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Cloning and characterization of an unknown Carboxylesterase (Ces) in the liver

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Danksagung

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“Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand.“ - Albert Einstein

Abstract

One of the main functions of the liver is to synthesize very low-density lipoproteins (VLDLs), a process which relies primarily on the availability of triglycerides (TGs). Adipose triglyceride lipase (ATGL) is a major player in TG catabolism in adipose and non-adipose tissues. However, liver-specific ATGL-deficiency has no significant impact on VLDL-TG synthesis, indicating that VLDL-TG formation does not apparently depend on ATGL-mediated TG catabolism. It has been suggested that the TG pool used for VLDL lipidation most likely resides in the ER lumen and that luminal lipases hydrolyze these TGs. Carboxylesterase 1 (CES1) has been suggested to participate in hepatic VLDL-TG formation. However, CES1-deficiency interferes merely moderate with hepatic VLDL-TG formation, suggesting the involvement of other currently unknown TG lipases. Recent studies discovered Ces2c as a potential TG-hydrolase in the gut and liver. The aim of my diploma thesis was to study the potential role of Ces2c in liver lipid metabolism.

Ces2c expressed in COS-7 cells markedly increased *in vitro* TG hydrolytic activity. In accordance with this observation, the overexpression of Ces2c in COS-7 cells led to a reduced incorporation of radiolabeled fatty acids (FAs) into cellular TGs. Concordant with a potential role of Ces2c in luminal TG catabolism, microscopy of a GFP-tagged Ces2c construct in COS-7 cells revealed that Ces2c localizes to the ER. To assess the impact of Ces2c overexpression on VLDL secretion, oleic acid (OA) pulse-chase analysis was employed. These studies revealed that Ces2c expression in AML12 hepatocytes led to significantly lower label accumulation within TGs. Moreover, TG-secretion was significantly elevated in Ces2c expressing cells.

Together, data suggest that Ces2c is a novel, currently unknown TG hydrolase, which possibly plays a role in VLDL assembly via hydrolysis of luminal TGs and the provision of FAs as substrate for VLDL-TG formation.

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1. Aim of the study

The aim of this study was to examine the potential role of Ces2c in TG catabolism *in vitro* and *ex vivo*.

2. Introduction

The liver is the second-largest organ in the human body, comprising about 1/50 of the adult's body weight. The liver is involved in many essential processes like metabolism, immunity, digestion and the storage of important nutrients within the body. Accordingly, the liver is central for human survival and many disorders are linked to changes in liver metabolism and function.

2.1. The different roles of the liver and liver cells in the human body

The liver plays a significant role in the body's immune response. Kupffer cells are resident macrophages of the liver, which capture and digest bacteria, fungi, parasites and cellular debris. Therefore, the liver is capable of cleaning large volumes of blood very quickly.

Additionally the liver is the main organ for the detoxification of the human body. Hepatocytes play an essential role in whole body detoxification as they monitor blood contents and eliminate potentially toxic substances before harm can be caused. To remove potentially toxic substances the liver is equipped with substrate-specific and substrate-nonspecific elimination mechanisms.

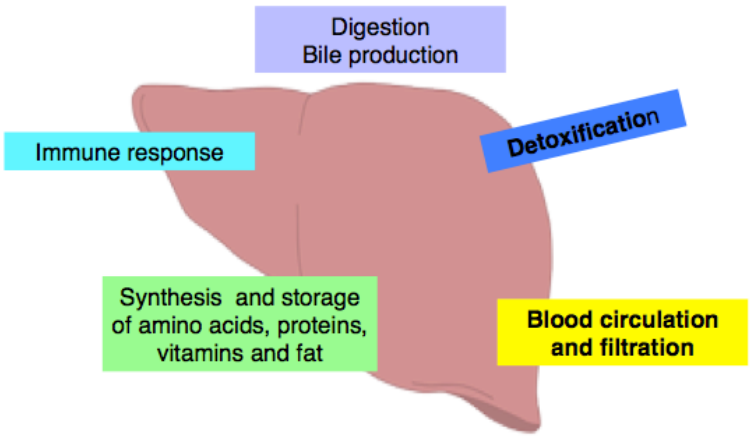


Figure 1: Various functions of the liver in whole body metabolism and homeostasis

The liver is involved in the digestion and the storage of many different nutrients. Moreover, the liver takes part in the detoxification of the human body by eliminating possibly toxic substances. Additionally, it plays a significant role in the body's immune response.

The liver plays an active role in digestion through the production of bile, which promotes the breakdown of lipids in the small intestine. Additionally, hepatocytes are required for the catabolism of carbohydrates, lipids, and proteins and to deliver substrates and intermediates for the formation of numerous biologically active components of the body.

Glucose-enriched blood enters the liver through the hepatic portal vein. Hepatocytes absorb large quantities of glucose and store them as glycogen. Glycogen is a short-term energy reservoir that in turn can be used to quickly release large amounts of glucose when plasma glucose levels are low. In addition to glycogen the liver also stores many essential vitamins and minerals.

FAs, which pass through the liver are also absorbed by hepatocytes and are used to generate energy in the form of ATP. Moreover, hepatocytes are capable of producing lipids like cholesterol, phospholipids and lipoproteins. Lipids are in general transported in the circulation as lipoprotein particles. These particles consist of TG, phospholipids, cholesterol, retinol and different apolipoproteins. There are four major types of lipoproteins, chylomicrons, VLDL, low-density lipoprotein (LDL), and high-density lipoprotein (HDL). These lipoprotein particles can be separated on basis of their density, but also by the types of apolipoproteins they contain.

2.2. Overview of lipoprotein secretion into the circulation

Chylomicrons and VLDLs are TG rich particles. Their main function is to deliver TGs to cells in the body. Enterocytes of the small intestine synthesize chylomicrons from absorbed lipids. VLDL particles on the other hand are synthesized and secreted by the liver. While in the circulation, TGs are hydrolyzed from chylomicrons and VLDL particles through the action of lipoprotein lipase, which is found on the surface of endothelial cells. Lipoprotein lipase metabolizes TGs to FAs and monoglycerides (MGs), which enter the cells by diffusion where they can be used to generate energy or re-synthesized into TGs and stored within intracellular lipid droplets (LDs). Mobilization of TGs from VLDL particles leads to the formation of intermediate density lipoprotein (IDL). The liver can either rapidly clear these particles or they are further processed to LDL particles. LDL particles are denser compared to VLDL and IDL and contain apolipoprotein AI and AII. The function of LDL particles is to deliver cholesterol to the cells, where it is either used for membrane formation, or for steroid hormone synthesis. LDL clearance is then

mediated by the interaction of apolipoprotein B100 (apoB100) with the LDL receptor. LDL binds to a specific LDL receptor and is internalized by receptor-mediated endocytosis. The LDL particles are hydrolyzed in endolysosomes, which leads to the release of cholesterol. The liver does not only synthesize cholesterol, but it also removes cholesterol from the body as a component of bile. HDL particles participate in a process known as reverse cholesterol transport. HDL particles are synthesized and secreted by the liver and the small intestine. They transport excess cholesterol from the cells back to the liver.

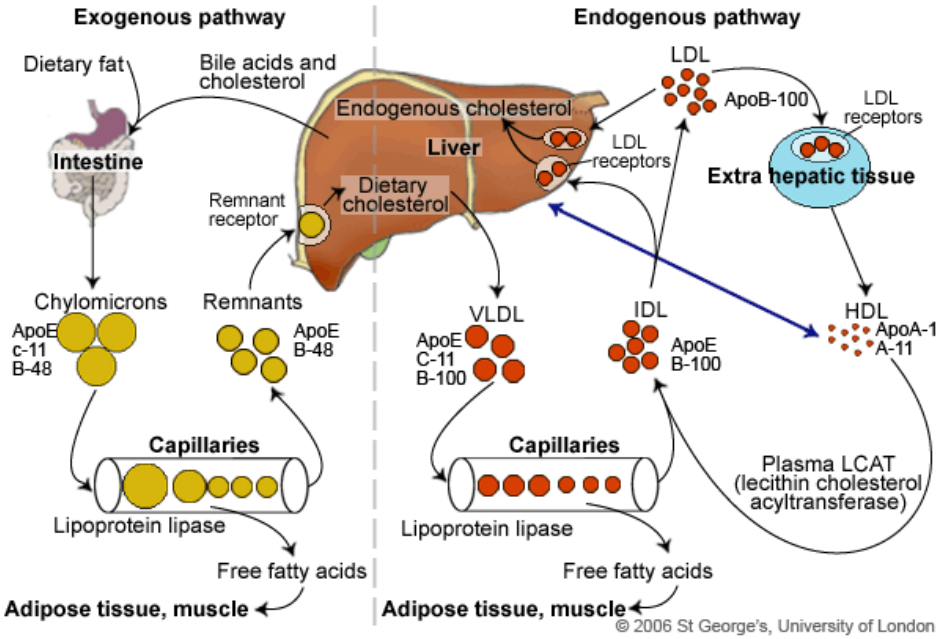


Figure 2: Overview of lipoprotein secretion into the circulation

Chylomicrons are assembled and secreted by the intestine, whereas VLDL particles are assembled and secreted from the liver. VLDL/chylomicron particles carry amphipathic lipids on the surface and hydrophobic lipids in the inner core. Additionally, these particles contain one molecule of apoB as well as apoE. While in the circulation, TGs are hydrolyzed from chylomicrons and VLDL particles through the action of lipoprotein lipase. This leads to the formation of IDL particles, which can either be rapidly cleared by the liver or can be continuously processed to become LDL particles. LDL particles are hydrolyzed in endolysosomes, which leads to the release of cholesterol.

2.3. VLDL biogenesis in the liver

The biogenesis of VLDL particles and their secretion into the circulatory system by the liver is a complex process, which is not fully understood. However it is known that TG availability is an important regulatory factor. The current model of VLDL assembly proposes two important steps. In the first step, newly synthesized apoB is lipidated during its translocation across the ER membrane into the lumen of the ER. This leads to

the formation of the primordial apoB particle. In the next step, a bulk of core lipids from luminal lipid droplets (LLDs) is added to the primordial apoB particle (Lehner et al. 2012).

2.3.1. Proposed model for VLDL assembly

TGs are in general synthesized on the ER membrane from diglycerides (DG) and activated FAs (FA-coenzyme A) by diacylglycerol transferase 1 (DGAT1). DGAT1 is expressed ubiquitously, with the highest mRNA levels found in the small intestine, liver and adipose tissue (Yen et al. 2008). The overexpression of human DGAT1 in rat hepatoma McA-RH7777 cells leads to increased cellular TG accumulation. Moreover DGAT1-overexpressing mice exhibit increased VLDL secretion (Yamazaki et al. 2005).

It is assumed that newly synthesized TGs initially accumulate within the ER bilayer, forming a blister like TG aggregate (Khandelia et al. 2010). LDs may bud off either to the cytosol or the lumen of the ER. LDs are in general composed of a core of neutral lipids, primarily TGs, surrounded by a monolayer of phospholipids and lipid droplet-associated proteins, which differ from cytosolic lipid droplets (CLDs) to LLDs (Brasaemle et al. 2004). The directionality of LD budding is in part regulated by the presence of specific LD-coat proteins.

Perilipin 2 (Plin2), a CLD protein has been implicated to take part in CLD formation. Plin2-deficient mice exhibit a 60% reduction in hepatic TG content and are resistant to diet-induced fatty liver. However, Plin2-deficiency does not interfere with hepatic lipogenesis and VLDL secretion. Normal VLDL secretion rate in the presence of reduced total liver TG content in Plin2-deficient liver is explained by the accumulation of TGs specifically in the ER, where VLDL particles are assembled. Given the concept that LDs form from the outer leaflet of the ER, the reduction of cytosolic TG content with a simultaneous accumulation of TGs in the ER of Plin2-deficient cells suggests an involvement of Plin2 in CLD formation (Chang et al. 2006).

Microsomal triglyceride transfer protein (MTTP) is a heterodimeric lipid transfer protein, which consists of a 55-kDa subunit and a 97-kDa subunit possessing lipid transfer abilities. Abetaliproteinemia in humans is caused by mutations in the gene coding for the 97 kDa subunit of MTTP (Atzel & Wetterau 1993). *In vitro*, MTTP mediates the transfer of neutral and polar lipids between microsomal membranes (Wetterau, J.R., and Zilversmit

1985). It has been shown that MTTP is required for the secretion of apoB containing lipoproteins from hepatocytes (Gordon 1997). The hepatic inactivation of MTTP in mice leads to a striking reduction in VLDL-TG and a large reduction in VLDL/LDL and HDL cholesterol levels. The inactivation of MTTP in the liver led to a 95% reduction of MTTP mRNA levels and a concomitant 95% reduction of apoB100 levels. Therefore it can be assumed that a complete hepatic knock down of MTTP would block VLDL secretion (Raabe et al. 1999). Moreover, ultrastructure analysis of MTTP knock out liver revealed the absence of lipid particles within the ER/Golgi lumen, whereas lipid bodies of VLDL size could be observed in control mice (Raabe et al. 1999). Therefore it has been suggested that MTTP plays a critical role in the movement of TGs into the lumen of the ER, thereby facilitating LLD formation.

It has been shown that TGs are not transferred to primordial apoB particles en bloc but are rather delivered via a process involving lipolysis and re-esterification (Wiggins & Gibbons 1992a; Lankester et al. 1998). It has been proposed that TGs in LLDs are hydrolyzed by lipases and that the more readily bilayer soluble products (for example DGs) are re-esterified by DGAT1 to TGs. TGs that are generated through this process are then used to lipidate primordial apoB particles. Again, MTTP plays an essential role in the assembly of VLDL particles aiding the co-translational recruitment of TGs by apoB. In a second step, a bulk of core lipids from LLDs is transferred to the primordial apoB particle independently of MTTP function (Rustaeus 1998).

Another protein, which has been implicated to participate in VLDL assembly, is cell death-inducing DFFA-like effector b (Cideb), which is localized to the cytosolic side of TG synthesis on the smooth ER and on LDs. Cideb contains an apoB-binding and a LD association domain, both of which are required for the secretion of TG-enriched VLDL particles. Cideb-deficient mice exhibit increased TG levels and decreased VLDL secretion. Based on these observations, it has been proposed that Cideb binds to apoB of the VLDL precursor particle and mediates the lipidation of the particle. It has been suggested that the TGs used for this process are either derived from CLDs or from the ER (Ye et al. 2009).

Additionally it has been observed that Plin2 protein levels are markedly up regulated in Cideb-deficient mice. Hepatic Plin2 knock down in Cideb-deficient mice led to reduced TG accumulation and increased VLDL-TG secretion. Accordingly, the knock down of

Plin2 rescued the phenotype caused by Cideb-deficiency (Li et al. 2012). However, it has to be elucidated, how VLDL secretion is increased in the absence of the negative regulator Plin2 and the positive regulator Cideb, which seems paradox. Additionally, further research has to be conducted how Cideb, a cytosolic protein mediates the lipidation of primordial apoB particles in the lumen of the ER.

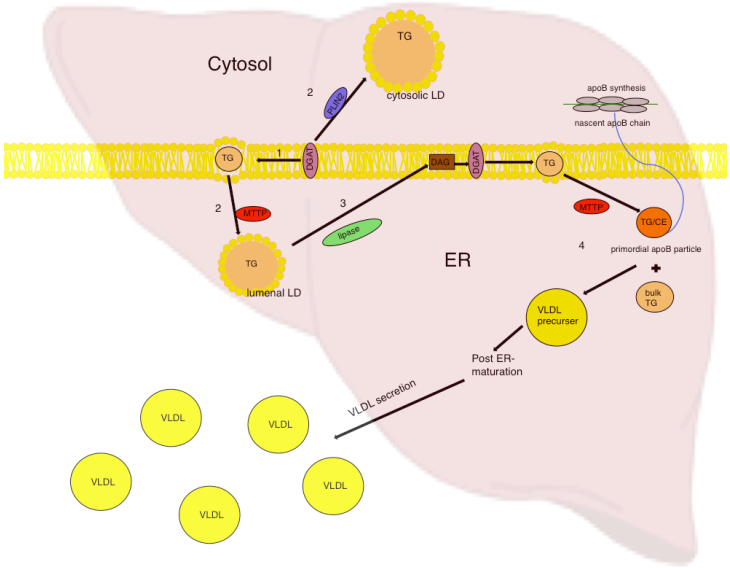


Figure 3: Proposed model for VLDL particle assembly

This model for VLDL assembly proposes two important steps. TGs are in general synthesized on the ER membrane from DGs and FA-CoA by DGAT1 (1). Whether LDs bud of to the cytosol or to the lumen of the ER is at least in part regulated by the presence of LD-specific coat proteins (2). It has been proposed that MTTP and Plin2 are involved in this process. TGs, which are stored in the lumen of the ER, are hydrolyzed by luminal lipases (3) to DGs, which are more readily bilayer soluble. These DGs are then re-esterified to TGs. Solely TGs, which are generated through lipolysis and re-esterification, are then used to lipidate the primordial apoB particle. Again, MTTP is crucial for VLDL assembly, aiding the co-translational recruitment of TGs by apoB. As a next step, a bulk of core lipids is transferred to the primordial apoB particle (4). The resulting VLDL precursor exits the ER and the maturation of VLDL particles is achieved through ER/Golgi trafficking. Mature VLDL particles are then secreted from the liver.

There are several open questions that have to be addressed regarding the synthesis of LLDs; the lipases involved in hydrolysis of luminal TGs, the mechanism of bulk lipidation of primordial apoB particles and even the cellular location of this process. Nonetheless, there is experimental evidence supporting the aforementioned model.

Hormone-sensitive lipase (HSL) and ATGL, a cytosolic lipase, which catalyzes the initial step in TG breakdown, are responsible for more than 95% of the lipolytic activity in white adipose tissue (Schweiger et al. 2006). However, in non-adipose tissue other neutral

lipases seem to be relevant for TG catabolism, since ATGL-deficient mice exhibit substantial remaining hepatic TG hydrolase activity (Reid et al. 2008). Additionally, liver-specific knock down of ATGL in mice has no apparent impact on VLDL biogenesis, although the assembly of hepatic VLDL particles requires the mobilization of a substantial amount of TGs (Wu et al. 2011). It is therefore conceivable that the TG store, which is used for VLDL lipidation, actually resides in the lumen of the ER and that luminal and not cytosolic lipases are responsible for the hydrolysis of these TGs.

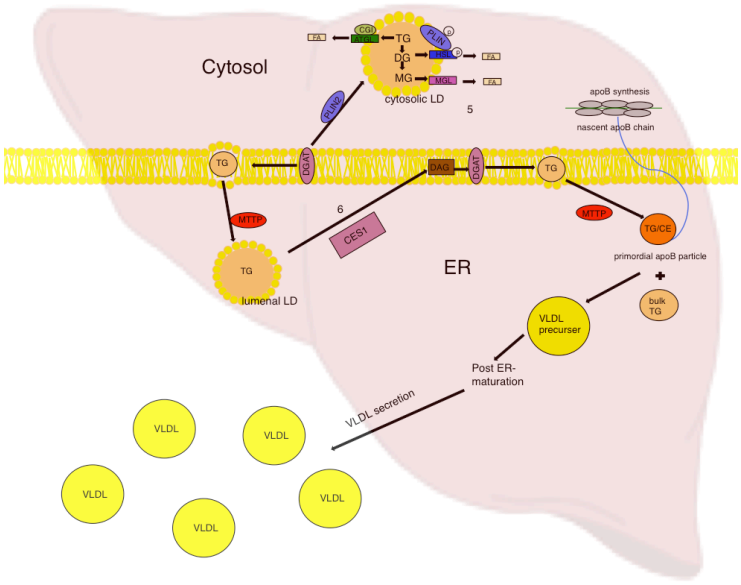


Figure 4: Proposed model of VLDL particle assembly

ATGL, a cytosolic lipase, catalyzes the initial step in TG breakdown, hydrolyzing TGs to DGs and FAs. DGs are then broken down to MGs and FAs by HSL. MGs are hydrolyzed by MGL to glycerol and FAs (5). CES1, a luminal lipase, has been proposed to catalyze the breakdown of luminal TGs (6). However, hepatic CES1-deficiency interferes merely moderate with VLDL-TG formation.

CES1, an ER localized lipase, has been implicated to take part in hepatic TG mobilization for VLDL particle assembly (Dolinsky et al. 2001). Liver-specific CES1-deficient mice exhibit decreased plasma TG and apoB100 levels. However, if CES1 is indeed the rate-limiting enzyme in the process of VLDL-TG formation, one would expect a much more severe phenotype, considering that CES1-deficient mice do not develop hepatic steatosis and still secrete VLDL particles. Therefore it is conceivable that other lipases, probably other Ces, might also be involved in the mobilization of hepatic TGs for VLDL assembly.

2.4. General characteristics of carboxylesterases (CEs)

CEs are abundantly present in different organisms ranging from bacteria to humans. CEs are α/β hydrolase family members. They have broad substrate specificity and mediate the hydrolysis of esters, amides and thioesters using a catalytic serine present within a catalytic triad (Serine-Histidine-Glutamic acid triad) (Redinbo & Potter 2005). Mammalian CEs are in general localized to the ER of many tissues. They contain an N-terminal hydrophobic signal peptide, which marks them for trafficking through the ER. In addition, a HXEL signal sequence, present at the C-terminus of the protein, ensures retention within the cell. These enzymes are involved in detoxification or metabolic activation of various drugs, environmental toxicants and carcinogens. In many cases, CEs determine the pharmacokinetic behavior of therapeutic drugs, which contain ester or amid bonds (Satoh & Hosokawa 1998).

Humans possess six CEs genes, which include one pseudogene. Mice, in contrast, harbor 20 CEs genes grouped into five isoenzyme classes based on their sequence similarity and gene structure. The symbol “CES” is used to refer to human genes and the symbol “Ces” to refer to mouse and rat genes. The CES or Ces gene name is followed by a number, which describes the gen family (e.g., CES1). For a family, where multiple genes were identified, a capital letter (human genes) or a lower-case letter (mouse and rat genes) was added after the number to further describe the gene.

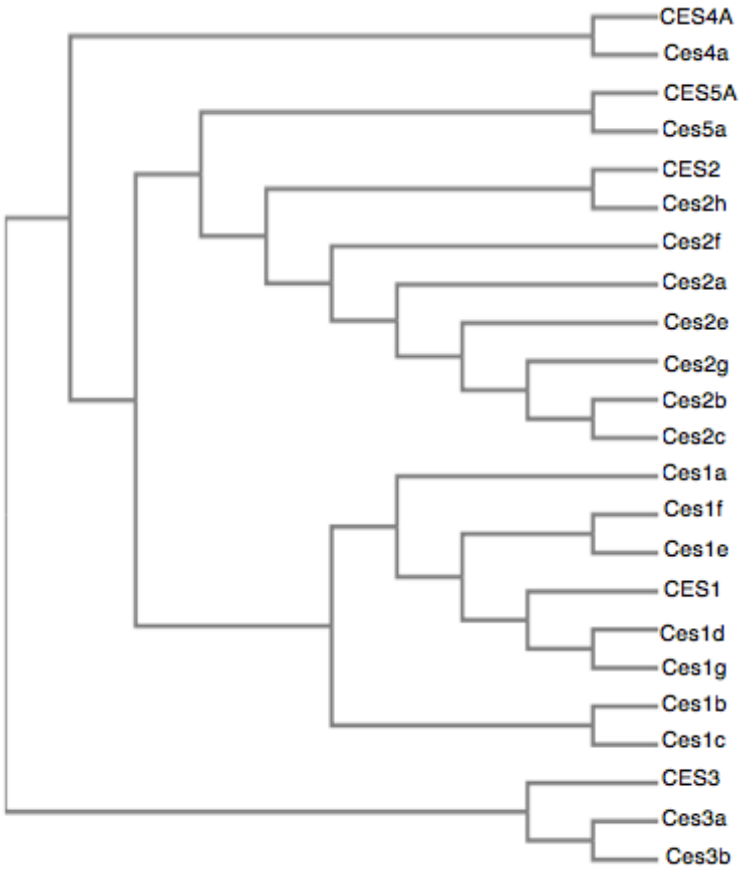


Figure 5: Phylogenetic tree of human and murine CEs genes

Each branch of the tree is labeled with the gene name and followed by a number, which describes the gene family. For families, which comprise multiple genes, a letter was added after the number to further describe the gene.

3. Materials

3.1. Media

LB medium	10 g/l peptone from casein 10 g/l NaCl 5 g/l yeast extract
LB – agar plates	10 g/l peptone from casein 10 g/l NaCl 5 g/l yeast extract 15 g/l agar
Dulbecco's modified Eagle's medium high glucose (DMEM)	commercially available 10 % fetal calf serum (FCS) 100 U/ml penicillin/ 100 µg/ml streptomycin
Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12	commercially available 10% FCS 100 U/ml penicillin/ 100 µg/ml streptomycin 5 µg/ml insulin/ 5 µg/ml transferrin / 5 ng/ml selenium 40 ng/ml dexamethasone
SOC-medium	commercially available
NZY broth	10 g of NZ amine (casein hydrolysate) 5 g of yeast extract add deionized H_2O to a final volume of 1 liter 12.5 ml of 1 M $MgCl_2$ 12.5 ml of 1 M $MgSO_4$ 20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)

3.2. Buffers and Solutions

1 x TAE buffer (pH 7.2)	40 mM Tris/HCl 50 mM EDTA 7 % glacial acetic acid
10% APS	1 g ammonium persulfate in 10 ml
100 mM potassium phosphate buffer (pH 7)	310 µl 1 M dipotassium hydogenphosphate buffer 690 µl 1 M potassium dihydrogenphosphate buffer dilute to 10 ml
1000x protease inhibitor (Pi)	20 mg leupeptin 2 mg antipain 1 mg pepstain 1 ml DMSO sterile filtered
1x PBS (pH 7.3)	140 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
1x TST (pH 7.4)	50 mM Tris/HCl 0.15 M NaCl 0.1% Tween 20
3 M K-acetate/ 5 M CH_3COOH	147.2 g 3 M K-acetate 150.1 g 5 M CH_3COOH in 500 ml
4 mM oleic acid	24.3 mg 8 mM oleic acid/ 10 ml 1x PBS 1.62 g 2.7 mM FFA free BSA/ 10 ml 1x PBS heat to 37 °C; add BSA solution drop wise to oleic acid (1:1)
4x lower buffer	0.5 M Tris

(pH 8.8)	0.4% SDS stored at 4°C
4x SDS loading buffer (pH 6.8)	0.2 M Tris 10% β-Mercaptoethanol 8% SDS 40% glycerin bromophenol blue (tip of a spatula)
4x upper buffer (pH 6.8)	0.5 M Tris stored at 4°C
5x DNA loading dye	9% glycerol 2.5 mg/ml bromophenol blue 2.5 mg/ml xylen-cyanol
Agarose 1%	300 ml ddH_2O 3 g agarose 6 ml 50x TAE 20 μl ethidium bromide
BMP substrate buffer	100 mM NaCl 50 mM Tris/HCl pH8 5 mM CHAPS 1 mM EDTA ddH_2O
BSA	10 mg/ml
BTP buffer (pH 7)	125 mM bis-tris propane
CAPS transfer buffer (pH 11)	10 mM CAPS 10% methanol
Coomassie destaining solution	10% from 80% acetic acid 30% methanol
Coomassie staining solution	50% ethanol 7.5% from 80% acetic acid

	0.25% coomassie-brilliant blue R250
NEB cut smart buffer (New England BioLabs Inc.)	
HSL buffer (pH 7)	0.25 M sucrose 1 mM EDTA 1 mM DTT 1x Pi
Lysis buffer I	50 mM glucose 25 mM Tris pH 7.5 10 mM EDTA
Lysis buffer II	0.2 M NaOH 1% SDS
Metafectene (Biontex)	
NEB T4 DNA Ligase Reaction buffer (New England BioLabs Inc.)	
Phusion GC Reaction Buffer (Finnzymes)	10x concentrate
Sevac	chloroform : isoamylalcohol 24:1
Substrate-no-buffer	100 mM KCL 1 mM EDTA 5 mM CHAPS 2.5% BSA
TGH assay buffer (pH 7)	100 mM potassium phosphate buffer 50 mM CHAPS

3.3. Primers

Table 1: PCR Primers

The primers used for cloning are listed below including their sequence and specificity for restriction enzymes.

Primer	Sequence	Specificity
Ces2c/pFLAG_forward	5'-TTA CGG GAA TTC ACC ATG ACA CGG AAC CAA CTA CAT AAC-3'	*EcoRI*
Ces2c/pFLAG_reverse	5'-CCG GTT TCT AGA AAG CTC CCT GTG CTT GTC CT-3'	*XbaI*
Ces2c lacking HREL_forward	5'TTA CGT GCG GCC GCC CAC CAT GAC ACG GAA CCA ACT ACA TAA C -3'	*NotI*
Ces2c lacking HREL_reverse	5'CCG GTT TCT AGA CTT GTC CTG AGA AGC CTT TAG C -3'	*XbaI*
Ces2c-FLAG-HREL_forward	5'TTA CGT GCG GCC GCC CAC CAT GAC ACG GAA CCA ACT ACA TAA C -3'	*NotI*
Ces2c-FLAG-HREL_reverse	5'CCG GTT GAT ATC CTA AAG CTC CCT GTG CTT GTC ATC GTC GTC CTT GTA A -3'	*EcoRV*
Ces2c-GFP-HIEL_forward	5’ TTA CGT CTC GAG CCA CCA TGA CAC GGA ACC AAC TAC ATA AC ‘3	*XhoI*
Ces2c-GFP-HIEL_reverse	5’ CCG GTT ACC GGT GGC TTG TCC TGA GAA GCC TTT AGC ‘3	*AgeI*
Ces2a_forward	5'-TTA CGG GAA TTC ACC ATG CCA TTG GCT AGA CTT CC -3'	*EcoRI*
	5'-CCG GTT TCT AGA CAG CTC TGC	*XbaI*

Ces2a_reverse	ATG CTT GTC C -3'	
Ces2b mRNA_forward	5’ CGG AGC CAA ATG CAT AAC TG ‘3	*EcoRI*
Ces2b mRNA_reverse	5’ AAG GGT ACT AAA GCT CCG TGT G ‘3	*XbaI*
Ces2b_forward	5'-TTA CGG GAA TTC ACC ATG CCA CGG AGC CAA ATG -3'	*EcoRI*
Ces2b_reverse	5'-CCG GTT TCT AGA AAG CTC CGT GTG CTT GTC CT -3'	*XbaI*
Ces2e_forward	5'-TTA CGG GAA TTC ACC ATG CCA CTA TAC AAA CTT CTT GG-3'	*EcoRI*
Ces2e_reverse	5'-CCG GTT TCT AGA CAA CTC TTT GTG CCT CTC CT-3'	*XbaI*
Ces2f_forward	5'-TTA CGG GAA TTC ACC ATG CCA GTG CAC AGA CTT CCT-3'	*EcoRI*
Ces2f_reverse	5'-CCG GTT TCT AGA TAC AGC CTT GAT TTT ATC GTG ATT T-3'	*XbaI*

3.4. Vectors

The pECFP-N1 vector (Richard Lehner) was used for localization studies in COS-7 cells via fluorescence microscopy analyses (see Figure 6). A CMV promoter regulates the expression of the inserted gene. The *CFP* gene is located downstream of the multiple cloning site (MCS), thus creating a C-terminal tag for the inserted gene. Furthermore, the ER-retention signal sequence (HIEL) is located C-terminal of the *CFP* gene, thereby ensuring localization to the ER. As a selection marker a kanamycin resistance gene is provided, which is under the control of a SV40 promotor.

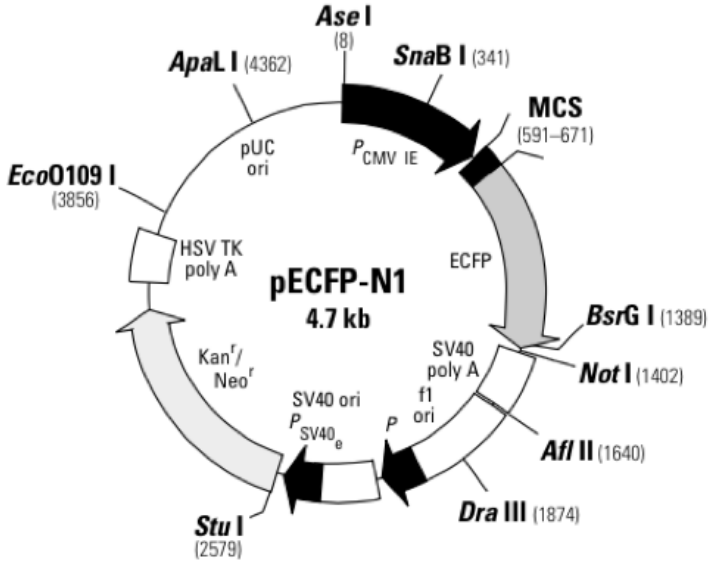


Figure 6: The pECFP-N1 expression plasmid

The CMV promoter regulates the inserted gene. Furthermore the vector contains an *ECFP* gene, which acts as a C-terminal CFP-tag. Moreover the plasmid contains a kanamycin resistance gene, which is under the control of the SV40 promoter.

The C-terminal pFLAG-CMV-5.1 plasmid (Sigma-Aldrich) was used for protein expression (see Figure 7). The FLAG-tag is located downstream of the MCS and is C-terminally linked to the inserted gene of interest, which is regulated by a CMV promotor. Additionally, the vector contains an ampicillin resistance gene.

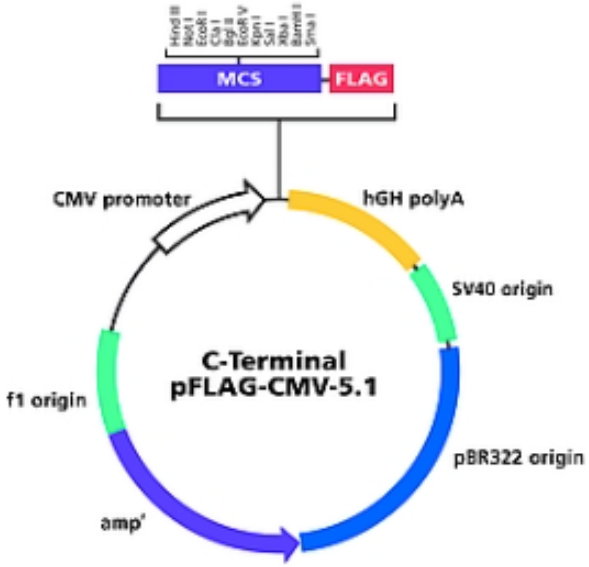


Figure 7: The pFLAG-CMV-5.1 expression plasmid

The vector contains a CMV promoter, a FLAG-tag downstream of the MCS and an ampicillin resistance gene.

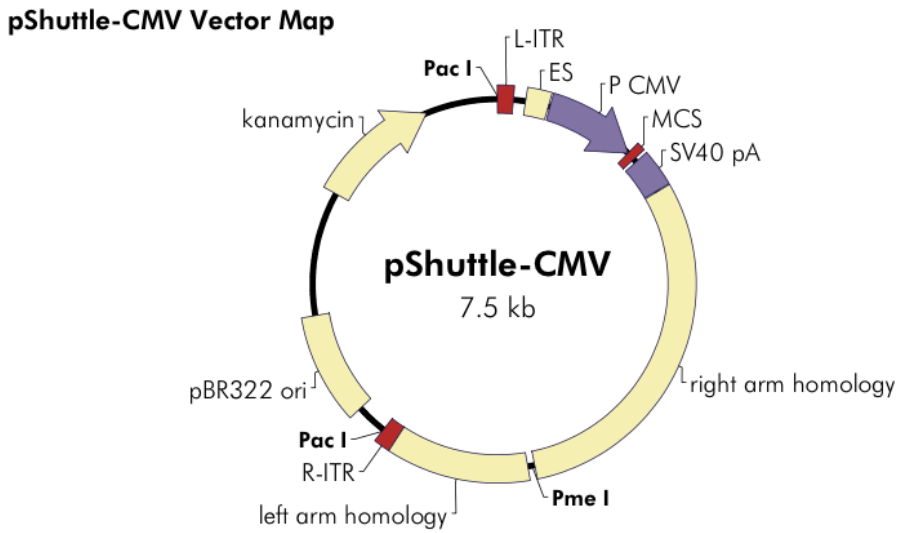


Figure 8: The pShuttle-CMV expression plasmid

The vector contains a CMV promoter and stretches of sequence similarity with the pAdEasy-1 plasmid (right and left arm). Moreover the vector contains an ampicillin resistance gene.

The pShuttle-CMV plasmid contains a MCS regulated by the CMV promotor and is suitable for insertion of a large gene of interest. The right and the left arm are stretches of sequence homology with the pAdEasy-1 plasmid, where the homologous recombination occurs. The R-ITR and L-ITR regions consist of short inverted terminal repeats, which are important for the replication of the viral DNA (see Figure 8).

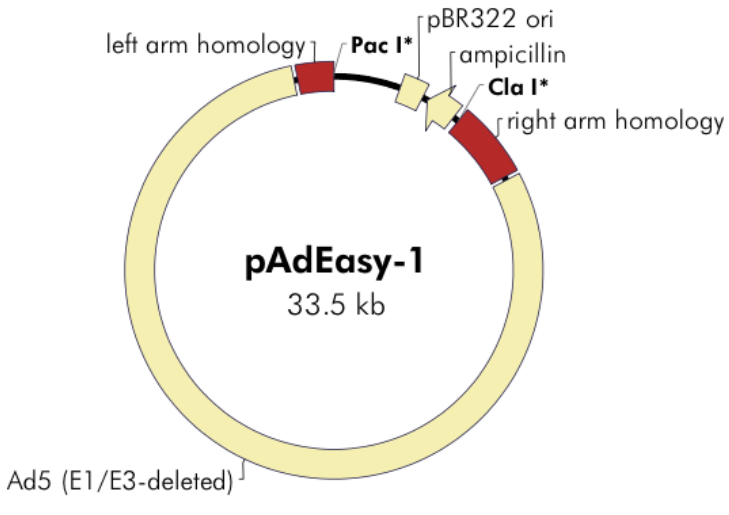


Figure 9: The pAdEasy-1 expression plasmid

The pAdEasy-1 plasmid contains most of the human adenovirus serotype 5 and is deleted for the *E1* and *E3* gene. Additionally, the pAdEasy-1 plasmid contains an ampicillin resistance gene, which is lost after recombination with the pShuttle vector.

The pAdEasy-1 vector contains most of the human adenovirus serotype 5 (Ad5) genome. However, the plasmid is deleted for the *E1* and *E3* gene, which creates space for foreign DNA coincidently eliminating the self-replication capabilities. Therefore, the virus is defective for replication and incapable of producing infectious viral particles. Moreover, the pAdEasy-1 plasmid contains an ampicillin resistance gene, which is lost after recombination with the pShuttle vector (see Figure 9).

3.5. Enzymes

Table 2: Restriction enzymes

The restriction enzymes used for cloning are listed below.

Enzyme	Company
EcoRI-HF	NEB, Frankfurt am Main, Germany
XhoI	NEB, Frankfurt am Main, Germany
NotI-HF	NEB, Frankfurt am Main, Germany
AgeI-HF	NEB, Frankfurt am Main, Germany
XbaI	NEB, Frankfurt am Main, Germany
EcoRV-HF	NEB, Frankfurt am Main, Germany
PmeI	NEB, Frankfurt am Main, Germany
PacI	NEB, Frankfurt am Main, Germany

3.6. Bacterial strains

- ***E. coli* DH5α**

  This strain is frequently used for cloning applications because it stably integrates the insert and provides high efficiency transformation. (New England BioLabs Inc, Frankfurt am Main, Germany).

- ***E. coli* BJ5183-AD-1**

  This E. coli strain is pre-transformed with the pAdEasy-1 plasmid and used for recombinant adenovirus generation. Moreover the cells are *recA* proficient and provide the machinery necessary to execute the recombination event (Agilent Technologies, Santa Carla, USA).

- ***E. coli* XL10-Gold ultracompetent cells**

  These cells allow the efficient transformation of large plasmids. Therefore they are used to amplify the recombinant pAdEasy-1 plasmid (Agilent Technologies, Santa Carla, USA).

3.7. Cell lines

COS-7

The COS-7 cell line was derived from the kidney of the African Green Monkey, *Cercopithecus aethiops*. The cells themselves resemble fibroblast cells in humans. The cell line has been established from CV-1 cells, which have been transformed by an origin-defective mutant of SV40 coding for wild-type T antigen (Cell-line-service.de 2014).
Medium$^{+/+}$: Dulbecco's modified Eagle's Medium containing 4.5 g/L glucose (DMEM; Invitrogen; Hofer, Austria) 10% FCS and 1% penicillin/streptomycin mix.
HSL-buffer: 0.25M saccharose, 1mM EDTA, 1mM DTT, and pH: 7.0

AD-293

Stratagene's AD-293 cell line is a derivative of the commonly used HEK293 cell line, with improved cell adherence and plaque formation properties. HEK293 cells are human embryonic kidney cells transformed by sheared adenovirus type 5 DNA.1 AD-293 cells, like HEK293 cells, produce the adenovirus E1 gene in *trans,* allowing the production of infectious virus particles when cells are transfected with E1-deleted adenovirus vectors such as the pAdEasyTM-1 vector (Chem-agilent.com 2014).
Medium$^{+/+}$: Dulbecco's modified Eagle's Medium containing 4.5 g/L glucose (DMEM; Invitrogen; Hofer, Austria) 10% FCS and 1% penicillin/streptomycin mix.
HSL-buffer: 0.25 M saccharose, 1 mM EDTA, 1 mM DTT, and pH: 7.0

HepG2

HepG2 are adherent, epithelial-like cells growing as monolayers and in small aggregates. The HepG2 cell line was derived from the liver tissue of fifteen-year-old male with differentiated hepatocellular carcinoma. They secrete plasma proteins, such as albumin, transferrin, fibrinogen, a-2-macroglobulin, plasminogen (HepG2.com 2014).
Medium$^{+/+}$: Dulbecco's modified Eagle's Medium containing 4.5 g/L glucose (DMEM; Invitrogen; Hofer, Austria) 10% FCS and 1% penicillin/streptomycin mix.

HSL-buffer: 0.25 M saccharose, 1 mM EDTA, 1 mM DTT, and pH: 7.0

AML12

The AML12 (alpha mouse liver 12) cell line was established from hepatocytes from a mouse (CD1 strain, line MT42) transgenic for human TGF alpha (ATTC.org 2014).
Medium$^{+/+/+/+}$: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 containing 4.5 g/L glucose (DMEM; Invitrogen; Hofer, Austria) 10% FCS, 1% penicillin/streptomycin mix and 5 µg/ml insulin/ 5 µg/ml transferrin / 5 ng/ml selenium 40 ng/ml dexamethasone.
HSL-buffer: 0.25 M saccharose, 1 mM EDTA, 1 mM DTT, and pH: 7.0

McA-RH777

Derived from the Morris hepatoma 7777 (obtained from a male Buffalo strain rat). The original tumour was induced with N-2 fluorenylphthalamic acid. Particularly interesting for oncogeny studies since the cells produce alpha-fetoprotein (AFP) (SigmaAldrich.com 2014).
Medium$^{+/+}$: Dulbecco's modified Eagle's Medium containing 4.5 g/L glucose (DMEM; Invitrogen; Hofer, Austria) 10% FCS and 1% penicillin/streptomycin mix.
HSL-buffer: 0.25 M saccharose, 1 mM EDTA, 1 mM DTT, pH: 7.0

3.8. Antibodies

Table 3: Antibodies for western blot analysis

Primary and secondary antibodies are shown.

Primary antibody	Secondary antibody
Anti-His N-terminal (Amersham) 1:5000 5 % milk, 1xTST	Anti-mouse HRP conjugated (GE healthcare) 1:10000 5 % milk, 1xTST
Anti-FLAG-HRP (Sigma) 1:5000 2 % milk, 1xTST	
Anti-GAPDH (Cell signaling) 1:10000 5 % milk, 1xTST	Anti-rabbit HRP conjugated goat IgG (Vector Laboratories) 1:10000 5 % milk, 1x TST

3.9. Standards

For the examination of DNA-products, Gene Ruler1kb DNA Ladder (Thermo Scientific, Waltham, USA) was used. For western blot analysis Precision Plus Protein Standards All blue (Bio-Rad, Hercules, USA) was used.

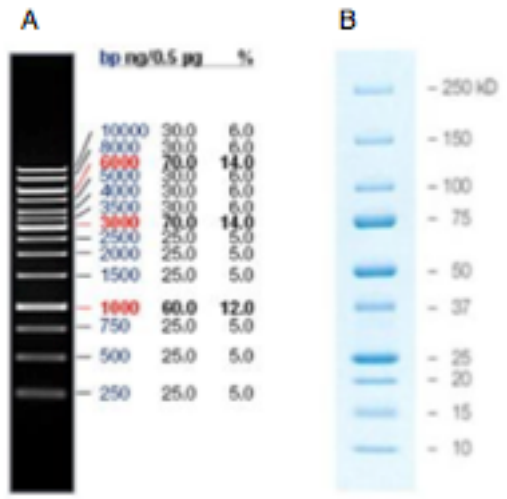


Figure 10: Used Standards

A: Gene Ruler1kb DNA Ladder **B:** Precision Plus Protein Standards All blue

3.10. Kits

- **Pierce (Rockford, USA)**
  BCA (bicinchoninic acid) Protein Assay Kit
- **GE Healthcare (Chalfont St Giles, UK)**
  ECL Plus Western Blotting Detection System
- **Wako Chemicals GmbH (Neuss, Germany)**
  NEFA-HR(2) Wako R1a and NEFA-HR(2) Wako R1b reagents
- **QUIAGEN N.V. (Venlo, The Netherlands)**
  NucleoBondXtra Plasmid Purification
  QIAprep Spin Miniprep Kit (250)
  PCR clean up Gel extraction
  NucleoBond® Xtra Midi / Maxi
- **NEB (Frankfurt am Main, Germany)**
  Q5 site-directed Mutagenesis Kit

4. Methods

4.1. General information about adenovirus generation

To shed light on the role of Ces2c in the assembly of VLDL particles an adenovirus, for Ces2c expression in different liver cell lines was generated using the pAdEasy™ XL Adenoviral Vector System (see Figure 11).

The system was chosen, because it makes use of the efficient homologous recombination machinery of *E. coli* to produce a recombinant adenovirus, which is capable of infecting a broad range of cell types. As a first step the gene of interest is cloned into the pShuttle-CMV vector, which contains stretches of sequence homology with the pAdEasy-1 plasmid, where homologous recombination occurs. The gene of interest containing pShuttle-CMV vector is then introduced into *E. coli* BJ5183 cells, which are pre transformed with pAdEasy-1 plasmids. Moreover BJ5183 cells are *recA* proficient and supply the machinery necessary to execute the recombination event between shuttle vector and pAdEasy-1 vector. The pAdEasy-1 vector is deleted for the genes *E1* and *E3*, which creates space for foreign DNA on the one hand and eliminates self-replication capabilities of the adenovirus on the other hand. The recombined adenovirus plasmid is then amplified in XL10-Gold ultracompetent cells. Since the *E1* gene is essential for assembly of infectious virus particles and pAdEasy-1 plasmids lack the *E1* gene, it is complemented *in vivo* by adenovirus packaging cells (AD-293 cells). The amplified, recombined, adenovirus plasmid is introduced into AD-293 cells and infectious virus particles are generated.

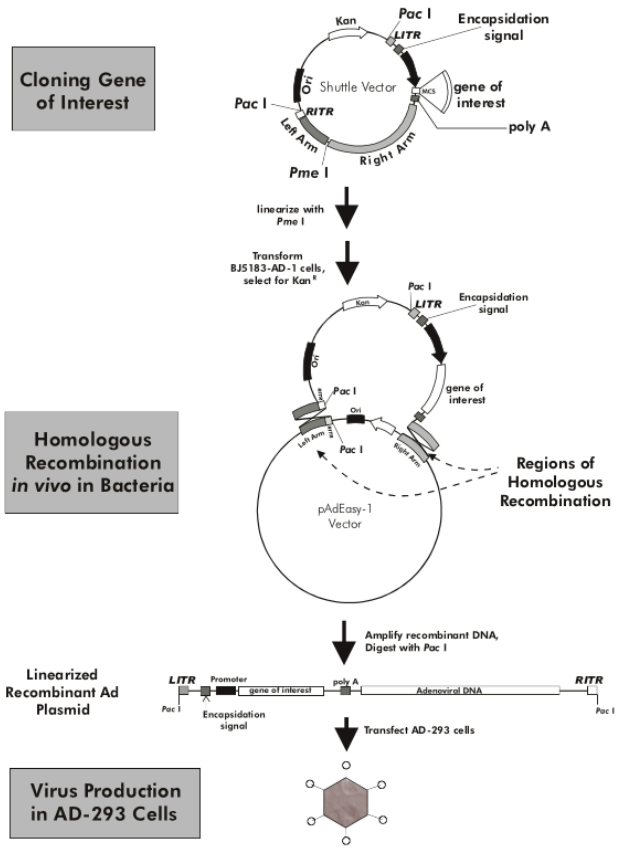


Figure 11: Production of a recombinant adenovirus using the AdEasy™ XL adenoviral vector system

Firstly, the gene of interest is cloned into the pShuttle-CMV plasmid. The shuttle vector is then linearized using *Pme*I restriction and transformed into BJ5183-AD-1 competent cells, where homologous recombination with the adenoviral pAdEasy-1 vector occurs. Kanamycin-resistance transformants were picked and identified by restriction digestion. The homolog recombinant is produced in bulk using the recombination-deficient XL10-Gold strain. To produce infectious virus particles *in vivo*, the purified construct is digested with *Pac*I and used to transfect AD-293 cells, which complement the *E1* gene for self-replication capabilities.

4.2. Cloning

To study the function of a specific protein, the protein has to be expressed in the cell of interest. A widely used method to achieve this goal is molecular cloning. The term molecular cloning refers to a process by which recombinant DNA molecules are produced and transformed into a host, where they are replicated (Neb.com 2014). Briefly, the coding region of your gene of interest is amplified and inserted into an expression vector via restriction enzyme sites. The expression vector is then transformed into host cells for amplification.

4.2.1. Polymerase chain reaction (PCR)

Before starting the cloning procedure a sufficient amount of your gene of interest has to be generated. Additionally, it is important to add restriction enzyme sites to both ends of the coding region for the following restriction. For the amplification of your gene of interest the PCR is your method of choice. The key components of every PCR are primers, which have sequence complementary to the target region, the template DNA itself and a DNA polymerase, which enzymatically assembles the new DNA strand from

nucleotides. For the following restriction enzyme digestion it is essential to create PCR primers, which generate a restriction site sequence at both ends of the PCR product.

In the course of this work several PCR were carried out using different Primer mixes and template DNAs. The first PCR was performed to amplify the complete coding sequence (CDS) of Ces2c (see Table 1).

Table 4: PCR Ces2c, Ces2c lacking HREL and Ces2c-FLAG-HREL

To amplify the coding region of Ces2c from murine intestine, Ces2c/pFLAG or Ces2c lacking HREL/pFLAG and to add specific restriction sites to the CDS different PCRs were conducted. Stated below are the different reaction components and the temperature programs, which were used.

	Ces2c	**Ces2c lacking HREL**	**Ces2c-FLAG-HREL**
template	intestine first-strand cDNA	1:100 dilution Ces2c-pFLAG Maxiprep	1:dilution Ces2c lacking HREL pFLAG Mini
buffer GC	3 µl	3 µl	3 µl
primer	1 µl of 20 pmol/µl forward and reverse primer mix	1 µl of 20 pmol/µl forward and reverse primer mix	1 µl of 20 pmol/µl forward and reverse primer mix
dNTPs 10 mM	0.5 µl	0.5 µl	0.5 µl
phusion polymerase	0.3 µl	0.3 µl	0.3 µl
temperature program	1 cycle at 98°C for 2 minutes followed by 36 cycles at 98°C for 30 seconds and 55°C for 1 minute. The final extension was performed at 55°C for 10 minutes.	1 cycle at 98°C for 2 minutes followed by 34 cycles at 98°C for 30 seconds and 62°C for 30 seconds and 72°c for 1 minute. The final extension was performed at 72°C for 10 minutes.	1 cycle at 98°C for 2 minutes followed by 34 cycles at 98°C for 30 seconds and 62°C for 30 seconds and 72°c for 1 minute. The final extension was performed at 72°C for 10 minutes.

To ensure the localization of Ces2c to the ER, a deletion mutant of Ces2c, which carries the coding region for the C-terminal four amino acids (HREL) long ER retention signal, C-terminal of the FLAG-tag, instead of N-terminal of the tag was generated by PCR. Therefore two PCRs using different primer mixes and templates were performed. The first PCR used forward and reverse primer to amplify the Ces2c CDS lacking the sequence for the C-terminal ER retention signal HREL (see 3.3 Primers). The primers,

which were used for the second PCR, contained the FLAG-tag sequence followed by the ER retention sequence (HREL) and a Stop Codon (see Table 1, 4).

To assess whether homologous Ces of the Ces2 gene family exhibit similar activity as Ces2c, PCRs were performed to amplify the CDS of Ces2a, Ces2b, Ces2e and Ces2f. The Ces2b CDS was amplified using two different PCR reactions, since PCR reactions amplifying merely Ces2b CDS were unsuccessful. The first set of primers used, was homologous to mRNA sequences outside of Ces2b CDS. The product of the first PCR reaction was then used to amplify solely the Ces2b CDS (Table 1, 5).

Table 5: PCR Ces2a, Ces2b mRNA and Ces2b

To amplify the coding region of Ces2a murine intestine cDNA was used. To amplify Ces2b two different PCR reactions were conducted. Stated below are the different reaction components and the temperature programs, which were used.

	Ces2a	**Ces2b mRNA**	**Ces2b**
template	intestine first-strand cDNA	liver first-strand cDNA	1:100 dilution Ces2b mRNA PCR
buffer GC	3 µl	3 µl	3 µl
primer	1 µl of 20 pmol/µl forward and reverse primer mix	1 µl of 20 pmol/µl forward and reverse primer mix	1 µl of 20 pmol/µl forward and reverse primer mix
dNTPs 10 mM	0.5 µl	0.5 µl	0.5 µl
phusion polymerase	0.3 µl	0.3 µl	0.3 µl
temperature program	1 cycle at 98°C for 2 minutes followed by 36 cycles at 98°C for 30 seconds and 68°C for 1 minute. The final extension was performed at 55°C for 10 minutes.	1 cycle at 98°C for 2 minutes followed by 34 cycles at 98°C for 30 seconds and 68°C for 30 seconds and 72°c for 1 minute. The final extension was performed at 72°C for 10 minutes.	1 cycle at 98°C for 2 minutes followed by 34 cycles at 98°C for 30 seconds and 68°C for 30 seconds and 72°c for 1 minute. The final extension was performed at 72°C for 10 minutes.

To study the localization of Ces2c, the Ces2c CDS lacking the ER-retrieval sequence HREL was amplified via PCR (Table 1, 6).

Table 6: PCR Ces2e, Ces2f and Ces2c-GFP-HIEL

To clone Ces2e into the pFLAG vector the Ces2e CDS amplified using Ces2e/HisMax Maxiprep as template. Ces2f was amplified from intestine first-strand cDNA and Ces2c-GFP-HIEL from Ces2c/pFLAG Maxiprep. Stated below are the different reaction components and the temperature programs, which were used.

	Ces2e	Ces2f	Ces2c-GFP-HIEL
template	Ces2e/HisMax Maxiprep	intestine first-strand cDNA	Ces2c/pFLAG Maxiprep
buffer GC	3 µl	3 µl	3 µl
primer	1 µl of 20 pmol/µl forward and reverse primer mix	1 µl of 20 pmol/µl forward and reverse primer mix	1 µl of 20 pmol/µl forward and reverse primer mix
dNTPs 10 mM	0.5 µl	0.5 µl	0.5 µl
phusion polymerase	0.3 µl	0.3 µl	0.3 µl
temperature program	1 cycle at 98°C for 2 minutes followed by 36 cycles at 98°C for 30 seconds and 68°C for 1 minute. The final extension was performed at 55°C for 10 minutes.	1 cycle at 98°C for 2 minutes followed by 34 cycles at 98°C for 30 seconds and 55°C for 30 seconds and 72°c for 1 minute. The final extension was performed at 72°C for 10 minutes.	1 cycle at 98°C for 2 minutes followed by 34 cycles at 98°C for 30 seconds and 62°C for 30 seconds and 72°c for 1 minute. The final extension was performed at 72°C for 10 minutes.

4.2.2. Gel electrophoresis

To confirm the amplification of the desired construct, the PCR products were analyzed by agarose gel electrophoresis. Agarose gel electrophoresis is a method used to separate DNA according to size and proteins according to charge and size. The molecules are separated by the aid of an electric field. Negatively charged nucleic acid molecules migrate toward the anode in an electric field, where the migration flow is solely determined by the size and conformation of the molecules. Therefore shorter molecules move faster and migrate farther through the pores of the gel than larger ones. The migration rate is also influenced by the conformation of the nucleic acid molecules. Supercoiled DNA moves faster than the relaxed or linear molecule, because it is more compact. To visualize the gel separated DNA molecules, ethidium bromide is added to the buffer as well as to the agarose gel. Ethidium bromide intercalates into the DNA and exhibits fluorescence when exposed to UV-light.

DNA samples were mixed with 6x DNA loading dye and pipetted into slots of a 1.5% or 0.8% agarose gel, which contained ethidium bromide. Standard Gene Ruler^{TM} 1 kb DNA Ladder was used to analyze the size of the DNA fragments. The samples were separated in 1x TAE buffer in an electrical field for 30 – 45 minutes at 90 V and 200 mA. Following the separation the gel was photographed under UV-light using Master VDS (Pharmacia Biotech, Upsala Sweden). If the samples were needed for further cloning procedures the bands were cut out of the gel and purified using PCR clean up kit (see 3.10 Kits).

4.2.3. Restriction digestion

Restriction enzymes are capable of cleaving double stranded DNA at specific regions generating either characteristic blunt or sticky ends. The recognition sites are unique for every enzyme and have a length of four to eight nucleotides. Most restriction enzymes digest DNA asymmetrically across their recognition sequence, which results in a single stranded overhang on the digested end of the DNA fragment. These overhangs allow the vector and the insert to bind to each other. Usually two different restriction enzymes are used for adding an insert into a vector, one enzyme on the 5' end and a different enzyme on the 3' end. The use of two different enzymes ensures the insertion of the insert in the correct orientation and additionally prevents the vector from ligating to itself during the ligation process. If the sticky ends on either side of the vector are compatible with each other, the vector is much more likely to ligate to itself rather than to the desired insert.

In the course of this work restriction enzymes were used to digest vectors generating sticky ends, which were complementary to the overhangs of the digested insert (see Table 2). The generated overhangs are ultimately fused by the ligation reaction. Additionally restriction enzymes were used to verify the correct incorporation of inserts into plasmids via specific restriction fragment patterns. The purified, in ddH_2O solubilized DNA, as well as the plasmid were digested at 37°C shaking at 550 rpm for 2 hours. Each restriction reaction consisted of the following components: 15 μl purified PCR product, 3 μl 10 x cut smart buffer, 1-5 μl vector DNA and 1 μl of each restriction enzyme used. Each digestion was brought to a final volume of 30 μl with ddH_2O.

4.2.4. Dephosphorylation of the vector

To prevent re-ligation of the digested vector, newly generated 5'ends of the plasmid were dephosphorylated using calf intestine phosphatase (CIP). 1 µl CIP was added to the digested vector and incubated for 30 minutes at 37°C. To verify successful digestion while additionally removing the restriction enzymes, samples were separated by agarose gel electrophoresis and purified out of the gel using PCR clean up kit (see 3.10 Kits).

4.2.5. Ligation

The last step in the construction of a recombinant plasmid is connecting the gene of interest with the vector. This is accomplished by covalently connecting the sugar backbone of the two DNA fragments. Therefore the 3'hydroxyl group of one fragment is covalently linked through the formation of a phosphodiester bond with the 5'phosphate group of the other fragment. To achieve an efficient ligation of the vector with the insert vector to insert ration needs to be 1:3. Therefore 1 µl of digested vector and digested insert was separated on a 1.5% agarose gel. The concentration of vector and insert could then be estimated. The T4 DNA ligase was used to ligate the overhanging, cohesive, sticky ends. The ideal temperature for the T4 DNA ligase reaction is 16°C, however it works over a range of temperatures. Therefore the ligation was performed at 20°C for 2 hours.

4.2.6. Transformation

Transformation is the process by which foreign DNA is introduced into a cell. Transformation of bacteria with plasmids is important for both storing and replicating plasmids. To enable cells to take up circular vector DNA they have to be made competent. The method for the preparation of competent cells depends on the transformation method used and the transformation efficiency required. Because of this, nearly all plasmids, even those designed for use in mammalian cells, carry both a bacterial origin of replication and an antibiotic resistance gene to use as a selectable marker in bacteria.

4.2.6.1. Chemically competent cells

An aliquot of chemically competent cells (*E. coli* DH5α), which are stored at -80°C, was thaw on ice for 5 minutes. Generally 25 µl of competent cells were used for one

transformation reaction. 3 µl of the ligation reaction was added to the cells and gently mixed by flicking the bottom of the tube a few times. Prior to transformation, 2 µl β-mercaptoethanol were added to 50 µl of XL10-Gold ultracompetent cells and incubated for 10 minutes on ice, while swirling every 2 minutes. 2 µl of *Pac*I linearized pShuttle plasmid were added to the cells and gently swirled. The competent cell/DNA mixture was always placed on ice for 30 minutes. The cells were then heat shocked by placing the bottom ½ of the tube into a 42°C water bath for 30 seconds. Following the heat shock the cells were incubated on ice for 5 minutes. The cells were suspended in either 250 µl pre-warmed SOC-media (*E. coli* DH5α) or 950 µl NZY broth (XL10-Gold ultracompetent) and grown in 37°C shaking incubator for 1 hour (see 3.1 Media). In the course of this work two different strains of chemically competent cells were used.

- *E. coli* DH5α
- XL10-Gold ultracompetent cells

4.2.6.2. Electrocompetent cells

Generally 25 µl of electro competent cells were mixed with 3 µl of ligation sample (*E. coli* DH5α) or 5 µl *Pme*I linearized pShuttle vector (BJ5183-AD-1) and transferred into a 2 mm electroporation cuvette (PeqLab Biotechnologie GmbH, Erlangen, Germany). Bio-Rad MicroPulser™ (Bio-Rad Laboratories Inc., Hercules, USA) with 2.5 kV and 200 W was used. After electroporation, 200 µl warm LB medium was added to the cells, which were then transferred in a 1.5 ml tube and incubated for 1 h at 37°C 180 rpm in the shaking incubator for regeneration (see 3.1 Media).

In the course of this work one strain of electro competent cells was used.

- BJ5183-AD-1

4.2.7. Miniprep

To identify positive bacteria clones, which took up the recombinant plasmid, aliquots of transformed cells were plated onto 10 cm LB agar plates, containing the appropriate antibiotic and incubated at 37° overnight. Depending upon the resistance gene of the plasmid either ampicillin or kanamycin were used as selection markers. The following morning single colonies were picked and over night culture (ONC) of 5 ml liquid LB media, containing 100 µg/ml of the appropriate antibiotic were inoculated. The liquid culture is generated because it is capable of supporting a higher density of bacteria and is therefore used to grow up sufficient numbers of bacteria, necessary to isolate enough

plasmid DNA. The ONCs were grown overnight at 37°C under constant shaking (180 rpm). After overnight growth, cells were centrifuged at 3000 rpm for 10 minutes. The cell pellet was suspended in 200 µl lysis buffer I and transferred into a sterile a 1.5 ml tube. 200 µl of lysis buffer II were added and mixed by inverting the tube 4 – 6 times. Afterwards, 150 µl 3 M potassium acetate were added, mixed by inverting and incubated on ice for 1 minute. The mixture was centrifuged for 5 minutes at 13000 rpm and the supernatant was transferred into a new 1.5 ml tube. 400 µl phenol and 250 µl sevac were added, the sample was vortexed and again centrifuged for 1 minute at 13000 rpm (see 3.2 Buffers and Solutions). Following centrifugation the upper phase was transferred into a new 1.5 ml tube. The same volume of isopropanol was added and briefly vortexed. The mixture was centrifuged. The pellet was washed with 500 µl 70% ethanol and then dried at 37°C. Finally, the pellet was dissolved in 50 µl ddH_20 with 2 µg/ml RNAse for 15 minutes at 37°C at 300 rpm.

4.2.8. Maxiprep

To isolate a sufficient amount of plasmid, a Maxiprep was performed. Therefore 5 ml LB medium containing the appropriate antibiotic were inoculated with a single colony and incubate at 37°C over day (ODC) with shaking. After 6-8 hours the ODC was transferred to 250 ml antibiotic containing LB medium and incubate at 37°C overnight with shaking. To isolate the plasmids the NucleoBond® Xtra Midi / Maxi Kit was used (see 3.10 Kits). The plasmid was dissolved in 100-200 µl H_2Odd and the concentration was measured.

4.2.9. Sequencing of isolated DNA

Isolated DNA (isolation via Miniprep or NucleoBondXtra Plasmid Purification Kit) was sequenced by Microsynth (Balgach, Switzerland) and sequence results were controlled with ClustalW2 alignment.

4.2.10. Site-directed Mutagenesis

To visualize the localization of Ces2c intracellularly a GFP-tagged Ces2c mutant was generated. Richard Lehner kindly provided a pEGFP-N1 vector, carrying the *hTGH* gene tagged with GFP. To ensure the retrieval of hTGH, the vector carries the ER retention sequence of hTGH (HIEL) C-terminal of the GFP-tag. Since Ces2c possesses a HREL sequence instead of a HIEL retrieval sequence a site directed mutagenesis was

performed. Therefore the Q5 site-directed Mutagenesis Kit was used. The procedure was carried out as described in the manual. Briefly, a PCR with the provided Q5 Hot Start High-Fidelity 2X Master Mix was performed (see Table 7). After the PCR, the amplified product was added to the provided Kinase-Ligase-*DpnI* enzyme mix and incubated for 5 minutes. Afterwards plasmids were transformed into *E. coli* DH5α competent cells, plated on agar plates containing kanamycin and grown overnight at 37°C. Clones were picked and DNA was isolated via Miniprep (see 4.2.7 Miniprep). The DNA was sequenced (see 4.2.9 Sequencing of isolated DNA) and DNA isolation from positives clones was performed using Maxiprep protocol (see 4.2.8 Maxiprep).

Table 7: Site-directed mutagenesis of Ces2c-GFP-HIEL to Ces2c-GFP-HREL

A PCR was performed to mutate the HIEL sequence of the Ces2c Mini to an HREL sequence. The mutated product was transformed into *E. coli* DH5α. Stated below are the different reaction components and the temperature programs, which were used.

	Ces2c-GFP-HREL
Template	Ces2c-GFP-HIEL
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl
Primer	1.25 µl of 10 µM forward and reverse primer
Nuclease-free water	9.0 µl
Temperature program	1 cycle at 98°C for 30 seconds followed by 25 cycles at 98°C for 10 seconds and 65°C for 30 minutes. The final extension was performed at 72°C for 3 minutes.

4.3. Cell culture

4.3.1. Cultivation of cells

Cells were cultivated in culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) and stored in an incubator at 37°C, 5% CO_2 and 95% humidity. When a confluence of 80 – 90 % (COS-7, HepG2, AML12, McA-RH777) or 50 – 70 % (AD-293) of the cells was achieved, $DMEM^{+/+}$ or $DMEM/F12^{+/+/+/+}$ medium was removed from the flasks and cells were washed with 10 ml 1x PBS buffer (see 2.2 Buffers and Solutions). 3 ml 0.05% Trypsin-EDTA solution (Invitrogen – Life Technologies, Carlsbad, USA) was added and cells were incubated in the incubator at 37°C until they were completely detached from the flask surface. To stop the trypsinization reaction 10 ml $DMEM^{+/+}$ or $DMEM/F12^{+/+/+/+}$

medium was added and the resulting cell suspension was transferred in a 50 ml tube and centrifuged for 3 minutes at 1000 g. For sub-culturing purposes the resuspended cells were splitted according to the manufactures splitting ratio. Therefore the appropriate volume of the cell suspension was aliquoted into the flask, which contained 13 ml $DMEM^{+/+}$ or $DMEM/F12^{+/+/+/+}$ medium.

To seed cells at a certain density the viable cell amount was calculated with the CASY 1 cell counter and the cells seeded accordingly.

Table 8: Seeding density of different cell lines

Amount of cells seeded into different culture dishes for distinct experiments.

	50 mm dish	8-well plate	6-well plate	10 culture dish
COS-7	225.000	15.000	$1.5*10^5$	$9*10^5$
AD-293			$2*10^5$	$7.5*10^5$
HepG2			$4*10^5$ - $6*10^5$	
AML12			$1.5*10^5$ / $2*10^5$	
McA-RH777			$6*10^5$	

4.3.2. Transfection and harvesting of DNA constructs in COS-7 cells

4.3.2.1. Generation of cell lysates for expression control of DNA constructs on protein level

To simply verify the expression of a generated DNA construct, cells were seeded into 6-well plates and transfected using Metafectene. Briefly, 24 hours prior to transfection the cells were treated with trypsin and seeded into 6-well plates with a density of 150.000 cells per well. For the transfection, 1 μg of the DNA construct per well was added to 50 μl $DMEM^{-/-}$. The DNA/medium mix was added to 5 μl Metafectene in 50 μl $DMEM^{-/-}$, mixed through inverting and incubated for 20 minutes at room temperature (RT).

Metafectene is a polycationic transfection reagent, which forms aggregates with DNA, allowing fusion with the cell membrane. In the meantime, the culture medium of the cells was changed to 500 μl $DMEM^{+/-}$. 100 μl of the DNA/Metafectene mixture were added to the cells and incubated for 4 hours at 37°C. After 4 hours the transfection medium was substituted with 2 ml $DMEM^{+/+}$. 24 hours after transfection the medium was aspirated and

the cells were harvested with 200 µl 1x SDS. The lysate was then denaturated at 99°C for 5 – 10 minutes and an aliquot (usually 20 µl) was used for western blot analysis.

4.3.2.2. Generation of COS-7 cell lysates

Briefly, 24 hours prior to transfection cells were treated with trypsin and seeded into 10 cm culture dishes with a density of 900.000 cells per dish. For transfection, 6 µg of the DNA construct was added to 300 µl $DMEM^{-/-}$. The DNA/medium mix was added to 30 µl Metafectene in 300 µl $DMEM^{-/-}$, mixed through inverting and incubated for 20 minutes at RT. In the meantime, the culture medium of the cells was changed to 2 ml $DMEM^{+/-}$. 600 µl of the DNA/Metafectene mixture were added to the cells and incubated for 4 hours at 37°C. After 4 hours transfection medium was substituted with 6 ml $DMEM^{+/+}$. Transfected cells were washed with 1x PBS buffer. 2 ml 1x PBS were added and cells were scraped from the surface of the dish with a sterile cell scraper. The suspension was transferred into a 15 ml tube and centrifuged for 3 minutes at 1000 g. The resulting cell pellet was dissolved in 200 µl HSL buffer (see 3.2 Buffers and Solutions). The cells were homogenized by sonication (4 x 5 seconds with a low amplitude) on ice. Nuclei and unbroken cells were removed by centrifugation at 1000 g at 4°C for 5 minutes. The protein concentration of the supernatant was measured using Bradford reagent and adjusted with HSL-buffer to a concentration of 2 mg/ml (see 4.4.2 Bradford). The cell lysates were stored at -20°C.

4.3.3. Fractionation of COS-7 cells

Ces2c, LacZ, Ces2c-GFP containing COS-7 cell lysates were prepared as described in 4.3.2.2 Generation of COS-7 cell lysates. Briefly, 2x 10 cm culture dishes per DNA construct were seeded at a density of 900.000 cells and transfected using Metafectene. 24 hours or 48 hours after transfection cells were scraped off and transferred to a sterile tube. After the centrifugation step the cell pellets were resuspended in 400 µl HSL buffer. The cells were either disrupted by sonication or homogenized using Potter-Elvehien homogenizer and centrifuged at 1000 g at 4°C for 5 minutes. 250 µl of the supernatant were used for ultracentrifugation at 100 000 g at 4°C for 1 hour, the remaining supernatant was stored at -20°C. Once again the supernatant, which contained the cytosolic fraction, was collected. The pellet, which contained the microsomal fraction was washed two times with 1x PBS and resuspended in 200 µl HSL buffer by repeatedly passing through a 21-gauge needle. The protein concentration was determined using

Bradford reagent (see 4.4.2 Bradford). 15 µg of total protein, cytosolic protein and microsomal protein were used for western blot analysis.

4.4. Measuring Protein content

In the course of this work two different methods were used to measure total protein content.

4.4.1. BCA protein assay

BCA protein assay is a copper-based protein assay for quantitation of total protein. BCA is compatible with samples that contain up to 5% detergents. Additionally it responds more uniformly to different proteins than Bradford and is 100 times more sensitive. The BCA Protein Assay combines the protein-induced biuret reaction with the highly sensitive and selective colorimetric detection of the resulting cuprous cation by BCA. There are two steps involved. First is the biuret reaction, where the reduction of cupric ion to cuprous ion leads to a color change. The second step is the chelation of two BCA molecules with the cuprous ion, resulting in an intense purple color. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

Standard curves are most commonly used to determine the concentration of a substance such as protein. A BSA stock solution was used to generate a calibration curve (1 mg/ml; 0.5 mg/ml; 0.25 mg/ml; 0.125 mg/ml; 0.0625 mg/ml). For dilution of BSA stock solution the same buffer was used as for diluting proteins. The protein concentration of each sample as well as the dilution points of the calibrations curve was measured as duplicates. Therefore 20 µl of the sample were pipetted into a microtiter plate, 200 µl BCA mix added and incubated for 30 minutes at 37°C. Afterwards the absorption was measured at 562 nm using Biotrak II Plate Reader (Amersheam Biosciences, Cambridge, UK). To generate the standard curve, concentration was plotted on the X-axis, and assay measurement on the Y-axis. To analyze the data, assay measurement of the unknown substance is located on the Y-axis. The corresponding value on the X-axis corresponds to the concentration of substance in the unknown sample.

4.4.2. Bradford

To determine protein concentrations in samples such as cell lysates, a Bradford protein assay was performed. The measurement is based on binding of Coomassie™ Brilliant Blue G-250 dye to basic amino acids in the acidic environment of the reagents. In the acidic environment of the reagent, proteins bind to the Coomassie dye. This results in a spectral shift of the dye from an absorbance maximum at 465 nm to an absorbance maximum at 610 nm. The Coomassie dye-protein complex is measured at 595 nm.

The samples were diluted with the respective buffers. A BSA stock solution was used to generate a calibration curve (1 mg/ml; 0.5 mg/ml; 0.25 mg/ml; 0.125 mg/ml; 0.0625 mg/ml). All samples and standard dilutions were mixed with 200 μl 1:5 diluted Bio-Rad Protein Assay reagent. After short incubation at RT, absorption at 595 nm was measured with the plate reader. Double determinations were done for each sample and the standard. The standard curve was calculated via Microsoft Excel®, which was then used to determine the protein concentration in the samples.

4.4.3. SDS PAGE

Polyacrylamide gel electrophoresis (PAGE) is a method to separate proteins based on their size. Sodium dodecyl sulfate (SDS), an anionic detergent, which attaches to proteins and linearizes them, is used to generate a negative charge of all proteins. Proteins can then be separated solely based on molecular weight.
Two different kinds of gels are used for the SDS-Page, the separating and stacking gel. The stacking gel contains chloride ions, which migrate quickly through the gel. The electrophoresis buffer on the other hand contains glycine ions, which migrate more slowly. The protein molecules are trapped in a sharp band between these ions. The separating gel has a smaller pore size, a higher pH and a higher salt concentration, which leads to the ionization of glycine. The proteins are then separated based on size.

The separating gel was pipetted between a clean glass plate and an aluminum silicate plate, which were held in place through fixing with clamps. To avoid evaporation butanol was placed on top of the gel. As soon as the separating gel was hardened, the stacking gel solution was added on top and a comb was placed in the stacking gel.

Protein samples were mixed with 4x SDS loading buffer and heated for 10 minutes at 99°C. The samples were briefly centrifuged and loaded onto the gel. Additionally, 5 µl of Precision Plus Protein All Blue Standard were loaded on the gel to determine protein size. The gel was running with 20 mA for about 1 hour in 1x SDS-PAGE buffer (see 3.2 Buffers and Solutions) until the front reached the bottom. When the SDS PAGE was finished the proteins were then blotted on a membrane (see. 4.4.4.1 Blotting and Sandwich construction).

Table 9: Composition of the SDS-Page gels

The separating gel was prepared in a final volume of 40 ml and the stacking gel in 5 ml.

Components	10% separating gel solution (40 ml)	5% stacking gel solution (5 ml)
ddH_2O	16.3 ml	2.95 ml
4x upper buffer	-	1.25 ml
4x lower buffer	10 ml	-
30% acryl amide	13.3 ml	0.75 µl
10% SDS	400 µl	50 µl
Tetramethylethylendiamin (TEMED)	36 µl	6.5 µl
10% APS) (ammonium persulfate)	108 µl	20 µl
0.5% Bromphenol blue	-	3 µl

4.4.4. Western Blotting analysis

Western Blotting analysis is a method, which enables the detection of the expression levels of selected proteins in the expression systems.

4.4.4.1. Blotting and sandwich construction

After the separation of the proteins via SDS-PAGE, the separating gel was blotted onto a polyvinylidene fluoride membrane (Carl Roth GmbH, Karlsruhe, Germany). Therefore the stacking gel was removed and the separating gel and the membrane were sandwiched between filter paper and a sponge. Air bubbles were removed to ensure complete protein

transfer. The “sandwich” construct was blotted at 200 mA for 1 hour in a blot chamber, containing 1x CAPS transfer buffer.

4.4.4.2. Blocking and antibody detection

After the blotting, the membrane was blocked with 10% milk in 1x TST for 2 hours at RT or overnight at 4°C. (see 3.2 Buffers and Solutions). Following the blocking, the membrane was incubated with the primary antibody for 1 hour at RT (see 3.8 Antibodies). The primary antibody was removed and the membrane was washed three times with 1x TST for 10 minutes. The membrane was incubated with the secondary antibody for 1 hour at RT and once again washed 3 times with 1x TST for 10 minutes. For antibody detection the ECL™ Western Blotting System (GE Healthcare Amersham, Buckinghamshire, UK) was used.

4.4.4.3. Coomassie Blue staining

To confirm that the same amounts of protein were used, the membrane was stained with Coomassie Blue staining solution and destained with Coomassie destaining solution (see 3.2 Buffers and Solutions). After drying under the vent, the proteins were visible as blue fragments.

4.5. Generation of recombinant adenovirus

AD-293 cells were transfected using “ProFection Mammalian Transfection System” from Promega. The cells were treated with trypsin and seeded into 10 cm culture dishes with a density of 750.000 cells per dish. 3 hours prior to transfection medium was aspirated and replaced with 6 ml fresh $DMEM^{+/+}$. For transfection of a 10 cm culture dish 10 - 20 μg *Pac*I digested plasmid DNA from XL-Gold cells were mixed with 62 μl $CaCl_2$ solution and brought to a final volume of 500 μl with sterile water. 500 μl HBS were added to a sterile tube and gently vortexed. The previously prepared DNA/$CaCl_2$ solution was added drop wise to the HBS containing tube. The solution was incubated for 30 minutes at RT. After 6 hours incubation transfection medium was substituted with 6 ml $DMEM^{+/+}$. The cells were cultivated for 7 – 10 days in the incubator. The medium was changed regularly. As soon as holes appeared in the cell layer or the cell layer detached from the plastic, fresh medium was added to the existing medium. These cells were used to prepare the primary viral stock.

4.5.1. Primary Virus stock

The growth medium from adenovirus-producing AD-293 dishes was removed and stored at -20. The cells were washed with 5 ml 1x PBS. To harvest the cells, 1 ml of 1x PBS was added to each dish and the cells were scratched off with a cell scraper. The cell suspension was transferred to a sterile tube and centrifuged at 3000 g for 3 minutes at RT. The supernatant was removed and the pellet was resuspended in 1 ml 1x PBS. The cells were subjected to four rounds of freeze/thaw by alternating tubes between liquid nitrogen and a 37°C water bath, vortexing briefly after each thaw. The cellular debris was collected by centrifugation at 12000 g for 10 minutes at RT. The supernatant, which contained the primary virus stock was transferred to a sterile tube and stored at -20.

4.5.2. Secondary Virus stock

To verify the successful assembly of an infectious virus, AD-293 cells were transduced using the primary virus stock, digested and the viral DNA was detected by PCR.

AD-293 cells were treated with trypsin and seeded into a 175 cm culture flask with a density of 2.000.000 cells per flask. To transduce the cells, the primary virus stock was diluted in 10 ml $DMEM^{-/-}$. The growth medium was removed from the flask and the diluted primary virus stock was added. After 2 hours of incubation 10 ml $DMEM^{+/+}$ were added to the cells. The cells were cultivated for 2-3 days in the incubator.
The growth medium from adenovirus-producing AD-293 flask was collected and centrifuged at 1000 g for 10 minutes at RT. The supernatant was transferred into a fresh tube and stored at -20°C. The cell pellet was resuspended in 1 ml 1x PBS and subjected to four rounds of freeze/thaw. The cells were flushed from the surface of the culture flask, transferred to a sterile tube and centrifuged at 3000 g for 10 minutes at RT. The supernatant was removed and the pellet was resuspended in 0.5 ml 1x PBS. The cells were subjected to four rounds of freeze/thaw. The supernatant from the cells of the culture flask and the supernatant form the cells of the medium were pooled and transferred to a sterile tube and stored at -20.

To verify the assembly of an infectious virus a PCR was conducted using 2 μl or 5 μl of the secondary virus stock or the medium as template DNA (Table 1).

4.5.3. Virus Maxiprep

To generate a sufficient amount of viral DNA for the purification, the viral DNA was amplified *in vivo*.

AD-293 cells were treated with trypsin and seeded into a 175 cm culture flask with a density of 2.000.000 cells per flask. 15 culture flasks were seeded. To transduce the cells, the secondary virus stock was diluted in 150 ml $DMEM^{-/+}$. Prior to the infection the medium was removed and 10 ml of diluted secondary virus stock were added to each flask. After 2 hours of incubation 13 ml $DMEM^{+/+}$ were added to each flask. After 1 day of incubation, medium was aspirated and cells were flushed of the surface using 3 ml 1x PBS. The collected cells were unified and centrifuged at 3000 g for 10 minutes at RT. The supernatant (Virus Maxiprep) was removed and the pellet was resuspended in 3 ml 1x PBS and stored at – 80°C.

4.5.4. Adenovirus purification

Prior to purification of adenovirus by ultracentrifugation, the Virus Maxiprep was subjected to four rounds of freeze/thaw and centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was collected in a sterile tube.

A discontinuous gradient was prepared by adding 3 ml 1.25 g/ml CsCl solution to an ultracentrifuge tube. 3 ml 1.40 g/ml CsCl solution were layered underneath the 1.25 g/ml CsCl solution. Approximately 5 ml of crude virus concentrate was carefully layered on top of the discontinuous CsCl gradient. The sample was centrifuged at 35000 rpm at 22°C for 1 hour. After the ultracentrifugation two bands of DNA, which were located in the center of the gradient were visible. The upper band contained nicked circular DNA and linear bacterial DNA. The lower, larger band consisted of closed circular plasmid DNA. To collect the plasmid DNA the tube was pierced below the plasmid DNA band with a 21-gauge needle. To separate empty capsid form the infectious virus a second ultracentrifugation step was performed. Therefore 8 ml 1.33 g/ml CsCl solution were added to a sterile tube. The previously collected adenovirus band was carefully layered on top of the CsCl gradient. The sample was centrifuged at 35000 rpm at 22°C for 20 hours. To collect the infectious adenovirus, the tube was pierced below the plasmid DNA band with a 21-gauge needle. Since the purified adenovirus contains CsCl, which is toxic to animals, dialysis was performed to remove CsCl. Therefore the adenoviral suspension

was injected into a dialysis slide and dialyzed overnight in a solution of $MgCl_2$ and Tris/HCL. The following morning the virus was collected in a sterile tube.

4.5.5. Determination of plaque forming units (pfu)

4.5.5.1. Absorbance measurement

Pfu were determined by absorbance measurement at 260/280 nm. Prior to the measurement 10 µl of the purified virus were diluted with 10 µl 1 x PBS, and 20 µl 0.2% SDS.

4.5.5.2. Plaque assay

A reliable method to quantify the infectious virus concentration is the plaque assay. Viral plaque assays are used to determine pfu/ml in a virus sample. A confluent monolayer of host cells is infected with the virus at varying dilutions, which leads to the amplification of virus particles. To prevent the virus infection from spreading to all cells in the culture dish, the cells are covered with medium, which contains a low percentage of agarose. After 7-10 days infected cells will lyse and spread the infection to the adjacent cells, which in turn undergo the same infection/lyse cycle. This leads to the formation of a plaque, which can be seen visually or with the microscope. The plaques are counted manually and then used to calculate the virus stock concentration (pfu/ml) under consideration of the dilution factor. The pfu/mL represent the number of infective particles within the sample and is based on the assumption that each plaque formed is representative of one infective virus particle.

AD-293 cells were seeded into 6-well plates at a density of 200.000 cells per well. Upon 90% confluence cells were infected with serial dilutions of the adenovirus Maxiprep as either duplicates (Ad-Ces2c) or triplicates (Ad-LacZ). The dilutions prepared ranged from 10^{-2} to 10^{-12}. Briefly, 10 µl of Virus Maxiprep were added to 990 µl sterile 1x PBS and gently mixed by pipetting up and down (10^{-2} dilution). 10 µl of the 10^{-2} dilution were once again mixed with 990 µl 1x PBS to generate the 10^{-4} dilution. This procedure was continued until the highest dilution (10^{-12}) was generated. Prior to infection the medium of the AD-293 6-well plates was aspirated and changed to 1 ml $DMEM^{+/+}$. To obtain the desired virus dilutions, ranging from either 10^{-5} to 10^{-13} (Ad-LacZ) or 10^{-5} to 10-^{12} (Ad-Ces2c), 10 µl or 100 µl of the previously prepared serial dilutions were added directly to the medium of the cells. 24 hours after infection the medium was aspirated and the cells

were overlaid with a sterile 0.4% agarose/medium solution. Briefly, a sterile 4% agarose solution was prepared and stored at 65°C. The agarose solution was diluted 1:10 with pre-warmed (37°C) plaque medium and homogenized by shaking. 1 ml of the agarose/medium solution was then quickly but gently added to the cells. The plates were incubated at RT until the agarose became solid (approximately 15 minutes). After 2 days of incubation at 37°C the overlayering process was repeated.

The first plaques emerge rather quickly at lower dilutions (5-7 days). At higher dilutions it takes 10-14 days for plaques to become visible. After an incubation period of 14 days the cells were stained with 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT). MTT can be used to stain living cells, since their mitochondrial dehydrogenases convert the yellowish/brownish MTT to a blue water-insoluble MTT formazan derivative. Therefore MTT stains the living cells blue while the plaques, which consist of dead cells remain colorless. 300 µl of MTT solubilized in 1x PBS was added to the cells and incubated for 2-3 hours at 37°C. After the incubation period the plaques of the two highest dilutions were counted, averaged and used to calculate the concentration of the virus Maxiprep (pfu/ml).

4.6. Assays

4.6.1. TG hydrolase-Assay

The measurement of TG-Hydrolase activity of recombinant proteins (see 4.3.2.2 Generation of COS-7 cell lysates) is based on the release of ^{3}H-labeled oleic acid from artificial, radioactive isotope-labeled triolein. The TG-substrate was always prepared as a master mix for every sample. For each sample 1.67 mM non-radioactive triolein and 10 µCi ^{3}H-TO/ml were mixed in an assay tube resistant to organic solvents. The solvent was brought to complete dryness under a stream of nitrogen. The dried substrate was dissolved in assay buffer (200 mM KPi, 50 mM CHAPS). Initially not more than 2 ml assay buffer were added and the substrate was sonicated (3 x for 30 seconds with an output power of 20%) on ice. After sonication, the remaining assay buffer was added. For each reaction 50 µg protein in 100 µl HSL buffer was used. LacZ was always used as negative control and HSL buffer as blank. Every protein sample was prepared as triplicates.

100 µl TG substrate were added to each sample and incubated for 1 hour in a water bath at 37°C under constant shaking. The reaction was terminated by the addition of 3.25 ml extraction solution I. 1.05 ml extraction solution II were added and the sample was vortexed vigorously. The sample was centrifuged at 1000 g for 10 minutes. 200 µl of the upper phase were transferred to 2 ml scintillation cocktail. The radioactivity of the free FAs was measured in the TRI-CARB 2300 TR liquid scintillation analyzer. To determine the specific substrate activity, 10 µl TG substrate were measured via liquid scintillation. The activity of the substrate was determined as counts per minute (cpm/nmol FS).

4.6.2. pH optimum

In vitro TG hydrolase activity assays were performed to determine the pH optimum of Ces2c enzymatic activity. The TG-substrate was always prepared as a master mix for every sample. For each sample 1.67 mM non-radioactive triolein and 10 µCi ^{3}H -TO/ml were mixed in an assay tube resistant to organic solvents. The solvent was brought to complete dryness under a stream of nitrogen. The dried substrate was dissolved in 50 mM CHAPS. The substrate was sonicated (3 x for 30 seconds with an output power of 20%) on ice. After the sonication the remaining assay buffer was added. For each reaction 50 µg protein in 100 µl HSL buffer was mixed with 50 µl buffer (200 mM potassium acetate pH 4; potassium acetate pH 5; potassium acetate pH 6; KPi pH 6; KPi pH 7; KPi pH 8; Tris/HCL pH 8; Tris/HCL pH 8). LacZ was used as negative control and HSL buffer as blank. Every protein sample was prepared as triplicates.
50 µl TG substrate were added to each sample and incubated for 1 hour in a water bath at 37°C under constant shaking. The reaction was terminated by addition of 3.25 ml extraction solution I. 1.05 ml extraction solution II were added and the sample was vortexed vigorously. The sample was centrifuged at 1000 g for 10 minutes. 200 µl of the upper phase were transferred to 2 ml scintillation cocktail. The radioactivity of the free FAs was measured in the TRI-CARB 2300 TR liquid scintillation analyzer. To determine the specific substrate activity, 10 µl TG substrate were measured via liquid scintillation. The activity of the substrate was determined as counts per minute (cpm/nmol FS).

4.6.3. MGH-Assay

To measure MG hydrolase activity, different amounts of protein were incubated with the MG substrate and the released free FA were measured using the commercially available

NEFA C Kit. Briefly, 10 µg protein in a volume of 50 µl HSL buffer were incubated with 50 µl MG substrate for 30 minutes in a water bath at 37°C under constant shaking. The MG substrate consisted of 2 mM racemic 1,3-oleoylglycerol in 200 mM KPI buffer pH 7.0 containing 1 mM EDTA, 6.4 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 600 mM NaCl and was prepared by sonication. LacZ containing COS-7 lysates were used as a negative control and MGL containing COS-7 lysates were used as a positive control. Each reaction was carried out in triplicates. The reaction was stopped by heat inactivation at 75°C for 10 minutes. The release free FAs were measured enzymatically from aliquots using the commercially available NEFA C Kit. Briefly, 50 µl of each sample were pipetted into separate wells of a 96-well plate. Each sample was measured as duplicates. 150 µl NEFA 1 were added and incubated for 10 minutes at 37°C. Afterwards 75 µl NEFA 2 were added and the absorbance was measured at 562 nm. A glycerol stock solution was used to generate a calibration curve (25 nmol; 12.5 nmol; 6.25 nmol; 3.13 nmol; 1.56 nmol; 0.78 nmol).

4.6.4. Substrate screen

This method was used to identify additional, potential substrates of Ces2c. The solvents of the substrate solutions were brought to dryness under a stream of nitrogen. The dried substrate was dissolved in Substrate-no-Buffer (5 mM CHAPS, 1 mM EDTA, 100 mM KCL, 2.5% BSA) to achieve a concentration of 2.5 mM. The resuspended substrate was sonicated for 5 seconds with low amplitude. For the assay, 800 µg protein in a total volume of 1.2 ml BTP buffer (Bis-Trispropan 125 mM pH 7) were used. The Assay was performed in a 96-well, flat-bottomed microtiter plate. To each microtiter plate well, 10 µl of the sample (15 µg protein), standard or blank (BTP) was added manually. 10 µl of the different substrates (Table 10) were added and incubated for 30 minutes at 37°C. Each sample was measured as duplicates. The release free FAs were measured enzymatically from aliquots using the commercially available NEFA C Kit. 150 µl NEFA 1 added and incubated for 10 minutes at 37°C. Afterwards 75 µl NEFA 2 were added and the absorbance was measured at 562 nm.

Table 10: Different substrates

The substrates were prepared at a concentration of 2.5 mM.

1,2-dioctanyl-*sn*-glycero-3-phosphocholine	PC-C8
1,2-dioleoyl-*sn*-glycero-3-phosphate (sodium salt)	PA
1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine	PE
1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt)	PG
1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt)	PS
1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol) (ammonium salt)	PI
1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (sodium salt)	CL
sn-[2,3-dioleoyl]-glycerol-1-phospho-*sn*-1'-[2',3'-dioleoyl]-glycerol (ammonium salt)	BDP (S,S)
1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-arachidonoyl (ammonium salt)	NAPE
1-(1Z-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphocholine	PLasmPC
1,2-dioctanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt)	MCPG
1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine	LPC
1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (sodium salt)	LPA
1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine	LPE
1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt)	LPG
1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-L-serine (sodium salt)	LPS
L-α-lysophosphatidylinositol (Liver, Bovine) (sodium salt)	LPI
sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-1'-(3'-oleoyl-2'-hydroxy)-glycerol (ammonium salt)	BMP(S,s)
Methyl-palmitate	MP
Ethyl-palmitate	EP
2-propyl palmitate	PP
1-Butyl palmitate	BP
1-(9Z-octadecenoyl)-rac-glycerol	MO
1-2-dioleoyl-*sn*-glycerol	DO (rac)
1,2,3-(9Z-octadecenoyl)-glycerol	TO-C18:1
Glyceryl trioctanoate	TO-C8
retinyl palmitate	RE
cholest-5-en-3ß-yl octadecanoate	CO

Arachidyl laurate	WE
1,2-diacyl-3-O-β-D-galactosyl-*sn*-glycerol	MGDG
1-O-hexadecyl-2-O-methyl-*sn*-glycerol (PMG)	PMG
1-oleoyl-N-heptadecanoyl-D-*erythro*-sphingosine	O-Ac-Cer

4.6.5. Acylcarnitine hydrolase activity

To verify the published (Furihata et al. 2003) acylcarnitine hydrolase activity of Ces2c, an *in vitro* assay was performed using again the commercially available NEFA C kit to measure the released free FA. 50 µg protein in 50 µl HSL buffer was incubated with 50 µl acylcarnitine substrate for 30 minutes in a water bath at 37°C under constant shaking. 2 mM palmitoyl-carnitine-chlorid was used as substrate and was either prepared by sonication in KPi buffer pH 7 or in assay buffer consisting of 200 mM KPi buffer pH 7.0 containing 1 mM EDTA, 6.4 mM CHAPS and 600 mM NaCl. LacZ containing COS-7 lysates were used as a negative control and liver lysates were used as a positive control. Each reaction was carried out in triplicates. The reaction was stopped by heat inactivation at 75°C for 10 minutes. The released free FAs were measured enzymatically from aliquots using the commercially available NEFA C Kit. Briefly, 50 µl of each sample were pipetted into separate wells of a 96-well plate. Each sample was measured as duplicates. 150 µl NEFA 1 added and incubated for 10 minutes at 37°C. Afterwards 75 µl NEFA 2 were added and the absorbance was measured at 562 nm. A glycerol stock solution was used to generate a calibration curve (25 nmol; 12.5 nmol; 6.25 nmol; 3.13 nmol; 1.56 nmol; 0.78 nmol).

4.6.6. Phospholipase activity assay

To check for possible Phospholipase hydrolase activity of Ces2c an *in vitro* assay was performed using the commercially available NEFA C kit to measure the released free FA. Therefore 50 µg protein in 100 µl HSL buffer were incubated with 100 µl phospholipase substrate for 1 hour in a water bath at 37°C under constant shaking. The substrate consisted of 1 mM dioleoyl–PC in 100 mM KPi buffer pH 7 with 2% BSA. Briefly the dioleoyl-PC substrate, which was stored in $CHCl_3$, was brought to dryness under a stream of nitrogen. The dried substrate was then dissolved in KPi buffer and sonicated three times for 30 seconds with 30 seconds pause in between. After the sonication BSA was added to achieve a concentration of 2%. LacZ containing COS-7 lysates were used

as a negative control. Each reaction was carried out in triplicates. The released free FAs were measured enzymatically from aliquots using the commercially available NEFA C Kit. Briefly, 50 µl of each sample were pipetted into separate wells of a 96-well plate. Each sample was measured as duplicates. 150 µl NEFA 1 added and incubated for 10 minutes at 37°C. Afterwards 75 µl NEFA 2 were added and the absorbance was measured at 562 nm. A glycerol stock solution was used to generate a calibration curve (25 nmol; 12.5 nmol; 6.25 nmol; 3.13 nmol; 1.56 nmol; 0.78 nmol).

4.6.7. p-Nitrophenyl assays

To measure esterase activity spectrophotometric assays, using p-nitrophenyl esters of various chain length FAs as substrate, were performed. Short chain esters are water-soluble and therefore their hydrolysis provides a measure of esterase activity. The assays are based on the release of p-nitrophenolat, which is measured spectrophotometrically at 405 nm.

4.6.7.1. p-Nitrophenylacetat (PNPA) assay

100 µg protein in 100 µl HSL buffer were pipetted into a 96-well plate. The samples were assayed as triplicates whereby LacZ was used as negative control. 100 µl PNPA substrate were added to the sample and incubated for 1 minute at 37°C. The substrate consisted of 3 mM PNPA in 100 mM KPi pH 7.4. The extinction was measured at 405 nm.

4.6.7.2. p-Nitrophenylvalerate (PNPV) assay

100 µg protein in 50 µl HSL buffer were pipetted into a 96-well plate. The samples were assayed as triplicates whereby LacZ was used as negative control. 200 µl PNPV substrate were added to the sample and incubated for 1 minute at 37°C. The substrate consisted of 3 mM PNPV in 75 mM Tris/HCL buffer pH 8.0. The extinction was measured at 405/620 nm.

4.6.8. Determination of the optimal multiplicity of infection (MOI) for adenovirus infection

To analyze the influence of Ces2c overexpression on intracellular TG/CE levels (HepG2, McA-RH777 and AML12) and TG/FA/CE secretion (McA-RH77, AML12) AML12 or

HepG2 cells were transduced with different adenoviruses (LacZ, Ces2c, ATGL). Prior to experiments the optimal MOI for adenovirus transduction was determined.

The MOI describes the number of virus particles needed to infect one cell and differs greatly between different cell types. Therefore, it is necessary to determine the optimal MOI when transducing cells for the first time. To determine the amount of virus needed for the transduction of a 6-well with a specific MOI the following formula was used:

$$virus\ amount\ (ml) = \frac{desired\ MOI * number\ of\ cells}{pfu/ml}$$

Depending on the cell line used, the cells were seeded into 6-well plates at different densities (Table 8). Prior to infection the medium was aspirated and substituted with 500 µl DMEM or DMEM/F12$^{-/-}$, containing the previously calculated amount of adenovirus. After 2 hours of incubation the medium was removed and replaced with 2 ml DMEM$^{+/+}$ or DMEM/F12$^{+/+/+/+}$. 1 or 2 days after transduction, the cells were harvested with 200 µl 1x SDS and 20 µl of the sample were used for western blot analysis. The lowest MOI at which all cells show transgene expression was then used for further experiments.

4.6.9. Microscopy in COS-7 cells

To assess Ces2c localization, microscopy was performed using a GFP-tagged Ces2c construct (see Table 6). Briefly, 24 hours prior to transfection the cells were treated with trypsin and seeded into 8-well plates or 50 mm dishes at a density of either 15.000 cells (8-well plate) or 225.000 cells (50 mm dish).

The cells were transfected with either Ces2c-GFP or co-transfected with Ces2c-GFP and the ER-marker. To transfect or co-transfect an 8-well either 100 ng of the Ces2c-GFP construct or 50 ng of the Ces2c-GFP construct and 50 ng of the ER-marker were added to 50 µl DMEM$^{-/-}$. The DNA/medium mix was added to 1 µl Metafectene in 50 µl DMEM$^{-/-}$, mixed through inverting and incubated for 20 minutes at RT. In the meantime the culture medium of the cells was changed to 100 µl DMEM$^{+/-}$. 100 µl of the DNA/Metafectene mixture were added to the cells and incubated for 4 hours at 37°C. After 4 hours the transfection medium was substituted with 300 µl DMEM$^{+/+}$.

For the transfection or co-transfection of a 50 mm dish either 1500 ng of the Ces2c-GFP construct or 750 ng of the Ces2c-GFP construct and 750 ng of the ER-marker were added to 75 µl $DMEM^{-/-}$. The DNA/medium mix was added to 5.5 µl Metafectene in 75 µl $DMEM^{-/-}$, mixed through inverting and incubated for 20 minutes at RT. In the meantime the culture medium of the cells was changed to 750 µl $DMEM^{+/-}$. 150 µl of the DNA/Metafectene mixture were added to the cells and incubated for 4 hours at 37°C. After 4 hours the transfection medium was substituted with 3 ml $DMEM^{+/+}$.

24 hours after transfection the cells were incubated with or without 400 µM OA for 20 hours 20 minutes prior to microscopy LDs were stained using a LD marker diluted 1:100.

4.6.10. Measurement of intracellular TG-content in COS-7, HepG2, McA-RH77 and AML12 cells and TG secretion in AML12 and McA-RH777 cells

To validate the TG hydrolase activity of Ces2c observed in TGH *in vitro* assays (see 5.3 Ces2c-enriched cell lysates exhibit a 13 fold increase in TG hydrolase activity *in vitro*). OA pulse-chase analysis protocols were employed. The cells were either transfected or transduced and pulse labeled with ^{3}H labeled oleic acid followed by a variable-length chase period. Cellular and medium lipids were then extracted and run on a thin-layer chromatography plate. The TG spots were scraped of the plate and counted in the liquid scintillation counter. Additionally the protein content was measured to regard lost cells.

4.6.10.1. Transfection or transduction and OA treatment

COS-7 cells

Briefly, COS-7 cells were seeded into 6-well plates (Table 8), and transfected with either Ces2c, pFLAG or ATGL in triplicates. pFLAG was used as a negative control and ATGL as a positive control. To measure the TG content, transfected COS-7 cells were incubated with $DMEM^{+/+}$, to which 10% OA as well as 0.5 µCi/ml ^{3}H labeled OA were added. After 20 hours the medium was discarded and the cells were washed twice with 1 ml 1x PBS. The cells were chased in fresh $DMEM^{-/-}$ supplemented with 2% FA free BSA for 4 hours.

HepG2, McA-RH777 and AML12 cells

To analyze the influence of Ces2c overexpression on intracellular TG/CE levels (HepG2, McA-RH777 and AML12) and TG/FA/CE secretion (McA-RH777, AML12), HepG2, McA-RH777 or AML12 cells were infected with different adenoviruses (Ad-LacZ, Ad-Ces2c, Ad-ATGL).

Depending on the cell line used, the cells were seeded into 6-well plates at different densities (Table 8). Prior to transduction, the medium was aspirated and substituted with 500 μl DMEM$^{-/-}$ or DMEM/F12$^{-/-/-/-}$, containing the previously calculated amount of adenovirus. After 2 hours of incubation the medium was removed and replaced with 2 ml DMEM$^{+/+}$ or DMEM/F12$^{+/+/+/+}$. 1 or 2 days after transduction the cells were used for different experiments.

HepG2 cells

Briefly, HepG2 cells were seeded into 6-well plates (Table 8), and transduced with either Ad-Ces2c (500 MOI) or Ad-LacZ (100, 500 MOI) in triplicates. Ad-LacZ was used as a negative control. 24 hours after the transduction, HepG2 cells were incubated with DMEM$^{+/+}$, to which 400 nM OA as well as 0.5 μCi/ml ^{3}H labeled OA were added. After 20 hours the medium was discarded and the cells were washed twice with 1 ml 1x PBS. One set of 6-well plates was used for intracellular lipid extraction. The other set of 6-well plates was chased in fresh DMEM$^{-/-}$ supplemented with 2% FA free BSA for 4 hours before intracellular lipids were extracted.

AML12 cells

Briefly, AML12 cells were seeded into 6-well plates (Table 8), and transduced with either Ad-Ces2c (1000 MOI) or Ad-LacZ (20 MOI) in sextaplucates. Ad-LacZ was used as a negative control. 24 hours after the transduction AML12 cells were incubated with DMEM/F12$^{+/+/+/+}$, to which 400 nM OA as well as 0.5 μCi/ml ^{3}H labeled OA were added. After 4 hours or 20 hours of pulse labeling the medium was collected and extracted (see 4.6.10.3 Extraction of medium lipids). The cells were washed twice with 1 ml 1x PBS and chased in fresh DMEM/F12$^{-/-/-/-}$ supplemented with 2% FA free BSA for 6 hours. After different time periods the medium was collected and extracted. The cells were washed twice with 1 ml 1x PBS and 1 ml DMEM/F12$^{-/-}$ supplemented with 2% FA free BSA was added.

McA-RH777 cells

Briefly, McA-RH777 cells were seeded into 6-well plates (Table 8), and transduced with either Ad-Ces2c (500 MOI) or Ad-LacZ (20 MOI) in sextaplucate. Ad-LacZ was used as a negative control. 24 hours after the transduction McA-RH777 cells were incubated with $DMEM^{+/+}$, to which 400 nM OA as well as 0.5 μCi/ml ^{3}H labeled OA were added. After 20 hours the medium was collected and extracted. The cells were washed twice with 1 ml 1x PBS and chased in fresh $DMEM^{-/-}$ supplemented with 2% FA free BSA for 6 hours. After different time periods the medium was collected and extracted. The cells were washed twice with 1 ml 1x PBS and 1 ml $DMEM/F12^{-/-}$ supplemented with 2% FA free BSA was added.

4.6.10.2. Extraction of intracellular lipids

After the chase period the cells were washed twice with 1x PBS. To extract the cellular lipids 1 ml freezing Hexane/Isopropanol (3:2) was added to each well. After shaking for 5 minutes at RT the supernatant was transferred into a 2 ml Eppi. The extraction was repeated under the same conditions. The supernatant of the second extraction was unified with the first. To eradicate the solvent the collected supernatants were dried under a stream of nitrogen. The samples were stored at -20°C until they were used for thin layer chromatography (TLC).

4.6.10.3. Extraction of medium lipids

The previously collected medium was centrifuged at 1000 g for 10 minutes to remove dead cells. The supernatant was transferred to a 12 ml tube and extracted according to the method of Folch (Folch et al. 1957). Briefly, 5 volumes of chloroform/methanol/glacial acetic acid (66/33/1 v/v) were added to 1 volume medium, briefly vortexed and centrifuged at 3000 rpm for 15 minutes. The aqueous phase was removed and the organic phase was dried under N_2.

4.6.10.4. TLC

The TLC chamber was saturated for 1 hour with the solvent (hexan:diethylether:glacial acetic acid, 70:29:1). The TLC plates were cut in half and a straight line was drawn across the plate with a pencil. 10 μl of inert standard were spotted on the drawn line. The dried lipids were re-suspended in 50 μl chloroform and applied to the spots of inert standard. The solvent was completely evaporated off. The TLC plate was placed in the

chamber so that the spots of the samples did not touch the surface of the eluent in the chamber. The plate was removed from the chamber before the solvent front reached the top. The solvent was completely evaporated off before the plates were placed in a jar containing iodine crystals. The separated lipid classes were immediately marked with a pencil. The marked TG bands were cut out after iodine evaporated, transferred to 8 ml of scintillation cocktail and counted in the beta counter. The counts per minute were normalized to the protein concentration.

4.6.10.5. Measurement of total protein concentration

To determine the total protein concentration, 1 ml of 1% SDS 0.3 M NaOH was added to each well of the 6-well plates. The plates were pivoted for 6 hours at RT. The total protein concentration was measured using BCA Protein assay kit (see 4.4.1 BCA Protein Assay).

5. Results

5.1. Cloning of Ces2c

To characterize the murine Ces2 family member Ces2c, the CDS was amplified via PCR from stomach cDNA and inserted into the pFLAG-CMV-5.1 vector. The vector is used to express proteins in mammalian systems, while adding a C-terminal FLAG-tag, which is used for protein detection. Since most Ces possess an N-terminal ER signal sequence, a C-terminal tag was used to ensure proper localization and to avoid potential disturbance of the signal sequence.

To amplify Ces2c CDS via PCR, a specific PCR program (see Table 4) and primers (see 3.3 Primers) were used. The resulting PCR products (Figure 12 A) were separated by agarose gel electrophoresis and eluted from the gel. Afterwards, the cDNA and the pFLAG-CMV-5.1 vector were digested with the restriction enzymes *XbaI* and *EcoRI*-HF (Figure 12 B). The digested insert and vector were purified using Gel-extraction kit (see 3.10 Kits) and ligated. After transformation into *E. coli* DH5α cells, the recombinant plasmid was isolated using a standard protocol. To verify successful ligation of the insert with the vector, the plasmid DNA was digested using *EcoRI*-HF and *XbaI* and separated by gel electrophoresis (Figure 12 C). One positive clone was picked and sequenced by Microsynth (Balgach, Switzerland).

Sequencing results were analyzed using ClustalW2. Therefore the CDS of Ces2c was aligned with the sequencing result, to verify the correct insertion and the absence of mutations. To isolate the plasmid on a larger scale, a Maxiprep was performed. The resulting plasmid was again sequenced.

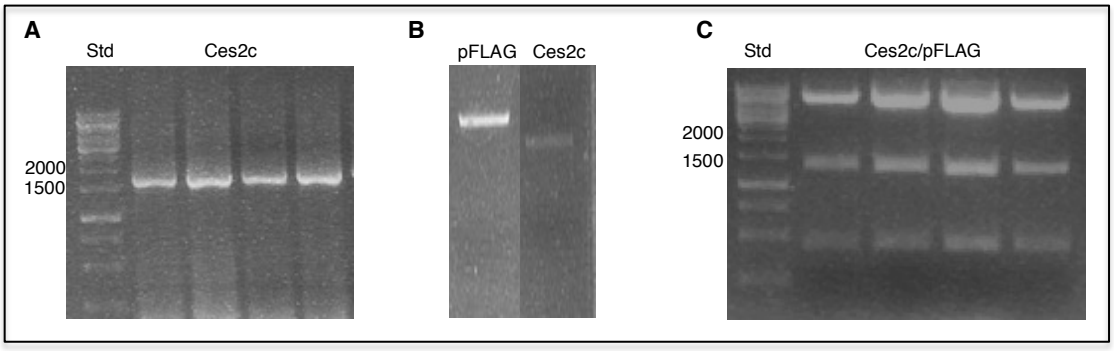


Figure 12: Amplification and restriction enzyme digest of Ces2c cDNA

A) Ces2c was amplified from stomach cDNA via PCR. The PCR products were separated by gel electrophoresis using a 1.5% agarose gel. Ces2c was detected at the expected size of 1685 bp.

B) Ces2c PCR products were purified from the agarose gel and digested with the restriction enzymes *XbaI* and *EcoRI*-HF at 37°C for 2 hours.

C) The digested PCR products and the digested vector were ligated for 2 hours at 22°C. The vector/DNA construct was transformed into *E. coli* DH5α cells. The transformed cells were selected on ampicillin containing LB-agar plates. To verify the presence and the correct size of the insert DNA, 4 plasmids were isolated via Miniprep and digested using *XbaI* and *EcoRI*-HF. The DNA fragments were separated by gel electrophoresis using a 1.5% agarose gel and visualized under UV-light.

5.2. Validation of Ces2c expression on the protein level

To validate protein expression of Ces2c, COS-7 cells were transfected with the plasmid obtained by Maxiprep isolation. The lysates were then used for western blot analysis (Figure 13). The signal obtained for the recombinant protein correlates with the expected size of Ces2c (59.5 kDa).

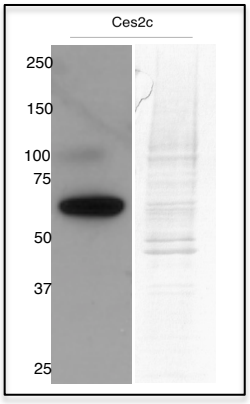


Figure 13: Western blot analysis of Ces2c protein expression in transfected COS-7 cells

COS-7 cells were transfected using Ces2c/pFLAG. The cells were harvested and sonicated. 20 µg protein was separated by SDS-Page and blotted onto a nylon membrane. FLAG-tagged proteins were detected using anti Flag antibody. Coomassie Blue staining of the membrane confirmed successful protein transfer.

5.3. Ces2c-enriched cell lysates exhibit a 13-fold increase in TG hydrolase activity *in vitro*

To investigate whether Ces2c exhibits TG hydrolytic activity, *in vitro* assays were performed using cell lysates prepared from transfected COS-7 cells. For that purpose, COS-7 cells were transfected with either Ces2c/pFLAG or LacZ/HisMax, as negative control. TG hydrolase activity assays were carried out using a micellar TG substrate including ^{3}H-labeled triolein as tracer. Briefly, TG and triolein were mixed in an assay tube and brought to dryness under a stream of nitrogen. The dried substrate was dissolved in assay buffer (200 mM KPi, 50 mM CHAPS) and sonicated to generate a micellar substrate.

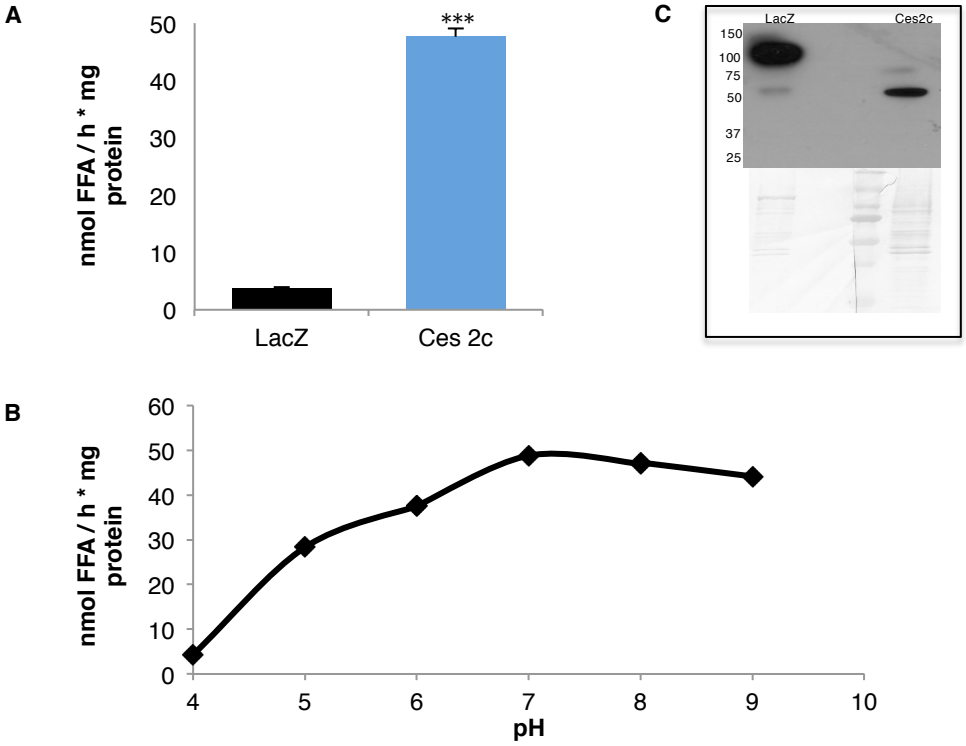


Figure 14: Ces2c-enriched cell lysates exhibit a 13-fold increase in TG hydrolase activity *in vitro*

A) COS-7 cells were transfected with Ces2c/pFLAG or LacZ/HisMax. For each reaction, 50 µg protein solubilized in 100 µl HSL buffer was incubated with 100 µl ^{3}H-labeled TG substrate at 37°C for 1 hour. FAs were extracted after termination of the reaction and counted via liquid scintillation. Data are shown as means ± standard derivation of n = 3. Statistical significance was analyzed by Student´s t-test ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***).

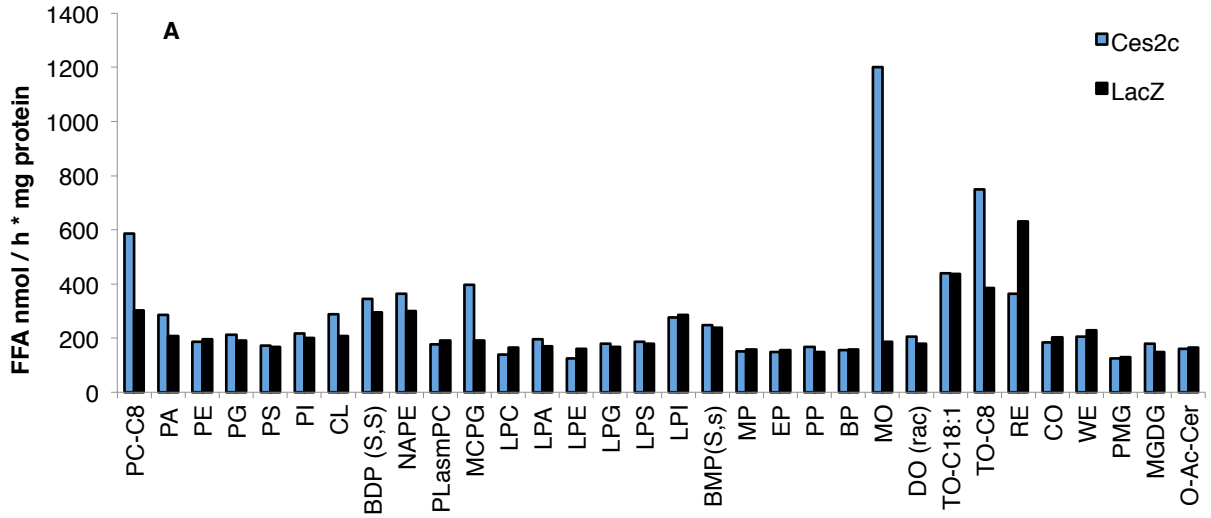
B) pH optimum curve for Ces2c TG-hydrolytic activity.

C) Protein levels were examined by western blot analysis to confirm the expression of Ces2c/pFLAG and LacZ/HisMax. 20 µg protein was loaded onto the gel. Coomassie Blue staining of the membrane confirmed successful protein transfer. FLAG-tagged proteins were detected using an anti-Flag antibody. To detect His-tagged proteins, a primary anti-His antibody and secondary anti-Mouse HRP conjugated antibody was used.

As shown in Figure 14 A, Ces2c-enriched cell lysates exhibited a 13-fold increase in neutral TG hydrolase activity compared to LacZ containing cell lysates. To determine the pH optimum for Ces2c enzymatic activity, *in vitro* TG hydrolase activity assays were performed using different buffers. Therefore 50 µg protein in 100 µl HSL buffer were mixed with 50 µl buffer ranging from pH 4 to pH 9 (see 4.6.2 pH optimum). The assays were performed using a micellar TG substrate with ^{3}H-labeled triolein as tracer. Ces2c exhibits a broad pH optimum ranging from pH 7 to 9, with maximum activity at pH 7 (Figure 14 B).

5.4. Ces2c substrate screen

To search for additional Ces2c substrates, a screen applying several lipid substrates was performed using Ces2c-enriched COS-7 cell lysates (see 4.6.4 substrate screen). Briefly, 10 µg protein in 15 µl BTP buffer were incubated with 10 µl substrate (32 different substrates; Table 10) and released FAs were measured with a commercially available kit (Figure 15 A).



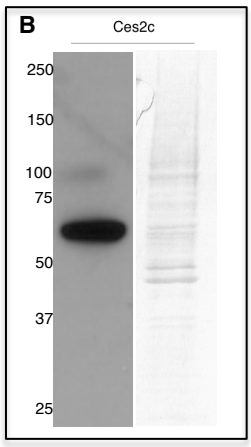


Figure 15: Ces2c exhibits hydrolytic activity toward PC, TO-C8 and MGs

A) COS-7 cells were transfected with the Ces2c/pFLAG expression plasmid and Ces2c-enriched cell lysates were incubated with several lipid substrate. 10 µg protein in 15 µl BTP buffer was incubated with 10 µl of a specific lipid substrate at 37°C for 30 minutes and released FAs were measured t. Data are shown as means of n = 2.

B) Protein levels were examined by western blot analysis to confirm expression of Ces2c/pFLAG. 20 µg protein were used for western blot analysis. Coomassie Blue staining of the membrane confirmed successful protein transfer. FLAG-tagged proteins were detected using an anti-Flag antibody.

Analysis of the different lipid substrates indicates that Ces2c exhibits potential hydrolytic activity towards PC-C8, TO-C8 and MGs (Figure 15 A).

5.5. Ces2c exhibits hydrolytic activity toward MGs *in vitro*

The marked increase in hydrolyzing MGs in the substrate screen approach prompted me to further investigate MG hydrolytic activity. Therefore, an *in vitro* assay was performed using racemic 1,3-oleoylglycerol as substrate incubated with a Ces2c-enriched COS-7 cell lysate (Figure 16 A). As a control, a cell lysate enriched with recombinant MGL was assayed. Briefly, 10 µg protein in 50 µg HSL buffer were incubated with 50 µl 1,3-oleoylglycerol substrate and released FAs were measured enzymatically (see 4.6.3 MGH Assay). Ces2c-enriched COS-7 cell lysates exhibited a significant increase in MG hydrolase activity compared to LacZ-enriched lysates. However, hydrolytic activity of Ces2c towards the MG substrate was moderate compared to the activity of MGL-enriched cell lysates (Figure 16 A).

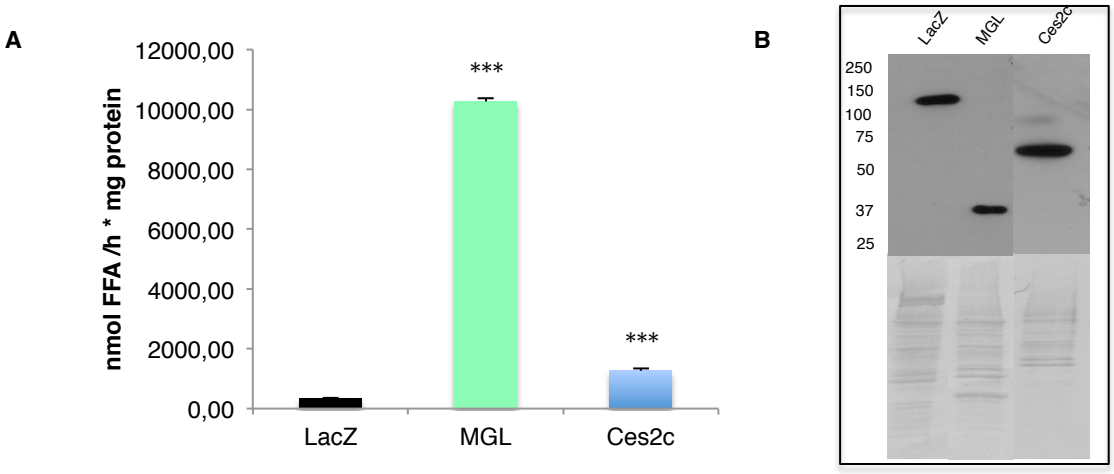


Figure 16: Ces2c exhibits moderate hydrolytic activity towards MGs compared to MGL

A) COS-7 cells were transfected with Ces2c/pFLAG, LacZ/HisMax and MGL/HisMax to overexpress the respective protein. For each reaction, 10 µg protein in 50 µl HSL buffer were incubated with 50 µl MG substrate at 37°C for 30 minutes. Released FAs were measured enzymatically using a commercial kit. Data are shown as means ± standard deviation of n = 3. Data are shown as mean + SD. $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ *** compared to LacZ.

B) Expression of recombinant proteins was examined by western blot analysis. 20 µg protein were used for western blot analysis. Coomassie Blue staining of the membrane confirmed successful protein transfer. FLAG-tagged proteins were detected using an anti-Flag antibody. To detect His-tagged proteins a primary anti-His antibody and secondary anti-Mouse HRP conjugated antibody was used.

5.6. Ces2c does not exhibit *in vitro* phospholipase activity

Furthermore, Ces2c was examined for phospholipase activity. For that purpose, COS-7 cells were transfected with either Ces2c/pFLAG or LacZ/HisMax and cell lysates were generated. Dioleoyl-PC was used as substrate and released FAs were measured (Figure 17 A). Ces2c-enriched COS-7 cell lysates exhibited no significant difference in phospholipase activity compared to LacZ-enriched lysates (Figure 17 A).

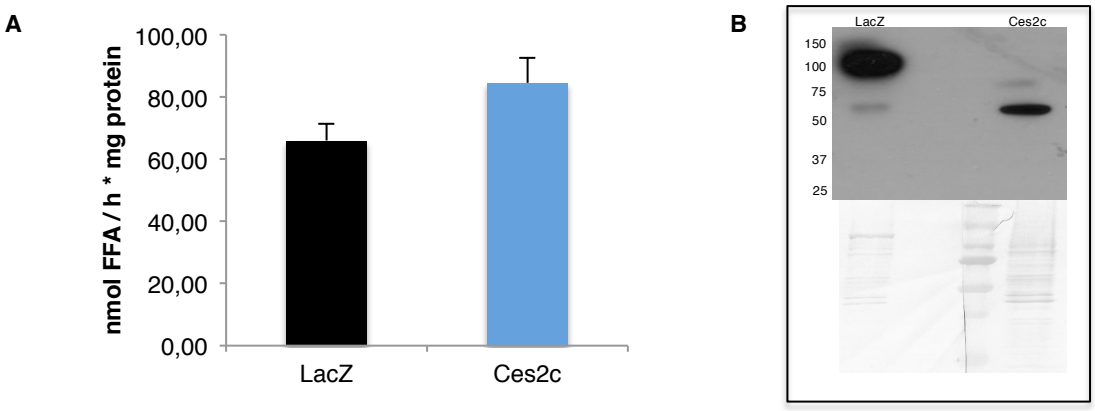


Figure 17: Ces2c does not exhibit phospholipase activity

A) COS-7 cells were transfected with Ces2c/pFLAG and LacZ/HisMax as control. For each reaction, 50 μg protein in 100 μl HSL buffer were incubated with 100 μl phospholipid substrate for 1 hour at 37°C. Released FAs were measured enzymatically. Data are shown as means ± standard deviation of n = 3. $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

B) Expression of recombinant proteins was examined by western blot analysis. 20 μg protein was loaded onto the gel and Coomassie Blue staining of the membrane confirmed successful protein transfer. Recombinant proteins were detected using an anti-Flag and anti-His antibody, respectively.

5.7. Ces2c-enriched cell lysates exhibit hydrolytic activity towards acylcarnitine

Ces2c was originally described as an acylcarnitine hydrolase ((Furihata et al. 2003). To confirm this finding, *in vitro* assays were performed using palmitoyl-carnitine-chloride as substrate. Briefly, 50 μg protein in 50 μl HSL buffer were incubated with 50 μl substrate and FA release was measured enzymatically (see 4.6.5 Acylcarnitine hydrolase activity assay).

Compared to LacZ-enriched COS-7 cell lysates, lysates prepared from Ces2c overexpressing cells exhibited a significant increase in acylcarnitine hydrolytic activity

and consequently the release of FAs from the substrate (Figure 18 A). Liver cell lysates were used as positive control.

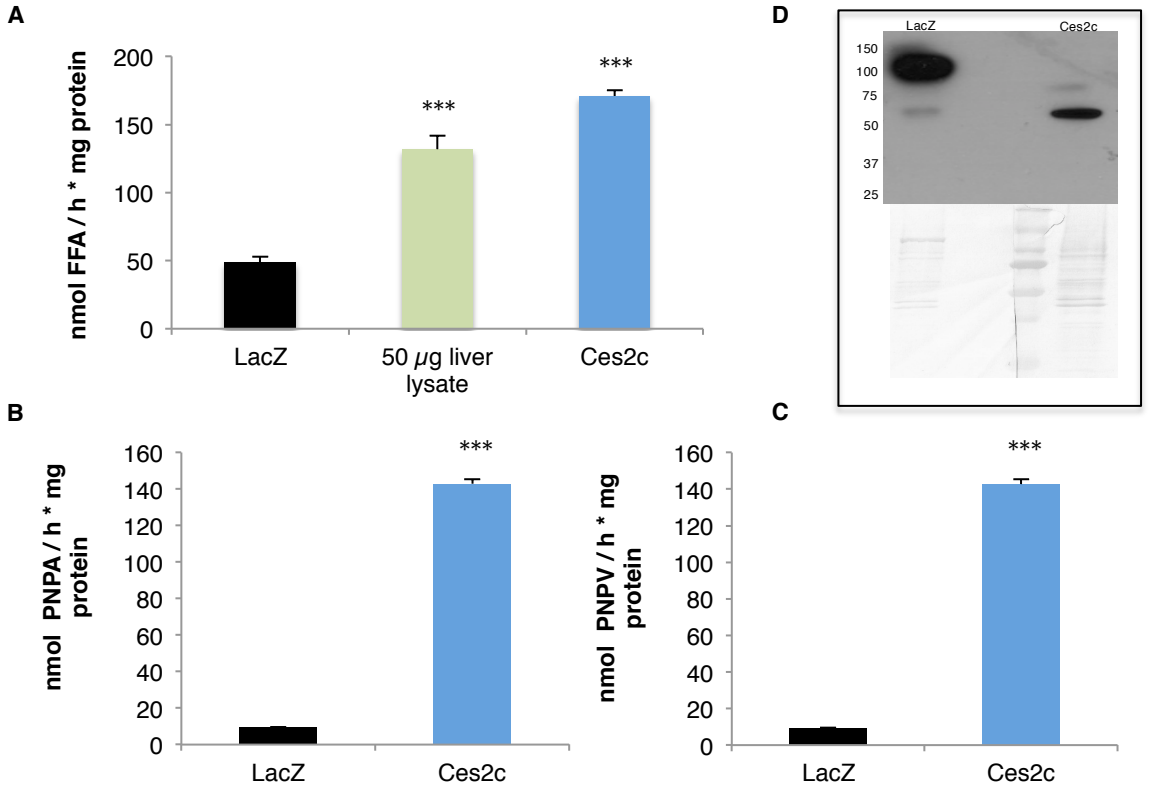


Figure 18: Ces2c exhibits hydrolytic activity towards acyl-carnitine, PNPA and PNPV

A) COS-7 cells were transfected with Ces2c/pFLAG and LacZ/HisMax for the subsequent preparation of cell lysates with enrichment of the respective proteins. For the acylcarnitine hydrolase assay 50 µg protein in 50 µl HSL buffer were incubated with 50 µl acylcarnitine substrate. Released FAs were measured enzymatically. Data are shown as means ± standard deviation of n = 3. $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

B) 100 µg protein in 100 µl HSL buffer were mixed with 100 µl PNPA substrate and incubated for 1 minute at 37°C. FA release was determined enzymatically. Data are shown as means ± standard derivation of n = 3.

C) 100 µg protein in 50 µl HSL buffer were incubated with 200 µl PNPV substrate for 1 minute at 37°C. Released FAs were measured enzymatically Data are shown as means ± standard derivation of n = 3.

D) Protein levels were examined by western blot analysis to confirm expression of Ces2c/pFLAG and LacZ/HisMax. 20 µg protein was loaded onto the gel and Coomassie Blue staining of the membrane confirmed successful protein transfer. FLAG- and His-tagged proteins were detected using anti Flag antibody and anti-His antibody, respectively.

Moreover, enzymatic activity towards PNPA and PNPV, which are artificial esterase substrates, was measured. In brief, PNPA or PNPV were added to Ces2c-enriched COS-7 cell lysates and the formation of p-nitrophenol was determined by measuring the optical dense at 405 nm. Hydrolytic activity toward PNPA as well as PNPV was observed (Figure 18 B, C).

5.8. Studies investigating Ces2c localization

5.8.1. Ces2c localizes to the ER

CEs are in general localized to the ER and typically contain a N-terminal hydrophobic signal peptide, which marks them for trafficking through the ER. Additionally, CEs harbor a C-terminal consensus sequence, which ensures retention within the ER. Confocal laser scanning live cell imaging was performed to examine the localization of Ces2c.

For that purpose, GFP-tagged Ces2c was co-expressed with an ER-marker protein in COS-7 cells. To prevent the GFP-tag from masking the ER retention signal (HREL) the GFP-tag was added N-terminal to the ER retention signal. Additionally, GFP-tagged Ces2c, transfected cells were incubated with 0.4 mM oleic acid to induce LD formation, which may be affected by Ces2c overexpression. LDs were stained by incubating the cells with Lipid TOX^{TM} Deep Red Neutral Lipid stain (Invitrogen, Carlsbad, USA).

GFP-tagged Ces2c co-localized with the ER marker protein (Figure 19), supporting the concept that Ces2c localizes to the ER. The expression of Ces2c had no apparent influence on the number of LDs in COS-7 cells. However, confocal laser scanning live cell imaging might not be a suitable method, since changes in LD number are quantified by eye and are therefore rather subjective.

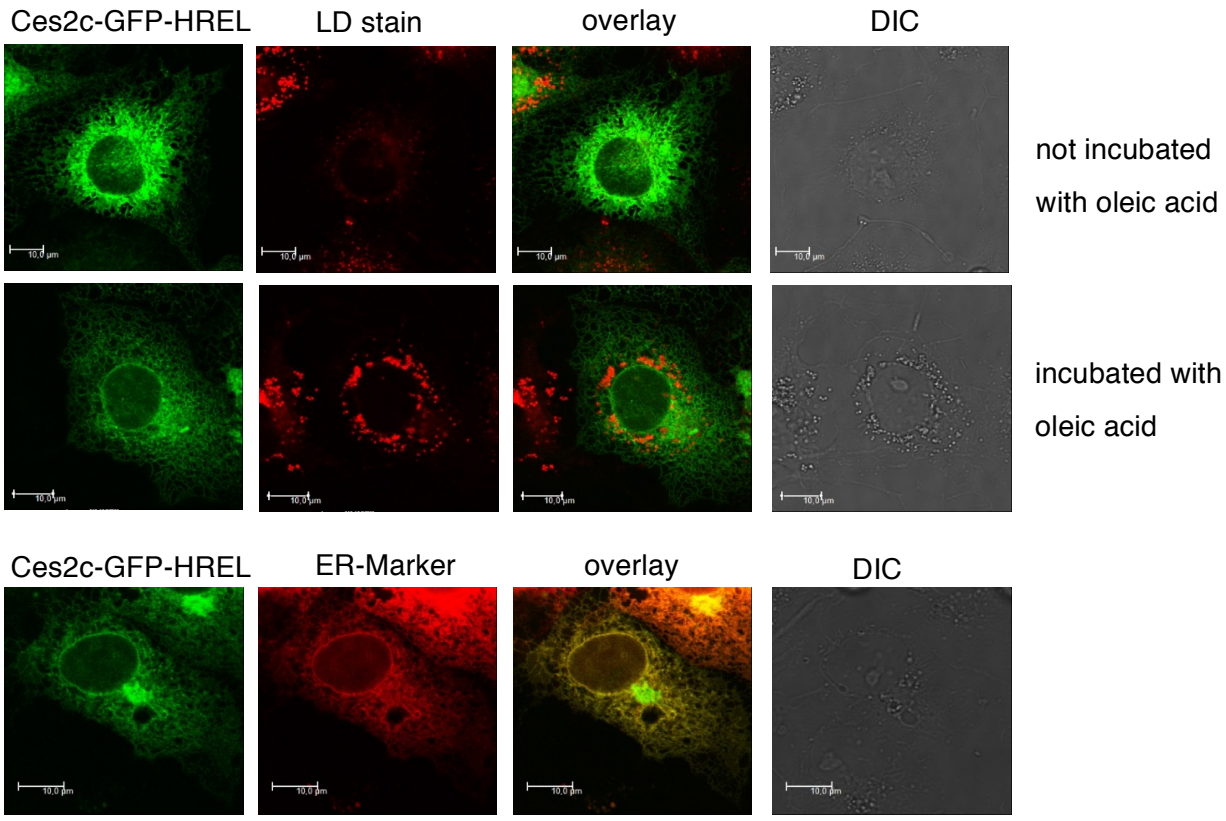


Figure 19: Ces2c localizes to the ER

COS-7 cells were either transfected with GFP-tagged Ces2c or co-transfected with the GFP-tagged Ces2c and an ER marker protein. GFP-tagged Ces2g was used as control (data not shown). LD formation was induced by incubation with 0.4 mM oleic acid for 20 hours. The subcellular localization of the proteins was determined by confocal laser-scanning live cell imaging. Green fluorescence of GFP-tagged Ces2c is shown in the first row. The second row either shows lipid staining or fluorescence of the ER marker. LDs were visible by staining with LipidTOX^{TM} Deep Red. Overlay images are shown in the third row and indicate co-localization of GFP-tagged Ces2c with the ER marker. DIC images are shown on the outermost row. Scale bar: 10 µm.

5.8.2. Cell fractionation to further examine Ces2c localization in COS-7 cells

To confirm data obtained by confocal laser scanning live cell imaging, cell fractionation experiments were conducted. Therefore COS-7 cells were transfected with FLAG-tagged Ces2c, LacZ/HisMax (negative control) and GFP-tagged Ces2c (positive control). Membrane and cytosolic fractions were isolated and the protein levels were determined by western blot analysis. To verify the purity of cytosolic and ER fractions, western blot analysis was performed using an anti-GAPDH antibody (as cytosolic marker) and an anti-Ireα antibody (ER marker), respectively.

As shown in Figure 20, the cell fractionation delivered no clear data about Ces2c localization. Neither FLAG-tagged Ces2c nor GFP-tagged Ces2c was enriched in the membrane fraction (Figure 20 A). Moreover, both, FLAG-tagged and GFP-tagged Ces2c were equally distributed in the cytosolic and membrane fraction, respectively (Figure 20 A).

Considering that cytosolic GAPDH was equally abundant in the cytosolic and membrane fraction indicates cross contaminations during preparation of cellular fractions (Figure 20 B). To further validate the purity of the fraction an anti-Ireα antibody was used as a marker for membrane proteins. However, Ireα could not be detected may due to a defective antibody. Experiments were repeated and showed a similar picture.

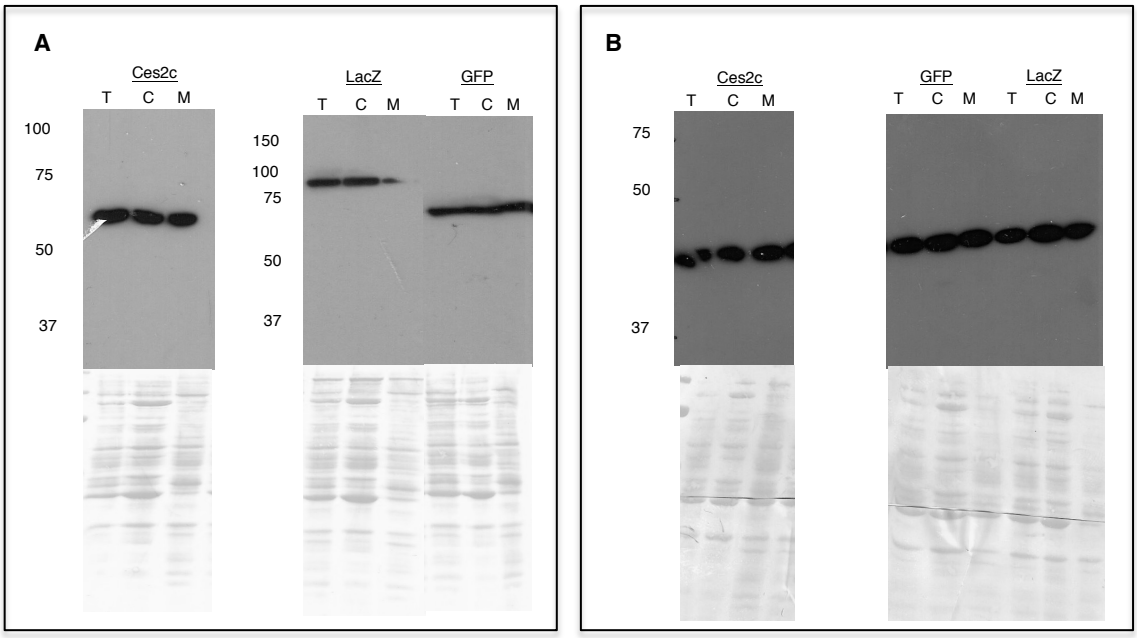


Figure 20: Cell fractionation to further address cellular localization of Ces2c

A) COS-7 cells were either transfected with FLAG-tagged Ces2c, GFP-tagged Ces2c (positive control) and LacZ/HisMax (negative control). Western blot analysis was performed with membrane and cytosolic fractions using 7.5 µg protein respectively. Coomassie Blue staining of the membrane confirmed successful protein transfer.

B) To validate the purity of the fractions and GAPD protein expression was determined as a cytosolic marker.

5.9. Cloning of Ces2a, Ces2b, Ces2e and Ces2f

The murine Ces2 family comprises of 7 genes with highly conserved DNA sequences. To determine if homologous Ces2 family members also exhibit TG hydrolase activity *in vitro*, or if the activity is specific for Ces2c, the CDS of Ces2a, Ces2b, Ces2e and Ces2f was

amplified via PCR from different tissues (see Table 5 & 6) and inserted into the pFLAG-CMV-5.1 vector. Ces2b shares 91% identity with Ces2c whereas Ces2a, Ces2e, Ces2f and Ces2g share 82-85% identity.

Ces2 family members were amplified with CDS-specific primers and PCR products were separated and eluted from the agarose gels. Afterwards the cDNA and the pFLAG-CMV-5.1 vector were digested with the restriction enzymes *XbaI* and *EcoRI*-HF and fragments were ligated and transformed into *E. coli* DH5α cells. Recombinant plasmids were isolated and successful cloning was investigated by *EcoRI*-HF/*XbaI* digestion and separation of the generated fragments. Clones were sequenced and DNA was purified by Maxiprep.

In vitro TG hydrolase activity assays were performed using a micellar TG substrate with ^{3}H-labeled triolein as tracer (Figure 21 A). Therefore Ces2a, Ces2b, Ces2c, Ces2e, Ces2f, Ces2g and LacZ cell lysates were generated. Briefly, 50 µg protein in 100 µl HSL buffer were incubated with 100 µl radiolabeled triolein substrate for 1 hour. FAs were extracted and the radioactivity was counted.

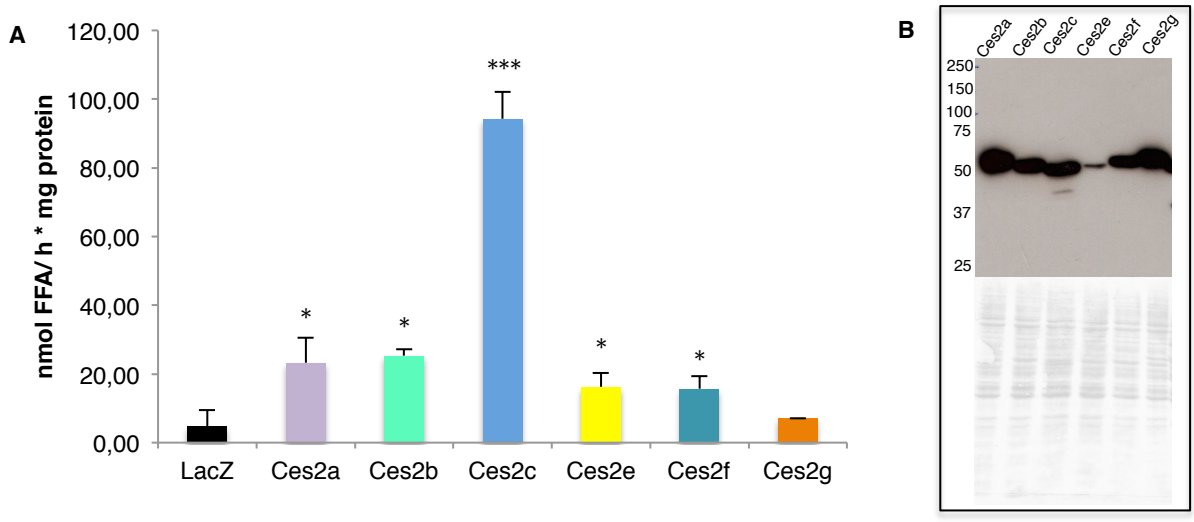


Figure 21: Measuring of TG-hydrolase activities of Ces2 family members

A) COS-7 cells were transfected with LacZ/HisMax, Ces2a/pFLAG, Ces2b/pFLAG, Ces2c/pFLAG, Ces2e/pFLAG, Ces2f/pFLAG, Ces2g/pFLAG. For each reaction 50 µg protein in 100 µl HSL buffer were incubated with 100 µl ^{3}H-labeled TG substrate at 37°C for 1 hour. FAs were extracted after the termination of the reaction and counted via liquid scintillation. Data are shown as means ± standard derivation of n = 3. $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

B) Protein levels were examined by western blot analysis to confirm expression of LacZ, Ces2a, Ces2b, Ces2c, Ces2e, Ces2f and Ces2g. 20 µg protein were used for western blot analysis. Coomassie Blue staining of the membrane confirmed successful protein transfer. FLAG- and His-tagged proteins were detected using specific antibodies.

Except for Ces2g-enriched COS-7 cell lysates, Ces2a-, Ces2b-, Ces2e- and Ces2f–enriched cell lysates showed a significant increase in TG hydrolase activity compared to LacZ control (Figure 21 A). However, activities were relatively low compared to Ces2c TG hydrolytic activity (15 fold increase). Western blot analysis confirmed equal expression of Ces2a, Ces2b, Ces2c, Ces2f and Ces2g (Figure 21 B). One should note that Ces2e was poorly expressed. Nonetheless, previous experiments applying higher protein concentrations of Ces2e showed relatively low TG-hydrolytic activities compared to the activity of Ces2c (data not shown).

5.10. Ces2c expression leads to decreased TG levels in COS-7 cells

To further study the role of Ces2c in lipid metabolism, the incorporation of radiolabeled FAs into cellular TGs was examined in COS-7 cells transfected with Ces2c (Figure 22 A). Briefly, COS-7 cells were seeded into 6-well plates and transfected with Ces2c/pFLAG, empty pFLAG as a negative control and ATGL/HisMax as a positive control. Subsequently, transfected cells were pulse labeled with ^{3}H-labeled OA for 20 hours and chased for 4 hours with BSA containing medium. Following the chase period, cellular lipids were extracted and TG levels were examined (Figure 22 A) (see 4.6.10 Measurement of intracellular TG-content in COS-7, HepG2, McA-RH77 and AML12 cells and TG secretion in AML12 and McA-RH777 cells).

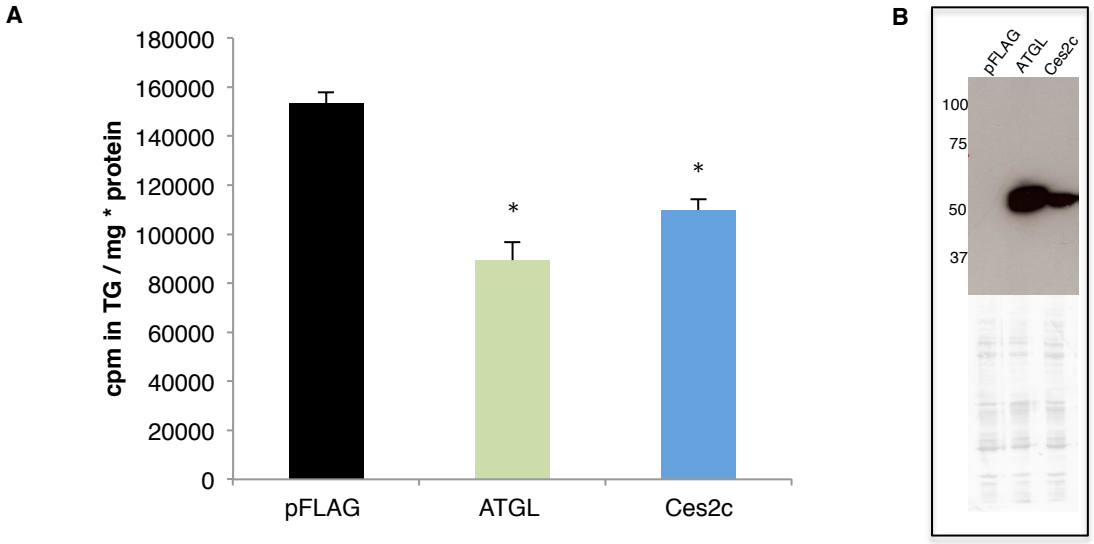


Figure 22: Ces2c expression in COS-7 cells leads to a significant decrease in TG levels

A) COS-7 cells were transfected with Ces2c/pFLAG, pFLAG (negative control) and ATGL/HisMax (positive control). The cells were pulse labeled with ^{3}H-labeled OA for 20 hours. Following pulse labeling, the cells were chased for 4 hours with DMEM$^{-/-}$ containing 2% BSA. TG levels were examined after the pulse labeling and the chase period. Data are shown as means ± standard derivation of n = 3. $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

B) Western blot analysis to confirm protein expression of Ces2c and ATGL. Cells were harvested after the chase period in 200 µl 1x SDS and 20 µl was used for western blotting. Coomassie Blue staining of the membrane confirmed successful protein transfer and the respective recombinant proteins were detected by antibodies specific for FLAG- and His-tagged recombinant proteins.

Results indicate that Ces2c participates in cellular TG-break down considering that incorporation of radioactivity was lower in Ces2c-transfected cells compared to LacZ control (Figure 22 A).

5.11. Generation of a Ces2c-recombinant adenovirus and infection of cell lines

5.11.1. Cloning of an Ces2c-recombinant adenovirus (Ad-Ces2c)

To further study the role of Ces2c in liver lipid metabolism, a recombinant adenovirus was generated using the pAdEasy™ XL Adenoviral Vector System from Stratogene (Santa Carla, USA). Therefore, the Ces2c/pFLAG construct was cloned into the pShuttle-CMV vector and subsequent homologous recombination transferred the insert into the pAdEasy-1 plasmid of *E. coli* BJ5183-AD-1 cells. The Ces2c/pAdEasy-1 construct was enriched in *E. coli* XL10 gold cells.

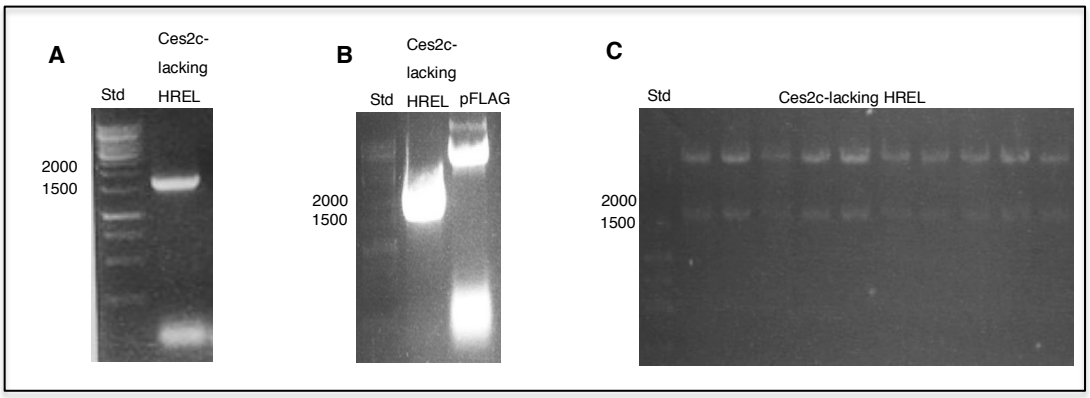


Figure 23: Amplification and restriction digest analysis of Ces2c lacking HREL

A) Ces2c lacking HREL was amplified from diluted Ces2c/pFLAG Maxiprep DNA via PCR. The PCR products were separated by gel electrophoresis using a 1.5% agarose gel. Ces2c was detected at the expected size of 1685 bp.

B) Ces2c PCR products were purified from the agarose gel and digested with the restriction enzymes *NotI*-HF and *XbaI* at 37°C for 2 hours.

C) The digested PCR products and the digested vector were ligated for 2 hours at 22°C. The vector/DNA construct was transformed into *E. coli* DH5α cells. To confirm proper cloning, plasmids were isolated and digested using *XbaI* and *NotI*-HF. The DNA fragments were separated by gel electrophoresis using a 1.5% agarose gel and visualized under UV-light.

To ensure Ces2c-localization to the ER, a deletion mutant of Ces2c, which carries the ER-retention signal C-terminal of the FLAG-tag was generated by PCR. In the first step, the Ces2c CDS lacking the sequence for the C-terminal ER retention signal (HREL) was amplified using specific primers (Table 4). The *NotI* restriction site was added to the fw primer and the *XbaI* restriction site to the rv primer. PCR products were separated by gel electrophoresis and purified from the gel (Figure 23 A). The pFLAG-CMV-5.1 vector and the purified PCR product were digested with *NotI*-HF and *XbaI* restriction enzymes (Figure 23 B). After the digestion, the vector and the PCR insert were separated by gel electrophoresis, ligated and transformed into *E. coli* DH5α cells. Proper cloning was confirmed by restriction enzyme digestion with *NotI*-HF and *XbaI* and separation of fragments via agarose gel electrophoresis (Figure 23 C).

A second PCR was performed to amplify the Ces2c CDS, lacking the ER retention signal sequence. For that purpose, Ces2c lacking HREL/pFLAG DNA was used as template. The reverse primer used for the PCR contained the FLAG-tag sequence followed by the ER retention sequence, a stop codon and an *EcoRV* restriction site. Purified PCR products and the pShuttle-CMV vector were digested using *NotI*-HF and *EcoRV*

restriction enzymes (Figure 24 A). After ligation the Ces2c/pShuttle construct was transformed into *E. coli* DH5α cells and proper cloning was confirmed by *NotI*-HF and *EcoRV* digestion and separation of the DNA fragments. The 1685 bp fragment (Figure 24 B) represents the insert and positive clones were sequenced by Microsynth (Balgach, Switzerland) (Figure 24 B).

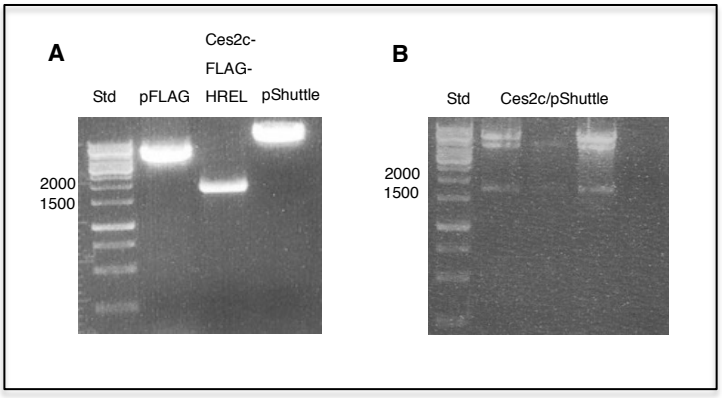


Figure 24: Amplification and restriction analysis of Ces2c-FLAG-HREL

A) Ces2c-FLAG-HREL was amplified from Ces2c lacking HREL/pFLAG Mini via PCR. The PCR products were separated by gel electrophoresis using a 1.5% agarose gel. Ces2c PCR products were purified from the agarose gel and digested with the restriction enzymes *NotI*-HF and *EcoRV* at 37°C for 2 hours.
B) Digested PCR products and pShuttle-CMV vector were ligated for 2 hours at 22°C and an aliquot was transformed into *E. coli* DH5α cells. Positive clones were identified by *NotI*-HF and *EcoRV* restriction enzyme digestion and separation of generated DNA fragments.

5.11.2. Homologous recombination of the recombinant pShuttle-CMV vector with the pAdEasy-1 plasmid

The Ces2c/pShuttle construct isolated by Miniprep was desalinated and linearized by digestion with *PmeI*. The ends of the digested vector were dephosphorylated using *CIP* and separated and eluted from a 1.5% agarose gel. 80 ng of linearized Ces2c/pShuttle were transformed into *E. coli* BJ5183-Ad cells and selected on kanamycin containing LB-agar plates. 10 of the smallest colonies were picked, streaked on LB-agar plates and ONCs were inoculated. Isolated plasmids were digested with *PacI* to verify homologous recombination of the pShuttle-CMV vector with the pAdEasy-1 plasmid. Digested products were separated on a 0.8% agarose gel. Homologous recombination between the linearized pShuttle-CMV vector and the pAdEasy-1 plasmid in BJ5183-AD-1 cells delivers a fragment of 3 kb or 4.5 kb after *PacI* digestion (Figure 25 B).

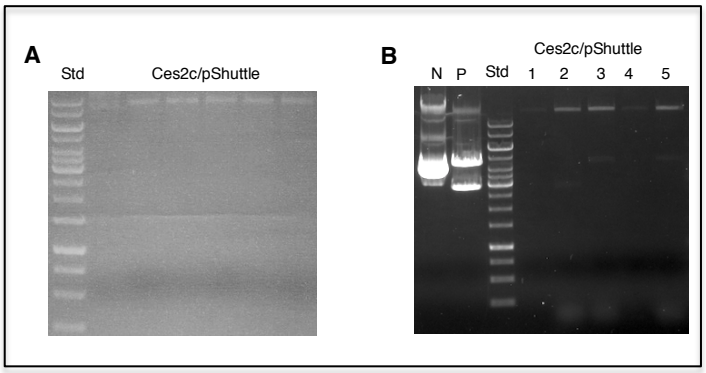


Figure 25: Digestion of Ces2c plasmid with *PmeI* and *PacI* after homologous recombination

A) Ces2c/pShuttle constructs were digested with *PmeI* at 37°C for 2 hours to linearize the plasmid. Digested plasmids were separated by gel electrophoresis and purified from the agarose gel. *E. coli* BJ5183-Ad cells were transformed with the linearized plasmid and selected on kanamycin containing LB-plates. ONCs were inoculated and Minipreps were performed to isolate the recombined Ces2c/pAdEasy-1 plasmids.

B) To confirm proper recombination**,** isolated Ces2c/pAdEasy-1 plasmids were digested with *PacI*. The digested plasmids were separated on a 0.8% agarose gel. *PacI* restriction of recombinant pAdEasy-1 plasmid should yield a fragment of 30 kb and a smaller fragment of either 3 kb or 4.5 kb, depending on the site of recombination. *PacI* digested LacZ/pShuttle was used as a positive control (P) and LacZ/pShuttle was used as a negative control (N).

Figure 25 B shows the *PacI* digested plasmids. Plasmid 1 and 4 were negative, whereas vector 3 and 5 showed a band at 4.5 kb and plasmid 2 a band at 3 kb, indicating a successful homologous recombination. Plasmid 2 and 3 were used for further cloning procedures (Figure 25 B).

5.11.3. Amplification of recombinant pAdEasy-1 plasmids in *E. coli* XL10 gold cells

For further amplification, aliquots of the two recombinant pAdEasy-1 plasmids were transformed into *E. coli* XL10 Gold cells. The cells were selected on kanamycin containing LB-agar plates. Per transformation reaction, 6 colonies were picked and streaked onto LB-agar plates. ONCs were inoculated and plasmids were isolated using Miniprep protocol. To verify successful transformation, plasmids isolated by Miniprep were digested with *PacI*. All of the digested plasmids exhibited a band at 3 kb or 4.5 kb after gel electrophoresis. Two recombinant plasmids were picked and enriched using ODC and ONC cultures.

5.11.4. Generation of an infectious virus for efficient intracellular Ces2c expression

Since the pAdEasy-1 vector is deleted for the *E1* gene, which is essential for the assembly of infectious virus particles, AD-293 cells were used complementing the *E1* gene *in vivo*. The final step in adenovirus generation comprises the enrichment and purification of viral DNA from AD-293 cells.

The infectiosity of the generated adenovirus and expression efficiency of Ces2c were determined on the basis of infection of different liver cell lines.

To transfect AD-293 cells, the linearized pAdEasy-1 plasmid was used. 10-20 *µ*g pAdEasy-1 plasmids isolated from *E. coli* XL10 gold cells were digested with *PacI* and separated by agarose gel electrophoresis to verify complete digestion. The digestion products were eluted from the gel, precipitated and resolved to increase the purity.

5.11.4.1. Transfection of AD-293 cells

AD-293 cells were transfected using the calcium-phosphate method. Briefly, cells were seeded into 10 cm culture dishes and transfected with 10 - 20 *µ*g *PacI* digested Ces2c/pAdEasy-1 plasmid mixed with $CaCl_2$. Then cells were cultivated for 7 – 10 days in the incubator and after appearance of plaques, cells were harvested and homogenized to generate the primary virus stock, which was used to additionally infect AD-293 cells as described above. The presence of viral DNA in the secondary virus stock was investigated via PCR with Ces2c gene specific primers.

The secondary virus stock and the cell culture medium of AD-293 cells were used as template for the PCR. A DNA band at 1685 kb was detected in both PCR reactions, indication the successful generation of infectious viruses. For the enrichment and purification process, the secondary virus stock of AD-293 cells transfected with the pAdEasy-1 from Maxiprep 3 was used, since the intensity of the PCR bands was increased compared to AD-293 cells transfected with pAdEasy-1 Maxiprep 2 (Figure 26).

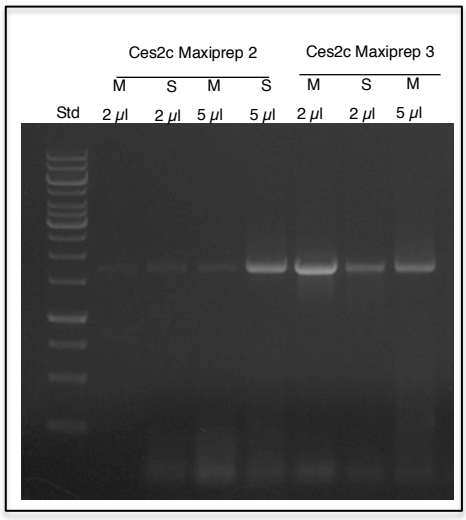


Figure 26: PCR products from secondary virus stocks and culture medium of infected AD-293 cells with Ces2c specific primer

The secondary virus stock of AD-293 cells (S) infected with the primary virus stock of Ad-Ces2c Maxiprep 2 or the primary virus stock of Ad-Ces2c Maxiprep 3 as well as the cell culture medium of the cells (M) was used as template to detect the viral DNA via PCR. 5 µl or 2 µl of the template and *Ces2c* gene specific primers were used for the PCR. The PCR products were separated by gel electrophoresis using a 0.8% agarose gel and visualized under UV-light.

After the enrichment and purification process, the concentration of the viral DNA was determined by absorbance measurement. The concentration of the Ces2c adenovirus accounts for $1.25 * 10^{-11}$ pfu/ml.

5.11.5. Determination of the optimal MOI

The MOI describes the number of virus particles needed to infect one cell and differs markedly between different cell types. Therefore the optimal MOI was determined for initially transduced cells. Therefore, HepG2 cells were seeded into 6-well plates and transduced with different MOI (300, 500, 800, 1000, 2000, 5000) of Ad-Ces2c virus. After 24 or 48 hours cells were harvested and used for western blot analysis. As shown in figure 27 A, increasing amounts of virus led to elevated Ces2c expression levels. Ces2c was detected at the expected size of 59.5 kDa using an anti-FLAG antibody. Moreover, Ces2c expression increased over timer comparing levels at 24 hours and 48 hours, respectively. For further experiments a MOI of 500 was used.

McA-RH777 hepatocytes were seeded into 6-well plates, infected with increasing amounts of virus and harvested after 24 and 48 hours, respectively. For western blot

analysis, 20 µl of cell lysate was used. Similar to HepG2 hepatocytes, increasing MOI led to elevated Ces2c expression over time (Figure 27 B).

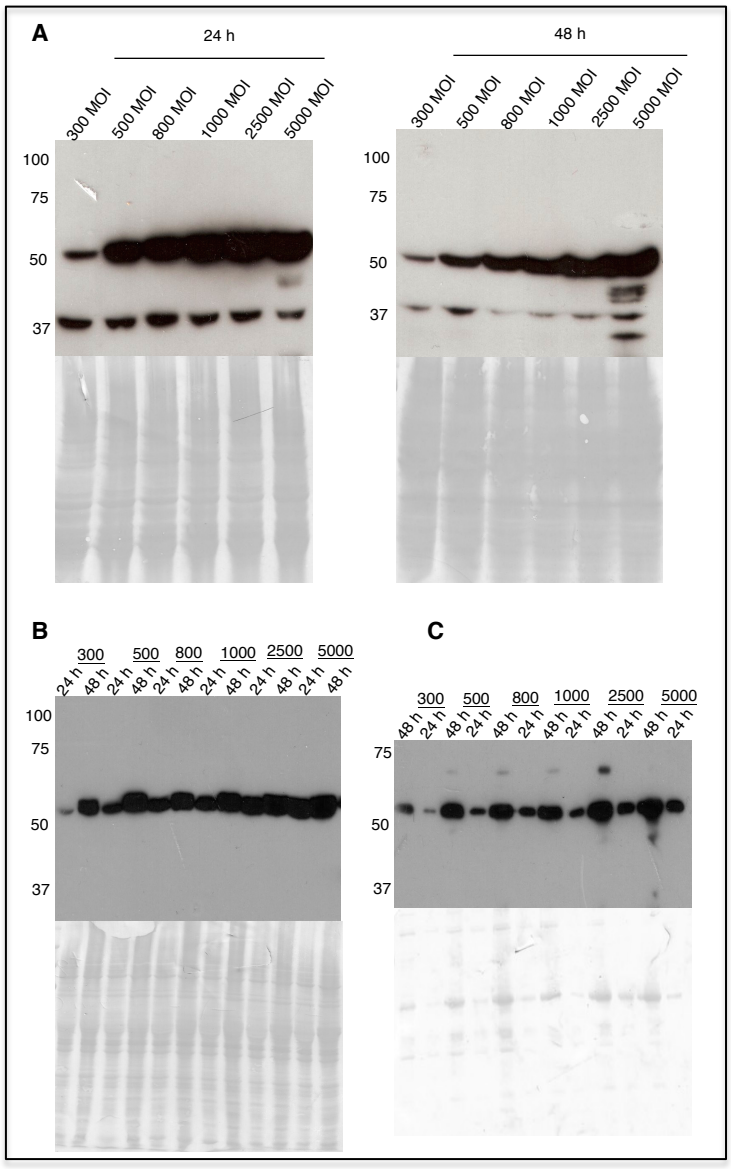


Figure 27: Optimal MOI determination for Ad-Ces2c transduction of different liver cell lines

A) HepG2 cells were seeded into 6-well plates and transduced with different MOI (300, 500, 800, 1000, 2000 and 5000) of Ad-Ces2c virus. After 24 hours or 48 hours cells were harvested, diluted 1:10 and 12 µl of the cell lysate was used for western blot analysis.

B) McA-RH777 cells were seeded into 6-well plates and transduced with different MOI (300, 500, 800, 1000, 2000 and 5000) of Ad-Ces2c virus. After 24 hours or 48 hours cells were harvested and 20 µl cell lysate was used for western blot analysis.

C) AML12 cells were seeded into 6-well plates and transduced with different MOI (300, 500, 800, 1000, 2000 and 5000) of Ad-Ces2c virus. For western blot analysis, 20 µl of the 24-hour samples and 25 µl of the 48-hour samples were used.

Next, AML12 hepatocytes were seeded into 6-well plates and transduced with different amounts of Ces2c virus (300, 500, 800, 1000, 2000, 5000). Again, cells were harvested after 24 hours or 48 hours and used for western blot analysis. Similarly, increasing MOI led to elevated expression levels of Ces2c. However, compared to HepG2 cells, AML12 hepatocytes exhibited decreased expression of Ces2c over time (Figure 27 C). For further experiments, a MOI of either 500 or 1000 was used.

5.11.6. Expression of Ad-Ces2c in HepG2 cells

The incorporation of radiolabeled FAs into cellular TGs was examined in HepG2 cells. Therefore, HepG2 cells were seeded into 6-well plates and transduced with Ad-Ces2c using 500 MOI and Ad-LacZ as a negative control using 100 MOI. Cellular TG pools were pulse labeled for 20 hours using ^{3}H-labeled OA. After the pulse labeling, intracellular lipids were extracted and TG levels were examined from one set of 6-well plates (Figure 28 A). Other sets of 6-well plates were pulse chased for 4 hours with BSA containing medium and cellular TG levels were determined.

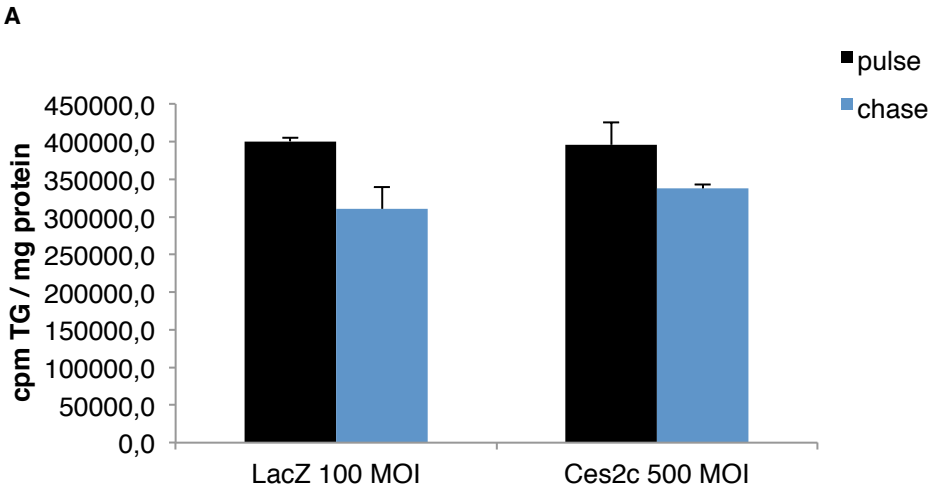
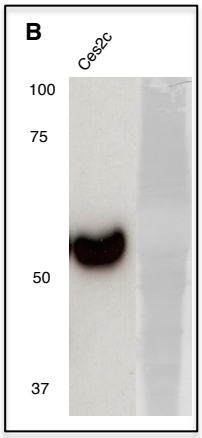


Figure 28: Ces2c expression in HepG2 hepatocytes does not cause a significant decrease in cellular TG levels



A) HepG2 cells were seeded into 6-well plates and transduced with either 100 MOI Ad-LacZ or 500 MOI Ad-Ces2c. Cells were pulse labeled with ^{3}H-labeled OA for 20 hours. Following the pulse labeling, cells were chased for 4 hours with DMEM$^{-/-}$ containing 2% BSA. Cellular TG levels were measured after the pulse labeling and the chase period. For that purpose cellular lipids were extracted either after the pulse or the chase, separated by TLC and quantified by liquid scintilation. Data are shown as means ± standard derivation of n = 3. $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

B) Protein levels were examined by western blot analysis to confirm the expression of Ces2c. Cells were harvested after the chase period using 200 µl 1x SDS and 20 µl was used for Western blot analysis. Coomassie Blue staining of the membrane confirmed successful protein transfer and Ces2c was detected by antibodies specific for FLAG-tagged recombinant proteins.

These studies revealed that Ces2c expression in HepG2 cells led to lower label accumulation within cellular TGs at the end of a 4-hour chase period compared to cellular TG levels after 20 hours of pulse labeling (Figure 28 A). The incorporation of radioactivity

into cellular CE was unchanged (data not shown). The cellular TG mass declined following OA deprivation indicating an involvement of Ces2c in TG catabolism (Figure 28 A).

However, compared to LacZ control, TG levels of Ces2c-transduced HepG2 cells were similar. Moreover, the incorporation of radioactivity was also decreased in LacZ control after a 4-hour chase period compared to TG levels after 20 hours of pulse labeling (Figure 28 A). These findings prompted me to use lower amounts of Ad-LacZ in the following experiments. Additionally, pfu were determined performing a plaque assay.

5.11.7. Plaque Assay

The absorbance measurement previously used to determine Ces2c pfu/ml does not differentiate between infectious virus particles and empty capsids. Therefore, pfu determined with this method are not representative of actual infective particles within the sample, which prompted me to perform a plaque assay (Figure 29). The advantage of the plaque assay is that pfu determined by this method, more likely represent the number of infective particles in the sample assuming that each plaque represents a single infective virus particle.

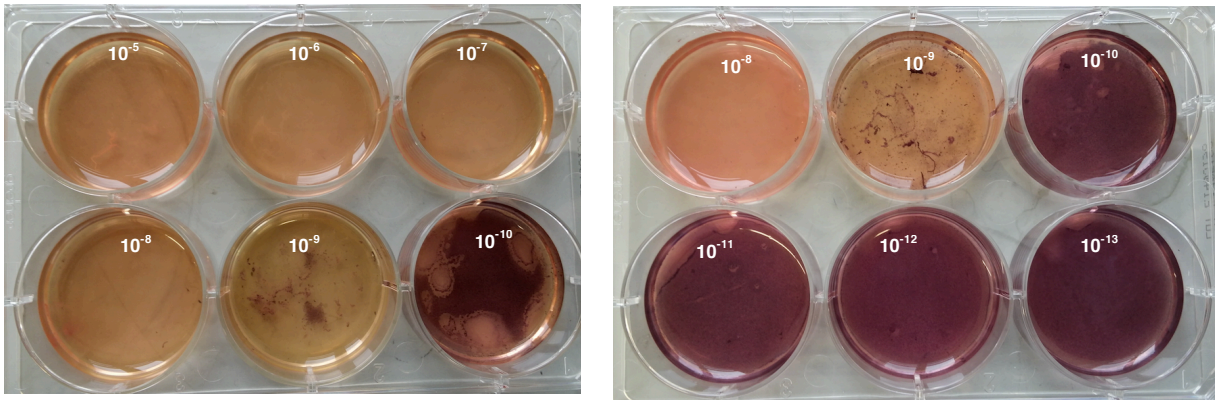


Figure 29: Plaque Assay of Ad-LacZ-transduced cells

AD-293 cells were seeded into 6-well plates and transduced with serial dilutions of Ad-LacZ. The dilutions prepared ranged from 10^{-5} to 10^{-13}. 24 hours after transduction, cells were overlaid with plaque medium. MTT was used to stain living cells after an incubation period of 14 days.

AD-293 cells were seeded into 6-well plates and transduced with serial dilutions of the adenovirus Maxiprep Ad-Ces2c or Ad-LacZ. The dilutions prepared ranged from 10^{-2} to 10^{-12}. Briefly, 10 μl of the Virus Maxiprep were added to 990 μl sterile 1x PBS and gently mixed by pipetting up and down (10^{-2} dilution). To obtain the desired virus dilutions,

ranging from either 10^{-5} to 10^{-13} (Ad-LacZ) or 10^{-5} to 10^{-12} (Ad-Ces2c), 10 µl or 100 µl of the previously prepared serial dilutions were added directly to the medium of the cells. 24 hours after transduction, cells were overlaid with sterile 0.4% agarose/medium solution. After 2 days of incubation at 37°C, the overlaying was repeated. First plaques emerged quickly at lower dilutions (5-7 days). After 10-14 days, plaques at higher dilutions became visible. After an incubation period of 14 days, cells were stained with MTT. MTT stains living cells blue, while plaques, which consist of dead cells, remain colorless. The two highest dilutions were counted, averaged and used to calculate the concentration of the virus Maxiprep (pfu/ml). The titer of the Ad-Ces2c virus stock was determined as 1.5 * 10^{10} pfu/ml (data not shown) and the titer of Ad-LacZ as 6 * 10^{11} pfu/ml (see Table 25).

Table 11: Difference in pfu/ml determined via plaque assay and absorbance measurement

Pfu determined via absorbance measurement do not differentiate between infectious virus particles and empty capsids. Pfu determined by plaque assay more likely represent the number of infectious particles.

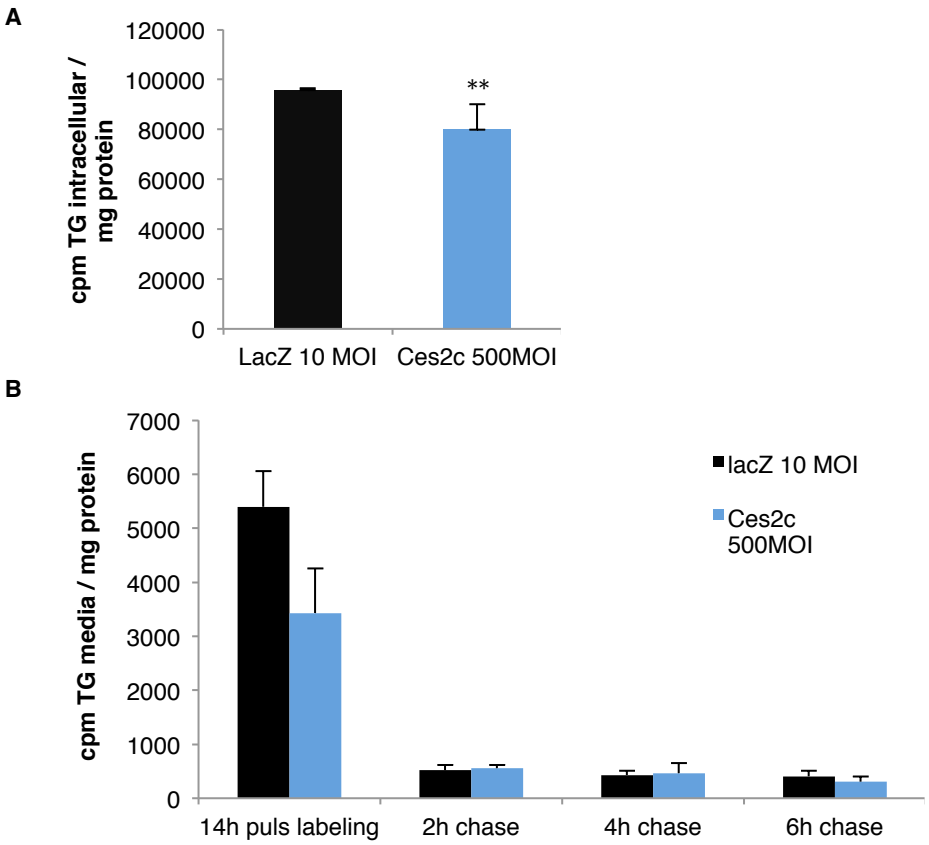
	pfu/ml determined via plaque assay	pfu/ml determined via absorbance measurement	quotient
Ad-Ces2c	1.5 *10^{10}	1.25 *10^{11}	0.12
Ad-LacZ	6 *10^{11}	1 *10^{12}	6

Comparing the quotients of Ad-Ces2c titer determined by plaque assay and absorbance measurement to the Ad-LacZ quotient, it is obvious that there are 5-fold more infectious particles in the Ad-LacZ virus Maxiprep compared to the Ad-Ces2c virus Maxiprep (Table 11). Accordingly, this suggests to use the pfu/ml determined via plaque assay to calculate the amount of virus Maxiprep DNA needed to transduce cells with a distinct MOI. Note that for the following experiments, the pfu/ml determined by absorbance measurement were used.

5.11.8. McA-RH777 hepatocytes transduced with Ad-Ces2c exhibit decreased intracellular TG levels

McA-RH777 hepatocytes were seeded into 6-well plates and transduced with Ad-Ces2c at 500 MOI and Ad-LacZ at 10 MOI. The cells were pulse labeled for 14 hours with ^{3}H-labeled OA. The cells were then chased for different time periods with BSA containing medium. After the pulse labeling, a chase period of 2, 4 and 6 hours, the medium was

collected and lipids were extracted according to the method of Folch. Extracted lipids were then separated by TLC and the radioactivity of medium TGs (Figure 30 B), FAs and CEs (data not shown) was measured. Additionally, cellular TG (Figure 30 A) and CE levels (data not shown) were also examined after 6-hour incubation with cold medium.



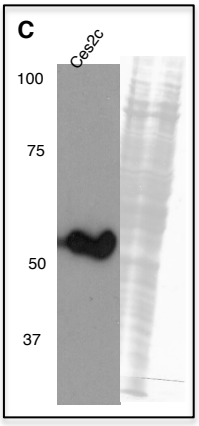


Figure 30: Ces2c expressing McA-RH777 hepatocytes exhibit a significant decrease in cellular TG levels concomitant TG secretion was not elevated

A) McA-RH777 cells were seeded into 6-well plates and transduced with either 10 MOI Ad-LacZ or 500 MOI Ad-Ces2c. Cells were pulse labeled with ^{3}H-labeled OA for 14 hours. After pulse labeling, cells were chased for 6 hours with DMEM$^{-/-}$ containing 2% BSA. Incorporation of radioactivity into cellular TGs was examined after the chase period. For that purpose cellular lipids were extracted and separated by TLC. Cellular TG levels were quantified by liquid scintillation.

B) After a 2-hour, 4-hour and 6-hour chase period, the medium was collected, lipids were extracted and separated by TLC. The radioactivity of medium TGs was counted by beta counter. Data are shown as means ± standard derivation of n = 6. $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

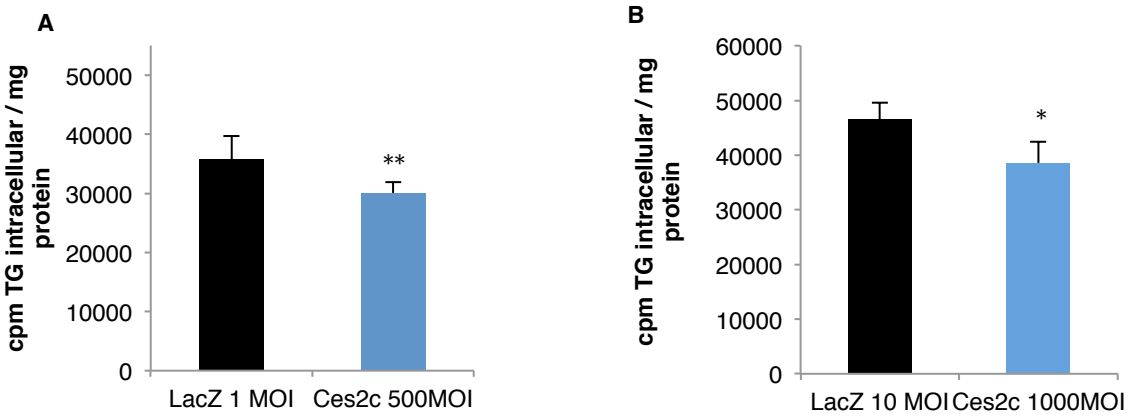
C) Protein levels were examined by western blot analysis to confirm the expression of Ces2c. Cells were harvested after the chase period using 200 µl 1x SDS and 20 µl was loaded onto the gel for Western blot analysis. Coomassie Blue staining of the membrane confirmed successful protein transfer and Ces2c was detected by antibodies specific for FLAG-tagged recombinant proteins.

5.11.9. Increased TG-secretion paralleled by reduced intracellular TG levels in Ad-Ces2c-transduced AML12 cells

To further address the potential role of Ces2c in cellular TG-mobilization and hepatic VLDL-TG formation, pulse-chase experiments applying ^{3}H-labeled OA were performed in Ces2c-transduced AML12 hepatic cells (Figure 31). AML12 hepatocytes were seeded into 6-well plates and transduced with Ad-Ces2c (500 or 1000 MOI) and Ad-LacZ (1 or 10 MOI) as control. To determine whether Ces2c overexpressing hepatocytes exhibit increased TG-secretion, cells were incubated in the presence of ^{3}H-labeled OA for 4 hours or 20 hours to radiolabel the intracellular TG pool. After the pulse period, cells were chased for different time periods with BSA containing medium. At indicated time points, the medium was collected, lipids were extracted and separated by TLC. Medium TG (Figure 31 C, D), FA and CE levels (data not shown) were quantified by liquid scintillation. Additionally cellular TG levels (Figure 31 A, B) and CE levels (data not shown) were examined after the designated chase periods.

When AML12 hepatocytes were pulse labeled for 20 hours, TG-secretion was significantly increased during both, the pulse and by 2-hour chase compared to control cells (Figure 31 C). In contrast, when the cells were pulse labeled for 4 hours, TG-secretion was significantly increased in Ces2c expressing hepatocytes after a 6-hour chase period (Figure 31 D). These results indicate that AML12 hepatocytes start to secrete apoB100 and apoB48 after approximately 10 hours when supplemented with OA. Therefore, a pulse-labeling period of 20 hours may be more representative to study VLDL-secretion. Nevertheless, Ces2c-expression in AML12 hepatocytes led to increased TG secretion (+50%), suggesting an involvement of Ces2c in VLDL-TG formation (Figure 31 C, D). Independent of the duration of the pulse labeling period, the secretion of FAs and CEs from Ces2c expressing cells was not significantly altered compared to control cells expressing LacZ (data not shown).

Additionally cellular TG levels were examined after the designated chase periods (Figure 31 A, B). In both set ups, cellular TG levels were significantly reduced in Ces2c-transduced cells compared to control cells, while cellular CE levels were unaffected (data not shown).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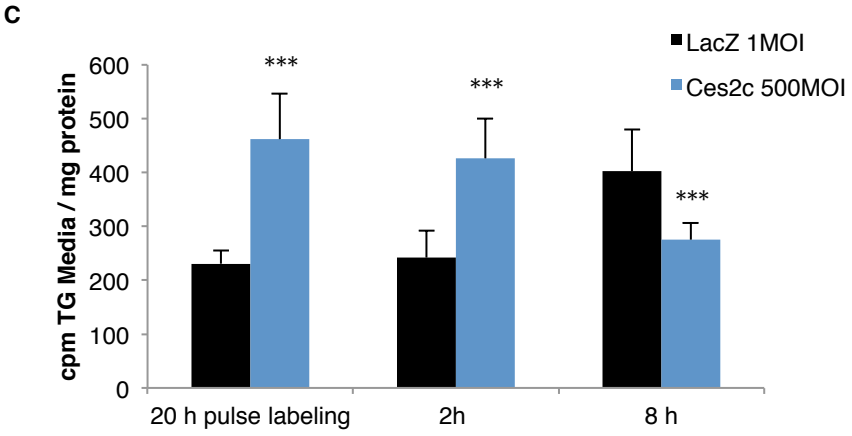
C
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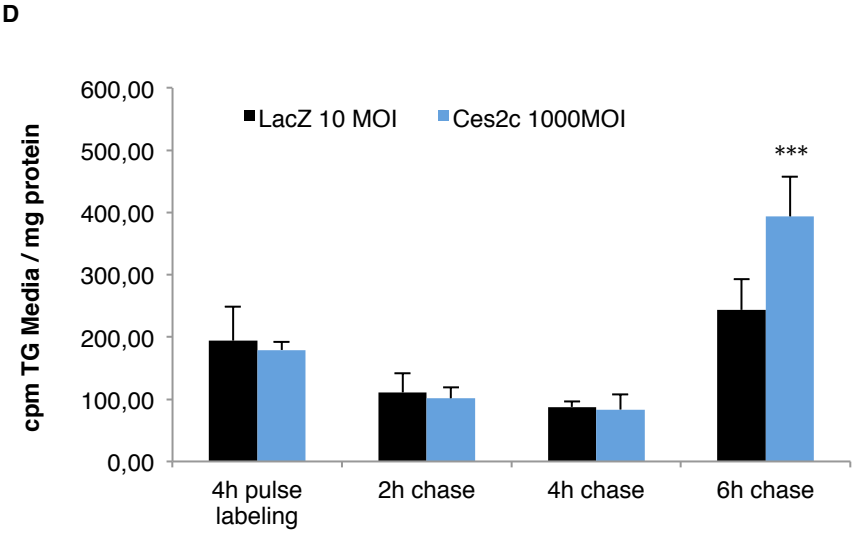
***
LacZ 1MOI
Ces2c 500MOI
20 h pulse labeling
2h
8 h


D
cpm TG Media / mg protein
600,00
500,00
400,00
300,00
200,00
100,00
0,00
LacZ 10 MOI
Ces2c 1000MOI
***
4h pulse labeling
2h chase
4h chase
6h chase

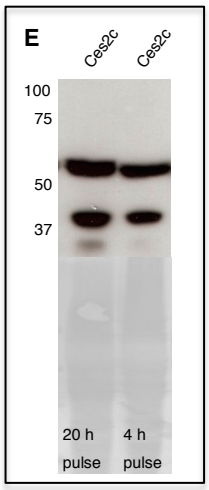


Figure 31: Ces2c expression in AML12 hepatocytes leads to a significant decrease in TG levels and a significant increase in TG secretion

AML12 cells were seeded into 6-well plates and transduced with either 1 MOI Ad-LacZ and 500 MOI Ad-Ces2c **(A, C)** or 10 MOI Ad-LacZ and 1000 MOI Ad-Ces2c **(B, D)**. The cells were pulse labeled with ^{3}H-labeled OA for 20 hours **(A, C)** or 4 hours **(B, D).** Following pulse labeling, the cells were chased for different time periods with DMEM$^{-/-}$ containing 2% BSA.

A, B) Following the 8-hour **(A)** or 6-hour **(B)** chase period, cellular lipids were extracted and separated by TLC. Cellular TG levels were counted by liquid scintillation.

C, D) After the pulse labeling and a 2-hour and 8-hour chase period **(C),** the medium was collected, lipids were extracted and separated by TLC. Alternatively, the medium was collected after the pulse labeling and the incubation for 2 hours, 4 hours and 6 hours with cold medium, respectively **(D)**. The incorporation of radioactivity in TGs in the culture medium was quantified via liquid scintillation. Data are shown as means ± standard deviation of n = 6. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***).

E) Protein levels were examined by western blot analysis to confirm the expression of Ces2c. Cells were harvested after the chase periods using 100 µl 1x SDS and 20 μl were used for Western blot. Coomassie Blue staining of the membrane confirmed successful protein transfer and Ces2c was detected by antibodies specific for FLAG-tagged recombinant proteins.

6. Discussion

Currently, the mechanism of VLDL-TG formation is incompletely understood. The formation of mature VLDL particles is highly dependent on the availability of TGs. FAs used for VLDL-TG biosynthesis are mainly derived from stored TGs and to a very small extent from *de novo* synthesis (Wiggins & Gibbons 1992b; Yang et al. 1996; Yang et al. 1995). It is believed that an abundance of hepatic TGs provides excess substrate for VLDL synthesis causing VLDL overproduction. However, an increase in cytosolic TG level does not necessarily lead to increased VLDL secretion. For example, hepatic DGAT1 and DGAT2 overexpression caused an increase in cytosolic TG content without influencing the production rate of VLDL-TG (Millar et al. 2006). Similarly Plin2-overexpression led to increased TG accumulation with a concomitant decrease in TG secretion rate (Edvardsson et al. 2006). Recently it has been suggested that the TG pool used for VLDL-TG biosynthesis resides in the lumen of the ER (Alexander et al. 1976; Hamilton et al. 1998; Sundaram, Zhong, Khalil, et al. 2010; Sundaram, Zhong, Bou Khalil, et al. 2010; Qin et al. 2011). CES1, a luminal TG lipase has been implicated to be an important player in VLDL-TG formation via hydrolysis of TGs deposited in the ER lumen (Dolinsky et al. 2001). Liver-specific CES1-deficient mice exhibit decreased plasma TG and apoB100 levels but absence of hepatic steatosis (Lian et al. 2012). However, compared to liver-specific MTTP knock down, which provokes marked hepatic steatosis and impairs VLDL secretion (Raabe et al. 1999), the phenotype of CES1-deficient mice is relatively mild implicating that CES1 is not the rate-limiting enzyme in VLDL-TG biosynthesis. Altogether, these observations suggest that VLDL-TG synthesis depends on a currently unknown TG-lipase possibly located in the ER lumen.

The aim of my master study was to characterize the enzymatic activity of Ces2c and to examine the potential role of this lipase candidate enzyme in VLDL-TG formation. For that purpose, Ces2c was expressed in COS-7 cells and examined for TG hydrolase activity *in vitro.* Results revealed that Ces2c exhibits strong *in vitro* TG-hydrolytic activity and a broad pH optimum between pH 7 to 9 with maximum activity at pH 7. Since the Ces2 family comprises 7 very homologous genes, *in vitro* TG hydrolase activity assays were performed of homologous genes including Ces2a, Ces2b, Ces2e, Ces2f and Ces2g to further study the specific and unique role of Ces2c in TG-breakdown. However,

activities were moderate compared to Ces2c indicating that Ces2c hydrolyzes TGs with high specificity.

To further elucidate the role of Ces2c in TG catabolism *ex vivo*, FA incorporation and lipid mobilization assays were performed. Given that a protein is involved in TG synthesis, elevated incorporation of radioactively labeled FAs can be assumed compared to control cells. In contrast, an enzyme involved in TG hydrolysis is expected to increase cellular TG catabolism and consequently reduces incorporation of FAs into the cellular lipid pool. FA incorporation and mobilization assays indicate that Ces2c is involved in intracellular TG-breakdown, since the radioactivity in the cellular TG fraction was significantly reduced upon Ces2c overexpression in cells loaded with FAs when compared to LacZ control. In summary, Ces2c exhibits TG-hydrolytic activity *in vitro* and *ex vivo* supporting the concept that Ces2c is involved in TG catabolism.

To further characterize the enzymatic activity of Ces2c, a substrate screen was performed to may identify additional Ces2c substrates. This analysis showed that Ces2c also exhibits hydrolytic activity toward MGs. *In vitro* MG hydrolase activity assays were performed to further characterize the potential role of Ces2c in MG hydrolysis in addition to its TG-hydrolytic activity. However, activities were relatively moderate compared to activities observed for MGL, an established MG-lipase. In contrast to HSL, which acts as a TG- and DG-lipase, Ces2c exhibits no obvious activity towards a DG substrate (data not shown). Ces2c was originally described as an acylcarnitine hydrolase by Furihata *et al.* (Furihata et al. 2003) which could be confirmed when measuring acylcarnitine hydrolase activities in lysates prepared from COS-7 cells transfected with a Ces2c-expression vector. Moreover, enzymatic activity towards the artificial esterase substrates PNPA and PNPV demonstrates that Ces2c is a carboxyl esterase.

Mammalian CEs are typically localized to the ER (Satoh & Hosokawa 1998; Du 2002). To investigate the localization of Ces2c, confocal laser scanning live cell imaging was performed in cells expressing recombinant GFP-tagged Ces2c. In agreement with previous findings we could demonstrate that Ces2c localizes exclusively to the ER. However, these finding could not be verified by cell fractionation experiments which was most likely due to contaminations of membrane together with cytosolic fractions. Nonetheless, data suggest that Ces2c is an ER-localized protein. In addition, Ces2c is highly expressed in secretory organs like kidney and liver with highest expression levels

in the duodenum (Jones et al. 2013). Taken together, these results are in agreement with a potential role of Ces2c in the hydrolysis of TGs deposited in the lumen of the ER thereby supplying lipid substrates, i.e. DG and FAs, required for VLDL-TG biosynthesis.

To further support this assumption, a Ces2c recombinant adenovirus was generated to efficiently express Ces2c in hepatocyte cell lines to study the impact of Ces2c-overexpression on *ex vivo* TG homeostasis and secretion. HepG2 cells transduced with Ad-Ces2c exhibited a decrease in cellular TG levels after the chase period. However HepG2 control cells, which were transduced with Ad-LacZ exhibited a similar impact on cellular TG content and FA mobilization. We hypothesized that probably the MOI used for LacZ transduction was too high and leads to toxicity and changes in lipid homeostasis. Moreover it has been shown that HepG2 cells exhibit low rates of apoB secretion (Wu et al. 1996) questioning the utilization of HepG2 cells as appropriate cell line to investigate TG secretion .

Ad-Ces2c transduced McA-RH777 hepatocytes exhibited a significant decrease in cellular TG but not CE levels compared to LacZ control after a 6-hour chase period indicating a role for Ces2c in lipid secretion. However, the appearance of TG, FA and CE in the medium of Ces2c-transduced McA-RH777 cells after the chase period was comparable to LacZ control which arguments against a significant role of Ces2c in TG-breakdown to promote VLDL-TG formation. Nonetheless, these preliminary experiments have to be repeated and the experimental design has to be modified to make VLDL-TG release generally visible and robust. Notably, AML12 hepatocytes transduced with Ad-Ces2c and incubated with tritium-labeled OA prior to incubation with cold medium exhibited a significant decrease in cellular TG content paralleled by increased release of radioactively-labeled TG (+50%) compared to LacZ control. Although preliminary, these findings argue for a role of Ces2c in VLDL-TG biogenesis. As Ces2c is also efficiently expressed in the intestine, it is assumable that Ces2c could play a role in lipid absorption in the postprandial state, which awaits further investigation.

To summarize, data suggest that Ces2c is a novel, currently unknown TG hydrolase in the liver and possibly plays a role in VLDL assembly via hydrolysis of luminal TGs thereby providing FAs and DG for VLDL-TG biosynthesis.

7. Abbreviations

ApoB100	apolipoprotein B100
ATGL	Adipose triglyceride lipase
BCA	bicinchoninic acid
Bp	base pair
BSA	bovine serum albumin
CAPS	3 (cyclohexylamino)propanesulfonic acid
cDNA	complementary DNA
CDS	coding sequence
CEs	carboxylesterases
Ces	murine carboxylesterase
CES1	carboxylesterase 1
Ces2c	carboxylesterase 2 c
Chaps	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
Ci	curie
Cideb	cell death-inducing DFFA-like effector b
CIP	calf intestine phosphatase
CLDs	cytosolic lipid droplets
CoA	coenzyme A
Cpm	counts per minute
ddH_2O	distilled, deionized water
DG	diglyceride
DGAT	acyl-CoA:diacylglycerol acyltransferase
DMEM	dulbecco‟s modified eagle‟s medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FAs	fatty acids
FCS	fetal calf serum

fw	forward
g	gram
GFP	green fluorescence protein
h	hour
HDL	high-density lipoprotein
HEPES	4 (2 hydroxyethyl) 1 piperazineethanesulfonic acid
IDL	intermediate density lipoprotein
kDa	kilodalton
LacZ	gene of lactose operon coding for β-galactosidase
LB	lysogeny broth
LD	lipid droplets
LDL	low-density lipoprotein
LLDs	luminal lipid droplets
M	molar
MCS	multiple cloning site
MG	monoglyceride
mM	millimolar
MOI	multiplicity of infection
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MTTP	microsomal triglyceride transfer protein
NEB	New England BioLabs
NTP	nucleoside triphosphates
OA	oleic acid
ODC	over day culture
p	piko
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidyl choline
PCR	polymerase chain reaction
Pfu	plaque forming units
Plin2	perilipin 2
PNPA	p-nitrophenylacetate
PNPV	p-nitrophenylvalerate

RNase	ribonuklease
rpm	revolutions per minute
RT	room temperature
rv	reverse
SDS	sodium dodecyl sulfate
Sec	second
Std	standard
TAE	tris-acetate-EDTA
TEMED	tetra methyl ethylene diamine
TG	triglyceride
TLC	thin layer chromatography
TST	Tris-buffered saline supplemented with Tween 20
U	unit
UV	ultraviolet
v/v	volume per volume
VLDL	very low-density lipoprotein
µ	micro

8. References

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9. Table of figures