in the chicken

In our opinion, AADA might serve as HSL analogue in the chicken. Thus, I performed several experiments to test whether AADA has the ability to cleave esterase substrates such as para-nitrophenyl esters and 4-methylumbelliferylheptanoate (MUH). I also tested whether known interaction partners of HSL, which increase the activity of HSL, would also influence the activity of AADA. This would indicate an interaction between AADA and interaction partners of HSL. These experiments are described in the following section.

3.8.1 AADA activity against pNP-esters

I tested whether AADA cleaves a short chain acyl ester (para-nitrophenyl acetate) and also a long chain acyl ester (para-nitrophenyl dodecanoate); pNP-acetate is soluble in water, whereas to dissolve pNP-dodecanoate in water an emulsifying agent, such as Triton X-100 and/or gum arabic, was added.

In addition to test the activity of AADA against pNP esters, I also tried pre-incubating AADA for 1h at room temperature with StAR and FABP4, two proteins that were previously shown to interact with HSL. I also tried pre-incubating AADA with PMSF, a known lipase inhibitor which should abolish the activity of the enzyme against the substrate.

In Figure 3.22 the activities of AADA alone, StAR alone, or AADA pre-incubated with StAR against pNP-acetate is shown. StAR alone had no activity against pNP-acetate, while AADA alone exhibited significant activity against this substrate. AADA pre-incubated with StAR showed higher activity against the substrate than AADA alone.

However, these experiments are of preliminary nature, and further experiments with modulated conditions (e.g. concentration curves with different concentrations of both AADA and StAR) are required.

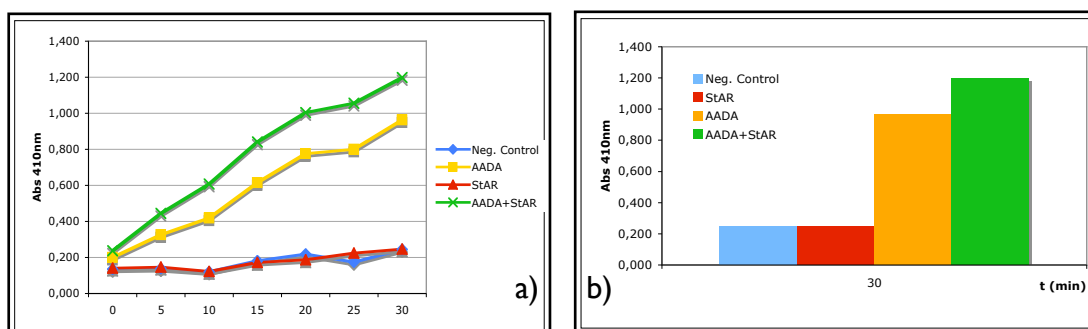


Figure 3.22 Activity of AADA and StAR against pNP-Acetate. {a) line graph; b) bar graph} 400 μ g AADA/ 5 μ g StAR protein/ both together, respectively, were mixed with a Tris buffer containing the substrate at a final concentration of 200 μ M. The cleavage of the substrate was monitored over 30 minutes at 410nm using a Victor V2 Spectrometer (Perkin Elmer). In b), the 30min time point, which is still in the linear range (see a)), is depicted. Neg. Control = light blue; StAR = red; AADA = yellow; AADA pre-incubated with StAR = green.

The increase in activity of AADA pre-incubated with StAR indicates that there might be some sort of interaction between the two molecules; however, when AADA was pre-incubated with FABP4 no increase in activity was observed (see Fig 3.23). Therefore, it is not probable that these two molecules interact. Both StAR and FABP4 showed no intrinsic activity against pNP-acetate (Fig. 3.22 and 3.23).

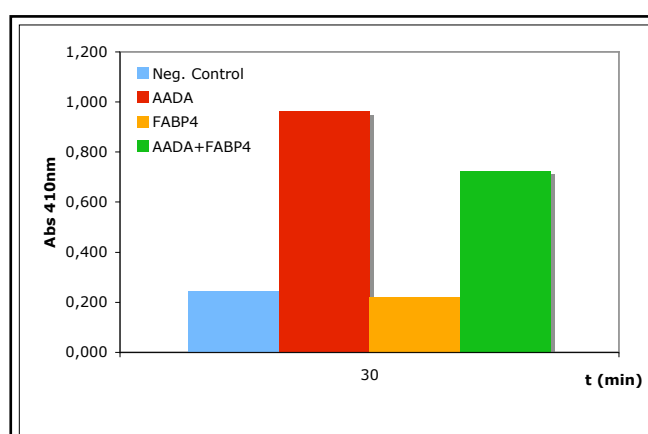


Figure 3.23 Activity of AADA and FABP4 against pNP-Acetate. 400 μ g AADA/ 5 μ g FABP4 protein/ 400ng AADA with 5 μ g FABP4, respectively, were mixed with buffer containing the substrate at a final concentration of 200 μ M. The cleavage of the substrate was monitored at 410nm using a Victor V2 Spectrometer (Perkin Elmer) over 30 minutes (also see Material and Methods). Here the 30min time point, which is still in the linear range, is depicted. Neg. Control = light blue; AADA = red; FABP4 = yellow; AADA pre-incubated with FABP4 = green.

As shown above, activity against a short chain acyl ester indicates esterase rather than lipase activity. To determine whether AADA may also have lipase activity, a long chain acyl ester, pNP-dodecanoate, was tested as substrate. However, no activity against this substrate was detectable (compare Fig. 3.24 with Figs. 3.22 and 3.23). It remains to be determined whether there is indeed a lack of lipase activity or assay conditions need to be altered.

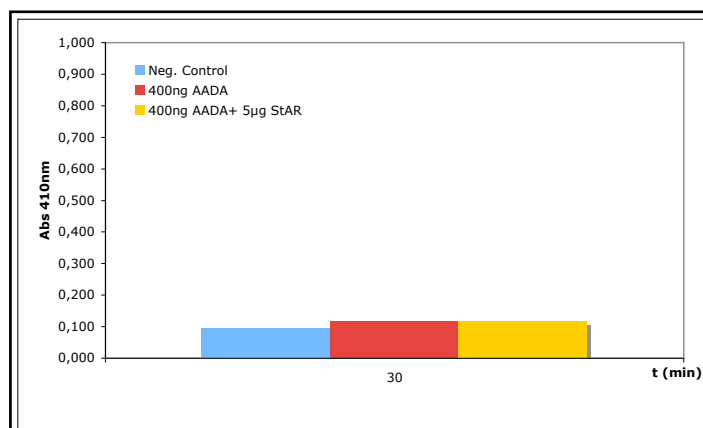


Figure 3.24 Activity of AADA and StAR against pNP-dodecanoate. 400µg AADA/ 5µg StAR protein/ 400ng AADA with 5µg StAR, respectively, were mixed with buffer containing the substrate at a final concentration of 200µM. The cleavage of the substrate was monitored at 410nm using a Victor V2 Spectrometer (Perkin Elmer) over 30 minutes (also see Material and Methods). Here the 30min time point, which is still in the linear range, is depicted. Neg. Control = light blue; AADA = red; AADA pre-incubated with StAR = yellow.

Finally, it was tested whether the activity of AADA and/or AADA pre-incubated with StAR would be abolished by pre-incubation with PMSF, a known lipase inhibitor.

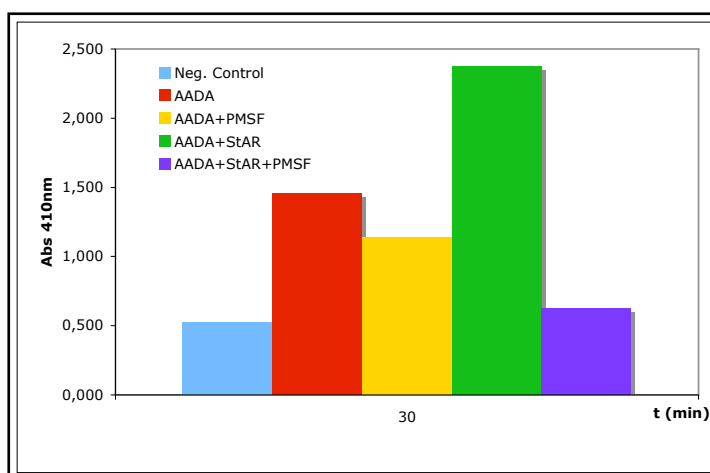


Figure 3.25 Effect of PMSF on AADA activity. 400µg AADA/ 400ng AADA with PMSF/400ng AADA with 5µg StAR/ 400ng AADA with 5µg StAR and PMSF, respectively, were mixed with buffer containing the substrate pNP-Acetate at a final concentration of 200µM. The cleavage of the substrate was monitored at 410nm using a Victor V2 Spectrometer (Perkin Elmer) over 30 minutes (also see Material and Methods). Here the 30min time point, which is still in the linear range, is depicted. Neg. Control = light blue; AADA = red; AADA pre-incubated with PMSF = yellow; AADA pre-incubated with StAR = green; AADA with StAR and PMSF = violet.

PMSF in fact had the ability to inhibit AADA activity against pNP-acetate by about one fifth (Fig. 3.25, red and yellow bars). If AADA had been pre-incubated with StAR and PMSF the inhibition was almost complete (Fig. 3.26, green and violet bars). As I showed before, StAR alone did not exhibit activity against the substrate, but StAR pre-incubation stimulated AADA activity in this experiment by about 0,6-fold (60%).

3.8.2 AADA activity against MUH

It was tested whether AADA exhibits activity against MUH, either alone or when pre-incubated with StAR and FABP4, respectively. As controls, also StAR and FABP4 alone were tested for activity against the substrate.

AADA and StAR alone showed almost the same activity against the substrate (Fig. 3.26, red and yellow bars). When AADA and StAR were pre-incubated, the activity against MUH increased about 2-fold (Fig. 3.26, green bar).

In the previously performed assay using pNP-acetate as substrate (Fig. 3.22, 3.23, and 3.25) StAR had not shown activity against the substrate, in contrast to its activity against MUH. I wanted to test whether PMSF inhibits the activity of StAR alone, as PMSF also inhibits AADA. PMSF indeed reduced StAR activity against MUH to levels of the negative control (Fig. 3.26, blue and purple bars).

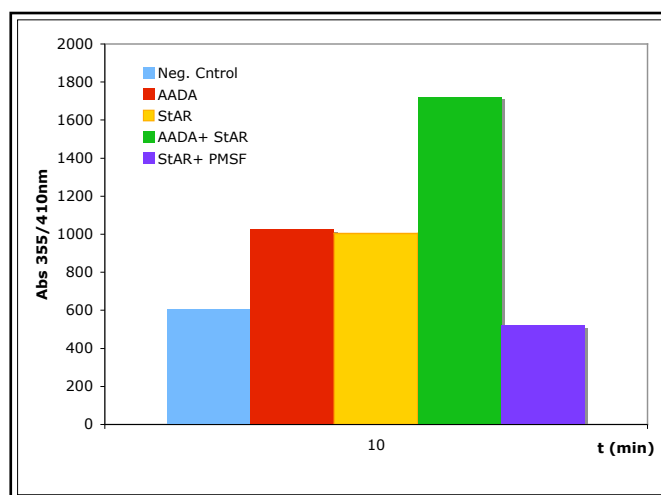


Figure 3.26 Activity of AADA and StAR against 4-MUH. 400µg AADA/ 5µg StAR/400ng AADA with 5µg StAR/ 5µg StAR and PMSF, respectively, were mixed with buffer containing the substrate at a final concentration of 100µM. Fluorescence was monitored at 355nm (excitation) and 460nm (emission) using a Victor V2 Spectrometer (Perkin Elmer) over 10 minutes (also see Material and Methods). Here the 10min time point, which is still in the linear range, is depicted. Neg. Control = light blue; AADA = red; StAR= yellow; AADA pre-incubated with StAR = green; StAR and PMSF = violet.

As for pNP-acetate, for MUH it was also tested whether pre-incubation with FABP4 affected AADA activity. FABP4 alone showed no activity against MUH (Fig. 3.27, red bar). AADA alone exhibited considerable activity against the substrate (yellow bar), but when AADA and FABP4 were co-incubated (green bar) activity levels - as in the assay using pNP acetate as substrate - did not reach or exceed the activity of AADA alone. Therefore, an interaction of AADA and FABP4 seems improbable.

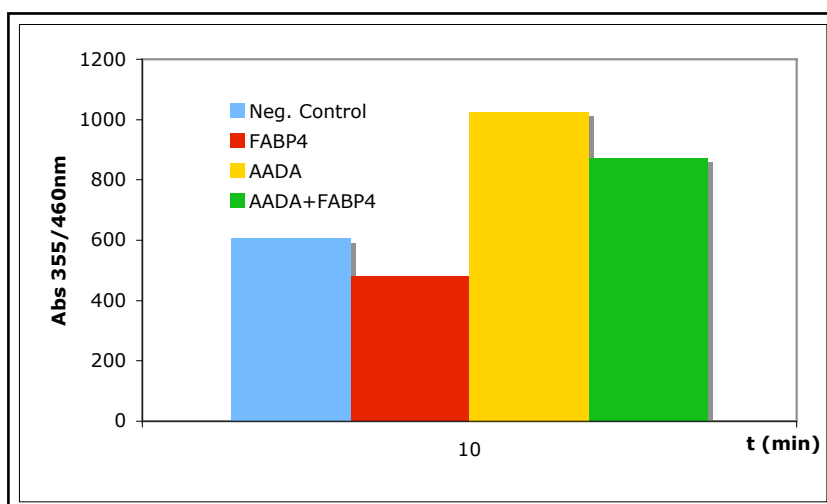


Figure 3.27 Activity of AADA and FABP4 against MUH. 400 μ g AADA/ 5 μ g FABP4/400ng AADA with 5 μ g FABP4, respectively, were mixed with buffer containing the substrate at a final concentration of 100 μ M. Fluorescence was monitored at 355nm (excitation) and 460nm (emission) using a Victor V2 Spectrometer (Perkin Elmer) over 10 minutes (also see Material and Methods). Here the 10min time point, which is still in a linear range, is depicted. Neg. Control = light blue; FABP4= red; AADA= yellow; AADA pre-incubated with FABP4 = green.

Taken the data of both esterase and lipase assays together, it can be concluded that chicken AADA has esterase activity (which has been shown for rodent AADA by Probst et al. 1994). Whether AADA also has lipase activity remains to be further elucidated, possibly by an assay that uses triglycerid as substrate since AADA has activity against MUH but not against pNP-dodecanoate. Whether StAR itself also has some sort of lipase activity should also be further examined, as StAR showed intrinsic activity against MUH (but not against other substrates) and could be inhibited by PMSF.

The preliminary experiments performed here form the basis for further, more detailed, studies on the enzymology of chicken AADA.

4. Discussion

4.1 Investigation of chicken PNPLA3

Patatin-like phospholipases have gained importance in the investigation of lipid metabolism when ATGL (PNPLA2) was shown to be the key lipase in the break-down of TG (Zimmermann et al. 2004) particularly because for long time HSL was thought to be the only enzyme important in this process. ATGL and PNPLA3 whose expression is induced by exactly opposite conditions, are now extensively investigated in mouse models and in clinical investigations in humans. Little is known, however, about patatin-like phospholipases in avian species such as the domestic chicken.

This work was focussed in part on PNPLA3. To investigate this family member, a polyclonal antibody against the chicken enzyme was raised, to facilitate investigations on PNPLA3 expression in the chicken at both the mRNA and the protein levels, employing qPCR and Western Blot, respectively.

The first striking results were obtained when PNPLA3 protein expression was monitored with the newly obtained antibody in various tissues. qPCR data on PNPLA3 expression in chicken was available (Saarela et al. 2008), which showed that PNPLA3 expression at the mRNA level was high in liver, muscle, and testis, but low in, e.g., small intestine, colon, and the yolk sac (Fig. 3.8). However, at the protein level, a different picture was observed: as expected from the qPCR data, expression was highest in the liver. But in muscle and testis, no PNPLA3 protein could be detected; however, in Western Blot experiments strong bands in small intestine and yolk sac tissue appeared (Fig. 3.10). These data suggest that PNPLA3 may serve as a protein promoting lipid storage: in the small intestine, a major site of lipid digestion, PNPLA3 may act, while dietary fat is digested, to recruit lipids to peripheral sites of lipid storage. Also, the high protein level of PNPLA3 in the yolk sac suggests a role in lipid storage: energy-rich yolk precursors have to be transported to and stored in the yolk, so that the embryo's energy supply is ensured - PNPLA3 could also affect these transport and storage processes. Finally, the fact that in RO liver and RO adipose tissue more PNPLA3 protein is present (Fig. 3.10) suggests that PNPLA3 is involved in promoting lipid storage.

As published before for humans and rodents (Johansson et al 2006; Moldes et al. 2006), we found that in the chicken, PNPLA3 expression also decreases upon fasting and increases again after refeeding at both the mRNA and the protein level. In livers of laying hens that were fasted for 18 hours and then refed for 24 hours, a sudden increase in PNPLA3 expression was observed. In these hens the level of mRNA expression even exceeded basal PNPLA3 expression (see. Fig. 3.9). In contrast, in fasted and refed RO hens, the decrease in PNPLA3 expression upon fasting is not followed by an increase upon refeeding (Fig. 3.11). The pattern for mRNA levels was also observed at the protein level (Fig. 3.10 and 3.12).

These data support a role for PNPLA3 in energy storage: in laying hens energy stores exhausted after fasting may have to be efficiently re-filled in the refeeding period, whereas RO hens having more energy stores, may not need to re-fill their reservoirs quickly and therefore may not up-regulate PNPLA3 expression to such a high extent as laying hens.

Interestingly, in hens that had received a fat diet for 3 weeks, PNPLA3 expression did not increase much, and expression of PNPLA3 mRNA is significantly lower in chickens on the fat diet than in fasted and re-fed chickens (Fig. 3.9 and 3.10). This expression pattern may be related to the so-called yo-yo effect observed in humans after restrictive diets: upon return to a former/normal life style after a period of low energy diet, often the body weight increases rapidly and may even surpass the weight before the diet. This may occur because the system interprets a low energy diet as famine, and when this diet stops, regulation is such as to restore all energy depots in anticipation of another period of scarcity. In comparison, if there is a constant small energy intake surplus, the body will also store some of the energy, leading to elevated body weight.

Many genes in lipid metabolism are differentially expressed in the female and male, possibly related to females having relatively more adipose tissue than males. Rooster liver expresses less PNPLA3 than laying hen liver. By treatment of roosters with oestrogen, I showed that this difference in PNPLA3 expression was abolished. Both at the mRNA and protein levels there was a significant increase in PNPLA3 expression when roosters were treated with estrogen for 48 or 72 hours, respectively (see Figs. 3.13 and 3.14).

A recently published paper showed that PNPLA3 in rodents is, as many other genes involved in lipid metabolism, regulated by Liver X Receptor (LXR) and SREBP signaling (Huang et al. 2010). To determine whether this was also true in the chicken, hens were treated with TO901317, an LXR agonist. In liver and white adipose tissue (WAT), a significant increase of PNPLA3 expression was observed in treated hens (Fig. 3.15). Thus, in the chicken PNPLA3 expression is also regulated via LXR/SREBP signaling. In rodents, SREBP was abolished to show that the regulation of PNPLA3 is not only dependent on LXR but also SREBP signaling; as knock-out methodology is not available for chickens, dependence of the LXR effects on SREBP cannot be demonstrated yet.

The immunohistochemical experiments in primary hepatocytes to localize PNPLA3 within the cell were of preliminary nature (see 3.16), but promising. Future studies are aimed at determining where in the cell PNPLA3 resides.

In conclusion, while a role in lipid storage for PNPLA3 has already been proposed, there are contradicting data on its putative function; e.g., although it is known that PNPLA3 possesses a G-X-S-X-G lipase motif and lipase activity *in vitro*, lipase activity could never be detected *in vivo* (Lake et al. 2005). My data on galline PNPLA3 support the hypothesis that this protein has a role in promoting nutrient storage rather than in lipolysis.

4.2 Investigations about chicken AADA

Although ATGL is present in the chicken and likely serves in breaking down TG, an analogue for HSL in the chicken has never been identified. It is unclear how DG are broken down in this organism. AADA was shown to act not only as a carcinogen-detoxifying enzyme, but also as a carboxylesterase (Probst et al. 1991), able to cleave substrates such as para-nitrophenyl acetate or 4-methyl umbelliferylheptanoate (MUH). Our working hypothesis therefore was that in the chicken AADA (which shows particular homology with mammalian HSL) takes over the role of HSL. We tested this hypothesis in several ways.

First, as AADA is primarily expressed in the liver, while HSL is mainly expressed in the adipose tissue, we searched for AADA-like proteins in the NCBI nucleotide database and obtained ten hits. Of these ten proteins „AADA-like protein 4“ and „Gallus gallus LOC429936 similar to LOC495387 protein“, had higher expression in adipose tissue than in liver. Nevertheless, the total mRNA level of these two proteins was much lower than the mRNA level of chicken AADA in liver and adipose tissue (see Fig. 3.20). For an HSL analogue, high expression in adipose tissue would be a crucial characteristic - from the data of this experiment, it is difficult to conclude whether or not AADA could be an analogue of HSL in the chicken, but future studies will focus on this point.

Second, it was monitored how AADA expression changes upon fasting and refeeding at both the mRNA and protein levels. One would expect that a lipase's expression increases upon fasting, as energy stores need to be mobilized, and decreases when food is supplied again. AADA showed, both at the mRNA level (quantitated with qPCR) and at the protein level (Western Blot), this expression pattern (Fig. 3.18 and 3.19). This supports the possibility that AADA could indeed act as a lipase and may substitute for HSL in the chicken. The above mentioned two AADA-like genes may be even stronger candidates for this role, and will be studied further.

In enzymatic assays using pNP-acetate, pNP-dodecanoate, and MUH as substrates it was tested whether AADA had activity against these substrates and whether the activity of AADA would change upon co-incubation with known HSL interaction partners (StAR and FABP4) or PMSF, a known esterase inhibitor.

AADA alone, and even more so if co-incubated with StAR, had significant activity against pNP-acetate (Fig. 3.22), while co-incubation with FABP4 did not result in any increase of AADA activity against pNP-acetate (Fig. 3.23). Co-incubation of AADA and AADA/StAR with PMSF resulted in inhibition of enzymatic activity against the substrate (Fig. 3.25). Both StAR and FABP4 showed no activity against the substrate.

No AADA activity was detectable against pNP-dodecanoate, with or without co-incubation with StAR. According to (Gilham and Lehner 2005), activity against short chain acyl esters like pNP-acetate indicates esterase activity rather than lipase activity and vice versa. Thus, it can be assumed that AADA has esterase rather than lipase activity.

In a second enzymatic assay that tests lipase activity using MUH (a fluorogenic compound) as a substrate, AADA showed activity, indicating that AADA exhibits lipase activity. This observation is compromised by the fact that StAR alone shows PMSF-inhibitable activity against this substrate (Fig. 3.26). No lipase motif is present in the StAR protein, and also blasting StARs' protein sequence does not give any other hits apart from START family members, thus, it remains unclear why StAR alone is able to cleave MUH.

Because StAR and AADA had activity against MUH it is difficult to say if the increase in activity against MUH observed when AADA and StAR are co-incubated is due to interaction of the two proteins or is merely an additive effect (Fig. 3.27). As for pNP-acetate as substrate, FABP4 did not influence or modulate AADA activity against MUH (Fig. 3.27).

Considering these results, the role of galline AADA remains unclear. However, as expected for an enzyme with lipase activity, AADA expression changes upon fasting and refeeding: the expression rises upon fasting when energy stores need to be mobilized and drops again when food is supplied. AADA-like proteins expressed in the adipose tissue could be found, but the regulation of these genes has not been analyzed yet.

AADA showed activity against pNP-acetate, a substrate whose cleavage more likely indicates esterase activity, and against MUH which indicates lipase activity, but not against pNP-dodecanoate, another substrate whose cleavage would suggest lipase activity. The lack of AADA activity against pNP-dodecanoate could, however, also be a result of yet insufficient experimental conditions. An interaction between AADA and FABP4, an interaction partner of HSL (Shen et al. 1999) was not detected. In enzymatic assays incubation of AADA with FABP4 had no effect on AADA activity, and also in ELISA experiments an interaction of these proteins could not be demonstrated (data not shown).

It is not yet possible to speculate on an interaction of AADA with StAR, a protein previously shown to interact with HSL (Shen et al. 2003). StAR had no endogenous activity against pNP-acetate, but co-incubation of AADA and StAR resulted in a significant increase of the activity of AADA. StAR alone already exhibited activity against MUH and so did AADA, therefore the increase in AADA activity against the substrate remains difficult to interpret. Moreover, in ELISA experiments an interaction between StAR and AADA could not be detected, and the same is true for Ligand Blotting experiments with these two proteins (data not shown).

To further elucidate whether or not AADA exhibits lipase activity will require the examination of its activity against true triglyceride molecules. To further investigate the interaction of AADA and StAR, StAR's activity against such substrates must be tested.

Also, it would definitely be interesting to further examine StAR's lipase activity despite the absence of common lipase features in StAR: the half-life of StAR is much longer than required for acute responses and it has been hypothesized that StAR might fulfill additional functions in the cell (Miller, 2007). Chicken AADA may offer a chance to shed light on this possibility.

5. References

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6. Abbreviations

A

α	alpha, anti
A	adenin
AADA	Arylacetamide Deacetylase
Apo	apolipoprotein
APS	ammonium persulfate
Amp	ampicillin
ATGL	adipocyte triglyceride lipase

B

bp	base pair
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C

C	cytosine
CE	cholesterolester
cDNA	complementary DNA
CGI-58	comparative gene identification 58
CaCl ₂	calcium chloride
C-terminus	carboxy-terminus

D

DG	diglyceride
DNA	deoxyribonucleic acid
DTT	dithiothreitol

E

EDTA	ethylenediaminetetraacetic acid
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum

F

FFA	free fatty acid
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G

G	guanine
gg	gallus gallus

H

HDL	high-density lipoprotein
HRP	horseraddish peroxidase
HSL	hormone sensitive lipase
hs	homo sapiens
His	histidine
h	hour

I

IPTG	isopropyl β -D thiogalactoside
IgG	immunoglobulin G

K

KCl	potassium chloride
kDa	kilo Dalton
kb	kilo bases

L

LD	lipid droplet
LDL	low-density lipoprotein
LB	luria broth
LDLR	low-density lipoprotein
LH	laying hen
LXR	liver X receptor

M

μ	micro
mM	milli molar
mA	milli ampere
mRNA	messenger ribonucleic acid
MG	monoglyceride
Mm	mus musculus

N

nm	nano meter
NTP	nucleoside triphosphate
N-terminus	amino terminus
Ni-NTA	nickel nitriloacetic acid

O

o.n.	overnight
OD	optical density

P

PNPLA	patatin-like phospholipase domain containing
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis

R

RT	room temperature
rpm	rounds per minute
RNA	ribonucleic acid
R/O	restricted ovulator
RT-PCR	reverse transcriptase polymerase chain reaction

S

SDS	sodium dodecyl sulfate
SNP	small nucleotide polymorphism
SREBP	sterol response element binding protein
StAR	steroidogenic acute regulatory protein

T

TG	triglyceride
T	thymine
TEMED	N,N,N',N'-Tetramethylethylenediamine
TBS	tris-buffered saline
TMB	tetramethylbenzidine

U

UV	ultra violette
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V

V	Volt
VLDL	very-low density lipoprotein
VTG	vitellogenin

Y

YS	yolk sac
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