# Cell Membrane Homeostasis in Mammals

## - *The roles of ADIPOR2 and TLCD1 & 2*

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*“Somewhere, something incredible is waiting to be known”*

- **Carl Sagan**

# ABSTRACT

Ten nanometers. That is the approximate thickness of the plasma membrane that separates the extracellular space from the entire cellular machinery. An asymmetrical bilayer consisting of predominantly phospholipids in which proteins and carbohydrates are anchored and exert their function. Together, these components determine the viscosity, thickness, fluidity, permeability and packing of the plasma membrane, which is constantly maintained within a near-optimal chemical composition for proper function, a phenomenon also known as membrane homeostasis. Dietary fatty acids become the building block for the major component of the membrane bilayer, namely the phospholipids, and its composition can reflect the composition in the food, giving some validity to the term "you are what you eat". To mitigate the huge variation in dietary fatty acids ending up in the membrane, it is reasonable to believe that regulatory mechanisms exist that adjust the fatty acid composition when required. Surprisingly, not much is known regarding how such adaptive responses regulate membrane fatty acid composition on a molecular level. Within this thesis, novel insights into such regulatory mechanisms are presented.

Founded on genetic modifications using CRISPR/Cas9, lipidomic analyses, and membrane fluidity measurements, we build upon previous work and provide further evidence implicating the mammalian adiponectin receptor 2 (ADIPOR2) as a master regulator of membrane homeostasis. Human cells that lack ADIPOR2 show a dramatic vulnerability to saturated fat (SFA) exposure, leading to a defective translational response, increased ER stress, impaired mitochondrial respiration, and importantly, a massive increase in SFA-containing phospholipids. By exploiting this phenotype, genetic ablation of ADIPOR2 in human and mouse pre-adipocytes led to cells with drastic elevation in SFA-containing phospholipids. These were deployed to study the effect of phospholipid saturation on insulin signaling *in vitro* and *in vivo*. Surprisingly, both human and mouse adipocytes showed normal insulin signaling despite excess SFA content in their phospholipids, thus highlighting the robustness of adipocytes in lipid handling.

Separately, genetic suppressors of SFA vulnerability in PAQR-2 (homolog to mammalian ADIPOR2) deficient *C. elegans* worms, revealed a novel protein dubbed FLD-1 that influences the amount of fluidizing, long-chain polyunsaturated fatty acids (PUFAs) in membrane phospholipids. Further studies in transgenic mice, lacking the mammalian homologs TLCD1 and TLCD2, led to elucidating the mechanism of action of these proteins, namely that they regulate the incorporation of monounsaturated fatty acids (MUFAs) at the sn-1 position of phosphatidylethanolamines. In parallel, another genetic suppressor to the SFA vulnerability of PAQR-2 knockouts was discovered, namely ACS-13, that was shown to regulate mitochondrial activation of long-chain PUFAs, a function conserved in the human homolog ACSL1.

Taken together, this thesis provides new insight into the mechanisms that regulate the fatty acid composition of cellular membranes and that are crucial for the ability of cells to maintain fluid membranes in the face of fluctuating levels of dietary fatty acids.



**Keywords:**
Membrane lipids, Membrane fluidity, Membrane homeostasis, Dietary lipids, Phospholipids, Genetics, Lipotoxicity, Lipidomics, ADIPOR2, TLCD1, TLCD2

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Uttrycket “du är vad du äter” återspeglar nog sanningen bäst när man talar om fett. Detta då det tunna höljet som omsluter alla kroppens celler, cellmembranet, är till mestadels uppbyggt av fett från kosten. Det finns dock ett problem med denna situation, nämligen hur fetter är uppbyggda. Fetter klassificeras, efter deras kemiska struktur, in i mättade eller omättade fetter. Mättat fett, främst från animaliska produkter så som kött och mejeriprodukter, packar ihop sig tätt och brukar vara fasta i rumstemperatur. Oljor från grönsaker och fet fisk är rika på omättat fett, vilka tenderar att packa sig glest och ger upphov till en vätska i rumstemperatur. Detta innebär att en kost rik på mättat fett kan leda till att cellmembranen blir ohälsosamt stela, medan för mycket omättat fett skulle leda till för rinniga cellmembran.

Celler kan lyckligtvis undvika dessa öden genom att modifiera halterna av mättat och omättat fett i cellmembranen. Specifika proteiner, inbäddade i cellmembranet, kan känna av när det omkringliggande membranet börjar bli för stelt och signalerar till cellen att konvertera mättat fett till omättat. Tidigare forskning har kartlagt några sådana proteiner, men många frågor kvarstår. Vad händer när dessa proteiner inte är närvarande eller slutar fungera? Finns det flera proteiner som kan justera cellmembraners fetter? Finns det mänskliga sjukdomar som kan härledas till problem i cellmembranens fettkomposition?

Avsikten med denna avhandling var att undersöka dessa frågor. Upptäckterna grundades i modellorganismen *C. elegans*, en 1 mm kort rundmask, där genmodifierade organismer som saknar proteinet PAQR-2 uppvisade en extrem känslighet för en kost rik på mättat fett. Detta protein tyder på att vara huvud-sensorn för ett stelare cellmembran och mutanten uppvisar gravt stela cellmembran. Genom att studera denna mutant kunde vi upptäcka två ytterligare proteiner som reglerar fettkompositionen i cellmembran; FLD-1 och ACS-13. FLD-1-proteinet begränsar mängden omättat fett i cellmembranen och ACS-13 vägleder omättat fett till förbränning i mitokondrierna; cellernas energikraftverk. När dessa proteiner slogs ut, ökade mängden omättat fett i cellmembranen vilket återställde fettsammansättningen i maskar som saknar PAQR-2.

Förmågan att justera cellmembranets fettkomposition är kritiskt för organismer som utsätts för olika slags fetter och är något som uppstod tidigt i evolutionen. Däggdjur, däribland människan, har även sådana proteiner. Genom studier på möss och mänskliga celler kunde vi utvärdera dessa proteiner även i en mänsklig kontext. Sensorn, motsvarande PAQR-2 i *C. elegans*, som känner av cellmembraners stelhet benämns ADIPOR2 i däggdjur. Detta arbete kunde påvisa liknande effekter även i mänskliga celler; när ADIPOR2 saknas leder detta till extremt stela cellmembran. Dessa celler är mycket känsliga för mättat fett. Likt vad vi visade för *C. elegans*, kunde de mänskliga motsvarigheterna till FLD-1 och ACSL-13, nämligen TLCD1, TLCD2 samt ACSL1 uppvisa en lindrande effekt på cellmembranets stelhet i celler som saknar ADIPOR2 och dessa proteiner. Studier i möss som saknar ett fungerande ADIPOR2 visade att celler i fettvävnader var rika på mättat fett. Trots detta visade mössen på fungerande insulin-signalering vilket tyder på att djur har en otrolig förmåga att kompensera för obalanser i cellmembraners fettsammansättning. Möss som saknar TLCD1 och TLCD2 hade en ökad mängd omättat fett i cellmembran i levern och uppvisar god hälsa. Denna ökning i omättat fett kan vara skyddande mot utveckling av den allvarliga leversjukdomen NASH, som tenderar att utvecklas hos människor med högt intag av mättat fett.

Sammanfattningsvis har detta arbetet lett till en utökad förståelse för celler och djurs förmågor att justera sin fettsammansättning vilket på sikt kan leda till medicinska behandlingar som avser att rätta till avvikelser i cellmembranet hos t.ex. diabetiker vars cellmembran är rika på mättat fett.

# PUBLICATIONS

This thesis is based on the following publications (* indicates shared first authorship)

**I.** **Membrane Fluidity is Regulated by the *C. elegans* Transmembrane Protein FLD-1 and its Human Homologs TLCD1/2**
Ruiz M., Bodhicharla R., Svensk E., Devkota R., Busayavalasa K., **Palmgren H.**, Ståhlman M., Borén J., Pilon M.
*Elife* 2018

**II.** **Evolutionarily conserved long-chain Acyl-CoA synthetases regulate membrane composition and fluidity**
Ruiz M., Bodhicharla R., Ståhlman M., Svensk E., Busayavalasa K., **Palmgren H.**, Ruhanen H., Borén J., Pilon M.
*Elife* 2019

**III.** **TLCD1 and TLCD2 regulate cellular phosphatidylethanolamine composition and promote the progression of non-alcoholic steatohepatitis**
Petkevicius K., **Palmgren H.**, Glover M., Ahnmark A., Andréasson A-C., Madeyski-Bengtson K., Kawana H., Allman E., Kaper D., Uhrbom M., Andersson L., Aasehaug L., Forsström J., Wallin S., Ahlstedt I., Leke R., Karlsson D., Löfgren L., Nilsson R., Pellegrini G., Aoki J., Sienski G., Pilon M., Bohlooly-Y M., Maresca M., Peng XR.
Under review at Nature Metabolism 2022.

**IV.** **Extensive transcription mis-regulation and membrane defects in AdipoR2-deficient cells challenged with saturated fatty acids**
Ruiz M.*, **Palmgren H.***, Henricsson M., Devkota R., Jaiswal H., Maresca M., Bohlooly-Y M., Peng XR., Borén J., Pilon M.
*BBA Molecular and Cell Biology of Lipids* 2021

**V.** **Elevation in adipocyte membrane phospholipid saturation does not compromise insulin sensitivity**
**Palmgren H.**, Petkevicius K., Bartesaghi S., Ahnmark A., Ruiz M., Nilsson R., Löfgren L., Glover M., Andréasson A-C., Andersson L., Kull B., Wallin S., Karlsson D., Hess S., Maresca M., Bohlooly-Y M., Peng XR., Pilon M.
Under review at Diabetes

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# INTRODUCTION

This thesis starts out by outlining fundamental aspects of biological membranes, their chemical makeup and homeostasis followed by how membranes change in human disease. Following this, the protein families of particular interest in this thesis are presented, namely the PAQRs, TLCs and ACSs.

## Membrane Homeostasis

### *Cellular membranes*

All over the biological world, a roughly 3 nm wide hydrophobic barrier separates the cellular environments serving as the boundary between life and death [1]. Structurally, cell membranes consists primarily of a lipid bilayer, containing proteins and signaling molecules that partly span or completely span the bilayer enabling interaction with components outside the cells and/or components within the cell. As such, the cell membrane is vital in several cellular processes such as controlling transport across the membrane [2], maintaining osmotic and ionic gradients [3,4], probing the extracellular milieu [5], regulating vesicular trafficking [6], and much more. Importantly, these processes rely on the biochemical and biophysical properties of the cell membrane to be finely attuned for optimal function [7]. How then, do cells control the biophysical phase behavior of membrane lipids and their interactions with membrane proteins, resulting in the unique compositions and manifold functionalities of their membranes?

Lipidomics, the main analytical chemistry analysis for cataloging sample membrane lipids, has identified thousands of different lipids within membranes of cells, with 5% of the human genome being attributed to lipid synthesis and remodeling [8]. Despite our increasing understanding of the specific function of many of these lipids, the functions of most remain unknown [1].

Generally, lipids accomplish three major functions. First, due to their reduced state, lipids are a potent source of storable energy and catabolic potential, mainly in the form of triacylglycerol esters or steryl esters within lipid droplets. Lipid droplets function as reservoirs for future caloric usage as well as providing fatty acids to membrane biogenesis as required. Secondly, the bulk of cellular membranes consists of polar (amphipathic) lipids, consisting of a hydrophilic and a hydrophobic region. Due to entropy-driven self-assembly by water, hydrophilic portions of lipids interact with other such portions and water, giving rise to spontaneous membrane formation. This main chemical feature of amphipathic lipids allowed the first cells to seclude their internal milieu from the external environment, which in turn allowed cells to further compartmentalize inner structures into separate organelles [9–11]. Besides providing a barrier between different environments, lipids allow membranes to undergo budding, fission, fusion, and tubulation, which are main features of cell division and membrane trafficking, as well as allowing the aggregation and dispersion of membrane-anchored proteins. Thirdly, lipids may act as internal messengers in signal transduction processes [12].

One prominent feature of membrane lipids is their phase behavior. Within membranes, lipids can form several types of semi-solid and fluid phases, determined by how each lipid arranges itself spatially and the freedom of its mobility with regards to its neighboring lipids. As such, a single lipid membrane can allow the existence of two or more fluid phases, separated by phase boundaries, which gives rise to differences in membrane organization; this view of the membrane is often termed the “fluid mosaic membrane model” [13,14]. Through high throughput lipidomics analyses, we have begun to understand how different lipids affect the structure of biological membranes.

### *Cell membrane lipids*

Structurally, eukaryotic lipid membranes are mostly comprised of glycerophospholipids composed of a hydrophilic glycerol-3-phosphate headgroup (either phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI),

phosphatidylglycerols (PG), or phosphatidic acid (PA)) and a diacylglycerol (DAG) containing saturated (SFA) and/or cis-unsaturated fatty acyl (UFA) moieties of varying length at the sn-1 and sn-2 positions of the glycerol. The chemical structure of both acyl chains and headgroups influences the biophysical properties of the membrane [15]. Typically, more than half of eukaryotic membrane phospholipids are PCs that spontaneously self-assembles as a planar lipid bilayers, where each PC exhibits an approximately cylindrical molecular geometry (Fig. 1A). The majority of PCs contains one cis-UFA, making them fluid at room temperature. PEs have comparatively small headgroups resulting in a conical geometry, which prohibits them from self-assembling into bilayers and are thus often found within one leaflet of the lipid membrane. Including PEs into PCs bilayers results in curvature of the membrane (Fig. 1A); this is important for processes such as fission, fusion and budding [16].

Another class of structural lipids are the sphingolipids, composed of a ceramide backbone; sphingomyelin (SM) and glycosphingolipids (GSLs) are the most abundant lipids in this class [17]. In contrast to PCs, sphingolipids have both cis- and trans-saturated fatty acyl chains, shaping them into narrower cylinders leading to even tighter packing and resulting in a solid gel-like phase. This packing can be disturbed by the insertion of sterols, predominantly cholesterol in mammals, achieving a more fluid region [18]. As cholesterol is non-polar, phospholipids will incorporate it within the fatty acyl chains to shield if from the polar headgroups, decreasing the energy in the system while simultaneously displacing the fatty acyl chains, increasing fluidity [19]. Similarly, the displacement of membrane lipids increases with the degree of desaturation in the fatty acyl chains where SFAs results in tight packing and long-chain polyunsaturated fatty acids (PUFAs) results in the most displacement, and thus higher fluidity (Fig. 1B) [20,21].

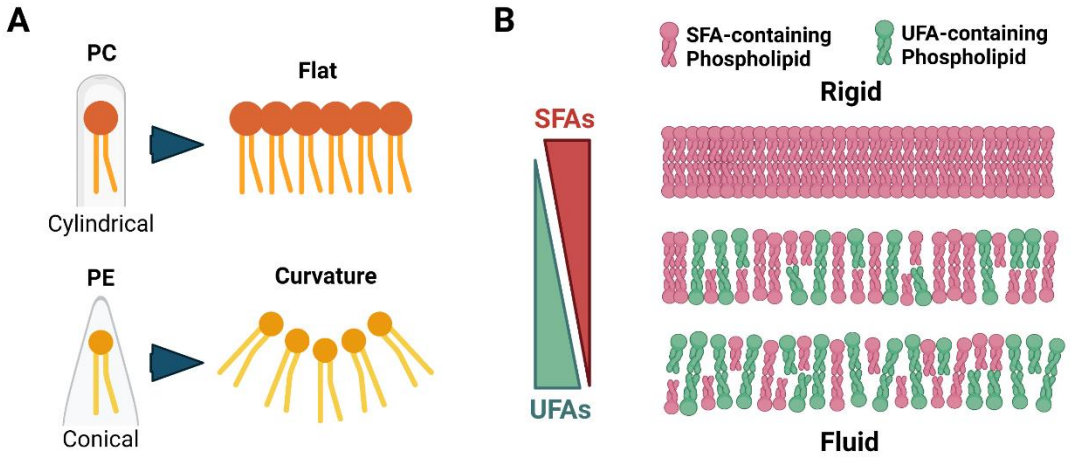


**Figure 1. Lipid membrane properties in mammalian cells**
**A.** Schematic representation of how PCs with a cylindrical configuration self-assemble into flat membranes while PEs, having a cone-like configuration, leads to curvature in the membrane. **B.** Schematic representation of the conformational fates of membranes rich in SFAs taking on a rigid structure while membranes rich in fluidizing UFAs lead to a more fluid membrane.

Finally, hydrolysis of glycerophospholipids and sphingolipids, induced by intracellular signaling, yields a myriad of messenger lipids, namely lyso-PCs (LPCs), lyso-PAs (LPA), PAs, DAGs, sphingosylphosphorylcholine (SPC), sphingosine (Sph), sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P), and ceramides. The fact that LPCs, LPAs, SPC, Sph, and S1P contain a single aliphatic chain allows them to detach from lipid membranes and subsequently signal through membrane-bound receptors [22]. Conversely, PAs, DAGs, C1P and ceramides are retained within the membrane allowing them to recruit cytosolic proteins [23]. Additionally, the local accumulation of membrane bound signaling lipids such as ceramides can profoundly affect the biophysical properties of the membrane, altering phase transitions and displacing cholesterol from within phospholipid bilayers [24–26].

### *Dietary lipids in membrane homeostasis*

Different cells require specific fatty acid compositions and distributions depending on the specific role of that cell. In order to meet such a variation in fatty acid demand, each cell must intrinsically be able to regulate its membrane composition and fatty acid pool. What makes this a challenge is the variety of ingested fatty acids from our diets. Ethnobotanical surveys estimate that more than 7000 different plant species have been used for human consumption at some point throughout history [27], all with unique fatty acid compositions. Indeed, the ratio of PUFAs to SFAs within one single food item can range from roughly 7 in the red seaweed *Gracilaria changii* to less than 0.04 in cow's milk [28].

Fat in human diets consists roughly of 95% triglycerides and 5% phospholipids [29]. Postprandially, dietary fats are spliced into micelle structures with the assistance of bile salts and phospholipids creating an emulsion of fat (Fig. 2A-B). Upon reaching the small intestine, ingested triglycerides and phospholipids are acted on by lipases, which results in free fatty acids, 2-monoacylglycerols and lysophospholipids. Enterocytes, lining the small intestine absorb these fatty acids via passive diffusion or by active transport via fatty acid transporters such as CD36. Intracellularly, the fatty acids are shuttled to the ER where they are incorporated into triglycerides anew and either stored as lipid droplets or packaged into chylomicrons, which are lipoproteins containing a main lipid core consisting mainly of triglycerides but also cholesterol and phospholipids (Fig. 2C) [30–32]. Chylomicrons are then secreted into the lymphatic system where the fatty acid composition with respect to triglycerides resembles the last meal [33]. Compositionally, chylomicron phospholipid content varies and is affected by both eating history and composition of biliary phospholipids, where dietary phospholipids varies between 2-8 g/day and biliary phospholipid production accounts for 10-20 g/day, containing mostly PCs [34,35]. Next, chylomicrons exit the lymphatic system and enter the main blood circulation where they can deliver fatty acids to tissues and cells. In parallel, previously stored fatty acids, secreted predominantly by adipocytes and hepatocytes, enter the circulation along with synthesized lipids via *de novo* lipogenesis. Circulating fatty acids are complexed into lipoproteins or bound to carrier proteins such as albumin or adiponectin [36,37], which are then accessible for uptake by cell throughout the body.

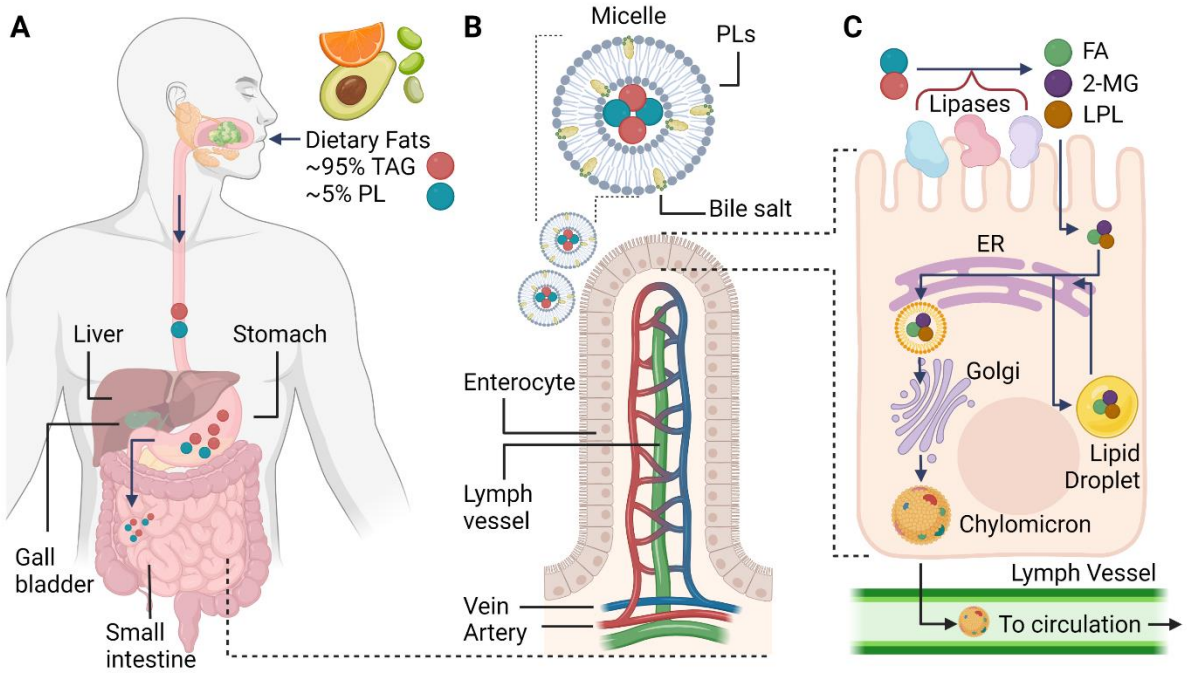


**Figure 2. Absorption of dietary lipids**
**A.** Schematic representation of the gastrointestinal (GI) tract showing ingested dietary fats with TAGs represented in red and phospholipids in blue. **B.** Schematic showing a closeup of the villi in small intestine with nearby micelles. A micelle closeup representation shows TAGs and phospholipids encapsulated by a micelle built from phospholipids and bile salts. **C.** Schematic closeup of an enterocyte

showing TAGs and phospholipids being broken down into fatty acyls, 2-monoacylglycerols and lysophospholipids by extracellular lipases. These products are taken up into the cell and either packaged into lipid droplets for later usage or packaged in the ER into vesicles transferred to Golgi for final packaging and protein coating into chylomicrons which are then released into the lymphatic vessel heading for the main circulation.

In mammals, most fatty acids originate from the diet, where approximately 90% of fatty acids in VLDLs are diet derived, and 10% are synthesized via *de novo* lipogenesis, mainly in the liver [38,39]. However, hepatic *de novo* lipogenesis is induced when total dietary carbohydrate intake exceeds body energy expenditure [40,41], with the end products being SFAs which in turn can result in hepatic insulin resistance and metabolic dysfunction [41,42]. Importantly, each cell can to some extent assert *de novo* lipogenesis and lipid remodeling based on its fatty acid needs, compensating for the varied fatty acid composition within the circulation at any given time, further highlighting the capabilities for cells to maintain membrane homeostasis. One notable exception is that PUFA composition in membrane phospholipids often reflect the specific PUFAs in the diet [43–45].

## *Eukaryotic lipid metabolism and distribution*

### *Mammalian cellular lipid metabolism*

The fatty acyl constituents of membrane lipids are either obtained from the diet or synthesized intracellularly using the substrates acetyl-CoA and malonyl-CoA in a process called *de novo* lipogenesis which ends with the 16 carbon SFA palmitic acid (PA, 16:0). Acyl-CoA-synthetases prime fatty acids into fatty acyl-CoAs enabling them for a diverse range of metabolic fates such as storage within lipid droplets, synthesis of membrane lipids, and energy extraction in mitochondrial β-oxidation [46]. Fatty acids undergo lipid remodeling by either elongation, desaturation or β-oxidation.

Desaturation introduces double bonds into the fatty acid and is mainly performed by stearoyl-CoA desaturase 1/5 (SCD1 and SCD5) in the ER, yielding mono-UFAs (MUFAs) from PA and stearic acid (18:0) (Fig. 3) [47,48]. Fatty acids can be classified by carbon chain length accordingly: short-chain fatty acids (SCFA) contain 2-6 carbons, medium-chain fatty acids (MCFA) contain 7-12 carbons, long-chain fatty acids (LCFA) contain 13-21 carbons, and very long-chain fatty acids (VLCFA) containing ≥ 22 carbons [48]. Chain length affects their biological function with VLCFA being important for processes such as skin barrier formation, myelin maintenance and sperm development and maturation [49]. Fatty acyl-CoAs are elongated by a four step process exerted by ER-localized enzyme complexes each including a fatty acid elongase (i.e. one of ELOVL1-7) which have different substrate specificities [48,50]. ELOVL1,3,4,6, and 7 elongate SFAs and MUFAs. ELOVL6 is specific for elongating PA-CoA into stearic acid but also act on shorter SFA-CoAs. ELOVL3 and ELOVL7 elongate saturated C16-C22 fatty acyl-CoAs with preference towards C18-CoAs [51]. ELOVL1 elongates C18:0-C:26:0-containing SFA-CoAs as well as C20:1 and C22:1 MUFAs and is the main elongase leading to C24 sphingolipids [52]. ELOVL4 elongates very long SFA-CoAs including >C26 fatty acyls in skin, in addition to >C26 PUFAs located in the brain, retina and spermatozoa [49]. Conversely, ELOVL2 and ELOVL5 are PUFA-restricted elongases with ELOVL2 acting generally on longer fatty acyl-CoAs than ELOVL5. ELOVL2 and ELOVL5 elongate C22-CoAs and C18-CoAs respectively (Fig. 3) [52].

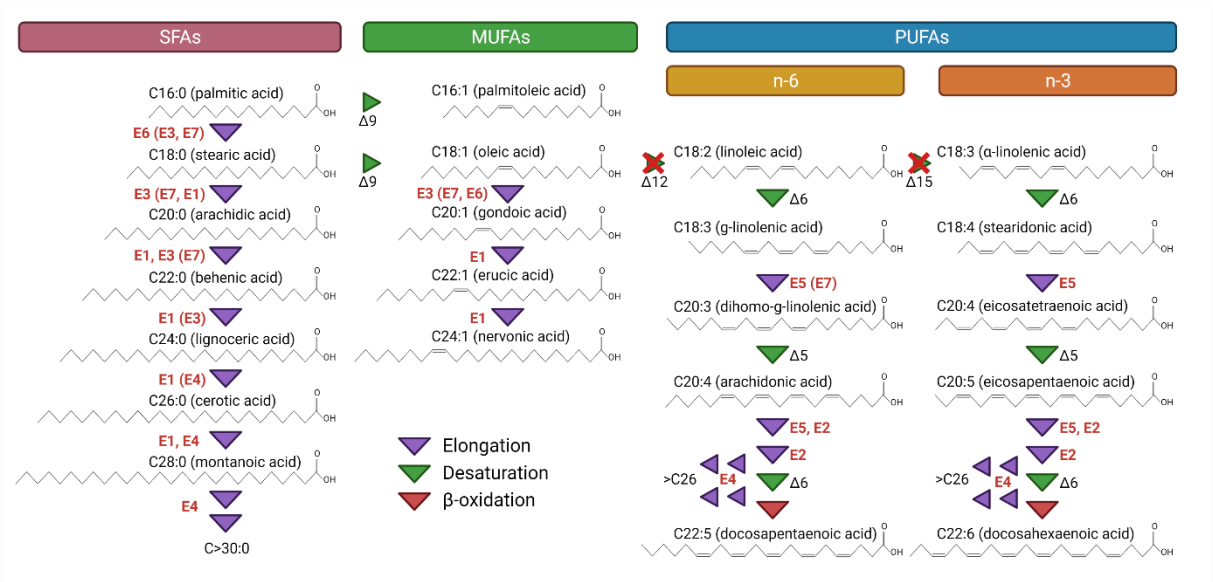


**Figure 3. Mammalian fatty acyl remodeling**
Human fatty acyl remodeling pathways acting through elongation, desaturation and β-oxidation are represented. Neither Δ12 nor Δ15 desaturases exist in humans and as such, n-3 and n-6 fatty acyls are not synthesized by desaturation. Ex and Δx denote ELOVLx and Δx desaturase respectively, where x represents the enzyme number. ELOVLs in parentheses indicate weak activities in the denoted directions. Adapted from Kihara et al. (2012).

A major difference between mammal and the nematode *C. elegans* in terms of lipid metabolism is the lack of desaturase pathways desaturating oleic acid (OA, 18:1) into linoleic acid (LA, 18:2) and further into α-linoleic acid (ALA, 18:3) in mammals, making these effectively essential fatty acids that must be supplied via the diet [53]. However, once obtained through diet, mammalian cells can elongate and desaturate these fatty acids via fatty acid desaturase 1 and 2 (FADS1/2), producing the highly fluidizing PUFAs arachidonic acid (ARA, 20:4), eicosapentaenoic acid (EPA: 20:5) and docosahexaenoic acid (DHA: 22:6), supplementing their levels above that obtained through the diet [54].

*Intracellular distribution of lipids*

Taking into consideration the myriad of combinations of aliphatic chains and headgroups, it is not surprising that thousands of different lipid species can be quantified within a single cell [8]. Fascinatingly, the distribution of phospholipids and sterols differs between organelles (Fig. 4). Partly, this can be attributed to the localization of lipid synthesis within the cell.

*Most lipids are synthesized in the ER*

The endoplasmic reticulum is the main lipid-synthesizing organelle within the cell [55], manufacturing most structural phospholipids and cholesterol, in addition to non-structural lipids such as triacylglycerols (TAGs) and cholesteryl esters. Ceramides, the precursors for sphingolipids, are also synthesized in the ER, including galactocylceramide (GalCer) specific to epithelial and myelinating cells which stabilizes apical membranes and myelin sheets (Fig. 4) [56]. Parts of the ER establish close contacts with mitochondria, forming mitochondria-associated membranes (MAMs) where specific enzymes involved in lipid synthesis resides [57], and separately also close contacts with the plasma membrane, forming plasma membrane-associated membranes (PAMs) where PSs and PIs are synthesized [58]. Thus, lipid synthesis is sub-compartmentalized within the ER. As for sterols, e.g. cholesterol, we note that the ER is also the main organelle for their synthesis, though the majority of sterols are then transferred to other organelles. As a result, the ER contains only small amounts of sterols and sphingolipids, which leads to a more fluid membrane [55].

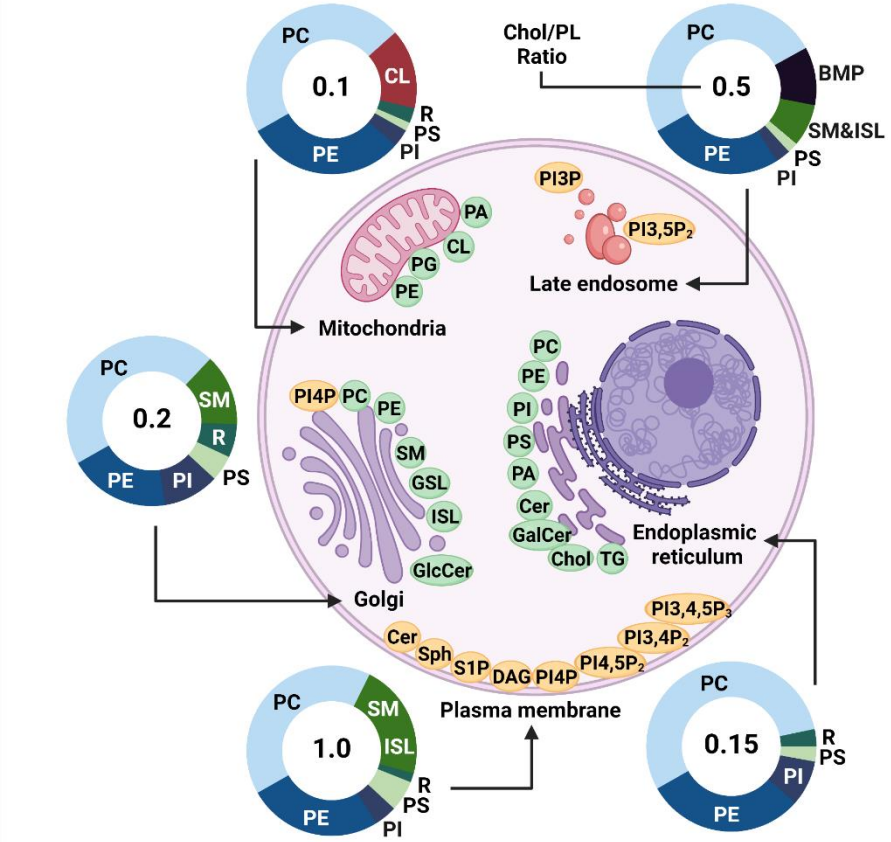


**Figure 4. Intracellular distribution of lipids**
Schematic showing organelle lipid composition in a general mammalian cell. Composition is represented as percentages of the total phospholipids. The center number represents the cholesterol/phospholipid ratio within that organelle. The main figure depicts organelles with their major synthesized lipids in green and resident signaling lipids in yellow. Note that the abundance of signaling lipids within a cell is <1% of total phospholipids except for ceramide. Glycerolipids made in the endoplasmic reticulum (ER) are PCs, PEs, PIs, PSs, PAs, ceramide (Cer), Galactocylceramide (GalCer), cholesterol (Chol), and triglycerides (TG). The Golgi stands for the main synthesis site for sphingomyelin (SM), complex glycosphingolipids (GSLs), glucosylceramide (GlcCer), and inositol sphingolipid (ISL) as well as producing PCs and PEs. Mitochondria synthesize roughly 45% of their own phospholipids, mainly PEs, PGs, PAs and cardiolipin (CL). Endosomes contain large levels of bis(monoacylglycero)phosphates (BMPs) in the inner membrane. PI(3,4,5)$P_3$, phosphatidylinositol-(3,4,5)-triphosphate; PI(3,5)$P_2$, phosphatidylinositol-(3,5)-biphosphate; PI(4,5)$P_2$, phosphatidylinositol-(4,5)-biphosphate; PI4P, phosphatidylinositol-4-phosphate; R, remaining lipids; S1P, sphingosine-1-phosphate. Adapted from Meer et al. 2009.

*The Golgi specializes in sphingolipid synthesis*

Next to the ER, a significant portion of lipid biosynthesis takes place in the Golgi. In mammals, the Golgi synthesizes sphingolipids such as sphingomyelin (SM), glucosylceramide (GlcCer), lactosylceramides (LacCer), and more complex glycosphingolipids (GSLs) [59], all exported primarily to the plasma membrane. PC and PE synthesis also occur in Golgi, with the final step in PC synthesis occurring in both ER and Golgi [60].

*The plasma membrane retains lipid signaling capability*

The plasma membrane is enriched in sterols and sphingolipids (Fig. 4), which pack tighter than the glycerophospholipids, resulting in a robust membrane able to handle mechanical stress. Despite not being able to autonomously synthesize its own structural lipids (these are ER- and Golgi-derived), the plasma membrane retains the capability of either synthesizing or degrading lipids involved in intracellular signaling cascades [61]. Plasma membrane turnover of SM is ample enough, allowing re-synthesis of SM from ceramide by local sphingomyelin synthase (SMS2), significantly contributing to local in addition to cellular SM levels [62,63].

*Mitochondria display bacterial-like lipid composition*

Mitochondria synthesize substantial amounts of lipids such as LPAs [64], which are primarily used for TAG production [65]. Additionally, mitochondria produce PAs and PGs, both of which are critical for PE and cardiolipin (CL) synthesis, a unique lipid class in mitochondria. Mitochondria supply other organelles with endogenously produced PEs, via a unique mechanism relying on decarboxylation of PS [66]. The inner mitochondrial membrane contains up to 25 mol% of CL in addition to PGs, which together with a high PE/PC ratio resembles membranes of the bacteria from which they are descendants and which could be necessary for oxidative phosphorylation [67]. Generally, mitochondria display low amounts of membrane-embedded cholesterol (Fig. 4). However, in unique cells involved in steroid hormone synthesis, mitochondria shuttles and metabolizes cholesterol in conjunction with the ER [68].

*Endosomes shift lipid composition during maturation*

Early endosomes reflect the plasma membrane with respect to membrane composition, but undergo a shift with maturation into late endosome and eventually lysosomes with decreased sterol and PS content exchanged for large increases in bis(monoacylglycero)phosphates (BMPs) [69]. BMPs are critical for endo/lysosomal functions such as fusion processes, multivesicular body synthesis and sphingolipid hydrolysis [70,71], with defects in BMP composition being linked to several diseases [72]. Several endosomal kinases and phosphatases act in concert, producing and hydrolyzing several PIs [61], such as PI3,5$P_2$ on late endosomes, PI4,5$P_2$ on plasma membranes, PI3P on early endosomes, and PI4P on the trans-Golgi network (Fig. 4) [73]. Thus, the PI composition shifts as endosomes mature allowing them to recruit specific protein partners involved in vesicle trafficking or cellular homeostasis [61]. Noteworthy is that membrane levels of signaling lipids are very low (<1%) [8] making them a challenge for scientific observations.

## *Asymmetrical lipid composition within cellular membranes*

Further stratification of membranes with respect to lipid composition within the individual leaflets of a bilayer reveals high degree of asymmetry in lipids facing the cytosol or the extracellular environment. One exception is the ER in the sense that lipids are symmetrically distributed within both leaflets in ER membranes whereas Golgi, plasma and endosomal membranes exhibit great asymmetry, with SM and GSLs located to the outer leaflet, and PSs and PEs enriched in the inner leaflet [74,75]. This asymmetry gives rise to significant functional consequences such as extracellularly exposed PSs on damaged cells, allowing coagulation proteins to assemble the enzyme thrombin on previously non-thrombogenic membrane surfaces, causing blood coagulation [76]. Additionally, accumulation of certain lipid species to the cytosolic leaflet can lead to specific membrane stress, which leads to membrane bending and eventually vesicle budding [77]. Several factors govern lipid asymmetry within membranes including biophysical barriers hindering spontaneous crossover of lipids from one leaflet to the other, retentive mechanisms that trap lipids within one leaflet, and the existence of transporter proteins that facilitate lipid translocation [74,77]. For many lipids, spontaneous flip-flopping between leaflets is slow and is greatly influenced by polarity, charge, headgroup and size of the lipid. For example, the flip-flopping half-life for PCs is expressed in hours to days [78,79], and that of complex GSLs is in the order of days. In contrast, the flip-flopping half-life for ceramides [80], sterols [81] and DAGs [82,83] is in the order of seconds. Additionally, lipids carrying a negative charge such as PGs and PAs can rapidly translocate between leaflets in cases when low pH neutralizes their charge [84].

### *Membrane fluidity*

The extent of molecular disorder and rate of molecular motion of lipids and proteins within a cellular membrane is often referred to as the fluidity of the membrane [85]. Membrane fluidity is directly impacted by the degree of fatty acyl saturation of membrane residing lipids [20] and generally decreases proportionally with increasing SFA levels. Additionally, membrane fluidity is impacted by intrinsic factors such as cholesterol concentration within the membrane [86,87], but also external factors such as osmolarity [85] and temperature [88], to which cells adapt by adjusting membrane composition. Proteins act as membrane property sensors and activate intracellular processes in response to changes in fluidity of the membrane.

### *Importance of membrane homeostasis regulation*

Maintaining an optimized lipid membrane is vital for a myriad of cell processes, which becomes increasingly evident as more disease states are shown to correlate with aberrant membrane composition, as described separately below. Importantly, many receptors, channel proteins and transporters demand precise interactions with lipids for proper function [89]. Elevated levels of SFAs within membranes induce c-Src clustering, which leads to JNK activation, a potent mediator of apoptosis [90]. Insulin receptor signaling increases with increased membrane fluidity [91]. Blood pressure is regulated partly in response to shear stress via TRPV channels, which are sensitive to membrane composition, specifically requiring phospholipids rich in PUFAs for proper activation [92–94]. Contrarywise, TLR4 receptor accumulation within compartmentalized lipid membranes called lipid raft is induced by low membrane fluidity, resulting in proinflammatory signaling [95,96]. Membrane fluidity-dependent phase separation also facilitates T cell mediated signaling [97]. Lastly, changes in cell membrane fluidity and tension results in altered conformational dynamics of membrane-bound GPCRs, which are involved in propagating mechanochemical signal transduction in endothelial cells [98]. These are but a few examples of how changes in membrane composition and biophysical parameters affect a range of cellular functions. Given its importance, one expect that robust mechanisms must exist to maintain membrane fluidity within a near-optimum range.

### *Sensors of membrane properties*

Several proteins able to react to changes in the membrane environment have been characterized. The bacterial DesK senses temperature-induced membrane packing defects leading to desaturation of membrane lipids [99]. Fungal Mga2 acts as a lipid-packing sensor in the ER, reacting to increased membrane saturation by conformational changes, leading to induced fatty acid desaturation [100]. An additional fungal protein, Opi1, is a major regulation of biosynthesis of membrane lipids such as PCs, PEs, PSs, and PIs which all require the precursor phosphatidic acid for synthesis. Phosphatidic acid binds directly to Opi1, inhibiting its translocation into the nucleus, but is released once phosphatidic acid is depleted, after which it can initiate biosynthesis of new membrane lipids [101]. In addition, several sensors are well documented in mammals and a few of them will be highlighted in further detail below. Note that the mammalian proteins ADIPOR1 and ADIPOR2 are emerging as a potential sensors of membrane properties but that they will be discussed at length later in the thesis.

#### *SREBP1 & SREBP2*

Cholesterol homeostasis is of vital importance in cells as it is crucial for plasma membrane structural stability. Importantly, excess cholesterol is detrimental as it forms solid crystals which rupture cells as well as being deposited in the arterial lining leading to atherosclerosis [102]. As cholesterol is mainly embedded within lipid membranes, there must exist a mechanism that senses the abundance of cholesterol and reacts by initiating a transcriptional response leading to the synthesis of more cholesterol or ameliorate excess accumulation. This sensing mechanism relies on the sterol response element binding proteins (SREBPs) which act both as biophysical lipid composition sensors and as transcription factors that can adjust cholesterol and lipid homeostasis [103,104]. The human SREBPs are all

membrane resident proteins and are divided into SREBP1a and SREBP1c as well as SREBP2. In order for the SREBPs to influence transcription, their $NH_2$-terminal domain must be released from their membrane location and enter the nucleus [104]. Generally, SREBP2 is considered the main sensor of cholesterol in the membrane and acts primarily by inducing cholesterol synthesis in situations of low membrane sterol content, while SREBP1a/1c control fatty acid desaturation and lipogenesis triggered by differences in PC abundance [105,106]. Natively, SREBP is found in complexes bound to the SREBP cleavage activating protein (SCAP), with both being linked to insulin-induced gene protein (INSIG) which retains the SREBP-SCAP complex within the ER [103]. With decreasing levels of membrane-bound cholesterol, ubiquitination of INSIG occurs allowing anterograde transport of SREBP-SCAP, via COPII-coated vesicles, to the Golgi. Golgi-residing protein and adipoQ receptor family 3 (PAQR-3) retains SREBP-SCAP allowing peptidases to cleave off the $NH_2$ domain, which transfers to the nucleus and transcriptionally initiates cholesterol and lipid synthesis [103,107]. Once cholesterol levels reach a certain threshold, SREBPs bind to SCAP once again, triggering conformational changes that prevent SREBP-SCAP to leave the ER, thus restoring homeostasis [108]. SREBP1 plays a crucial role in lipogenic gene regulation, and is predominantly activated by decreased levels of membrane PCs, which allows the GTPase ARF1 to initiate the cleavage of SREBP1 for downstream lipid regulation [109]. PUFAs have an inhibitory effect on SREBP1 activation as shown by decreased $NH_2$ release and down-regulated lipogenic gene expression [110,111].

*PCYT1A*

PCs are the most abundant phospholipid class in eukaryotic cell membranes and provide the bulk of structural lipids within the cell. As such, PC synthesis must be closely regulated with membrane-demanding processes such as cell proliferation in which new membranes are synthesized en masse. Two pathways exists that provide *de novo* synthesis of PCs, specifically the Kennedy pathway and the PE methyltransferase pathway [112]. The former stands for the dominant pathway for PC synthesis in eukaryotes and entails three enzymatic reactions finalized by condensation of DAG and choline into PCs. The rate-limiting step of this pathway is the creation of cytidine diphosphate (CDP)-choline by choline phosphate cytidylyltransferases (CCTs) [113] which are highly evolutionarily conserved in eukaryotes [114]. Mammals express two CCTs, namely PCYT1A and PCYT1B, where the former is ubiquitously expressed while PCYT1B is less widely expressed in addition to lacking the nuclear localization signal (NLS) that PCYT1A includes [112]. Membrane association and catalytic activation of PCYT1A/B is induced by conically-shaped membrane lipids such as PEs or DAGs, or by negatively charged phospholipids such as PSs or PAs *in vitro* [115–117]. It has been suggested that PCYT1A/B sense PC scarcity relative to curvature-inducing membrane lipids, resulting in initiation of PC synthesis, ameliorating the membrane stress caused by conically shaped membrane lipids [112]. Recent studies have confirmed this induction of PCYT1A/B *in vivo*, pinpointing their activity within the inner nuclear membrane where they are activated by packing defects. More specifically, elevation of membrane-bending lipids such as PEs leads to stored curvature elastic stress, which causes a conformational change in PCYT1A that activates it, thus inducing PC synthesis which in turn alleviates the membrane stress, achieving homeostasis [112].

*IRE1*

Proper folding of lipid membranes and proteins is a crucial process taking place within the ER. As such, the ER must sense and react accordingly in scenarios of defective protein folding [118] as well as perturbed membrane lipid composition [119]. IRE1 is a eukaryote-spanning class III sensor protein that attends to these processes, namely by regulating the ER unfolded protein response (ER-UPR) [118–120]. Generally, the ER-UPR initiates a down-regulation in protein synthesis and activation of chaperones of the ER-associated degradation (ERAD) apparatus while also elevating ER membrane biosynthesis [121,122]. ER stress induced by faulty membrane composition and/or protein folding errors leads to oligomerization of the single-pass membrane protein IRE1, which triggers trans-autophosphorylation of its kinase domain, followed by activation of its RNase domain. Said RNase domain goes on to splice the mRNA of the mammalian gene XBP1 of which the spliced XBP1 transcript is a transcription factor able to initiate a transcriptional response involving hundreds of genes, i.e. the

effectors of the ER-UPR [122,123]. Several lipid membrane disturbances have been linked to IRE1 activation such as low PE/PC ratio [124], elevated membrane cholesterol [125], increased lipid saturation [126], and depletion of inositol, a precursor molecule for PI synthesis [127]. Mechanistically, in *C. cerevisiae*, IRE1 is able to "sense" all these membrane abnormalities by the biophysical characterization of the surrounding bilayer. This process is thermodynamically driven: increased thickness of the ER, which correlates with membrane rigidity, favors oligomerization of IRE1, which has a short transmembrane-spanning domain, and thus initiating its activation [119].

## *Membranes role in disease*

The human diet may be the main factor leading to disability and premature death in the west [128]. As mentioned previously, a major source of cellular lipids are derived from dietary fat. It comes as no surprise that defects in membrane properties or disruptions in membrane homeostasis would feature in human diseases. A few diseases are highlighted below along with how membrane lipid composition defects have been associated with each. In essence, it is of critical importance that we further our knowledge in the interplay between membrane homeostasis and dietary fatty acids as this has substantial relevance for human health.

### *Coronary heart disease*

Heart disease is the main killer in western countries causing close to 400 000 deaths yearly in the United States alone [129]. Alterations in phospholipid-mediated signal transduction has been observed in myocardial ischemia-reperfusions, via up-regulation of phospholipase A2 ($PLA_2$) which promotes PC cleavage into lyso-PCs (LPCs). LPCs have arrhythmogenic properties in the heart [130]. Additionally, when exogenous levels of SFAs overwhelms the cellular capacity for storage and energy extraction, SFAs are channeled into phospholipid membranes, which leads to ER-UPR activation and progression of diastolic cardiac dysfunction [131]. The cardiomyocytes rely heavily on desaturase activity of SCD1 in order to mitigate the detrimental effects of SFAs, with SCD1-inhibition leading to cardiomyocyte apoptosis [132]. Clearly, dysfunctional lipid homeostasis contributes to the pathology of the leading killer of men and women [131].

### *Brain diseases*

Alzheimer's disease kills nearly 85 000 Americans every year making it the 6$^{th}$ leading cause of death [133]. The disease progression mainly involves the buildup of amyloid plaque in brain tissue. Increased PE synthesis in mitochondria-associated membranes in turn increases amyloid beta (Aβ) peptide amounts through an unknown mechanism in familial Alzheimer's disease where the Presenilin proteins are mutated [134,135]. Aβ can perturb cholesterol homeostasis by modifying its distribution within the cell membrane, changing membrane fluidity [136]. Reduced membrane fluidity has, in turn been attributed to multiple regions in brains of Alzheimer's patients [137,138].

Parkinson's disease is characterized by accumulation of cytosolic aggregates of α-synuclein (Lewy Bodies), which in turn leads to progressive loss of dopaminergic neurons in the brain [135,139,140]. α-synuclein-induced cytotoxicity is correlated with its potential in altering membrane properties by interaction with membrane PUFAs leading to rapid α-synuclein oligomerization [141]. In fact, membrane fluidity has a substantial impact on α-synuclein aggregation within the membrane where it localizes preferably to highly curved membranes [142]. Inhibiting the formation of the MUFA oleic acid (OA) has an ameliorating effect on α-synuclein cytotoxicity suggesting that SCD1 inhibition may be a promising treatment option in Parkinson's disease [143,144].

Huntington's disease is a fully penetrant neurodegenerative disease with fatal outcome caused by a CAG trinucleotide repeat in the huntingtin gene [145]. Pathogenic huntingtin promotes neural dysfunction by several mechanisms converging on mitochondrial disruption. Interestingly, mitochondria and plasma membranes in Huntington's patients are disproportionally fluid [146,147], which opens venues where membrane rigidification by e.g. cholesterol could be a potential treatment.

*Diabetes*

Incidence of type 2 diabetes, or diabetes mellitus, has increased nearly exponentially since the 80s leading to 20 million cases of type 2 diabetes in the US, with projections estimating that one in three Americans will be diabetic by 2050 [148]. Global excess mortality in adults attributable to type 2 diabetes is roughly 4 million deaths, yearly [149]. That SFA intake via diet has an impact on insulin sensitivity, which is decreased in type 2 diabetes, is a century-old concept [150]. The function of insulin, the main regulator of glucose uptake in cells, is impaired within hours after consuming a SFA-rich meal [151]. Largely, the higher the concentration of circulating SFAs is, the higher the risk of developing insulin resistance and further on type 2 diabetes [152]. In fact, increased SFA-content in membrane phospholipids has been linked to impaired insulin sensitivity [153–155]. Modifying the fatty acid composition of plasma membranes can influence insulin receptor accessibility, binding and action in multiple cell types [156–159]. Thus, it is not surprising that several independent studies have observed that cell membranes of diabetics are excessively rich in cholesterol [160], sphingomyelin [161], and SFAs [153,162], all resulting in decreased membrane fluidity. Two longitudinal studies followed thousands of healthy participants measuring the membrane lipid composition of red blood cells and found that people showing the most SFA-containing membrane lipids were most likely to develop diabetes later in life [156,163,164]. A state of low membrane fluidity impairs several aspects of proper glucose control, ranging from: perturbed GLUT4 transport [159], vesicular trafficking in β-cells [165], glucose transport [156], endocytosis [166], and metabolic rate control [167]. The notion that decreased membrane fluidity is a core feature in the pathophysiology of diabetes has been reviewed extensively through the years [159,168,169].

*High blood pressure*

High blood pressure is the number one risk factor for death worldwide [170], leading to around 7 million deaths each year [171]. Red blood cells, or erythrocytes, of hypertension patients have repeatedly been shown to exhibit decreased membrane fluidity, which decreases with increasing age, including other vascular cell types such as smooth muscle cells, lymphocytes and platelets [172–175]. A human intervention trial showed that oral supplementation of the long-chain PUFA EPA led to increased EPA-containing membrane phospholipids, with reduced blood pressure as a result [176], suggesting a direct impact of membrane lipid composition on the sodium-controlled mechanism regulating blood pressure. Even a mild exercise regime for 6 months led to increased erythrocyte membrane fluidity in hypertensive patients suggesting a potential treatment venue directly associated with membrane fluidity in hypertension.

*Cancer*

Cancer has been clearly linked with membrane lipid composition. Generally, most cancer cells depend profoundly on *de novo* lipogenesis (synthesis of lipids) to provide the rapidly dividing cells with constant supply of new membrane [177,178]. Palmitate, the major SFA in cells, is the end product of *de novo* lipogenesis and, as such, cancer cells continuously run a risk of producing excessively rigid cell membranes. Therefore, cancer cells critically rely on the desaturase machinery to maintain membrane homeostasis [178,179]. It therefore comes as no surprise that challenging cancer cells with exogenous PA impairs cancer cell growth, which could provide new venues of cancer treatments [180]. In fact, increased membrane fluidity is correlated with poor lung cancer progression [181]. As such, inhibiting desaturases could be a potential avenue of cancer treatment [182,183]. Conversely however, decreased membrane fluidity in cancer cells contributes to resistance to chemotherapeutic agents, likely because it reduces cell permeability [184,185], and protection from peroxidation damage from PUFA-induced oxidative stress [184].

*Aging*

Dying from old age might just be a myth. A study that analyzed over 42 000 consecutive autopsies concluded that the studied centenarians, people living past a hundred years, were found to have succumbed to diseases in 100 percent of the cases examined, despite being perceived, by their physicians to have been healthy prior to "dying of old age" [186]. Interestingly, cellular membrane lipid composition tends to deviate from the presumed optimum with increasing age, showing increased accumulation of SFAs, decreased MUFAs and PUFAs (containing two double bonds), as well as slight increases in PUFAs along with peroxidized byproducts [187,188]. In total this leads to a net increase in membrane lipid saturation with age [189,190] which could be a general phenotype of diseases at old age.

## The PAQR Proteins

The Progestin and AdipoQ Receptor (PAQR) proteins are an evolutionarily ancient family with homologs present organisms as diverse as mammals, nematodes, yeast [191] and even bacteria [192]. Humans express eleven PAQR proteins (PAQR1-11), where PAQR1 and PAQR2 correspond to ADIPOR1 and ADIPOR2, respectively. Essentially, PAQR proteins exhibit three main features namely: seven transmembrane domains, an inverted orientation with respect to GPCRs meaning that their N-terminus is cytoplasmic, and being a subclass of the CREST hydrolase protein family [193]. A brief overview of PAQRs in yeast, *C. elegans*, and the mammalian ADIPOR1/2 will be described below.

*Izh in yeast*

In yeast, the three Implicated in Zinc Homeostasis (Izh1-3) genes encode PAQR proteins homologous to the mammalian ADIPORs. Functionally, Izh1-3 controls abundancy of structural sterols within the membrane, which in extension can affect the membrane permeability of zinc ions [194]. Izh gene expression is controlled via an oleate response element (ORE) [195] with expression being suppressed by the fluidizing lipids UFAs oleic (C18:1) and linoleic (C18:1ω6) [196], and induced by SFAs [194,196]. In addition, Izh2 was shown to act as a ceramidase, which degrades ceramides into free fatty acids and secondary messenger sphingoid molecules [197]. Following this finding, human ADIPOR1 and ADIPOR2 were shown to possess a similar ceramidase activity when heterologously expressed in yeast, which could be induced further by addition of the adipokine adiponectin from an unspecified source [198,199].

Separately, transcriptomic analysis of mis-regulated genes in a Izh2 knockout mutant, revealed differences in several lipid metabolism genes affecting membrane homeostasis, namely inositol-3-phosphate (INO1), phosphatidylserine synthase (CHO1), TSC10 (essential in phytosphingosine synthesis), and ERG28, an ER membrane bound protein involved in ergosterol biosynthesis [200]. Furthermore, Izh2 expression is induced in scenarios of increased membrane rigidity where UFA content was modified [201] in addition to being suppressed, together with Izh4 expression, in scenarios of abundant UFAs [202]. To summarize, the Izh proteins can regulate membrane structural lipid composition, are induced by membrane rigidification and suppressed by UFAs, exert ceramidase activity, and can partially be functionally replaced by human ADIPORs.

*PAQR in C. elegans*

*C. elegans* expresses 5 distinct PAQR proteins, two of which appear to be functional orthologs of mammalian ADIPOR1/2, namely PAQR-1 and PAQR-2 [203]. Noteworthy is that *C. elegans* lacks a homolog to mammalian adiponectin, suggesting that such a ligand is not required for the regulation of PAQR-1 and PAQR-2. PAQR-1 deficiency leads to no clear phenotype though it enhances several phenotypes observed in the PAQR-2 knockout, indicating functional redundancy [203,204]. PAQR-2 deficiency leads to a withered tail tip, perturbed autophagy, shortened life span, reduced brood size and locomotion, and shows strong intolerance to rigidifying conditions such as cold exposure and dietary SFAs [203,205–207]. Significantly, PAQR-2 mutants display a strong excess in SFA-containing phospholipids in conjunction with decreased membrane fluidity *in vivo* [205,207,208]. An obligate

partner to PAQR-2 function, IGLR-2, was discovered in a forward genetics screen, cementing that both proteins must be present within cells in order to maintain membrane homeostasis when worms are challenged with cold exposure of SFA-rich diets [207,208]. IGLR-2 contains a large extracellular N-terminal domain including a single immunoglobulin region, several leucine-rich repeats, one transmembrane α-helix, and a substantial C-terminal domain [207]. Vague homology can be established between IGLR-2 and the mammalian LRIG proteins, which contains roughly 40 members [209]. As of now, no functional mammalian homolog to IGLR-2 has yet been identified. IGLR-2 has been described to locate to the plasma membrane where it physically interacts with PAQR-2 when regulating membrane homeostasis [207,210]. Certain evidence points to a direct interaction between IGLR-2 and PAQR-2 via their transmembrane domains, where IGLR-2 could potentiate displacement of the cytoplasmic domain of PAQR-2, allowing substrates into the active site [204,210]. Surprisingly, PAQR-1 function does not require IGLR-2, indicating that PAQR-1 could be constitutively active or interacts with a different partner [204].

Astoundingly, PAQR-2 and IGLR-2 were the only *C. elegans* proteins identified as essential in averting lethal membrane rigidification due to the SFA PA in an unbiased genetic screen [210]. Separate screens, aimed at identifying compensatory mutations to the SFA-vulnerability of PAQR-2 knockouts, exposed an independent downstream pathways in PAQR-2/IGLR-2 regulation. Said pathway engages up-regulation of desaturases by gain-of-function mutations in NHR-49 (homolog to mammalian PPARs), MDT-15 (homolog to mammalian mediator subunit MED15) and SBP-1 (homolog to mammalian SREBPs) [206]. However, this pathway alone is not sufficient to completely restore the defective membrane homeostasis in PAQR-2/IGLR-2 mutants (see Papers I and II discussed later in this thesis). Taken together, the experimental evidence shows that PAQR-2 in *C. elegans* is essential to allow worms to adaptively modulate membrane homeostasis in response to cold or SFA-rich diet exposure by increasing UFA-containing phospholipids in membranes.

*ADIPORs in mammals*

Initially, ADIPOR1 and ADIPOR2 were discovered as proposed receptors for the metabolically beneficial adipokine adiponectin, identified using a cDNA expression library screen aimed at isolating proteins that bound to fluorescent bacterially expressed recombinant adiponectin [211]. Structurally, the ADIPOR crystal structure is solved [212] and shows a barrel-shaped conformation (open towards the cytoplasm), harboring a cavity able to accommodate a fatty acid or fatty acid-like substrate and including a zinc-coordination site potentially stabilizing the structure and/or partaking in hydrolytic reactions such as the previously described ceramidase activity [213,214]. The ceramidase activity in the ADIPORs has is increased by exogenously added adiponectin, however the activity is low, and as such does not rule out that they might possess hydrolase activity for other neutral lipids [214]. Conformationally, several studies have reported that both ADIPOR1 and ADIPOR2 can form homo- and heteromultimers [211] with ADIPOR1 dimers observed in human cell lines such as HEK293, primary human cells such as HUVEC and human primary muscle tissue [215]. ADIPOR1 and ADIPOR2 homo- and heterodimers were shown to accumulate to similar levels on the plasma membrane and ER in HEK293AD cells [216]. However, subcellular localization of ADIPOR1 and ADIPOR2 may differ, at least in HeLa and HEK cells, with ADIPOR1 predominantly locating to the plasma membrane and the majority of ADIPOR2 located within the ER membrane [217,218].

Signaling downstream of ADIPOR1 and ADIPOR2 has been proposed to be via either or both of two major pathways: via AMP-activated protein kinase (AMPK) or via peroxisome proliferator activated receptor α (PPARα) signaling [219–222]. AMPK, a potent master regulator of metabolism, induces ATP formation during periods of low intracellular energy by phosphorylating a myriad of proteins, many of which are involved in lipid homeostasis [223]. PPARα, together with PPARγ and PPARδ, make up a family of nuclear receptors and act as ligand-activated transcription factors, which upon activation regulate a cascade of lipid metabolic processes such as synthesis, transport, and degradation [224,225]. Several hepatic and muscle metabolic benefits in *AdipoR1* overexpressing transgenic mice or cultured hepatocytes are suggested to be dependent on AMPK activation both *in vivo* and *in vitro* [219,221]. Conversely, the hepatic benefits reported in *AdipoR2* transgenic mice have been proposed to depend on

PPARα activation (not AMPK) and its downstream target genes [219], with similar effects observed in muscle albeit there also including AMPK activation [226]. The reported ceramidase activity of ADIPOR1 and ADIPOR2, which would generate sphingosine and consequentially the signaling molecule sphingosine-1-phosphate (S1P) [12], protects cells from SFA-induced apoptosis [213,214]. S1P generation and signaling is not AMPK-dependent *per se* but processes downstream of S1P could be AMPK-dependent [213].

A word of caution is warranted as several of the results mentioned in the above paragraph were obtained using two different sets of AdipoR1/2 KO mouse models (one made in Japan and one by Deltagen [211,227,228]) which often yielded contrasting phenotypes. Prominently, the Deltagen-generated *AdipoR1 Adipor2* double knockout is embryonic lethal at day 16.5 [229] while the Japanese double mutant is viable [211]. The Deltagen *AdipoR2* single knockout mouse has been validated [228], and studied extensively [229], and is the model used for the present thesis work and is described further in the Methods section below.

Several studies have described adiponectin-dependent ADIPOR signaling, including physiological functions [213,219–221,227]. Certain findings, correlating adiponectin and the ADIPORs, are debatable as recent findings describe no interaction between adiponectin and the ADIPORs [230], with adiponectin being shown to act as a lipid carrier protein which could explain its high concentrations in the circulation [37]. Interestingly, recent genome-wide CRISPR/Cas9 screens ranked *ADIPOR2* at 25$^{th}$ place out of roughly 3000 metabolism genes, as crucial for T-cell-derived Jurkat cells ability to mitigate PA toxicity [231], while ranking at 4$^{th}$ place out of around 20 000 genes for K562 cells capacity to ameliorate hypoxia-induced membrane rigidification [232], suggesting a role for *ADIPOR2* in human membrane homeostasis. Notably, neither screen identified adiponectin as a candidate for either suppression. As such, these results strongly indicate a role of the mammalian ADIPORs in membrane homeostasis, which would be in line with mirroring evidence in *C. elegans* and yeast, ultimately implicating the PAQR proteins as master regulators of membrane homeostasis.

## The TLC Proteins

Characterization of the genetic mutation leading to a condition progressive epilepsy with mental retardation led to the discovery of a missense mutation in the mammalian gene *CLN8* [233]. In humans, *CLN8* is ubiquitously expressed, encoding a multi-pass transmembrane (TM) protein localized predominantly to the ER, including in the ER-Golgi intermediate compartment [234]. Since then, CLN8 has been classified as one member of a large eukaryotic homologue family which encompasses two previously characterized protein groups, namely Lag1p in *S. cerevisiae* and the human translocating chai-associating membrane proteins (*TRAMs*). Lag1p in yeast has been reported as a regulator of aging and longevity [235] which together with its paralogue Lac1p, facilitate the transport of glycosylphosphatidylinositol (GPI)-anchored proteins from the ER to Golgi [236,237]. Yeast *lag1 lac1* double knockout mutants display severe growth defects correlated with depletion of sphingolipids, increased fatty acyl shuttling into glycerophospholipids in addition to polar ceramide accumulation [236]. At least two Lag1 and Lac1 homologs has since been identified in most animal species, with mammals containing six paralogs named the LASS (longevity assurance) genes. Note that although neither CLN8 nor TRAM1 were sufficient to rescue the ceramide defect in *lag1*/*lac1* double mutants, several human LASS proteins could [238,239], suggesting that a major function of the LASSes include ceramide metabolism.

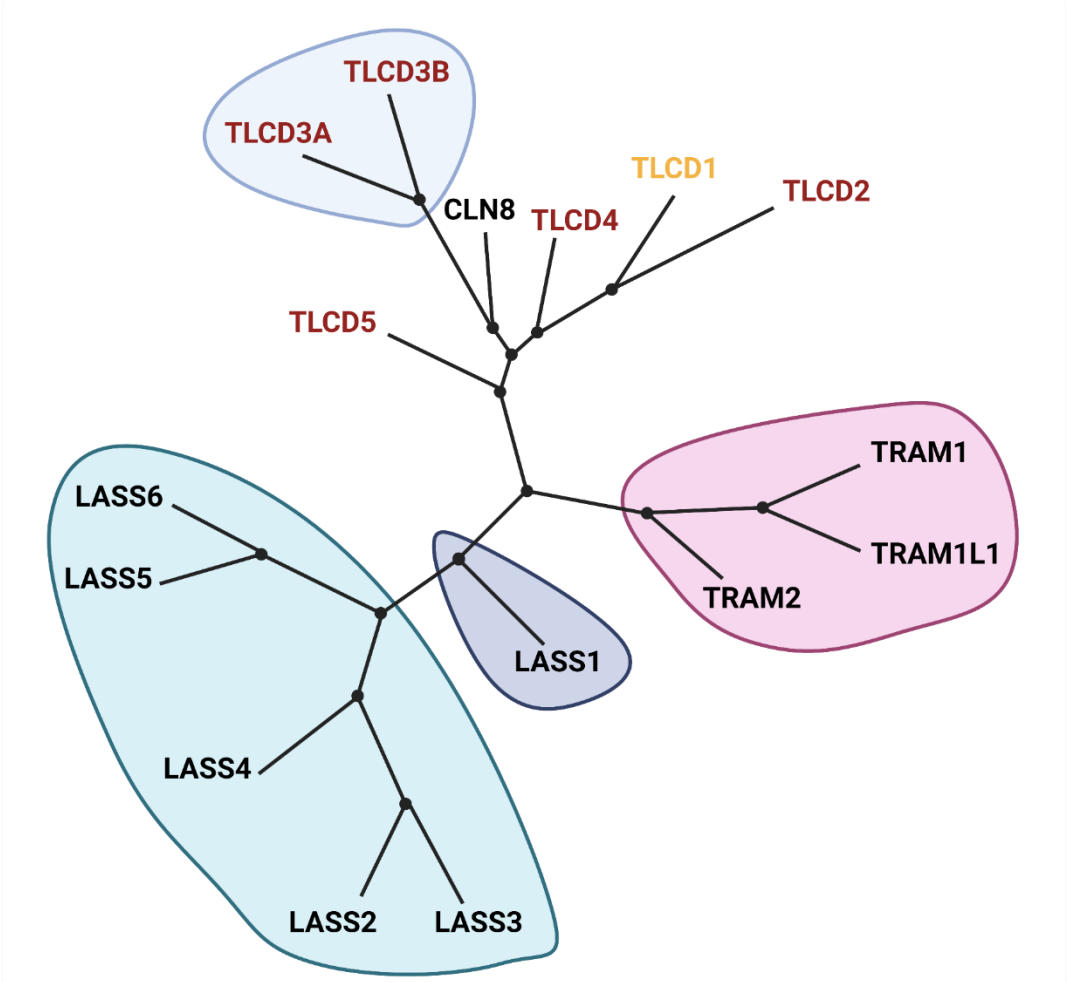


**Figure 5. Human TLCD Phylogenic Tree**
Phylogenic tree depicting all the TLC-containing proteins in humans. The phylogenic tree was generated by ClustalW alignment on one hundred data sets by Seqboot in the Phylip package. Trees were built with PromI (maximum likelihood, Phylip package), with a consensus tree by Consense (Phylip). Trees with identical topology was obtained using Neighbor Joining (ClustalW) with 1000 bootstrap values. Colored outlines represents branches with bootstrap values of 1000, indicating a high replication rate to the original phylogeny [240]. Non-colored branches showed no significant bootstrap values. Black fonts represent proteins of which their function is known or partly known. Yellow signifies few study observations and red font indicates genes with currently unknown function. Adapted from Pewzner-Jung et al. [241], with branch lengths preserved.

Sequence alignment efforts including 70 TRAM, Lag1p, CLN8, and other homologue sequences, identified an evolutionarily conserved region of five TM α-helices [242]. Said homologues are referred to as the TLC (TRAM-Lag1p-CLN8) proteins. There are 16 identified TLC domain-containing genes in humans, also conserved in mice: six LASSes (*LASS1-6*), three TRAMs (*TRAM1*, *TRAM2* and *TRAM1L1*), *CLN8*, and six novel genes named TLC domain containing 1 (*TLCD1)*, 2 (*TLCD2)*, 3A (*TLCD3A)*, 3B (*TLCD3B)*, 4 (*TLCD4)*, and 5 (*TLCD5*) of largely unknown functions [241]. Alignment analysis of the 16 human proteins yields 4 distinct branches (Fig. 5). TLCD3A/B cluster into one subgroup closest to CLN8, followed by a branch separating TLCD4, TLCD1 and TLCD2. Including the singled out TLCD5, these proteins cluster separately from the remaining TRAMs and LASSes [241]. The three TRAMs form a distinct subgroup relatively close to the LASSes and have been shown to control polypeptide translocation into the ER with some evidence pointing to it modulating exposure of translocating proteins to the cytosol [243].

The LASSes partition into two distinct groups with *LASS1* standing by itself and *LASS2-6* on a separate branch, which is consistent with LASS1 sharing closer homology with yeast proteins compared to the others (Fig. 5) [241,244]. The human *LASS2-6* genes are predicted to include a homeobox (Hox) domain which is not present in *LASS1*. By comparison, there are only 4 TLC genes in *Drosophila* containing a Hox domain, and no Hox domain is found in yeast and plant *LASS* genes [245]. Said Hox region is derived from sequence-specific transcription factors important for development, namely the homeobox proteins [246]. No evidence has been reported linking the Hox domain in LASS genes as a transcription factor, which together with the fact that several *LASS* proteins lack a Hox domain altogether suggests that the Hox domain present in some LASS genes may not act as a transcription factor required for their documented ceramide synthase activity [241]. All *LASS* genes, excluding *LASS3*, encode a minimum of two isoforms, one of which lacks part of the TLC domain. Mechanistically, not much is known about these short isoforms but they have been found in lymphomas and other tumor cells, where they may facilitate tumor recognition by the immune system, though this is of course not their natural function [247]. A splice variant of *LASS5* has been described to be involved in immune system detection of tumors [247]. Functionally, the mammalian *LASS* genes were first linked to ceramide synthesis by overexpression of *LASS1* leading to increased mammalian cell C18-ceramides [248]. Subsequently, LASS4 and LASS5 were described to utilize C18/20 and C16 acyl-CoAs, respectively, as substrates in the synthesis of ceramides [249]. LASS6 produces shorter (C14 and C16) acyl chain ceramides [244] while LASS3 produces C18 and C24 ceramides [250]. LASS5 was later described to exhibit direct ceramide synthase activity [251], supporting the claim that the mammalian LASS proteins are genuine ceramide synthases and account for all known ceramide synthesis, leading to a renewed nomenclature, namely LASS1-6 being renamed ceramide synthases CERS1-6 [252].

Lysosomal enzyme are manufactured in the ER, followed by maturation throughout the Golgi prior to being transferred to the endolysosomal system [253,254]. Recently, CLN8 has been described as necessary for functional transfer of lysosomal enzymes from the ER to the Golgi [255]. The cytosolic C-terminus of CLN8 is critical for export and retrieval signals for COPI and COPII-mediated vesicle transfer and mutations in this region leads to diminished levels of lysosomal enzymes, impairing lysosome biogenesis, eventually leading to Batten disease [255].

Functionally, and prior to the present thesis work, not much was known about the remaining TLC proteins, the TLCDs, except for one study linking TLCD1 as a positive regulator of calcium channel activity [256]. Spatially, all described TLC proteins have been localized to the ER membrane, except for one study localizing human TLCD1 to the plasma membrane [241,243,255,256].

## The ACS Proteins

Cells are provided with long-chain fatty acids (>16 carbons) via either directly from the diet, *de novo* lipogenesis or turnover of TAGs, phospholipids and cholesterol esters. The fates of these fatty acids range from degradation, incorporation/re-incorporation into complex lipids and eicosanoid synthesis, to esterification to proteins [46]. Additionally, long-chain FAs can activate transcription factors, partake in intracellular signaling and directly modulate enzyme reactions allosterically (Fig. 6). Besides lipid signaling and eicosanoid synthesis, activation of fatty acids is the central, initial reaction in fatty acid metabolism and is catalyzed by the thioesterification of CoAs onto fatty acids by Acyl-coenzyme A synthetases (ACSs). Mammals express 26 different ACSs of which 13 can activate long-chain fatty acids containing 16-22 carbons [257,258]. ACSs are named according to the chain length of their preferred fatty acid substrate. Short-chain acyl-CoA synthetases (ACSSs) activate acetate (2 carbons), propionate (3 carbons) and butyrate (4 carbons). Medium-chain acyl-CoA synthetases (ACSMs) favor fatty acids containing 6-10 carbons while occasionally activating longer-chain fatty acids. Long-chain acyl-CoA synthetases (ACSLs) act on fatty acids containing 12-20 carbons. Very-long-chain acyl-CoA synthetases (ACSVLs) may activate fatty acids with more than 20 carbons but favor 16 and 18 carbon lengths [259]. The last class contain the acyl-CoA synthetase bubblegums (ACSBGs) which prefer long-chain fatty acids [259,260]. Overlap of fatty acid preference between the different classes has been described, with individual isoforms within one class displaying preferences for specific saturation or

chain length. Chain length preference has been theorized to reflect the shape and size of the fatty acid binding site [261] with further studies reporting that specific amino acids within the site control the fatty acid preference [262]. Certain ACSVL isoforms can act on other substrates besides fatty acids. In particular, ACSVL6 (FATP5) activates bile acids [263], while ACSVL1 (FATP2) can activate 3α, 7α, 12α-trihydroxy-5β-cholestanoate [264].

ACSs contain homologous sequences within their catalytic domain sharing homology with a larger family of proteins including non-ribosomal peptide synthetases and luciferase [265]. Fascinatingly, the homology among ACSs and luciferase is such that a single residue mutation (L345S) provides *Drosophila* ACS with luciferase activity [266]. Functionally, a rotation of the C-terminal domain upon adenylation provides the conformational change which induces ACSs ability to form thioesters [267]. ACS-induced activation of fatty acids into fatty acyl-CoAs is an energetically demanding process, consuming the equivalent of two high-energy bonds [257]. Acyl-CoAs have a myriad of functions within the cell such as allosteric inhibitors of liver glucokinase [268], acetyl-CoA carboxylase (ACC) [269], HMG-CoA reductase [270], phosphofructokinase-1 [271], and hormone sensitive lipase (HSL) [271], while also stimulating transport vesicle release [272]. Additionally, long-chain acyl CoAs are potent detergents, forming micelles with the CoA moiety facing the aqueous phase, but is kept at low intracellular concentrations to inhibit self-aggregation [273]. Due to their amphipathic composition, acyl-CoAs can affect lipid membrane integrity by acting as detergents, increasing permeability of small molecules such as citrate and sucrose [274].

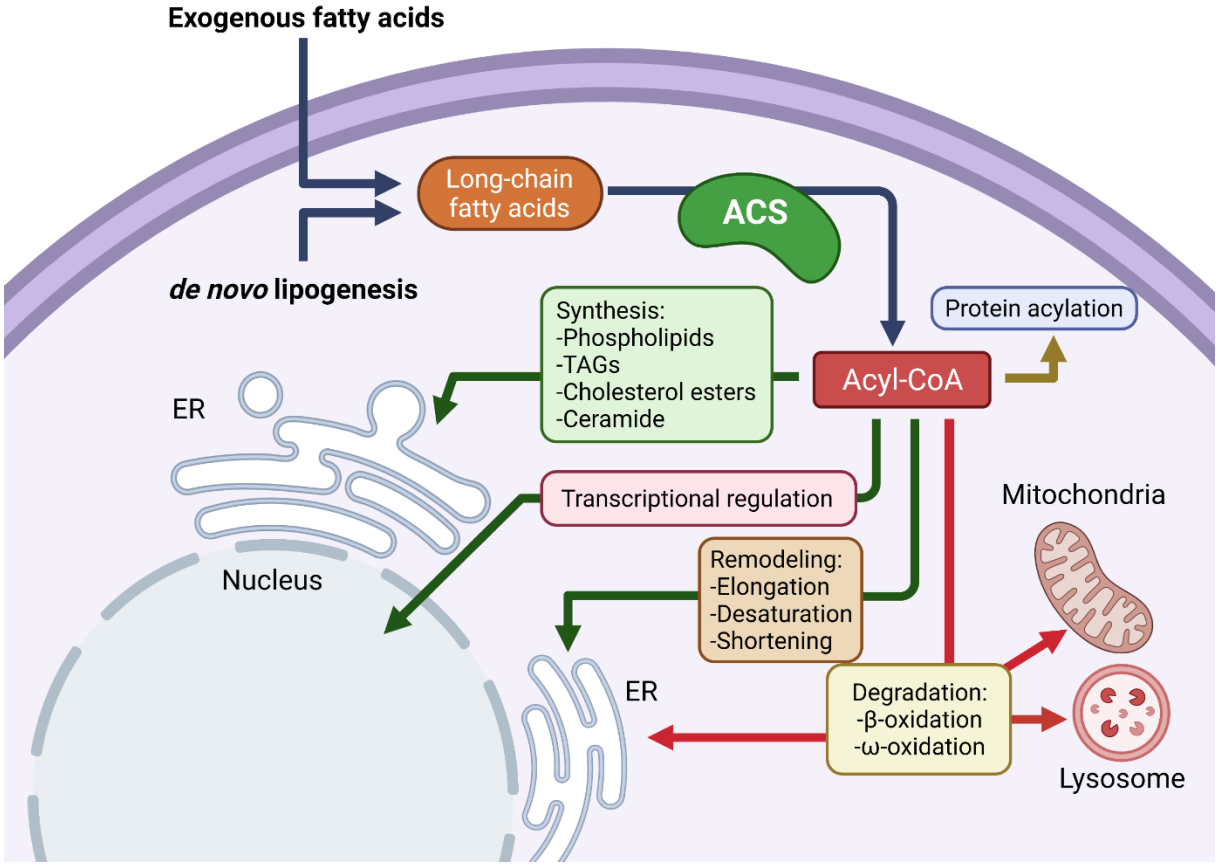


**Figure 6. Metabolic fates of long-chain fatty acids**
Exogenously or endogenously (*de* novo lipogenesis) acquired long-chain fatty acids are activated by one of 13 acyl-CoA synthetase (ACS) isoforms into fatty acyl-CoAs. These acyl-CoAs can be shuttled to different fates such as degradation by oxidation (in mitochondria, lysosomes and ER), remodeling, transcriptional regulation, protein modification, and can also serve as substrates for complex lipid synthesis in the ER. TAGs, triacylglycerols; ER, endoplasmic reticulum. Adapted from Grevengoed et al. 2018.

Chemically, fatty acids are carboxylic acids with (long-chain) hydrocarbon side groups. In mammals, long-chain fatty acids containing 16 or 18 carbons are the major fatty acid constituents, with differing degrees of saturation. A small fraction of the total fatty acid pool consists of 20 carbon fatty acids, such as 20:4ω6 and 20:5ω3, which are precursors for eicosanoid synthesis [46]. It remains controversial regarding exactly how fatty acids are transported into cells. Whether it is via protein transport or via flip-flop across the plasma membrane, ACS-induced CoA thioesterification prevents fatty acids leaving the cell. Even ACSVLs/FATPs have been implicated in fatty acid transport across the plasma membrane [275].

Spatially, different ACS isoforms are found in various cellular compartments with e.g. ACSL1 isoforms detected on the plasma membrane, ER, nucleus, mitochondria, peroxisomes, lipid droplets, and GLUT4 vesicles [276–281]. Subcellular location of ACSL1 may change depending on cell type and splice variants, which could help explain the many observed locations [282]. Additionally, ACS isoforms could relocate depending on physiological conditions changing as is observed for FATP1 which translocates from the ER to the plasma membrane upon insulin stimulation [283]. In support of the association between function and location, hepatic ACSL1 has been observed on ER and mitochondria which reflects its action on neutral lipid synthesis and fatty acid oxidation [284], while cardiac ACSL1 locates to mitochondria, consistent with a large impact on fatty acid oxidation [285]. Multi-enzyme complexes control a substrate's translocation through any of several possible pathways [286]. Therefore, the subcellular location of an ACS might govern the fate of its activated fatty acyl-CoAs by immediate interaction with proteins controlling downstream processing of fatty acyl-CoAs. For example, ACSL1 co-immunoprecipitates with carnitine palmitoyltransferase 1A (CPT1a) and voltage-dependent anionic channel (VDAC) on the outer mitochondrial membrane [287]. CPT1a catalyzes fatty acyl-CoA conversion into an acyl-carnitine, which is required for mitochondrial uptake and, consequently, for oxidation in mitochondria [288]. Thus, by complexing with CPT1a and VDAC, ACSL1 can facilitate the transport of fatty acyl-CoAs to CPT1a. Similarly, on the ER, initiation of complex lipid synthesis may also be mediated by ACSL1 and the responsible acyltransferases [46]. In other instances, ACSs can provide a local increase in the concentration of their fatty acyl-CoA products, resulting in an increase of available substrate for downstream processes.

*ACSL1*

The most studied ACS isoform is ACSL1 which is abundantly expressed in liver, white and brown adipose tissue, heart, and skeletal muscle [289]. ACSL1 exerts different tissue-specific functions as deduced from the study of tissue-specific knockouts. Liver-specific ACSL1 knockout mice exhibited a 50% decrease in total ACSL activity alongside a 30% decrease in hepatic acyl-CoA content, including a 20% reduction in oleate (18:0) incorporation into TAGs [284]. Increases in 16:0 PCs and PEs were observed at the expense of 18:0-containing phospholipids suggesting a specific effect on oleate-specific phospholipid composition [284]. Additionally, hepatic long-chain acyl-carnitines were reduced by 50% in the liver-specific ACSL1 knockout, indicating impaired trafficking of fatty acyl-CoAs into oxidation and TAG pathways. Taken together, these results suggest that ACSL1 does not shuttle its fatty acyl-CoAs into a specific pathway or that, due to its intracellular location in both ER and mitochondria, ACSL1 might divert its products into both degradation and synthesis pathways.

On the other hand, both cardiac-, white adipose- and brown adipose-specific ACSL1 knockouts indicate that ACSL1-derived acyl-CoAs are predominantly shuttled to β-oxidation [285,290]. These knockout tissues display roughly a 80-90% decrease in total long-chain ACS activity alongside strongly impaired fatty acid oxidation, without perturbing TAG nor phospholipid composition. Brown adipose-specific ACSL1 knockout mice exhibit impaired body temperature regulation at 4°C, likely due to the impaired fatty acid oxidation [290]. Despite up-regulation of *Acsl3* transcripts in both brown adipose and heart tissue, this isoform does not manage to supply ample levels of fatty acyl-CoAs for oxidation and thermogenesis. Correspondingly, white adipose-specific ACSL1 knockouts display a 50% decrease in 18:1 oxidation without altering fatty acid composition in TAGs and phospholipids, while exhibiting 40% larger white adipose depots compared to controls [290].

Macrophages from diabetic humans and mice exhibit up-regulated metabolism of arachidonic acid (20:4ω6) accompanied by increased inflammation and atherosclerosis [291]. In contrast to the findings in ACSL1-deficient liver, adipose tissue and heart, ACSL1 ablation in macrophages was not shown to impair fatty acid oxidation or neutral lipid accumulation [291]. In fact, the macrophage-specific ACSL1 deficiency led to reduced levels of arachidonic acid-CoAs accompanied by inhibited synthesis of prostaglandin E2 ($PGE_2$), a product of arachidonic acid metabolism often increased in type I diabetic mice. This decrease has been hypothesized to be due to limited uptake and subsequent activation of arachidonic acid by ACSL1, leading to a protective phenotype with decreased inflammation [292]. An alternative hypothesis is that inflammation decreased because of decreased ACSL1-mediated activation of linoleic acid (18:2ω6), a precursor to arachidonic acid (Fig. 3) [293]. Interestingly, inflammatory signal induction in macrophages lead to markedly up-regulated levels of *Acsl1* transcripts [294]. Conversely, ACSL1 ablation in macrophages reduces lipopolysaccharide (LPS) induced remodeling of inflammatory lipid species [294]. In closing, these observations show that ACSL1 activity differs drastically in a tissue-specific manner.

# AIM

The aim of this thesis has been to further understand the mechanisms that allow cells to compensate for the huge variation in dietary fat by modulating the fatty acid composition of lipid membranes i.e. membrane homeostasis. The foundation had been laid with the identification of the PAQR-2 and IGLR-2 proteins in *C. elegans* as sensors of membrane fluidity [206,207], along with the first evidence showing similar functions in the human homolog ADIPOR2 [205]. As the evidence emerged linking ADIPOR2 to membrane homeostasis, in contrast or in addition to previous results suggesting it as one of the putative receptors to the adipokine adiponectin, several questions arose. Are there more proteins able to influence membrane homeostasis in *C. elegans*? If so, are they conserved in mammals and what function do they exert? Is the primary function of ADIPOR2 to maintain membrane homeostasis in mammalian cells? This thesis aimed at finding answers to these questions, which are described in detail herein.

# METHODS

Herein, descriptions of the analytical methods, used throughout this thesis, that directly assesses membrane fluidity are presented, followed by an introduction to the *in vivo* models used throughout this work, namely *C. elegans* and genetically modified mice.

## FRAP

Fluorescence recovery after photobleaching (FRAP) is the most commonly used method to directly assess the diffusion of molecules within biological membranes [295]. In essence, fluorescently labeled molecules, be it proteins or lipids, are irreversibly photobleached by a high-intensity laser beam targeting a small area of a cell or organ, which becomes void of fluorescence intensity. Continuously, the lateral diffusion of fluorescently labeled molecules into the bleached area is recorded until fluorescence is recovered. This yields quantitative approximations to the rate of mobility of fluorescent molecules and the mobile fraction; the portion of the molecules able to freely diffuse. The time it takes for half of the fluorescence to recover, i.e. t½, is the most common parameter used to convey the fluidity of molecules. In scenarios of decreased membrane fluidity, e.g. due to increased saturation of membrane phospholipids, it takes longer for the fluorescence to recover, i.e. slower movement, which leads to a larger t½. Conversely, in situations with increased fluidity, e.g. an increase in fluidizing PUFA-containing phospholipids, molecules diffuse faster, decreasing t½. FRAP can also be used to assess the movement of fluorescently labeled *in vivo* such as in *C. elegans* [296] which has been extensively used to quantify the decrease, or recovery, in fluidity due to cold-, glucose- and SFA vulnerability of the *paqr-2* mutants (see animal model section below).

## Membrane order assessed by Laurdan

Cellular membranes are complex in their chemical makeup, being made up of hundreds of lipid species in various forms. In addition, lipids assemble into complex structures, lipid rafts, that give rise to an asymmetrical distribution of lipids in the membrane [297]. Lipid rafts are so small (10-200 nm) that detecting individual rafts becomes impossible due to the resolution limit of conventional optical microscopes [298]. However, the biophysical parameter that give rise to raft domains, membrane packing order, can be visualized. Membrane order, or the degree of lipid packing, determines the thickness of the lipid bilayer and in extension, the rotational freedom of lipids therein. Increased membrane order occurs when lipids pack together tightly, which happens more readily with SFA-carrying phospholipids, which in turn excludes water and changes the local polarity of the region. Conversely, a decreased membrane order, e.g. in membranes rich in PUFAs, allows for more water within the bilayer, thus increasing the polarity. Molecular polarity-sensitive fluorescent probes exist that leverage these variation in bilayer properties to quantify membrane order. The most widely used probe is Laurdan (6-lauryl-2-dimethylamino-napthalene) developed by Weber and Farris [299]. Laurdan, when incorporated within lipid membranes, emits light at different wavelengths depending on the proximal water content (i.e. membrane order), which results in striking images where differences in membrane order can be visualized within a single cell. Additionally, the Laurdan dye is suitable for high throughput imaging capture, and analysis by automated scoring scripts [298], making it possible to screen thousands of cells in one go. Quantification results in a general polarization (GP) index score, where a high value indicates an increased membrane order, i.e. tighter lipid packing hence decreased membrane fluidity, with the opposite being true of lower GP values.

## Animal Models

The work presented in this thesis relied on several animal models to obtain new insight into proteins responsible for membrane homeostasis. Initial discoveries were made using the *paqr-2* and *iglr-2 C. elegans* loss-of-function mutants. These were used further when identifying *paqr-2* or *iglr-2* suppressor mutations in other proteins, leading to the discovery of *fld-1* (TLCD1 and TLCD2 in mammals) and *acs-13* (ACSL1 in mammals). Further investigation of the TLCDs required the generation of TLCD1/2 knockout mice while studies on ADIPOR2 (homolog to *paqr-2*) was done using previously generated ADIPOR2 KO mice. Each model is described below.

### *The paqr-2 and iglr-2 C. elegans mutants*

Early studies with the *C. elegans paqr-2* mutant led to the discovery that this gene is essential for the ability of the worms to adjust their membrane composition for growth at 15°C, which is a relatively low temperature well tolerated by wild-type worms; *paqr-2* mutants are said to be "cold-intolerant" [203,206]. In addition, a similar cold-intolerance phenotype was discovered in loss-of-function mutants of the *iglr-2* gene. Additionally, both the *paqr-2* and *iglr-2* mutants display a characteristic withered tail tip phenotype that is useful for keeping track of the mutant alleles during crosses [203,207]. IGLR-2 is a single-pass plasma membrane protein containing an extracellular region that includes one immunoglobulin domain along with several leucine-rich repeats. IGLR-2 and PAQR-2 are both required for the normal response to increased membrane rigidity, i.e. induction of fatty acid desaturation that restores membrane fluidity, and IGLR-2 and PAQR-2 physically interact during this process [203,206,207]. Furthermore, both *paqr-2(tm3410)* and *iglr-2(et34)* loss-of-function mutants show extreme vulnerability to diets rich in SFAs: mutants rapidly accumulate an excess of SFA-containing phospholipids accompanied by membrane rigidification and, ultimately, death. In addition, diets rich in glucose also lead to the demise of the *paqr-2(tm3410)* and *iglr-2(et34)* mutants, which was later discovered to cause an increased SFA/MUFA ratio in the dietary *E. coli* used as food source for lab-grown *C. elegans* [205]. Interestingly, all the null mutant phenotypes described can be partially or fully restored by secondary mutations that result in increased fatty acid desaturation, such as *mdt-15* [206], or even by addition of small amounts of nonionic detergents [206].

### *The TLCD KO mice*

In order to further our understanding of the mammalian TLCD1 and TLCD2 proteins, transgenic mice lacking these proteins were generated. The effort resulted in three transgenic knockout (KO) lines: TLCD1 KO, TLCD2 KO and a TLCD1/2 double KO. Said lines were generated using a previously published mRNA guided CRISPR/Cas9 methodology [300]. Genotypes were confirmed by fragment analysis and Sanger sequencing with edited loci regions further assessed by targeted locus amplification technology [301], which showed no genomic translocations or off-targets of the targeted loci. All lines were backcrossed with C57Bl/6N mice (Charles River) for at least 5 generations prior to any phenotyping. Prior to any investigation a thorough phenotyping was done on the TLCD1/2 DKO mice, revealing normal development with unaltered phenotypes with respect to standard behavioral and physiological assessments. Energy balance was comparable between TLCD1/2 DKO mice and wildtype littermate controls at room temperature and at thermoneutral housing conditions. No difference in glucose handling was observed on regular chow diet. In addition, no differences were observed for neither TLCD1, TLCD2 or TLCD1/2 DKO with respect to general health, whisker condition, dehydration, eyes, blink reflexes, teeth, nose discharge, mucous membranes, paw pads, fur condition, posture, movement, feces, mobility, rearing, nesting, grooming, sniffing, digging, climbing, passivity and general activity within the cage.

### *The ADIPOR2 KO mice*

To study the impact of phospholipid saturation on adipocyte biology, and in particular their ability to respond to insulin, we used ADIPOR2 KO mice that were previously generated by Deltagen (San Carlos, CA) in 2007. These mice were initially created as part of an effort to study the metabolic phenotypes of mice lacking *ADIPOR1* or *ADIPOR2* [228], and it was later found that the ADIPOR1/2 double KO mouse genotype is embryonic lethal by embryonic day 16.5, indicating an essential function of these proteins during development [229]. In contrast to the TLCD1/2 KO mice, the ADIPOR2 KO mice were generated by homologous recombination and insertion of targeting cassettes which did not impact downstream gene expression of flanking genes [228]. The ADIPOR2 KO founder was backcrossed six times with C57Bl/6 mice prior to intercrossing and subsequent phenotyping. Metabolically, both male and female ADIPOR2 KO mice tended to be leaner than wildtype controls on regular chow, with the difference more pronounced in females, while both showed complete resistance to high fat diet (HFD)-induced weight gain, which was not due to decreased food intake [228]. Another publication, using the same mice, showed similar findings albeit that ADIPOR2 KO mice gained weight on a HFD but not as much as wildtype controls [302]. No difference in fecal energy content was observed, suggesting that the ADIPOR2 KO mice were obesity resistant despite showing no differences in energy intake or fecal energy loss [228,302]. ADIPOR2 KO male mice displayed markedly reduced weights in epididymal white adipose tissue (WAT), perirenal WAT and brown adipose tissue (BAT), as well as circulating leptin levels compared to controls after being fed a HFD for 14 weeks [228]. Female ADIPOR2 KO mice showed reductions in adipocyte size in WAT with fewer vacuoles in BAT compared to wildtypes after being fed a HFD for 14 weeks [228]. Both male and female ADIPOR2 KO mice showed reduced liver triglyceride levels after a HFD compared to controls while having reduced circulating HDL cholesterol [228,302]. Additionally, ablation of *Adipor2* in *ApoE* knockout mice, known to be highly atherosclerotic, led to an amelioration of atherosclerotic development in the brachiocephalic artery [229]. Taken together, it can be concluded that ADIPOR2 KO mice are lean and unaffected by a HFD in terms of weight gain and obesity, and show a metabolically healthy phenotype with reduced hepatic steatosis compared to wildtype controls as well as a inhibitory effect on atherosclerosis development [228,229,302].

Lacking ADIPOR2 does not come without a cost: the knockout male mice show dramatic reductions in testis weight along with seminiferous tubule atrophy and aspermia, rendering them sterile. In addition, both male and female ADIPOR2 KO mice show enlarged brain weights compared to controls, though no gross morphological changes have been observed [228]. Additionally, ADIPOR2 KO mice fed a HFD for long periods of time (20 weeks), showed increased fasting glucose levels, correlated with

dysfunctional β-cell replication and neogenesis [302]. Together, this suggests that ADIPOR2 is vital for proper spermatogenesis and brain development [228] and that ADIPOR2 deficiency gradually leads to an overt diabetic phenotype after prolonged periods of HFD-feeding [302].

All experiments using this mouse model used male mice with littermate wildtype mice as controls.

# RESULTS AND DISCUSSION

The ambition of this thesis was to further explore the molecular mechanisms that influence and govern cellular membrane homeostasis. In paper I we used the power of forward genetics to identify proteins able to suppress the vulnerability of *paqr-2* and *iglr-2 C. elegans* mutants to perturbations in membrane composition, identifying the protein FLD-1 and its human homologs, TLCD1 and TLCD2, as proteins limiting the amount of long-chain PUFAs in phospholipids. Paper II describes an additional suppressor of *paqr-2* and *iglr-2* mutant phenotypes, namely a loss-of-function allele of *acs-13* and shows that ACS-13 and its human homolog ACSL1 are important for uptake of long-chain PUFAs into mitochondria. Following the discoveries of FLD-1 and TLCD1/2 as modulators of membrane lipid composition, paper III transitioned the TLCD1/2 effort into genetic mouse models, further elucidating the molecular mechanisms of TLCD1 and TLCD2, namely governing MUFA incorporation into the sn-1 position of phosphatidylethanolamines. In parallel to studying suppressors of membrane rigidification, questions remained regarding the function and mechanism of action of ADIPOR2, the human homolog of *C. elegans* PAQR-2, and Paper IV showed that lack of ADIPOR2 leads to massive transcriptional defects when cells are cultivated in the presence of SFAs, further cementing the need of ADIPOR2 in membrane homeostasis. Finally, by using the ADIPOR2 KO as a model system to enrich SFA-containing membrane phospholipids in human and mouse adipocytes, we studied if this would in turn impact adipocyte biology, namely its response to insulin. Each paper is discussed at length in the following sections, including results, discussion, conclusions and my individual contributions to each manuscript.

## Paper I: Membrane Fluidity is Regulated by the *C. elegans* Transmembrane Protein FLD-1 and its Human Homologs TLCD1/2

### INTENTION

An organism's ability to compensate for variations in degree of saturation of cell membranes, i.e. membrane homeostasis, was fundamental when the first single-cell organisms developed billions of years ago [9]. Crucially, the lipid composition of cellular membranes is vital for a myriad of cellular processes such as organelle homeostasis, vesicular trafficking and receptor signaling [7]. As such, it come as no surprise that several human diseases manifests with aberrations in membrane composition. Rigid membranes, rich in saturated fatty acids (SFAs), are a characteristic trait in diabetics [153,162,164,303], likely leading to faulty insulin signaling and microcirculation [168,304,305]. Additionally, there are strong correlations between membrane composition and cancer [306], where fluid membranes, rich in unsaturated fat, increase odds of developing lung cancer [181]. Surprisingly, not much is known regarding molecular mechanisms regulating membrane homeostasis despite its importance for basal cellular functions.

Lipotoxicity manifests as accumulation of SFAs in circulation or within cellular membranes [307–309]. Particularly, enrichment of SFA-containing phospholipids, leading to decreased membrane fluidity, is one way lipotoxicity causes deleterious effects [310]. Indeed, unbiased genome-wide CRISPR/Cas9 screens identified genes involved in SFA-incorporation into phospholipids as critical to prevent palmitic acid-induced lipotoxicity in human cells [231,311]. Mitigation of membrane rigidification in response to SFA-rich diets in *C. elegans* depends on the proteins PAQR-2 and IGLR-2 [205–207], and this function of PAQR-2 is conserved in its human homologs ADIPOR1 and ADIPOR2 [205,208]. *C. elegans* lacking PAQR-2 or IGLR2, e.g. *paqr-2(et3410)* and *igrl-2(et34)* null mutants, show striking vulnerability to SFAs: feeding SFA-rich diets to these mutants lead to increased SFA-containing

phospholipids, membrane rigidity and death [206,207]. Interestingly, this phenotype could be mitigated by addition of fluidizing non-ionic detergents, or by mutations in other genes that result in increased desaturation of fatty acids [206]. Since membrane homeostasis is a crucial feature of cellular health, we postulated that other regulatory pathways, mediating or protecting against SFA-induced lipotoxicity, were likely to exist.

## RESULTS

### Loss-of-function in *fld-1* suppresses cell membrane rigidification

Identification of mutations able to suppress the SFA sensitivity of the *paqr-2(tm3410)* and *iglr-2(et34)* mutants was done using forward genetics. More than 100 000 mutagenized haploid genomes were screened to identify mutations that allowed *paqr-2(tm3410)* or *iglr-2(et34)* mutants to grow in the presence of a SFA-rich diet and cold exposure, two major phenotypes of these mutants [207]. In addition, the double mutant *paqr-2(tm3410) mdt-15(et14)*, where *mdt-15(et14)* is a gain-of-function allele previously shown to suppress *paqr-2(tm3410)* mutant phenotypes by promoting fatty acid desaturation, was included in the screening effort to identify enhancers of the *mdt-15(et14)* allele that would likely act in a separate pathway. Together, 15 unique mutants, able to diminish the rigidifying effect of SFAs, were isolated. Eight were novel mutations within the same gene, dubbed *fld-1*, of previously unknown function. All mutations were confirmed to be loss-of-function mutations since wildtype *fld-1* provided as a transgene restored the SFA-sensitivity of the original mutants. Additionally, protein quantification, by Western blot, confirmed the absence of the FLD-1 protein in the novel *fld-1* mutants. That more than half of the isolated mutants were *fld-1* alleles suggests that it has an especially important role in preventing SFA-induced toxicity.

### Plasma membrane shows enrichment of FLD-1

The human genes *TLCD1* and *TLCD2* show the closest homology to *fld-1*, which codes for proteins containing a multiple-transmembrane region, characterized by a TLC (TRAM Lag1p CLN8) domain often found in proteins involved in membrane protein synthesis [242]. At the time of performing this work, TLCD1 had been suggested to have a putative regulatory function on calcium channels, located in the plasma membrane [256], with TLCD2 having an unknown function. This has since been studied further and the functional role of TLCD1 and TLCD2 in liver has been studied extensively; see paper III of this thesis. GFP-tagged FLD-1 transgenes, with no impact on the function of the protein, revealed a plasma membrane localization in *C. Elegans* throughout development to adults, corroborating the observations for TLCD1, that it locates to the plasma membrane.

### Mutations in *fld-1* remedies defects in membrane homeostasis

The only phenotype observed for the *fld-1* single mutant was elevation of glutathione S-transferase 4 (GST-4), which de-toxifies reactive oxygen species [312], when the mutant was exposed to linoleic acid, a long-chain PUFA, suggesting that loss of *fld-1* causes a defect in preventing peroxidation. Five different *fld-1* allele mutants were studied in terms of ameliorating the defects in membrane homeostasis of the *paqr-2(tm3410)* mutant. All mutants could suppress the main phenotypes of the *paqr-2(tm3410)* mutant, such as SFA- and cold intolerance as well as a shriveled tail tip defect [205–207]. One mutant, *fld-1(et48)* carried a splice donor site mutation, as well as being able to suppress all *paqr-2* and *iglr-2* mutant alleles tested, and as such was singled out for the future experiments. Four other *C. elegans* genes show significant homology to *fld-1*, including *Y48G8AL.13* & *F41H10.5* for which null mutant alleles were available at the *C. elegans* Genetics Center (CGC). Mild suppression of the cold intolerance was observed for these mutants but neither could suppress the SFA-intolerance of the *paqr-2(tm3410)* mutant, indicating only partial functional redundancy with *fld-1*.

### Different mechanism of *paqr-2* suppression observed in the *fld-1* mutant

Genetic interactions between *fld-1(et48)* and other *paqr-2* suppressor mutants were analyzed to try and determine by which mechanism it exerts its protective effect. Several of the known *paqr-2* suppressor mutants, e.g. *mdt-15(et14)*, *cept-1(et10)* and *hacd-1(et12)*, act by promoting increased fatty acid desaturation, and these showed further suppression of the *paqr-2* mutant glucose and PA vulnerability when combined with the *fld-1(et48)* mutation, suggesting that *fld-1(et48)* acts through a separate pathway. Fluorescence recovery after photobleaching (FRAP; see methods for details) experiments confirmed that the *fld-1(et48)* allele improved membrane fluidity in the *paqr-2(tm3410)* genetic background, and this without elevating the desaturase FAT-7 as seen in the other *paqr-2* suppressors, further indicating a separate mechanism of action for *fld-1* mutants. Note however that *fld-1(et48)* did require a functional desaturase activity in order to exert its protective effect since RNAi silencing of the desaturases *fat-6* and *fat-7* abolished the *fld-1(et48)* suppression of glucose intolerance in the *paqr-2* mutants.

Lipid composition assessment of PEs, the major phospholipid constituents in *C. elegans*, revealed that the *fld-1(et48)* and *mdt-15(et14)* mutations normalized the increased SFAs and decreased PUFAs observed in the glucose- and PA-exposed *paqr-2(tm3410)* mutant, with the *mdt-15(et14) fld-1(et48)* double mutant completely restoring these defects. MUFAs remained unaffected in all *fld-1(et48)* mutants. The changes in PE composition could not be pinpointed to a specific membrane compartment since the lipidomics analysis was performed on whole worms lysates. Taken together, these observations indicated that the *fld-1(et48)* mutation led to elevation of PUFA-containing PEs, similarly to *mdt-15(et14)* albeit via a different pathway in the *paqr-2* mutants.

**FLD-1 regulates the abundance of PUFA-containing phospholipids**

PUFAs readily undergo peroxidation [313–315], and this liability makes it reasonable to expect that there exists proteins controlling their abundance and promote their turnover. If FLD-1 would be such a regulator, ablation of it, such as in the *fld-1(et48)* mutant, could lead to slower PUFA turnover and thus remedy other mutants that lack PUFAs. One such mutant is the *fat-2* loss-of-function mutant, which cannot desaturate 18:1 lipids to 18:2, which is the main and only precursor for endogenous long-chain PUFA synthesis [316]. Indeed, the characteristic phenotype, stunted growth, of the *fat-2* mutant was alleviated when introducing the *fld-1(et48)* mutation, even in the *fat-2 paqr-2(tm3410)* double mutant. This is consistent with the hypothesis that the mutation in *fld-1* increases long-chain PUFA-containing phospholipid synthesis or diminish their turnover, leading to increased PUFA levels in phospholipids (particularly PEs) and helping to normalize PUFA levels in mutants such as *paqr-2, iglr-2* and *fat-2*.

**The mammalian homologs of *fld-1*, i.e. TLCD1 and TLCD2, regulate membrane phospholipid composition**

Tissue transcriptomics showed variable expression of the TLCDs throughout the human and rodent bodies. Mouse *Tlcd1* was predominantly expressed in adipose tissues, heart and liver while *Tlcd2* showed most expression in liver, small intestine and muscle, with similar observations seen in human tissues [317]. *TLCD1*- and *TLCD2*-silencing, using siRNA, caused no obvious defects in HEK293 cells. However, *TLCD*-silencing ameliorated the vulnerability of HEK293 cells to the rigidifying effects of PA, and reduced PA-induced apoptosis, suggesting that the function of these proteins may be conserved with that of FLD-1 in *C. elegans*.

*TLCD*-silenced cells showed no difference in PA-uptake, SFA-containing TAGs or neutral lipids accumulation compared to controls, suggesting no role in fatty acid uptake or storage. Expression of desaturases *SCD* and *FADS1/2/3* showed no up-regulation in *TLCD*-silenced cells, indicating no interaction between them; in fact, *SCD* was down-regulated compared to control cells suggesting a decreased demand for desaturation. As such, likely functions of TLCD1/2 are limitation of long-chain PUFA-containing phospholipid synthesis or of long-chain PUFA turnover, consistent with observations related to *C. elegans* FLD-1. This is also in line with lipid composition analyses, namely an elevation of long-chain PUFA-containing PEs (18:2, 18:3 and 22:6) in *TLCD1*-silenced cells and PUFA-containing PCs (18:2, 18:3 and 20:5) in *TLCD2*-silenced cells exposed to PA. Other lipid species remained unchanged, suggesting a specific effect of the TLCDs on long-chain PUFA-containing PEs and PCs.

Note that as in *C. elegans*, lipidomics analysis was done on whole cell lysates and thus curtail any effort in pinpointing a subcellular location of these lipidome changes.

The specific increase of the 20:5 eicosapentaenoic acid (EPA) in the phospholipids of *TLCD2*-silenced cells warranted a closer look since EPA is a potent membrane fluidizer: 1 µM EPA counters the rigidifying effect of 400 µM PA. *TLCD2*-silenced cells exposed to exogenous EPA showed attenuated incorporation of EPA-containing PCs and PEs, indicating that TLCD2 is not governing PUFA synthesis, echoing observations in *C. elegans*. As with SFA storage, no difference in EPA incorporation into TAGs was observed in *TLCD2*-silenced cells suggesting that TLCD2 does not affect PUFA uptake or storage.

In order to assess whether the TLCDs regulate the rate of PUFA-containing PE and PC synthesis or their turnover, *TLCD1/2*-silenced and control HEK293 cells were incubated with $^{13}$C-labeled linoleic acid (LA, 18:2) for 6 and 24 hours, followed by monitoring of incorporation into phospholipids and clearance rate. *TLCD2*-silencing led to elevation of LA-containing PCs and PEs, compared to controls, after 6 h of incubation which persisted in PEs after 24h. *TLCD1*-silenced cells showed increased PUFAs in PEs at 24 h with no changes in clearance of LA observed in neither knockdown setting compared to controls, suggesting that TLCD1/2 modulate the rate of synthesis of long-chain PUFA-containing phospholipids, especially PEs, rather than influencing their turnover.

**TLCD1/2 share common mechanisms of action as FLD-1**

Several of the observed, and described, genetic interactions of *fld-1* with desaturases and alleviation of membrane homeostasis disruptions could be mirrored in *TLCD1/2*-silenced HEK293 cells. *TLCD2*-, but not *TLCD1*-silenced cells, showed alleviation of disrupted membrane homeostasis in PA-treated *FADS2*-silenced cells compared to controls, where *FADS2* is a homolog of *fat-2* in *C. elegans*; this again indicates a desaturase-unrelated role for TLCD2. Furthermore, *TLCD2*-silencing alleviated the PA-induced rigidification of *ADIPOR2*-silenced cells, which is similar to the ability of *fld-1* to suppress *paqr-2* mutant phenotypes in *C. elegans*. In addition, *TLCD1/2*-silencing partly restored the membrane lipid composition aberrations observed in PA-treated *ADIPOR2*-silenced cells, again echoing the findings in *C. elegans*, further strengthening the evolutionarily conserved function of these proteins.

**DISCUSSION**

This body of work revealed the role of the novel protein FLD-1 in *C. elegans* to be a regulator of phospholipid composition in PCs and PEs, focused on long-chain PUFAs. Translationally, human TLCD1/2 were shown to behave similarly to FLD-1, revealing a highly conserved function of these proteins. Together, these proteins exacerbate SFA-induced membrane rigidification by limiting the amount of highly fluidizing long-chain PUFAs in cellular membranes. A small increase of fluidizing lipids in phospholipids, due to *fld-1* or *TLCD1/2*-silencing, can counteract the rigidifying effect of high doses of SFAs, which is in line with separate findings showing increased secretory capacity of membrane vesicles with increasing amounts of long-chain PUFAs [318]. The spectra of dietary fatty acids consumed is in turn reflected in most cell membranes. This is especially true for PUFAs, which are readily absorbed and incorporated into membrane phospholipids postprandially in *C. elegans* [319] and mammals [43,320,321]; clearly regulatory mechanisms must exist that monitor lipid composition with regard to PUFAs and compensate for dietary variation. The evidence generated in this work points to FLD-1 in *C. elegans* and TLCD1/2 in mammals as either acting by controlling PUFA turnover in phospholipids, in order to minimize peroxidation damage, or by regulating the rate of PUFA incorporation into phospholipids to counterbalance the variation in dietary PUFAs (Fig. 7).

Given that PUFAs are prone to peroxidation due to their chemical makeup, with severe consequences [313–315], it comes as no surprise that cells would have evolved mechanisms to cope with this phenomena. Indeed, increased oxidative stress was observed in the *fld-1* mutant when exposed to LA, suggesting that FLD-1 might have such a protective role in *C. elegans*. Further work would be necessary to exert a similar function in a mammalian context. However, the substantial increase in long-chain PUFA-containing phospholipids in EPA-treated *TLCD2*-silenced cells, in addition to no difference in

LA clearance with respect to TLCD-silencing, would suggest a role in incorporation rate of long-chain PUFAs into phospholipids. Nonetheless, their function would result in decreased membrane lipid peroxidation.

TLCD1/2, and by extension, FLD-1, belong to a TLC-family of proteins comprising16 members that all include a TLC domain [241]. Amongst these are the translocation-linked membrane proteins TRAM1, TRAM2 and TRAM1L1 [242], with TRAM1 postulated to modulate phospholipid bilayers by increasing packing of phospholipids [322], the ceramide synthases CERS1-6 [241], and CLN8, which is linked to sphingolipid metabolism [323] with depletion in the brain causing massive changes in phospholipid composition, including PEs, leading to progressing epilepsy and retardation [324,325]. A common denominator of these proteins are their subcellular localization, namely the ER and Golgi [241,242,324]. Our findings in *C. elegans*, showed a clear plasma membrane localization, using fluorescence imaging, of FLD-1, albeit no co-localization staining was done to rule out localization to the ER. Published work on TLCD1, also referred to as Calfacilitin [256], showed the importance of this protein in neural plate development in chicks and presented some evidence of a plasma membrane localization of TLCD1 in HEK293T cells. Future work (see paper III) would nevertheless show a clear localization of both TLCD1 and TLCD2 to the ER/Golgi with close proximity to mitochondria, which puts the TLCDs in line with the other TLC proteins in terms of subcellular residency. As such, the plasma membrane localization in *C. elegans* might be a unique feature of worm biology, perhaps due to their poikilotherm nature in adapting membrane composition to temperature differences [326], while the findings in HEK293T would require further co-localization experiments to confirm the plasma membrane location. With respect to molecular function, both FLD-1 and the TLCDs lack critical motifs required for roles as ceramide synthases or membrane translocation proteins. Rather, our observations on FLD-1 and TLCD1/2 suggest that they act as membrane-bound phospholipid remodeling proteins.

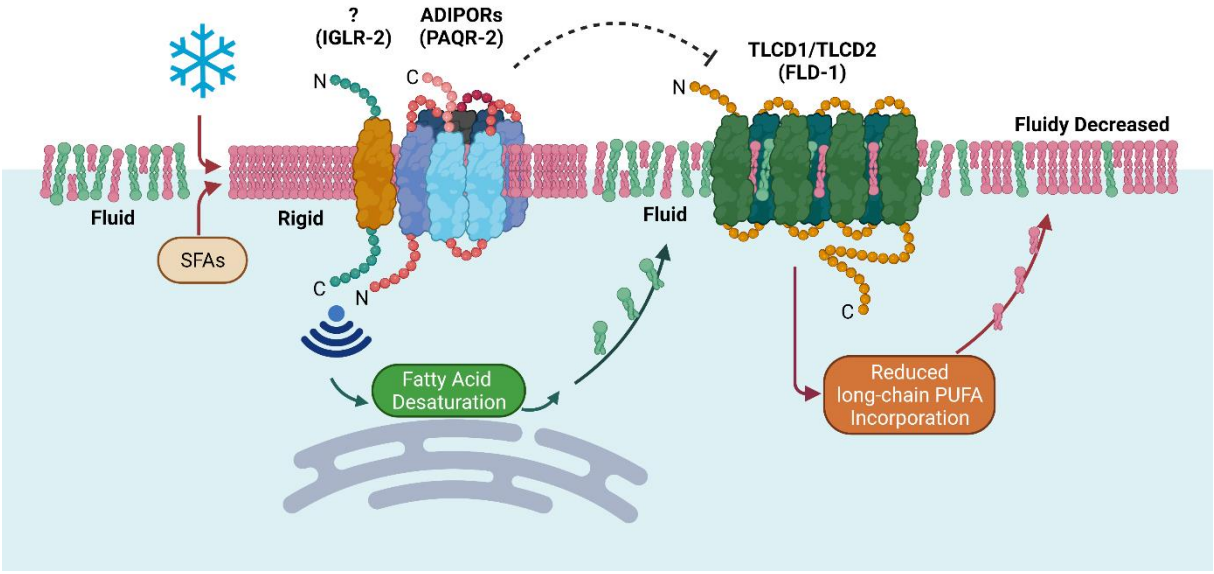


**Figure 7. Model of membrane fluidity regulation in mammals and *C. elegans*.**
Impaired membrane fluidity, either by cold exposure (*C. elegans*) or by SFA-rich diets, is sensed by the PAQR-2/IGLR-2 complex in *C. elegans* or the ADIPORs in mammals (no mammalian IGLR-2 ortholog has been identified so far), which initiates signaling for downstream fatty acid desaturation in membrane phospholipids, restoring fluidity. Paper I describes the TLCDs (FLD-1 in *C. elegans*) as a potentiator of decreased membrane fluidity: their proposed activity results in reducing available long-chain PUFAs for incorporation into membrane phospholipids. When TLCDs/FLD-1 are knocked out, more long-chain PUFAs become available for membrane incorporation, increasing fluidity. The dashed line suggests a possibility interaction between PAQR-2 and FLD-1 in *C. elegans* as loss-of-function mutations in *fld-1* suppressed *paqr-2* mutant phenotypes. However, there is no evidence suggesting a physical interaction between these proteins at this time.

Certain long-chain PUFAs, elevated in the *fld-1* mutant as well as in *TLCD1/2*-silenced HEK293 cells, might lead to other consequences besides increasing membrane fluidity, such as becoming signaling lipids, including pro-inflammatory eicosanoids [327,328]. Interestingly, this proved to be relevant when studying the TLCDs further (see paper III).

In closing, this work has started to reveal a novel molecular pathway regulating membrane phospholipid plasticity, namely that of FLD-1 in *C. elegans* and TLCD1/2 in mammals that regulate the levels of long-chain PUFAs in membrane phospholipids. These findings may open novel TLCD-based avenues to combat pathophysiological defects associated with membrane rigidity, as are found in diabetics [168,329] or ADIPOR1-induced retina defects [330,331].

**CONTRIBUTIONS**
For this work I contributed with the following:

- TLCD1 and TLCD2 expression profiling in mice: I performed everything regarding the ex vivo tissue transcriptional analysis from handling and termination of the mice up to and including the qPCR analysis.
- Data analysis: I processed and compiled all data for experiments mentioned above.
- Manuscript: I prepared graphs representing my contributions as well as proof-reading and commenting the manuscript.

## Paper II: Evolutionarily conserved long-chain Acyl-CoA synthetases regulate membrane composition and fluidity

### INTENTION

Following the discovery of the protective effect that *fld-1* loss-of-function alleles had on *paqr-2(tm3410)* and *iglr-2(et34) C. elegans* mutants fed dietary SFAs, questions remained regarding other potential proteins that could suppress the sensitivity to SFAs of the *paqr-2 a*nd *iglr-2* background. One mean of alleviating the excess of SFAs in membranes is for cells to increase desaturation of SFAs into fluidizing MUFAs or PUFAs, as with the *mdt-15(et14)* gain-of-function mutation that partly rescues the SFA sensitivity of the *paqr-2(tm3410)* and *iglr-2(et34)* [206,207]. By screening for enhancers of the *paqr-2(tm3410) mdt-15(et14)* in resisting the rigidifying effect of SFAs, novel proteins, able to influence membrane lipid order could potentially be identified.

### RESULTS

#### Loss-of-function of *acs-13* diminished the SFA vulnerability of the *paqr-2* mutant

Ethyl methanesulfonate was used to mutagenize *paqr-2(tm3410) mdt-15(et14)* double mutants, followed by isolations of F2 progeny able to mature into fertile adults under 72 h in a SFA-rich environment [205]. Six unique mutants were isolated after screening 50 000 haploid genomes, a subset of the 100 000 screened for the *fld-1* work (see paper I), of which four were amongst the previously identified *fld-1* loss-of-function mutants. The fifth mutant, was revealed to be a loss-of-function mutant of *acs-13(et54)*; *acs-13* encodes a *C. elegans* homolog of human long-chain fatty acid acyl-CoA synthetases (ACSL1, ACSL5 and ACSL6), which reside in the ER [332,333], peroxisomes [334–336] and/or the outer membrane of mitochondria [287,332,337]. Genomic analysis of the *acs-13(et54)*, pinpointed an amino acid substitution (glycine to arginine) at position 125 (G125R), localized on the cytoplasm-residing N-terminal region, predicted by homology as transmembrane domains native to ER and mitochondria. Confirmation of the *acs-13(et54)* as a loss-of-function mutant was shown by several means ranging from CRISPR/Cas9 disruption of *acs-13*, purchased clones from the *C. elegans* Genetics Center and the ameliorating effect that *acs-13(et54)* had on other *paqr-2* double mutants [338]. Loss of a*cs-13(et54)* by itself was a weak *paqr-2(tm3410)* suppressor of glucose (SFA) and cold intolerance, and also did not suppress the tail-tip defect characteristic of *paqr-2* mutants. Rather, the *acs-13(et54)* enhanced the

effects of the elevated UFA production caused by the *mdt-15(et14)* mutation to better suppress *paqr-2(tm3410)* mutant phenotypes. Thus, the *acs-13(et54)* mutation amplified the fluidizing effect of the PUFA linoleic acid (18:2) on the *paqr-2(tm3410)* mutant, suggesting a synergetic function of *acs-13(et54)* with available PUFAs.

**ACS-13 localizes to mitochondria and maintains mitochondrial morphology**

Restoration of wildtype ACS-13 in the *paqr-2(tm3410) mdt-15(et14) acs-13(et54)* triple mutants re-established the SFA vulnerability. Fusion of green fluorescent protein (GFP) to the C-terminus of ACS-13 revealed, by fluorescence microscopy, co-localization in mitochondria of intestinal and hypodermal cells. Protein quantification, by Western blot, showed enrichment in mitochondrial sub-cellular fractions, further suggesting a mitochondrial residency of ACS-13. Gene expression analysis, by qPCR, of *acs-13* showed similar levels between the *paqr-2* mutants and wildtype worms. However, *acs-13* was down-regulated in the *paqr-2(tm3410) mdt-15(et14)* double mutant, indicating transcriptional regulation downstream of MDT-15. Furthermore, fluorescence microscopy revealed abnormal morphologies of mitochondria in the *acs-13(et54)* mutants, with irregular inter-spacing and length variations of mitochondria in the mutant compared to wildtype. Taken together, these findings suggest a mitochondrial localization of ACS-13 where it contributes to normal morphology, and that *acs-13* transcription is regulated by MDT-15.

**Acyl-CoA synthetases can act synergistically to improve *mdt-15(et14)* rescue**

At least 26 different acyl-CoA synthase genes have been identified in humans [258] with more than 20 found in the *C. elegans* genome [339]. Phenotypes have only been identified for a few of them, such as *acs-20* and *acs-22* contributing to cuticle formation [340], and *acs-4* and *acs-5* regulating serotonin-induced fat reduction [341]. Generally, acyl-CoA synthetases add CoA to free fatty acids, thus priming them for different fates such as glycerolipid conjugation, making phospholipids and triacylglycerols, or energy generation by β-oxidation in mitochondria and peroxisomes. In order to assess the impact of other acyl-CoA synthetases on *paqr-2(tm3410)* suppression by *mdt-15(et14)*, transcript silencing using available RNAi inducing *E. coli* clones [342] was tested on the double mutant. Of the *acs* genes tested by RNAi, silencing of *acs-5* and *acs-15* (homologs to human *ACSL1/5/6*) proved to be the most potent enhancers of *mdt-15(et14)* suppression of SFA intolerance in the *paqr-2(tm3410)* mutant followed by *acs-16* (ACSF2 homolog). Previous characterization of ACSL1/5/6 could indicate similar functions of *acs-5* and *acs-15*, by having long-chain fatty acids (mostly PUFAs) as their preferred substrates, targeting them for transfer to mitochondria and/or peroxisomes [280,282,287,332,334,336,337]. Indeed, loss-of-function alleles of *acs-5(ok2668)*, was shown to enhance the suppression of *mdt-15(et14)* on *paqr-2(et3410)*, albeit not with the same magnitude as *acs-13(et54)*, which could explain why only *acs-13* was isolated in the forward genetics screen.

**Desaturase activity required for the enhanced effect provided by *acs-13(et54)***

As previously mentioned, *mdt-15(et14)* relies on desaturase activity to suppress the SFA vulnerability of *paqr-2(tm3410)* mutants, and *acs-13(et54)* therefore likely acts in a distinct pathway since it enhances the *mdt-15(et14)* mutation. Fluorescence microscopy assessing a GFP reporter for FAT-7, a Δ9 desaturase, revealed no elevation in the *acs-13(et54)* mutant as compared to the *mdt-15(et14)* mutant, suggesting that *acs-13(et54)* does not itself increase desaturation. Nevertheless, RNAi experiments show that the *acs-13(54)* mutation requires normal levels of the desaturases *fat-5*, *fat-6* and *fat-7* in order to enhance suppression of *paqr-2(tm3410)* mutant phenotypes in the *paqr-2(tm3410) mdt-15(et14) acs-13(et54)* triple mutant.

**PUFA-containing phospholipids are increased in the *acs-13(et54)* mutant**

*ACSL1*, a human homolog to *acs-13*, is known to prime long-chain fatty acids for mitochondria uptake [282,343]. As such, a conceivable mechanism driving the *acs-13(et54)* loss-of-function mutant phenotype could be diminished mitochondrial import of long-chain PUFAs, leading to their

accumulation in the cytoplasm where they would be available for activation by other acyl-CoA synthetases and shuttling into phospholipids, which leads to increased membrane fluidity, thus suppressing the rigidifying effect of the *paqr-2(tm3410)* mutant. Indeed, lipidomic analysis revealed increased amounts of PUFA-containing PEs and PCs in *acs-13(et54)* single mutants compared to wildtypes on normal growth medium. Similar increases have been observed in the *mdt-15(et14)* mutant [205], with a SFA-specific depletion of PUFA-containing PEs and PCs being a feature of the *paqr-2(tm3410)* mutant [207]. Analysis of PE composition due to *acs-13(et54)* showed a specificity of long-chain PUFAs being elevated (20:3, 20:4 and 20:5) at the cost of shorter SFAs and/or MUFAs (15:0, 16:0 and 16:1) in the single mutant compared to wildtype and on the *paqr-2(tm3410) mdt-15(et14)* background on normal growth conditions. Taken together, this suggests that *acs-13(et54)* exerts its ameliorating effect by elevating PUFA-containing phospholipids, which restores membrane homeostasis in the *paqr-2(et3410)* mutant, otherwise characterized by an excess of SFA-containing phospholipids.

**ACSL1 silencing counteracts the rigidifying effects of PA by maintaining membrane homeostasis in human cells**

The human long-chain fatty acyl CoA synthetases *ACSL1*, *ACSL5* and *ACSL6* share the highest sequence homology to *acs-13*. Transcriptionally, *ACSL1* was expressed 20-fold higher than *ACSL5* and *ACSL6* in human HEK293 cells. Membrane fluidity, assessed by FRAP, on *ACSL1*-, *ACSL5*- and *ACSL6*-silenced HEK293 cells showed no increase of fluorescence recovery (i.e. fluidity) under normal culture conditions compared to controls. However, treating HEK293 cells with PA causes decreased membrane fluidity, a phenotype that was suppressed in *ACSL1*-silenced cells, but not in *ACSL5* or *6*-silenced cells. This *ACSL1*-specific effect was corroborated, as assessed by general polarization index using the Laurdan dye; another method to monitor membrane fluidity [298], indicating an *ACSL1*-specific effect on membrane homeostasis.

Disrupting the expression of adiponectin receptor 2 (*ADIPOR2*), a human homolog to *paqr-2* in *C. elegans*, by siRNA silencing or CRISPR/Cas9 gene disruption, increases the vulnerability of HEK293 cells to rigidification by PA [344,345]. Interestingly, *ACSL1*-silencing led to a suppression of the PA vulnerability of *ADIPOR2*-silenced HEK293 cells, in line with *acs-13(et54)* suppressing the SFA sensitivity of the *paqr-2(tm3410) C. elegans* mutant and highlighting an evolutionarily conserved function of these proteins.

The endoplasmic reticulum unfolded protein response (ER-UPR) becomes activated in scenarios when membrane homeostasis is disrupted in cells, such as when challenged with SFAs [119,120,127]. *ACSL1*-silencing in HEK293 cells led to a decreased ER-UPR induction when treating cells with 400 µM PA as compared to non-targeting siRNA. Knockdown of *ACSL1* might induce transcriptional changes in other ACSLs, resulting in FAs being shuttled into pathways affecting membrane composition and, by extension, membrane fluidity. However, no substantial up-regulation was observed for *ACSL3/4/5/6* in *ACSL1*-silenced HEK293 cells treated with PA. In addition, no changes in *ACSL1* expression was observed in either *ADIPOR2*- or *TLCD1/2*-silenced HEK293 cells, indicating that *ACSL1* is not regulated by *ADIPOR2* or *TLCD1/2*.

**ACSL1 silencing elevates PUFA-containing phospholipids in human cells**

Similar to the findings with *C. elegans acs-13(et54)* mutants, lipidomics revealed increases in PUFA-containing PCs and PEs, with the strongest effect on long-chain PUFAs (20:4, 20:5, 22:5, and 22:6) in *ACSL1*-silenced HEK293 cells treated with PA. Increased PUFAs was also observed in lysophosphatidylcholines. *ACSL1*-silencing led to decreased in relative levels of ceramides, dihydroceramides and glucosylceramides, with lactosylceramides and sphingomyelins remaining unchanged. PUFA-containing TAGs showed no difference, as well as no changes in total abundance of PCs, diacyl PEs, alkenyl PEs, PIs, or PSs in *ACSL1*-silenced cells. Similar changes in PUFA-containing phospholipids, but lower in magnitude, was observed in ACSL1-silenced cells under normal culture conditions. In summary, *ACSL1*-silenced HEK293 cells showed specific elevations of PUFA-containing PCs and PEs, with decreased sphingolipids, when treated with SFAs.

### ACSL1 is enriched in mitochondria and controls mitochondrial homeostasis

Intracellular residency of ACSL1 varies between cell types [282] but several observations have located it to the mitochondria of brown adipocytes [290], cardiac myocytes [343], and hepatocytes where it was localized in the outer mitochondrial leaflet together with carnitine palmitoyltransferase I (CPT1) as part of a fatty acid import complex [280,287]. There, ACSL1 selectively activates long-chain PUFAs for mitochondrial import [343,346]. Mitochondrial isolation revealed, as with ACS-13 in *C. elegans*, a 20-fold enrichment of ACSL1, as compared to whole cell lysate, in mitochondria of HEK293 cells. As such, ACSL1 might play a similar role in long-chain FA import, for beta-oxidation or membrane homeostasis, in HEK293 cells. Mitochondria of *ACSL1*-silenced cells, treated with PA, showed an elevation of palmitoylcarnitine, with diminished levels of cardiolipins. In line with the depleted cardiolipins, several genes involved in cardiolipin synthesis (*CRLS1*) and remodeling (*TAFAZZIN* and *LCLATA1*) were down-regulated in the *ACSL1*-silenced cells post PA addition. Down-regulation of *TAFAZZIN* in the *ACSL1*-silenced cells corroborates published observation on an *Acsl1* knockout mouse model [343]. Similar to the morphological abnormalities observed for the *acs-13(et54)* mutant, the depleted cardiolipins in the *ACSL1*-silenced HEK293 cells would most likely distort mitochondrial morphology in this system, given their vital function in regulating mitochondrial structure [347–350]. Fascinatingly, mitochondrial respiration remained comparable between PA treated, ACSL1-silenced cells and controls, suggesting that the lipid composition did not perturb basal mitochondrial functions.

### Primary human cells are protected against SFA-induced rigidification by ACSL1-silencing

HEK293 cells, being a cancer cell-line with crucial aberrations in lipid metabolism [177,179,182], provides limitations when studying protein function in primary human cells. Thus, the effect of ACSL1-silencing was explored in primary human umbilical cord vein endothelial cells (HUVEC). As with the HEK293 cells, ACSL1-silenced HUVEC cells were able to maintain membrane fluidity, as assessed by the Laurdan dye method, in the presence of 400 µM PA, suggesting that this protective phenotype is indeed conserved in primary human cells.

## DISCUSSION

Our results provide further evidence that a primary mechanism of SFA-induced lipotoxicity relates to its effects on membrane composition since *acs-13* loss-of-function in *C. elegans* and ACSL1-silencing in human cells protect against SFA-induced toxicity by preventing membrane rigidification by elevating fluidizing PUFA-containing phospholipids (Fig. 8). Acyl CoA synthetases have previously been implicated in mediating vulnerability to SFA-induced toxicity [231]. Disruption of *ACSL3*, which activates SFAs, was ranked as the top protective mutation in a genome-wide screen, likely because the ACSL3 mutation hinders SFA-accumulation within phospholipids, resulting in decreased membrane fluidity. Conversely, *ACSL4*, which activates MUFAs, was ranked as the top sensitizing mutation that promotes SFA toxicity, likely because fewer fluidizing lipids are incorporated into phospholipids of that mutant, which aggravates the SFA-induced rigidification. In light of our results, a possible third arm connecting ACSLs to SFA-induced toxicity would be mediated by ACS-13 in *C. elegans* and ACSL1 in human, which primes long-chain fatty acids for mitochondrial uptake and/or lipid remodeling. Loss of ACS-13/ACSL1 leads to an accumulation and availability of PUFAs in the cytosol that can be incorporated into membrane phospholipids, providing a more fluid membrane.

The fate of different ACSL-activated acyl-CoA molecules can be partly attributed to the intracellular location of the specific ACSL [351]. In adipocytes, ACSL1 has not only been located to mitochondria [352], but to the plasma membrane [278], GLUT4 vesicles [281], and lipid droplets [353] as well, indicating a cell-wide need for this protein in adipocyte fatty acid metabolism. Indeed, ACSL1 stands for 80 % of the total ACSL-activity in adipose tissue [290], where it plays a crucial role in directing long-chain fatty acids for beta-oxidation in mitochondria. We have shown, for the first time, that the *C. elegans* homolog of ACSL1, namely ACS-13, locates to mitochondria where it exerts its function, indicating an evolutionarily conserved location of this acyl-CoA synthetase. This is in agreement with

the mutant *acs-13* having elevated PUFA-containing phospholipids in mitochondria, similar to what was observed in ACSL1-silenced human cells.

Ablation of ACSL1 could modify mitochondrial uptake of long-chain fatty acids, which would explain our findings in human cells, namely elevation of PUFA-containing PCs and PEs and aberrations in the mitochondria-specific cardiolipins and palmitoylcarnitine. These findings are further corroborated by a previous publication showing depleted PUFA-containing phospholipids, including cardiolipins in mitochondria of ACSL1 knockout cardiac myocytes [343]. A plausible explanation for the increased PUFA-containing phospholipids could be an accumulation of PUFAs in the cytosol, becoming available for other lipid fates such as phospholipid synthesis.

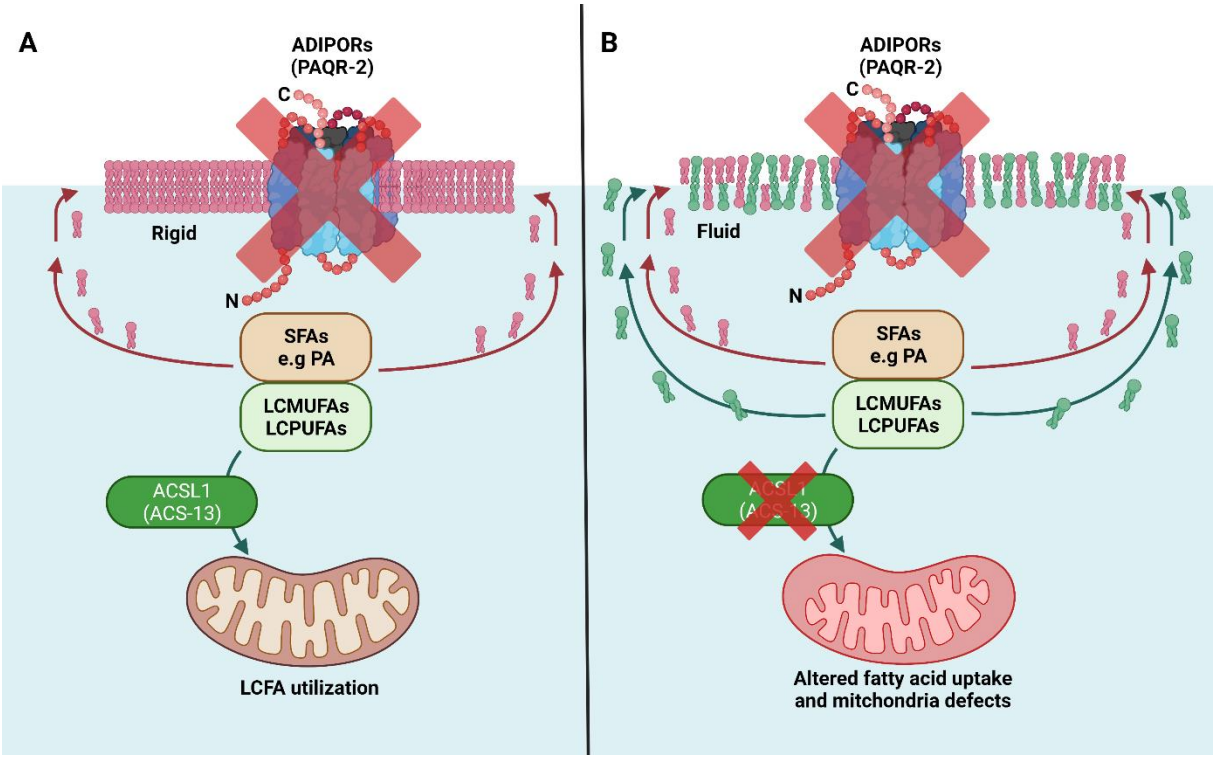


**Figure 8. Model explaining how loss of ACSL1/ACS-13 leads to increased membrane fluidity.** Under normal circumstances, mammalian ADIPOR2 (PAQR-2 in *C. elegans*) monitors membrane fluidity and is activated by membrane rigidity, at which point they signal to promote fatty acid desaturation and UFA-incorporation into membrane phospholipids, restoring fluidity. When ADIPOR2/PAQR-2 is missing, exogenous SFAs are excessively incorporated into membrane phospholipids, leading to rigid membranes. Under normal circumstances, mammalian ACSL1 (ACS-13 in *C. elegans*) activates long-chain fatty acids (LCFAs) such as MUFAs and PUFAs for mitochondrial utilization. When knocked out, this shuttle becomes impaired and LCMUFAs and LCPUFAs can be utilized for phospholipid synthesis which restores membrane fluidity in the ADIPOR/PAQR-2 knockouts.

No impairments to mitochondrial respiration was observed in the ACSL1-silenced human cells nor any growth defects in the *acs-13 C. elegans* mutant despite changes in lipid composition and morphology. This stands in contrast to previous publications showing that cardiolipins play a crucial role in mitochondrial respiration and morphology [343,354–357], however they might not be strictly mandatory as some organisms show functional respiration despite total cardiolipin depletion [358]. Likewise, the rare disease, Barth syndrome caused by a mutated *TAFAZZIN* gene, leads to whole-body depletion of cardiolipins [359], but patients are viable, with cells able to perform mitochondrial respiration [360]. If anything, this points to the robustness of mitochondria, being able to withstand significant insults to lipid composition, such as for ACSL1-silenced cells and in *acs-13 C. elegans* mutants.

ACSL1 is also located on peroxisomes [334–336] and inhibition of long-chain fatty acid uptake into peroxisomes, due to *ACSL1*-silencing, could explain some of our findings such as re-direction of PUFAs to phospholipid synthesis. Furthermore, ACSL1-mediated tethering of lipid droplets to the outer mitochondrial membrane can enable FA uptake from TAGs [332,351]. Even though we observed no differences in TAG composition in ACSL1-silenced HEK293 cells, hydrolyzed lipids from TAGs could be re-directed to phospholipid synthesis instead of mitochondrial uptake.

In closing, inhibition of ACS-13 in *C. elegans* or of its homolog ACSL1 in human cells, led to elevation of long-chain PUFA-containing phospholipids, which suppressed vulnerability to SFA-induced disruptions to membrane homeostasis, i.e. membrane rigidity. This is not without consequence however, as this also led to drastic changes in mitochondrial morphology and lipid composition.

**CONTRIBUTIONS**
For this work I contributed with the following:

- Protein detection by Western Blot: I performed all Western Blot experiments for the HEK293 cells.
- Bioenergetics: I performed all experiments assessing cellular respiration in the HEK293 cells.
- Data analysis: I processed and compiled all data for all experiments mentioned above.
- Manuscript: I prepared graphs representing my contributions as well as proof-reading and commenting the manuscript.

## Paper III: TLCD1 and TLCD2 regulate cellular phosphatidylethanolamine composition and promote the progression of non-alcoholic steatohepatitis

**INTENTION**

Building upon the body of evidence pointing to that *TLCD1* and *TLCD2* play a role in modulating MUFA/PUFA content in phospholipids, further mechanistic studies were warranted to increase the understanding of these proteins in mammalian systems. Alongside this, there was limited knowledge regarding how these proteins affect physiology. Lastly, nothing was known of the cellular and physiological consequences of TLCDs genetic ablation in mammals. However, the fact that no obvious phenotype was observed in the *fld-1 C. elegans* single mutant [361] suggested that cells and organisms could tolerate the loss of these proteins.

Previously we showed that siRNA-targeted silencing of *TLCD1* and *TLCD2* in human HEK293 cells led to an increase in SFA-containing PCs and a decrease in MUFA-containing PEs [361]. Exposure of these *TLCD* knockdown cells to 400 µM of PA led to extensive increases of PUFA-containing PEs, with several long-chain PUFAs specifically being upregulated in the *TLCD1* knockdown cells [361], suggesting a role of the TLCDs in PE composition.

Fatty acid composition of PEs is determined by the fatty acyl chain moiety bound to the sn-1 and sn-2 position of glycerol [135]. Currently, our understanding of how fatty acids are incorporated into PEs is limited to the sn-2 position, where the enzymes ACSL4 and LPCAT3 coordinate the integration of PUFAs [362,363]. Importantly, PEs are important precursors in eicosanoid synthesis, i.e. when the PUFA arachidonic acid is detached off the sn-2 position, then processed; eicosanoids are required for a myriad of cellular processes including modulation of inflammation [364,365]. Additionally, previous studies have shown SFAs or MUFAs to be commonly esterified to the sn-1 position of PEs [366] but the underlying mechanism of fatty acid replacement at this position is largely unknown.

When searching in an available genome-lipid association map [170] for specific loci potentially involved in regulating PE composition in mouse liver, one locus localized on chromosome 11 was strongly associated with several SFA- and MUFA-containing PEs. To our excitement, further investigation of this locus revealed that it corresponds to both *Tlcd1* and *Tlcd2*. Our previous study on the TLCDs

revealed high expression of both *Tlcd1* and *Tlcd2* in mouse liver [361]. Together, these observations led us down the path of further elucidating the cellular mechanisms of the TLCDs in liver.

## RESULTS

### TLCD1/2 double knockout mice have reduced hepatic MUFA-containing PEs

Comparable with several lipid remodeling and lipogenesis genes [367,368], *Tlcd1* and *Tlcd2* expression was down-regulated in the fasted state in mouse liver. In order to investigate if *Tlcd1* and *Tlcd2* modulate PE homeostasis, *Tlcd1* and *Tlcd2* single knockouts (KO) and *Tlcd1/2* double knockout (DKO) mice were generated. Lipidomics from liver revealed substantial decreases in MUFA-containing PEs in the Tlcd1 KO mice with similar results, albeit of lower magnitude, in the Tlcd2 KO mice. Specifically, PEs comprising the MUFAs palmitoleate (16:1) and oleate (18:1) in the sn-1 position were severely diminished in the Tlcd1/2 DKO mice, establishing the first link between the TLCD proteins and sn-1 composition of PEs. In order to minimize the effect of possible functional redundancy between *Tlcd1* and *Tlcd2*, the Tlcd1/2 DKO was used for further studies. In terms of total PEs, a decrease in total MUFA-containing PEs with a concordant increase in SFA-containing PEs was observed in the Tlcd1/2 DKO mice livers compared to WT controls. This was observed in all Tlcd1/2 DKO mice irrespective of age, sex or diet.

### TLCD1 and TLCD2 affect MUFA incorporation into PEs post-transcriptionally

Having established that the TLCDs are important for PE lipid composition, the next aim was to better understand the underlying molecular mechanism. In order to determine whether the changes in PE composition was due to intracellular uptake of MUFAs, TLCD1/2 DKO mice were orally gavaged with radiolabeled triolein, which is a TAG with three oleate (18:1) moieties. Accumulation of radioactivity in both plasma and tissues was comparable between TLCD1/2 DKOs and WT, indicating that Tlcd1/2 does not impact MUFA uptake. Next, we investigated whether the differences in PE composition was due to changes in circadian lipid metabolism, by fasting and re-feeding. No differences in PE composition was observed in TLCD1/2 DKO mice compared to controls and irrespective of feeding state, suggesting that this was not the reason for the disparity. Subsequently, looking into whether the TLCDs remodel PEs transcriptionally, hepatic transcriptomes were compared between TLCD1/2 DKO mice and WT. Again, the TLCD1/2 DKO mice showed no transcriptome differences compared to WT. In order to determine if TLCD1/2 exhibit their effect post-transcriptionally, PCs and PEs were measured in red blood cells that lack transcription [369]: we detected the same differences in PE composition of red blood cells as were seen in the livers of the TLCD1/2 DKO mice, indicating a post-transcriptional effect of these proteins.

### TLCD1 and TLCD2 function cell-intrinsically by promoting MUFA incorporation into PEs

Excised primary hepatocytes from TLCD1/2 DKO mice preserved the changed PE lipid profile after two days of cell culture, suggesting cell-intrinsic effects of the TLCD proteins. Cultured Tlcd1/2 DKO hepatocytes were then exposed to radio-labeled fatty acids, in a pulse-labelling experiment, to investigate the rate of incorporation of SFAs and MUFAs into PEs. Reduction in MUFA and increased SFA incorporation rates were observed in the Tlcd1/2 DKO hepatocytes at the sn-1 position of PEs compared to controls; no differences were seen in PCs, suggesting a PE specific effect. Synthesis of SFA- or MUFA-containing acylcarnitines was similar between genotypes, implying unchanged oxidation of exogenous fatty acids in mitochondria. SFA and MUFA incorporation into TAGs showed only minor differences between genotypes except for an increase in double-labelled 16:0/18:1/18:1 TAG in the Tlcd1/2 DKO hepatocytes, suggesting a potential shunt from PE incorporation to TAG storage in the double knockouts.

Fatty acid incorporation into phospholipids is regulated via the Lands' cycle [370], where fatty acyl-CoA molecules and lysophospholipid intermediates are acted on by lysophospholipid acyltransferases (LPLATs). In order to investigate whether the TLCDs act via LPLATs, synthesis rates of canonical

LPLAT reactions as well as custom sn-2-lysoPE acyltransferase reactions [371], aimed at specific species observed to be reduced in the Tlcd1/2 DKO mice, were analyzed in liver microsomes. No differences between genotypes were detected in the LPLAT assays, suggesting that TLCD1/2 act cell intrinsically and upstream of LPLATs when regulating MUFA-composition into the sn-1 position of PEs.

**TLCD1 and TLCD2 form an evolutionarily conserved lipid remodeling complex at the mitochondria**

Separately, three TLCD1/2 DKO clones were created using the human hepatocellular carcinoma cell line HepG2 in order to study whether the PE fatty acid composition effect is conserved in human cells. In line with the observations in mice hepatocytes, the TLCD1/2 DKO clones showed diminished amounts of MUFA-containing PEs, with enrichment of SFA-containing PEs compared to WT clones, indicating a conserved function in humans.

Stable HA-tagged TLCD1 and TLCD2 HepG2 and HeLa (superior for microscopy) cell lines were generated. Since GFP tags on the C-terminus of FLD-1, the *C. elegans* homolog of TLCDs, functioned well [361], HA-tags were also placed on the C-terminus of TLCD1 and TLCD2 in these cell lines. Fluorescence microscopy revealed HA-TLCD1 and HA-TLCD2 to be located in proximity to the ER/Golgi organelles, comparable to other lipid-remodeling proteins [372]. The interactome of both TLCD proteins, by proteomics and immunoprecipitation (IP), was mapped alongside the interactome of the similarly tagged *C. elegans* FLD-1, in order to study the evolutionary conservation between these proteins. Several proteins that co-purified with *C. elegans* FLD-1 and human HA-TLCD1 and HA-TLCD2 were proteins involved in mitochondrial respiration. In particular, several homologs of ATP synthase subunits precipitated in both the human and *C. elegans* systems. Additionally, the phospholipid synthesis and lipid remodeling proteins PHB and PHB2, which form a lipid remodeling complex at the mitochondria [373], ranked amongst the top HA-TLCD1/2 interacting proteins. Interestingly, PEs are especially enriched in mitochondria-associated membranes, where most mitochondrial phospholipid metabolism takes place [374]. Fluorescence microscopy revealed that HA-TLCD1 and HA-TLCD2 localize in close proximity to mitochondria, possibly near or on the mitochondria-associated membranes. Lipid composition of liver-derived mitochondria from Tlcd1/2 DKO mice reflected whole liver lipid composition in that MUFA-containing PEs were diminished, with a concordant increase of SFAs at the sn-1 position. Mitochondrial PE synthesis relies on PS precursors [375], but no changes in several mitochondrial PS species were observed in mitochondrial fractions nor whole liver, irrespective of genotype. Taken together, these results suggest that the TLCDs, and FLD-1 in *C. elegans*, exert their modulation of PE-composition in close proximity to mitochondria.

**Tlcd1/2 DKO mice show ameliorated NASH progression after HFD feeding**

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the west, with some estimates stating that the prevalence in the U.S adult population is around 25 % [376]. NAFLD, if not corrected, will progress into non-alcoholic steatohepatitis (NASH), followed by liver cirrhosis and eventually liver failure. The progression from NAFLD to NASH has been partly attributed to defective hepatic mitochondrial PE composition [377]. As such, we hypothesized that the Tlcd1/2 DKO mice may be protected against NASH progression. Initially, *in vitro* assessments were performed to investigate whether lack of *Tlcd1/2* impacts hepatocyte core gene expression in an induced inflammatory setting by LPS and PA [378]. No changes in *de novo* lipogenesis, hepatocyte marker or cytokine gene markers were detected in Tlcd1/2 DKO primary hepatocytes compared to controls, suggesting that deletion of *Tlcd1/2* had no impact on core hepatocyte gene expression in an inflammatory response setting *in vitro*.

Next, we investigated whether TLCD1/2 have a role in the development of NAFLD *in vivo* by placing both male and female Tlcd1/2 DKO mice on a high-fat diet (HFD). Mice placed on a HFD are known to develop systemic insulin resistance and hepatic steatosis which does not develop further into steatohepatitis and fibrosis [379,380]. Tlcd1/2 DKO mice gained weight similarly to control mice in addition to displaying similar body composition and fasting insulin levels. Circulating glucose and lipids

were also comparable between genotypes after the study. However, reduced liver weight, hepatic lipid content and serum ALT levels were observed in the Tlcd1/2 DKO HFD-fed mice compared to controls. Transcriptionally, down-regulation of *de novo* lipogenesis pathways was observed in Tlcd1/2 DKO females with markers involved in biosynthesis of fatty acids being down-regulated in both male and female Tlcd1/2 DKO mice compared to controls. No differences with respect to genotype was observed for fatty acid metabolism markers in either brown or epididymal white adipose tissue. Taken together, these results show that *Tlcd1/2* ablation ameliorated NAFLD development in HFD-fed mice.

The attenuated NAFLD development observed in HFD-fed Tlcd1/2 DKO mice suggests a protective effect also in NASH development. In order to induce NASH progression, Tlcd1/2 DKO mice and controls were fed a western diet (WD) for 32 weeks. No difference in weight gain was observed between genotypes and both genotypes showed normal glucose handling, assessed by oral glucose tolerance test, prior to termination. Tissue analysis revealed decreased liver weight, liver inflammation and serum TAGs in the Tlcd1/2 DKO mice compared to control. Reductions in plasma ALT levels, a biomarker of inflammation, and hepatic fibrosis was also observed in the Tlcd1/2 DKO livers compared to control. No changes in hepatic markers were observed in the chow-fed control cohort, and females resisted liver fibrosis development after the WD irrespective of genotype. Serum cholesterol and hepatic lipid levels were also normal in the Tlcd1/2 DKO mice after WD, but a reduction in hepatic macrovesicular steatosis was observed in the Tlcd1/2 DKO mice. WD led to increases in liver markers of inflammation, immune cell infiltration, fibrosis, and eicosanoid synthesis in the WT mice compared to chow-fed WT mice. These inflammation markers were down-regulated in the WD-fed Tlcd1/2 DKO mice compared to WD-fed WT mice, with stronger reductions observed in males. In contrast to observations on HFD-fed mice, no transcriptional changes in lipid metabolism genes were observed between genotypes on WD-fed mice. Transcriptional analysis of upstream regulators of pro-inflammatory cytokines also showed a down-regulation of several markers in the WD-fed Tlcd1/2 DKO mice livers compared to controls. Gene markers involved in lipid metabolism and remodeling were unchanged. In extension, decreased levels of the pro-inflammatory cytokines KC/GRO and IL-6, along with decreased spleen size, was observed in the WD-fed Tlcd1/2 DKO female mice compared to controls, suggesting lower systemic inflammation. Finally, as membrane PEs can provide substrates for inflammation-mediating eicosanoids [381], where hepatocyte-originated eicosanoids have been implicated in NASH progression [382], a panel of 19 eicosanoids were quantified in WD-fed mouse livers. No differences in eicosanoids levels were observed with respect to genotype. Taken together, *Tlcd1* and *Tlcd2* ablation led to a sex-dependent, reduction in NASH development with Tlcd1/2 DKO males exhibiting reduced fibrosis and liver damage and females showing a suppressed hepatic inflammatory phenotype compared to controls.

## DISCUSSION

This work began elucidating the molecular mechanisms and physiological roles of TLCD1 and TLCD2. Our tentative conclusion is that TLCD1/2 promote MUFA incorporation into the sn-1 position of PEs (Fig. 9). This regulation is important since MUFA-containing PEs are sensitive to peroxidation, which in turn can lead to ferroptotic cell death [362,383]. Our observations suggest that TLCD1/2 exert their function downstream of MUFA uptake into the cell and upstream of the LPLAT enzymes, which incorporate MUFA-CoAs into PEs. Further studies would need to be performed in order to pinpoint whether the TLCDs act by esterification of CoA onto MUFAs or if they impact lipid trafficking. Intriguingly, a recent publication showed that dual MUFA-containing PEs were synthesized from PS precursors in yeast [384]. Similar to our observations on TLCD1/2 DKO mice and human cells, genetically altered yeast models that lack lipid transport between the ER and mitochondria, displayed elevated SFA-containing PEs at the cost of reduced MUFA-containing PEs [384]. Based on our observed localization of TLCD1/2 to the ER/Golgi organelles with close proximity to mitochondria, it is conceivable that the TLCDs might be the mammalian mediators of this lipid transfer system. In addition, observations from our genetic HepG2 models, imply the TLCDs in lipid transport, as the TLCD1/2 DKO cells excessively decreased MUFA-containing PEs, while conversely, both HA-TLCD1 and HA-TLCD2 cells displayed an opposite effect, suggesting that PE biosynthesis might require TLCD1/2 while not being rate-limiting. Furthermore, CLN8, the closest TLCD1/2 mammalian paralog, is thought to facilitate the transport of lysosomal proteins between the ER and Golgi, and is also located

in the mitochondrial associated membrane [255]. Note however that neither TLCD protein was identified in the validated mammalian mitochondrial proteome, MitoCarta [385]. This may be explained by our observations that there is only partial interaction between the TLCDs and all mitochondria (Fig. 3C of Paper III), in addition to this potentially being a transient interaction, which could result in the TLCDs being unidentified during the rigorous filtering process [385]. Future studies are necessary to describe this potential link between mitochondria-associated TLCDs and lipid transport.

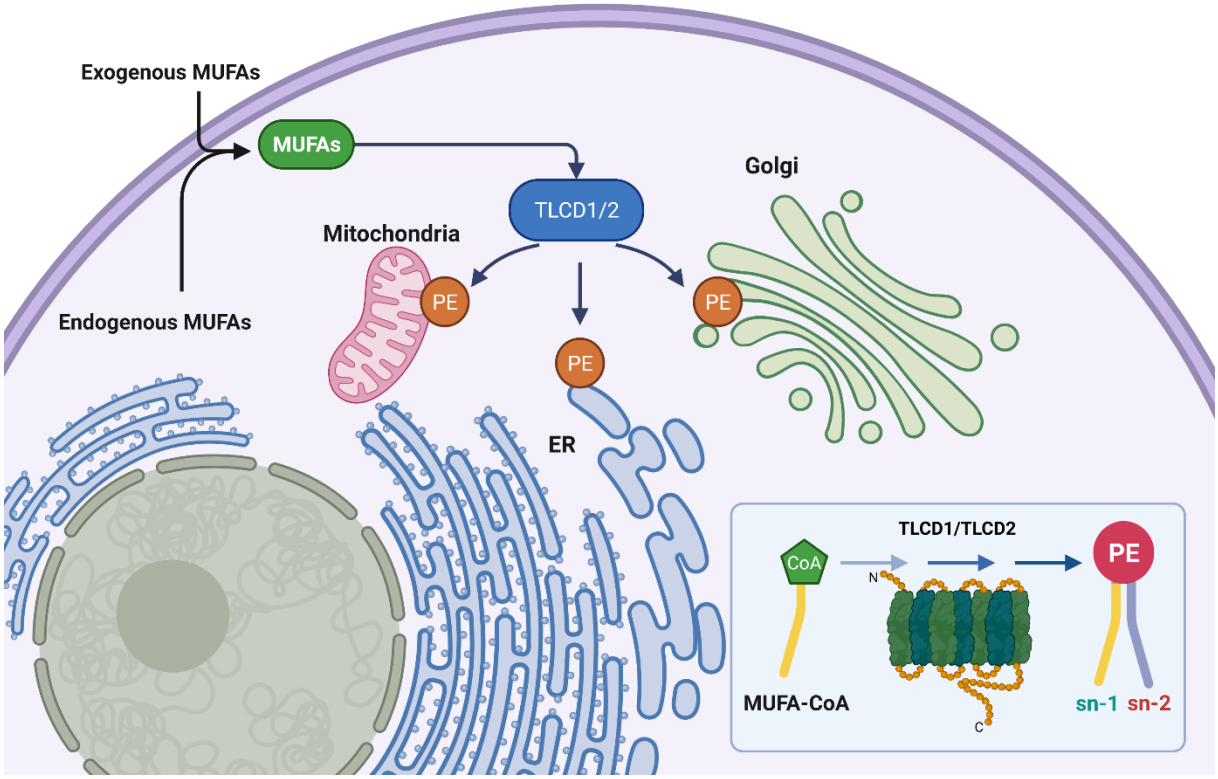


**Figure 9. Model explaining the putative action of the mammalian TLCDs.**
We hypothesize that the mammalian TLCDs act as lipid transport proteins, mainly targeting MUFAs for distribution to the three main sites of PE phospholipid synthesis, namely the ER, Mitochondria and Golgi. The TLCDs were shown to co-localize to these three organelles, and the TLCDs may control the supply of MUFAs to be incorporated into the sn-1 position of PEs. How this is achieved remains to be studied.

Our observations revealed stronger outcomes on PE composition when *Tlcd1* was deleted as compared to *Tlcd2* deletion, suggesting that TLCD1 could be the main modulator of PE composition with TLCD2 having potential additional functions. Further studies on the individual proteins are warranted to divulge their specific mechanisms of action and how they contribute to liver disease progression, as was observed in the Tlcd1/2 DKO mice.

Further, our data links TLCD1/2-mediated hepatocyte PE remodeling with NAFLD development and, by extension, NASH development. PE metabolism has been implicated in liver disease progression [386,387]. However, little is known regarding how PE lipid composition influences NASH development. Our results implicate the TLCD1/2 proteins in promoting a PE composition, namely MUFAs at the sn-1 position, favorable for liver disease pathogenesis *in vivo*, with the exact mechanisms as to how this is achieved still remaining unknown. As PE fatty acyl chains are precursors for pro-inflammatory eicosanoid synthesis [381], it is conceivable that the changed hepatic PE composition observed in WD-fed Tlcd1/2 DKO mice could impair liver eicosanoid production, which ameliorates NASH development. Despite not observing any differences in the panel of quantified eicosanoids, it cannot be ruled out that other species, not detected in the analysis, could show differences between Tlcd1/2 DKO mice and controls. With EPA-containing PEs decreased in Tlcd1/2 DKO mouse livers (Fig. S1E of Paper III), it is reasonable that undetected EPA-derived eicosanoids, such as 18-HEPE

[388], could be different with respect to genotype. Therefore, further studies are warranted in studying the TLCDs by means of pro-inflammatory eicosanoid signaling in liver disease progression, which could open avenues of disease prevention by modulating these proteins.

Considerable diet-dependent differences were observed in the Tlcd1/2 DKO mice. HFD-fed Tlcd1/2 DKO mice displayed reduced hepatic lipid accumulation, likely due to down-regulated transcription of *de novo* lipogenesis genes (Fig. S8E-G of Paper III). Mice fed a HFD are known to display increased insulin in circulation, which together with insulin known to induce *de novo* lipogenesis transcription [367], could explain that *Tlcd1/2* ablation would modulate hepatocyte response to insulin during obesity. Conversely, mice fed a WD do not develop systemic insulin resistance, but leads to liver inflammation and fibrosis [380]. Hepatic lipid accumulation was comparable between genotypes after WD feeding, indicating that TLCD1/2 can modify NASH development separate from *de novo* lipogenesis transcription and following lipid accumulation. Together with similar responses with respect to genotype in primary mouse hepatocytes to LPS and PA *in vitro*, more physiological cell culture systems, such as organoids and co-culture models [389] are warranted to further elucidate the pathological mechanisms of TLCD1/2.

The degree of amelioration with respect to NASH development in Tlcd1/2 DKO mice showed sex-dependent differences. Interestingly, hepatic PE metabolism differs between males and females, and phosphatidylethanolamine N-methyltransferase (PEMT), the enzyme catalyzing PE to PC conversion, is transcriptionally controlled by estrogen [390]. As a result, female mice display increased hepatic PE to PC conversion compared to male mice [391], which may explain the sex-dependent differences in liver disease development [392]. In addition to the altered PE composition of Tlcd1/2 DKO mice, several PCs are quantifiably different compared to controls (Fig. 1C of Paper III), predominantly PCs containing 22:6 PUFAs at the sn-2 position, of which the synthesis is attributed to the PEMT pathway [393]. As such, despite the similarities in hepatic PE composition between male and female Tlcd1/2 DKO mice, an increased PE to PC conversion in females could explain the sex-dependent observations in the NASH study.

Besides NASH progression, TLCD1 has been implicated in hepatocellular carcinoma where up-regulation of the gene was correlated to liver inflammation and increased morbidity [394]. Additionally, screens mapping cancer dependency highlight TLCD1 as a strong candidate (depmap.org/portal/gene/TLCD1) in cancer progression, potentially via dependency on PE remodeling. Finally, allergy-driven inflammation in the murine lung, due to eosinophils, ranks *Tlcd2* expression as the top ranking factor driving this phenotype [395]. In conclusion, our work classifies the TLCD1/2 proteins as key regulators of PE lipid composition *in vitro* and *in vivo*, which has the potential to shed new light on the pathology, and possibly lead to treatments, of advanced liver disease.

**CONTRIBUTIONS**

For this work I contributed the following:

- Mouse hepatocyte perfusions: I helped with the perfusion of mouse livers and subsequent cell preparation for *in vitro* experiments.
- Lipidomics: I helped with sample generation of mouse tissues.
- Fluorescence microscopy: I performed all fluorescence microscopy experiments for this manuscript.
- *In vivo* studies: I shared responsibility for all in vivo studies in terms of study plans, ethics, document preparation and reporting as well as performing genotyping, weekly weighing and cage changes.
- *In vivo* energy balance by CLAMS: I helped perform the CLAMS experiments, and I measured total fecal energy content by bomb calorimetry.
- Fasting & re-feeding experiment *in vivo*: I helped with weighing, and with the termination and tissue isolation.

- *In vivo g*lucose tolerance tests: I helped with weighing the mice, calculating glucose dose, taking blood samples for glucose and insulin. I performed the insulin ELISAs.
- Radiolabeled oleate uptake *in vivo*: I helped with weighing the mice, supporting the dosing, taking terminal blood samples, processing samples for scintillation counting.
- Mouse body composition by dual x-ray absorptiometry (DEXA): I performed the DEXA experiments.
- *In vivo* study on NASH development: I helped with the preparation, termination and weighing of tissues.
- Manuscript: I prepared graphs representing my contributions as well as proof-reading and commenting the manuscript.

## Paper IV: Extensive transcription mis-regulation and membrane defects in AdipoR2-deficient cells challenged with saturated fatty acids

### INTENTION

Previous work from the Pilon group had established the evolutionary conserved ADIPOR pathway as being essential for tolerating exogenous SFAs, from *C. elegans* to mammalian cells, by increasing UFAs into membrane phospholipids, counteracting the rigidifying effect of SFAs and restoring membrane fluidity [205,206,338,344]. All mammalian results linking the ADIPORs with membrane homeostasis regulation relied on siRNA to knock down the proteins in order to show the effect of SFAs on cells [344]. Questions remained, such as what cellular responses rely on ADIPOR2 function and how does ADIPOR2 interact with other genes involved in lipid metabolism? In order to further elucidate the role of ADIPOR2 in membrane homeostasis, an ADIPOR2 KO HEK293T cell line was generated using CRISPR/Cas9. Findings with this ADIPOR2 KO model were complemented by comparing with the effect of silencing other lipid metabolism genes in the response to SFA challenges.

### RESULTS

#### ADIPOR2 deficiency leads to increased membrane rigidification upon PA treatment in HEK293T cells

In order to assess the impact of ADIPOR2 in cell membrane homeostasis, HEK293T knockout clones were generated using CRISPR/Cas9, targeting and disrupting the *ADIPOR2* gene. The clones were verified and subsequently characterized in terms of growth. First, ADIPOR2 KO cells showed delayed growth in normal culture conditions compared to WT cells, but did eventually reach confluency. Furthermore, ADIPOR2 KO cells displayed sensitivity to exogenously added PA resulting in cell shrinkage and detachment. This vulnerability was reversable when co-adding low amounts of eicosapentaenoic acid (EPA), which is a known membrane fluidizer. Next, direct assessment of membrane packing order (fluidity) using both Laurdan dye generalized polarization index and FRAP showed that cellular membranes of ADIPOR2 KO cells suffered from increased packing, signifying rigidity compared to WT cells. Once more, this increase in rigidity could be ameliorated with the addition of EPA. Lipidomics, focused on membrane phospholipids, showed that PA-treated ADIPOR2 KO cells contained elevated levels of SFA-containing, and decreased levels of MUFA- and PUFA-containing PCs and PEs compared to WT cells, and that these phospholipid composition defects increased over time.

In parallel, *ADIPOR2*-knockdown in HEK293 cells using siRNA, showed that vulnerability to other SFAs besides PA, such as myristic acid (C14:0) and stearic acid (C18:0) that also caused increased membrane rigidity in the mutant cells. Additionally, atomic force microscopy, which quantifies mechanical stiffness on the atomic scale, showed increased rigidity in PA-treated *ADIPOR2*-knockdown cells compared to WT. Furthermore, knockdown of *ADIPOR2* in human umbilical vein endothelial cells (HUVECs), led to increased SFA-containing PCs at the expense of MUFAs and PUFAs. In summary,

the results indicate that ADIPOR2-mediated regulation of membrane homeostasis is an essential function in human cells when tolerating SFAs.

**ADIPOR2 is essential for normal cellular responses to SFAs**

In order to further study the consequences of *ADIPOR2* deficiency, RNA sequencing analysis was used to investigate the effect of PA treatment, hence membrane rigidification, in ADIPOR2 KO cells. Analysis of all observed transcripts (>50 000) showed clear differences due to treatment between genotypes. Mis-regulated genes increased with PA treatment time starting with 330 genes after 3 h up to 2861 genes after 24 h in ADIPOR2 KO cells compared to WT cells. Strikingly, the most significantly down-regulated gene in the ADIPOR2 KO cells after 24 h of treatment was *SCD*, which encodes the human Δ9 desaturase. Further analysis revealed 53 differentially expressed genes (up or down) at all timepoints (3, 9, 24 h) in the ADIPOR2 KO cells compared to WT treated with PA, with several being involved in cholesterol synthesis (e.g. *CYP51A1*), fatty acid metabolism (*FADS2*) and oxidative phosphorylation (e.g. *IDH2*, *NDUFA6*, *NDUFB1*). Examination of the top 50 most significantly variable transcripts within the whole data set revealed that said genes clustered into four distinct categories: 1) fatty acid and cholesterol metabolism, 2) starvation-induced genes, 3) genes consistently different between genotype, and 4) endoplasmic reticulum unfolded protein response (ER-UPR). Specifically, genes in category 1 were extensively down-regulated and category 4 were up-regulated in the PA-treated ADIPOR2 KO cells compared to WT.

**ER-UPR, lipid remodeling and energy metabolism pathways are impaired in ADIPOR2 KO cells**

Transcriptionally mis-regulated pathways can be identified by using gene set enrichment analysis (GSEA) [396]. GSEA, based on the available KEGG [397] and REACTOME [398] pathway collections, revealed that loss of *ADIPOR2* led to down-regulation of pathways involved in glycolysis/gluconeogenesis, ribosomes and metabolism by cytochrome P450 in all conditions. PA treatment specifically led to down-regulation of pathways involved in oxidative phosphorylation, steroid biosynthesis and propanoate metabolism in the ADIPOR2 KO cells compared to WT. Gene analysis of pathways responsible for membrane homeostasis revealed that ADIPOR2 KO cells show down-regulation of pathways such as biosynthesis of unsaturated fatty acids, cholesterol biosynthesis and acyl chain remodeling compared to WT as well as showing down-regulation of markers in respiratory electron transport, TCA cycle and glycolysis with genes in the ER-UPR being up-regulated. Quantification of the lipid remodeling proteins SCD and FADS2 showed decreased levels in ADIPOR2 KO cells treated with PA compared to WT. In line with the observed down-regulation of genes involved in cholesterol biosynthesis and oxidative phosphorylation, relative abundances of cholesteryl esters and mitochondrial respiration were diminished in the ADIPOR2 KO cells compared to WT. In total, the observations showed that *ADIPOR2* is crucial for a normal transcriptional response needed for membrane homeostasis and mitochondrial function when cells are exposed to SFAs. In summary, the results suggest that loss of *ADIPOR2* leads to deteriorated membrane homeostasis, impairing UFA and cholesterol regulation, leading to an increase in ER stress.

**Lack of ADIPOR2 resembles transcriptional inhibition of SREBP pathway**

The sterol response element binding proteins (SREBPs) are transcription factors regulating lipid synthesis, lipid storage and cholesterol regulation [103]. Interestingly, PA-treated ADIPOR2 KO cells phenocopied loss of SREBPs transcriptionally [399], showing down-regulation of several SREBP pathway genes after 24 h. Additionally, knockdown of *SREBF*s using siRNA in human cells showed reduced *SCD* expression, decreased membrane fluidity and increased SFA-containing phospholipids (PCs) at the cost of MUFAs compared to control cells, similar to observations of PA-treated ADIPOR2 KO cells. Down-regulation of the SREBP pathway in the ADIPOR2 KO cells is therefore a potential contributor to the defective membrane phenotype observed.

**ADIPOR2 is similar to SCD and FADS2 in terms of protecting cells from lipotoxicity**

Several genes, involved in fatty acid desaturation and UFA incorporation into membrane lipids, such as *SCD*, *FADS2* and *ACSL4*, have been identified as crucial in protecting cells from SFA toxicity [231,311]. Knockdown, using siRNA, of said genes was done in HEK293 cells and the effects compared with knockdown of *ADIPOR2*. Decreased membrane fluidity, as assessed by FRAP and Laurdan-derived GP index, was observed for all knockdown conditions, with *ADIPOR2*, *SCD* and *FADS2* leading to the largest effect. SFA-containing PCs and PEs were increased in all knockdown conditions at a cost of MUFAs with the strongest effects seen in knockdown of *ADIPOR2* and *SCD*. As expected, membrane rigidification was accompanied by ER stress as measured by the increase of ER-UPR markers in all knockdown conditions. Together, these results suggest that *ADIPOR2* is crucial in order to sustain normal membrane lipid composition and homeostasis in the presence of exogenous SFAs.

## DISCUSSION

In this work we explored the impact that loss of *ADIPOR2* has on membrane homeostasis in human HEK293 cells. Genetic ablation of *ADIPOR2* led to increased membrane rigidification, impaired growth and respiration, alongside a cascade of genes being transcriptionally mis-regulated in response to PA (Fig. 10). Of particular note, many genes involved in membrane remodeling such as the *SREBP*s and *SCD* were down-regulated, while ER-UPR markers were up-regulated indicating increased ER stress in ADIPOR2 KO cells compared to WT cells in the presence of PA. We postulate, based on current results and previous studies, that membrane homeostasis dysfunction is the primary consequence when *ADIPOR2* is removed, with increased ER-stress, growth and respiration defects being a secondary effect. Of note and in line with our results, respiration and, by extension, mitochondrial function, has been shown to be impaired with increased membrane rigidification [400]. Overall, excess SFA accumulation in most non-adipose cells, often leads to deterioration of cellular membranes, increased stress and finally, apoptosis [401]. As mentioned earlier, removal of *ACSL4*, an acyl-CoA synthetase promoting UFA incorporation into phospholipids, decreases the capability of human cells to tolerate exogenous SFAs, while loss of *ACSL3*, which assists in SFA incorporation in phospholipids, increased the tolerance to SFAs [231], further suggesting that membrane-rigidification is a primary mechanism of PA lipotoxicity.

Fascinatingly, *ADIPOR2* ranked, in terms of significance, 4$^{th}$ and 25$^{th}$ in two separately published, genome-wide CRISPR/Cas9 screens set out to discover genes needed to protect against PA toxicity or hypoxia-induced desaturase inhibition [231,232], strengthening the link between membrane rigidity and SFA-induced lipotoxicity. In line with our results, and the significance of *ADIPOR2* in the mentioned genome-wide screens, it becomes increasingly apparent that *ADIPOR2* plays as important a role in maintaining membrane homeostasis as *SCD*, *FADS2*, *SREBP1/2*, and *ACSL4*, when facing increased SFAs. Of note, the significance of *ADIPOR2* in maintaining membrane homeostasis outlined in this work is particularly interesting due to its functional overlap with its ortholog, *ADIPOR1* [344]. One would expect even more severe defects in membrane homeostasis with both genes ablated, perhaps explaining the reported synthetic lethality [229].

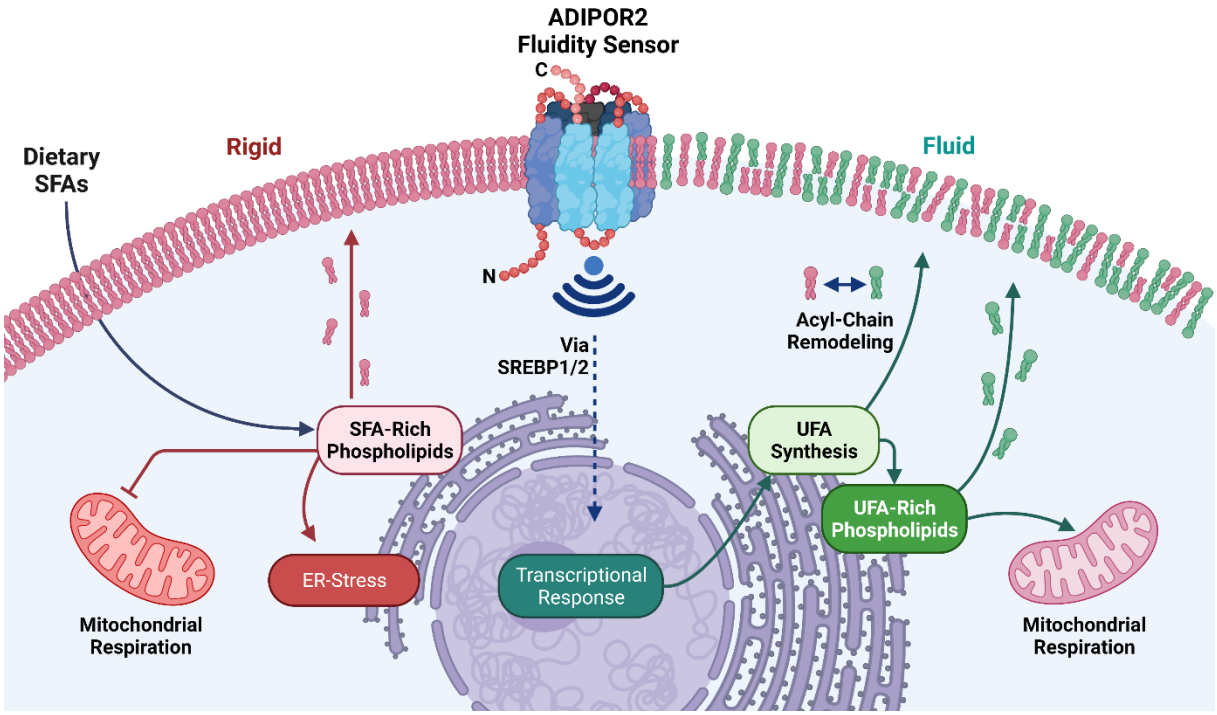


**Figure 10. Model explaining the membrane fluidity sensing function of mammalian ADIPOR2.** Dietary or endogenously produced SFAs ending up in phospholipids can lead to ER-stress, perturbed mitochondrial respiration and decreased membrane fluidity, which is intensified upon ADIPOR ablation. Normally, ADIPOR2 senses this increased rigidification and signals, via the SREBPs, to initiate a transcriptional response, activating lipid remodeling genes and inducing UFA synthesis that increases UFA-rich phospholipids, thus restoring membrane fluidity and mitochondrial respiration.

Our results add to the growing body of evidence supporting an evolutionary conserved role of *ADIPOR2* in membrane homeostasis [205,208,338,344,361]. However, both *ADIPOR2* and *ADIPOR1* have been proposed to act as ceramidases, modulating ceramide levels in lipid membranes as well as generating sphingosine-1-phosphate, a signaling molecule leading to a myriad of cellular effects [213,214,227,402]. In addition, separate evidence suggests that the ADIPORs might regulate cellular metabolism through activation of AMPK and/or PPARα [219–221]. As such, the ADIPORs are likely involved in several functions within the cell.

In conclusion, this study solidifies ADIPOR2 as one of the most crucial genes allowing cells to achieve membrane homeostasis in the presence of exogenous SFAs, while simultaneously revealing similarities between ADIPOR2- and SREBP-deficiency in terms of transcription and membrane composition. Uncertainty remains regarding whether ADIPOR2 acts upstream or in parallel to the SREBPs. Overexpression of SBP-1, the only *C. elegans* homolog to human SREBPs, alleviates *paqr-2* mutant phenotypes [206]. Thus, a relationship between ADIPOR2 and SREBPs is evolutionarily conserved throughout the 600 million years that separate nematodes and mammalian lineages; what a partnership!

**CONTRIBUTIONS**

For this work I contributed with the following:

- ADIPOR2 KO HEK293 generation: I performed the genetic inactivation of ADIPOR2 using CRISPR/Cas9, validated knockout efficiency, prepared samples for Sanger sequencing, and expanded the cells for all future experiments.
- Membrane rigidification by Laurdan: I performed all experiments in quantifying membrane packing order using Laurdan in the HEK293 cells.

- Fluorescence recovery after photobleaching (FRAP): I performed all FRAP experiments in the HEK293 cells.
- Protein detection by Western Blot: I performed all Western Blot experiments for the ADIPOR2 KO HEK293T cells.
- Bioenergetics: I performed all experiments assessing cellular respiration.
- Incucyte growth assay: I performed all growth assay experiments.
- RNA sequencing: I performed sample generation, RNA extraction, RNA validation, sequencing library preparation and quantification.
- Shotgun lipidomics: I generated and prepared all the samples sent for HEK293 lipidomics.
- Lipidomics: I performed sample generation, lipid extraction and lipid separation for the human cells and mouse adipose tissues.
- Data analysis: I processed and compiled all data for all experiments mentioned above excluding FRAP, RNA sequencing and lipidomics.
- Manuscript: I prepared graphs representing my contributions as well as proof-reading and commenting the manuscript.

## PAPER V: Elevation in adipocyte membrane phospholipid saturation does not compromise insulin sensitivity

### INTENTION

From paper IV we observed a clear link between ADIPOR2 and SFA-content in phospholipids in human cell lines and primary human cells, thus further establishing the translational link between PAQR-2 in *C. elegans* and the ADIPORs in human. We established that functional ADIPOR2 is required for cells to mitigate the lipotoxic effect of the exogenous SFA PA. Cells lacking ADIPOR2, when challenged with PA, showed increased SFA content in several phospholipid species, suffered impairments to oxygen consumption, showed massive mis-regulation of the transcriptome, culminating in cell shrinkage and detachment. In light of these results, a burning question was to investigate whether other cell types lacking ADIPOR2 would exhibit similar phenotypes. In order to assess this we turned to the cell type that is most likely to be robust to lipid challenges, namely the adipocyte.

Adipocytes play a central role in metabolic homeostasis, failure of which leads to disease [403]. Decreased insulin sensitivity, which can develop into type 2 diabetes, is one of the hallmarks of obesity in humans [404] and, by extension, of the obese adipocyte [403]. Dietary SFAs lead to decreased insulin sensitivity within hours after consumption [151]. Insulin signaling in adipocytes is initiated by the insulin receptor located on the plasma membrane, and is impacted by modulation of the plasma membrane lipid composition which influences insulin receptor accessibility, insulin binding and insulin action [156–159]. Given this, it was reasonable to hypothesize that an increase in membrane saturation could negatively affect adipocyte insulin signaling. Interestingly, adipocytes maintain the SFA content of their membranes within a narrow even in the insulin resistance phenotypes [405,406]. In contrast, the adipocyte UFA membrane composition, i.e. the identity of the UFAs present in phospholipids, readily changes in response to dietary interventions and genetic alterations of genes involved in lipid metabolism [91,407].

In the light of the literature mentioned and our previous results, we postulated that genetic ablation of *ADIPOR2* could potentially elevate SFA-containing phospholipids in human and mouse pre-adipocytes which would be retained after differentiation into adipocytes. As such we took the approach as to use the ADIPOR2 KO as a genetic model in which one could potentially study the effect of increased membrane rigidity in adipocytes, and how and if that would impact insulin signaling.

### RESULTS

#### *Human ADIPOR2 KO pre-adipocytes showed increased membrane rigidification*

In line with our previous work on ADIPOR2 [345], genetic ablation of the protein led to an increase in SFA-containing phospholipids in immortalized human pre-adipocytes. This increase was seen in the vehicle control state and was strongly elevated if the cells were treated with 200 µM of PA. The increase in phospholipid saturation was mainly seen in PCs, the most abundant phospholipid class in adipocytes [408], followed by PEs. Interestingly, the main phospholipid species driving this increase were PCs and PEs containing two PA moieties (16:0/16:0). Additionally, this specific species was also elevated in PIs and PGs, suggesting that one role of ADIPOR2 is to regulate the composition of saturated phospholipids. Alongside phospholipids, up-regulation of SFA-containing diacylglycerols (DAGs) was observed in the vehicle control, and not further impacted by PA. Direct impact on membrane fluidity, as quantified by the Laurdan dye method, showed increased membrane rigidity in ADIPOR2 KO pre-adipocytes when treated with ≥100 µM PA compared to control cells. Identical treatments also impaired basal respiration compared to controls, which is consistent with our previous observations in HEK cells [345]. Perturbations to membrane homeostasis is known to induce the activation of the endoplasmic reticulum unfolded protein response (ER-UPR) [409]. Most ER-UPR response genes were heavily up-regulated, compared to control cells, when treated with as low as 50 µM of PA. Wildtype cells also showed up-regulation of the ER-UPR but only at higher doses of PA (≥200 µM). These findings add to our previous results, in showing that the loss of ADIPOR2 leads to increased saturation of membrane phospholipids in human pre-adipocytes, which are also sensitive to PA-induced lipotoxicity.

***Differentiated human ADIPOR2 KO adipocytes retained increased phospholipid saturation but remained normal in terms of insulin response***

Having established the increase in membrane saturation in human pre-adipocytes, the cells were differentiated over 30 days to promote a white adipocyte-like phenotype. The ADIPOR2 KO cells differentiated as well as wildtype cells as measured by expression of lipogenic markers and development and accumulation of lipid droplets. When treating the adipocytes with PA, differences in lipogenic markers and elevation of the ER-UPR became apparent in the ADIPOR2 KO cells compared to control. Lipid remodeling genes, known to affect lipid composition, such as *SCD* and *ELOVL6* were down-regulated in the ADIPOR2 KO cells upon PA treatment. As seen in the pre-adipocytes, the ADIPOR2 KO adipocytes showed increases in SFA-containing phospholipids in normal culture conditions with further increases in the presence of 500 µM PA compared to control. The enrichment of SFA-containing phospholipids could, again, be attributed to 16:0/16:0-containing PCs and PEs. Up-regulation of SFA-containing DAGs was also observed in the ADIPOR2 KO adipocytes compared to control cells. To summarize, by disrupting the ADIPOR2 locus it was possible to obtain human adipocytes with increased levels of SFA-containing phospholipids, which could be further increased by treating the cells with PA.

We next wanted to investigate whether the increase in membrane saturation had an impact on adipocyte biology, with a specific focus on insulin signaling. To our surprise, the ADIPOR2 KO adipocytes retained normal insulin sensitivity as measured by insulin-induced phosphorylation of AKT. Furthermore, glucose uptake, which is one of the primary responses of adipocytes in the presence of insulin, was normal in the ADIPOR2 KO adipocytes, even in presence of 600 µM PA. The only observed difference was in terms of non-insulin mediated glucose uptake, which was increased in ADIPOR2 KO adipocytes when treated with 600 µM PA compared to controls, suggesting an increased energy demand. PA uptake was similar between genotypes, signifying that the increased saturation of phospholipids was not due to influx of PA. In terms of respiration, the ADIPOR2 KO adipocytes showed similar oxygen consumption as wildtype cells in the presence of PA, which was in contrast to the pre-adipocytes. In summary, these observations showed that insulin signaling remained normal in human ADIPOR2 KO adipocytes despite elevated levels of SFA-containing phospholipids.

***ADIPOR2 KO mouse adipocytes showed normal insulin response despite enrichment of SFAs in phospholipids***

Having established that ablation of *ADIPOR2* could be a suitable cell model system to study the effect of increased phospholipid saturation, it remained to be seen if this could be recapitulated *in vivo*. To address this question, we next turned to an ADIPOR2 KO mouse model previously characterized in

terms of whole-body metabolism [228]. First, we wanted to investigate whether the human adipocyte findings would translate to mouse adipocytes. Pre-adipocytes, from subcutaneous white adipose tissue (scWAT) of WT and ADIPOR2 KO mice, were isolated and treated with PA to assess membrane rigidification using the Laurdan dye method. As with the human pre-adipocytes, mouse ADIPOR2 KO pre-adipocytes showed increased membrane packing compared to WT when treated with PA. ER-UPR markers were also up-regulated in mouse ADIPOR2 KO pre-adipocytes in the presence of PA, compared to controls.

Knowing this, we proceeded to differentiate the pre-adipocytes, which showed similar degree of lipogenic markers expression and lipid droplet formation between genotypes. Next, we analyzed the lipidome of these cells. Again, we observed striking elevations of SFA-containing phospholipids in the ADIPOR2 KO adipocytes compared to controls. Once more, the species driving this increase were 16:0/16:0-containing PCs and PEs. These observations show a strong translationally conserved function of ADIPOR2, i.e. conserved between mouse and human, in controlling the saturation of phospholipids in adipocytes. Again as with the human adipocytes, insulin-induced AKT phosphorylation was unimpaired in the ADIPOR2 KO mouse adipocytes, and glucose uptake, both insulin-mediated and not, remained similar between genotypes irrespective of PA. No increases in ER-UPR could be measured in the ADIPOR2 KO mouse adipocytes. In essence, mouse ADIPOR2 KO adipocytes showed an excess of SFA-containing phospholipids, while retaining normal insulin signaling and glucose uptake despite the presence of exogenous PA.

***Adipose tissues from ADIPOR2 KO mice show elevated SFA-containing phospholipid levels and normal insulin signaling***

Having observed that the ADIPOR2 KO mouse adipocytes showed similar phenotypes as the human cells, we next studied the white adipose tissue (WAT) of ADIPOR2 KO mice. Previously described ADIPOR2 KO mouse phenotypes, such as an increased brain weight and decreased adiposity [228], were reproduced in our study. In line with the *in vitro* observations, increased SFA-containing phospholipid in both scWAT and epididymal adipose tissue (eWAT) were found in the ADIPOR2 KO mice compared to WT controls. Most notably, there was a two-fold increase in the abundance of saturated PCs in scWAT of the ADIPOR2 KO compared to WT. PE composition remained similar, in terms of total levels of SFAs, between genotypes but both 16:0/16:0 PE and PC were elevated in both fat depots of ADIPOR2 KO mice compared to WT. A total increase in DAGs was observed in scWAT of ADIPOR2 KO mice compared to WT with increases in SFA-, MUFA- and PUFA-containing DAGs. Also, the composition of SFA-containing triacylglycerols (TAGs), i.e. neutral lipids accumulated in lipid droplets and not membrane components, were similar between genotypes, indicating a specific role of ADIPOR2 in membrane homeostasis. In summary, and in line with the *in vitro* findings, lack of ADIPOR2 leads to increased amounts of SFA-containing phospholipids *in vivo*.

No differences in lipogenic, desaturation enzymes nor inflammatory markers could be detected by qPCR. Even ER-UPR markers were not up-regulated in the WAT of the ADIPOR2 KO mice, which contrasts with what was observed in human ADIPOR2 KO adipocytes. Taken together, the increase in SFA-containing phospholipids in ADIPOR2 KO mice does not seem to induce changes in lipid remodeling genes, nor to cause a stress response in WAT *in vivo*.

When investigating insulin signaling, no differences could be observed in the scWAT, eWAT or liver of both fasted male and female ADIPOR2 KO mice in terms of AKT phosphorylation in response to exogenous insulin. Other metabolic tissues, such as liver and muscle, also showed no differences in lipogenic, desaturation, inflammatory, and ER-UPR markers except for a slight up-regulation of *Acsl4* in muscle of ADIPOR2 KO mice compared to controls. Note also that BAT and liver were of normal weight in the ADIPOR2 KO mice. Quite remarkably then, the pronounced increase in SFA-containing phospholipids in ADIPOR2 KO cells is very consistent between human and mouse adipocytes as well as in mouse WAT, but does not perturb insulin signaling nor cause any obvious malfunction or stress responses in WAT and other tissues.

### *ADIPOR2 KO mice retain normal insulin sensitivity after a HFD*

In an attempt to further increase SFA-containing phospholipids in WAT, male ADIPOR2 KO mice were housed at thermoneutrality, minimizing SFA oxidation by BAT, and fed a SFA-rich diet (HFD) for four months. Despite normal bodyweights at the end of the study, the ADIPOR2 KO mice showed decreased scWAT and liver mass compared to controls. Importantly, increased amounts of SFA-containing phospholipids were again observed in the adipose tissues of ADIPOR2 KO mice compared to control. Once more, the increase was primarily driven by increased PCs, specifically 16:0/16:0 PC, and increases in 16:0/16:0 PE was also observed. Increased levels and saturation of DAGs was observed in scWAT of ADIPOR2 KO mice compared to controls. As with the chow-fed animals, TAGs composition was normal in the HFD-fed ADIPOR2 KO mice. Interestingly, the ADIPOR2 KO mouse defects in lipid composition and amounts were less pronounced on the HFD than on the normal chow diet. No differences in lipogenic, desaturation, inflammatory, and ER-UPR markers in scWAT, eWAT, liver, nor muscle were detected. Finally, a glucose tolerance test showed no differences in terms of glucose clearance or insulin levels between HFD-fed ADIPOR2 KO mice and WT. Taken together, and in line with the findings described above, we conclude that excess membrane phospholipid saturation in ADIPOR2 KO mice does not impair insulin function.

## DISCUSSION

In this body of work, *ADIPOR2* gene knockout models were used to increase SFA-containing phospholipids in the cellular membranes of adipocytes, both *in vitro* and *in vivo*, in order to investigate whether this impairs insulin signaling. Deleting *ADIPOR2* led to a striking two-fold elevation of SFA-containing PCs in human pre-adipocytes, human and mouse adipocytes and ADIPOR2 KO mouse WAT, revealing a consistent and conserved effect between human and mouse. Phospholipid SFA content, *in vitro*, could be increased three- to four-fold by treating ADIPOR2 KO adipocytes with PA. Remarkably, both human and mouse adipocytes, including mouse WAT from ADIPOR2 KOs, showed normal insulin responses despite elevation of SFA-containing phospholipids, indicating that increased saturation of phospholipids on its own does not impair insulin signaling in adipocytes.

However, the increased SFA-content of phospholipids is not without consequence for the cells. Both human and mouse ADIPOR2 KO pre-adipocytes exhibit increased rigidification and activation of the ER-UPR, likely resulting from perturbations in membrane homeostasis [410]. ER-UPR activation due to exogenous PA has been shown to lead to abnormal ER morphology and increased phospholipid saturation in yeast [125] and several mammalian cells [411–414]. Particularly, induction of XBP1, a major regulator of the ER-UPR, in PA treated mouse pre-adipocytes amplifies ER biogenesis and PC levels, principally PC 16:0/16:0 in the ER [415,416]. This is indeed what was observed in the human pre-adipocytes, where ADIPOR2 KO pre-adipocytes showed heightened responses. When quantifying membrane packing order directly the results are also quite striking. Both human and mouse ADIPOR2 KO pre-adipocytes show massive increases in general polarization (GP) index when treated with PA (Fig. 11). WT pre-adipocytes from human and mouse show no or minor increases in GP index when PA is present in the culture media while large swathes of crystal-like structures were apparent in the similarly treated ADIPOR2 KO cells. Judging from the images, increased rigidity was distributed throughout the cell cytoplasm rather than localized to one specific region such as the plasma membrane.

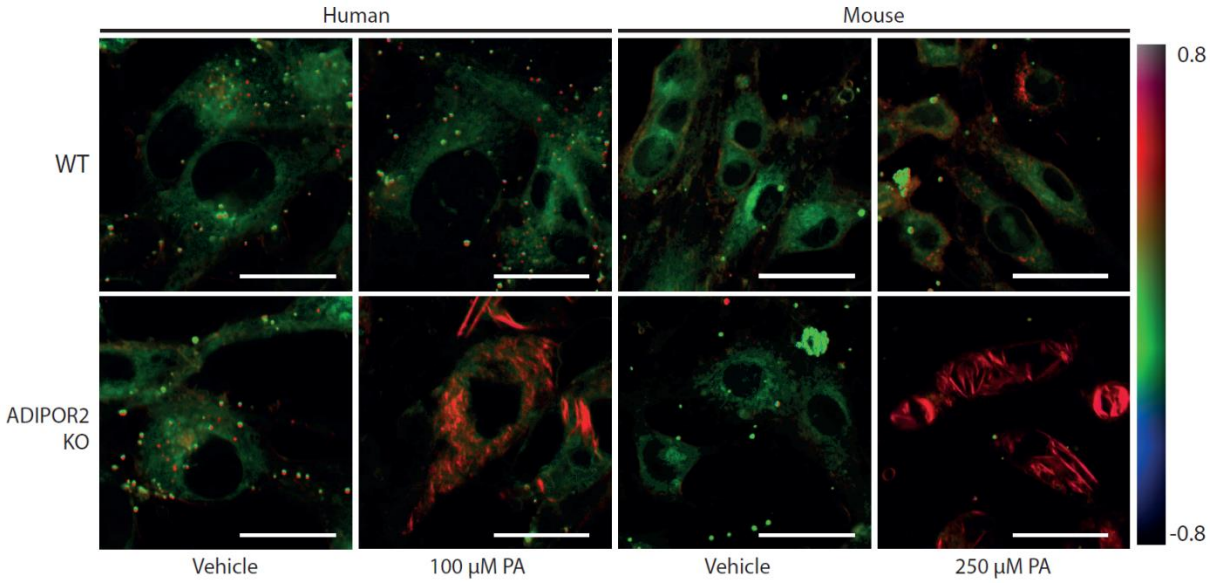


**Figure 11. Membrane packing order of human and mouse pre-adipocytes**
Representative pseudocolor images of Laurdan dye GP index for human and mouse WT and ADIPOR2 KO pre-adipocytes in full medium with either BSA (vehicle), 100 µM PA (human) or 250 µM PA (mouse) for 24 h. Red color signifies increased packing order (decreased fluidity), blue signifies decreased packing order (increased fluidity), while green represents normal packing order. Scale bars represents 40 µm.

Expanded ER, due to stress, can take unusual shapes, appearing crystalline in nature in several cell types [417–419]. Importantly, a limitation in this study was that lipidomics analyses were performed on whole cells and tissues, making it impossible to assign the subcellular location of the increased SFA-rich phospholipids. As such, a possibility exists that the SFA-containing phospholipids observed in the ADIPOR2 KO are localized primarily in the ER. Enlargement of ER size, due to membrane synthesis, alleviates ER-stress via UPR-mediated lipid biosynthesis in yeast [420] and is a coping mechanism to handle ER stress also in mammalian cells [421,422]. PCs followed by PEs are the main phospholipids in the ER [415,416] and an expansion of ER membrane would be accompanied by an increase in these species, consistent with our observations. To investigate this further, organelle fractionation of PA-treated ADIPOR2 KO pre-adipocytes could be performed to analyze organelle-specific lipidomes.

Human adipocytes are known to up-regulate the ER-UPR in response to exogenous PA [423] and ER-UPR activation in liver, muscle and adipose tissue is often associated with obesity and insulin resistance [424–426]. In our hands, cultured human ADIPOR2 KO adipocytes exhibited an activated ER-UPR accompanied by excess of SFA-containing phospholipids. However, when studying the ADIPOR2 KO mice, there was no difference in expression of lipid remodeling genes or in ER-UPR marker expression, despite the increased saturation in phospholipids, suggesting a milder response to insults to membrane homeostasis in mice. An explanation could be that there are pre-adipocytes left in the human adipocyte cell culture which gave rise to the ER-UPR activation. Other compensatory mechanisms, such as decreased plasmalogen content in phospholipids that is known to increase membrane fluidity [406], differential membrane protein concentrations or increased PUFA content in lipid species not measured in this work could help maintain membrane homeostasis and fluidity *in vivo*. In fact, merely increasing the concentration of PC headgroups, irrespective of fatty acid moieties, can restore membrane fluidity [406], which would counterbalance the rigidity caused by increased SFA-content in ADIPOR2 KO phospholipids.

In terms of neutral lipids, no differences in SFA-containing TAGs were observed between ADIPOR2 KO and WT mice. This could be expected since SFAs accumulate poorly into TAGs [427–429], but are readily incorporated into phospholipids and DAGs [410]. Accumulation of SFA-containing DAGs is a

known response to excess SFAs, leading to insulin sensitivity in human muscle cells [430,431]. Such increases in DAGs was observed for both human adipocytes and mouse scWAT, irrespective of diet. However, insulin signaling remained normal in ADIPOR2 KOs, suggesting that DAG saturation did not impair insulin sensitivity. As previously mentioned, due to the nature of the lipidomics analyses, subcellular location of the enriched SFAs observed in ADIPOR2 KO adipocytes and WAT cannot be specified. Therefore, a possibility remains that the plasma membrane, in which the insulin receptor is located, somehow maintains lipid composition or property homeostasis, with the excess of SFA-containing phospholipids perhaps being sequestered into other compartments such as the ER. As a last caveat, note that only a fraction of the cellular and tissue lipidome was quantified, leaving open the possibility that other non-detected lipid species could compensate for the increased SFAs in phospholipids and thus sustain physiologically desirable membrane properties.

Trying to further increase SFA-containing phospholipids in mouse adipose tissue with a HFD at thermoneutrality quite surprisingly led to the opposite outcome: the abundance of SFA-containing phospholipids was decreased in the ADIPOR2 KO mice fed a HFD rather than chow. One explanation could be that a low, but still significant, amount of mono- and poly-unsaturated fatty acids, which are known membrane fluidizers, in the HFD (8 % of energy, roughly ~ 0.45 kcal/g) could preferentially be incorporated into phospholipids. The strongest difference in phospholipid composition *in vivo* was observed in ADIPOR2 KO mice kept on chow, where unsaturated fat accounts for only ~3.6% of energy, which is clearly less than in the HFD. Note that diet-induced increases in SFA-containing phospholipids in rodents is usually extremely difficult to achieve, even on diets where SFAs account for 88% of the energy content [43]. The ADIPOR2 KO mouse used in this work is therefore a very promising model to study the effect of excess membrane SFAs *in vivo*.

In summary, we showed that ADIPOR2 deficiency in human and mouse pre-adipocyte and adipocytes as well as mouse WAT leads to an increased SFA content in membrane lipids, and that insulin signaling and glucose handling remain normal in adipocytes and mouse WAT in which there is an extensive increase in SFA-containing phospholipids.

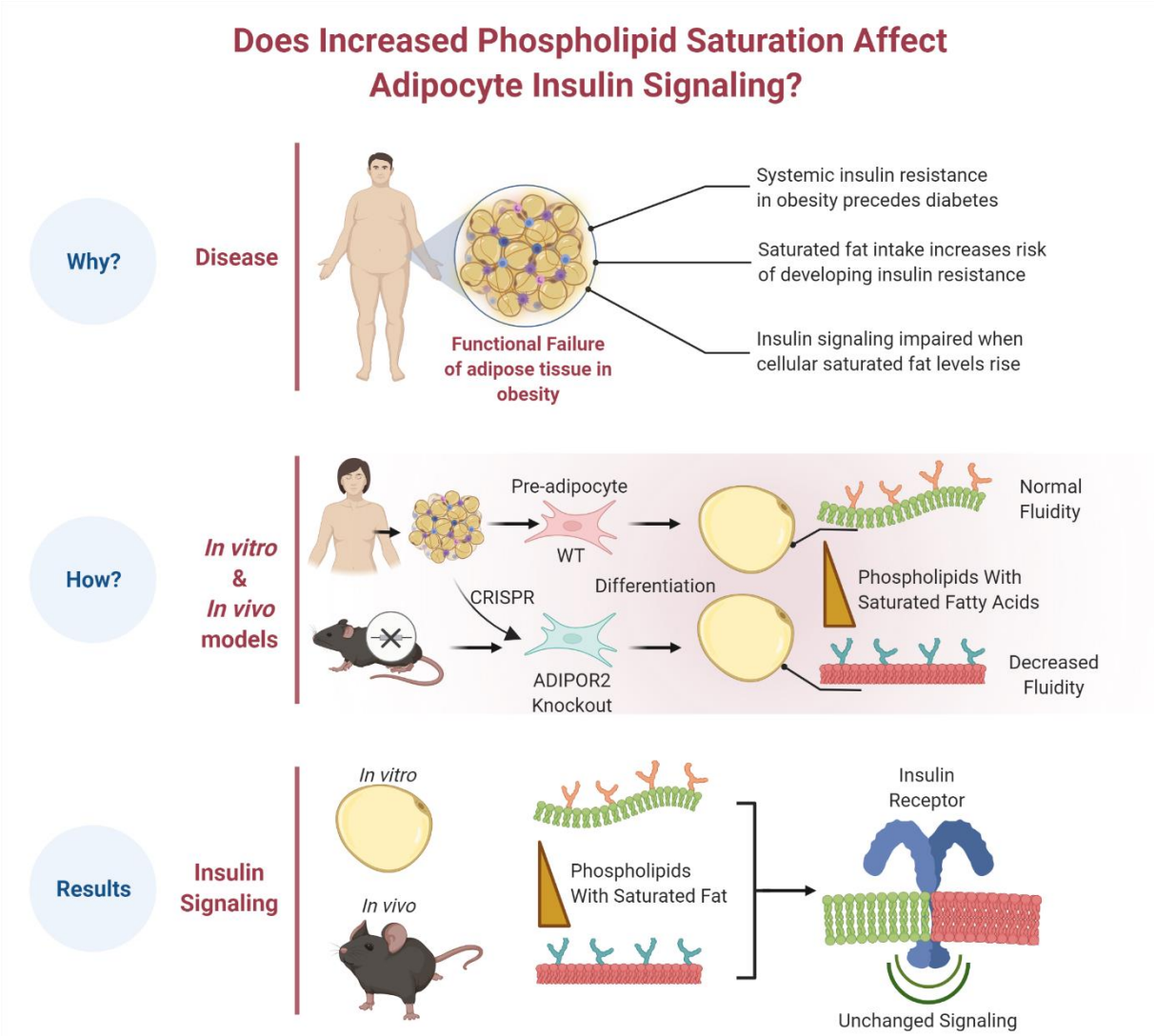


**Figure 12. Graphical abstract representing this work**

**CONTRIBUTIONS**

For this work I contributed with the following:

- Membrane rigidification by Laurdan: I performed all experiments in quantifying membrane packing order using the Laurdan dye.
- Fluorescence microscopy: I performed all fluorescence microscopy experiments assessing lipid accumulation.
- Bioenergetics: I performed all experiments assessing cellular respiration.
- Gene expression: I performed all gene expression analyses covering the human pre-adipocytes and adipocytes as well as all mice tissues.
- Lipidomics: I performed sample generation, lipid extraction and lipid separation for the human cells and mouse adipose tissues.
- Insulin signaling: I performed the insulin signaling experiments for the human adipocytes.
- Glucose uptake: I performed the glucose uptake experiments for the human adipocytes.
- PA uptake: I performed the PA uptake experiments for the human adipocytes.
- *In vivo* studies: I was responsible for all in vivo studies in terms of study plans, ethics, document preparation and reporting as well as performing genotyping, weekly weighing and cage changes.
- Insulin signaling in adipose and liver tissues *in vivo*: I weighed the mice, calculated insulin doses, helped with the termination and isolations of tissues.

- *In vivo g*lucose tolerance test on HFD: I weighed the mice, calculated glucose dose, took blood samples for glucose and insulin. I performed the insulin ELISA.
- *In vivo* study on HFD: I helped with the preparation, termination and weighing of tissues.
- Data analysis: I processed and compiled all data for this manuscript.
- Manuscript: I made all the figures and wrote the manuscript.

# FUTURE PERSPECTIVES

This body of work has continued the exploration into molecular mechanisms that govern lipid composition in cellular membranes, further cementing them as providing a core cellular feature, namely adjusting membrane lipid composition due to changes in influx of dietary fatty acids. This comes as no surprise since membrane homeostasis, the ability to intrinsically adjust membrane composition due to changes in the environment, has been a defining feature of the origin of the first cells [9–11]. Membrane homeostasis with respect to lipid composition is not a unique phenomenon throughout life as organisms can provide membrane homeostasis due to environmental changes such as temperature, pressure, pH [432–434]. All mammalian proteins studied in this work: ACSL1, ADIPOR2, TLCD1, and TLCD2, have homologs within *C. elegans* where they share similar functions, indicating strong evolutionarily conserved links across the 700 million years separating us.

## ACSL1

We have implicated mammalian ACSL1 and *C. elegans* ACS-13 as contributing to SFA-induced toxicity, at least in the context of ADIPOR2/PAQR-2 deficiency, i.e. when membrane homeostasis is impaired. ACSL1/ACS-13 natively promotes vulnerability to SFAs by shuttling long-chain PUFAs into mitochondria, making them unavailable for redirection into fluidizing membrane phospholipids. Further studies are required to elucidate the precise molecular mechanism by which ACSL1/ACS13 exerts its function. Silencing ACSL1 leads to an increase in long-chain PUFA-containing PCs and PEs, which counteracts the rigidification caused by exogenous SFAs. As such, ACSL1-silencing could be a potential therapeutic approach to maintain proper membrane composition in situations of increased rigidity. In terms of metabolic complications, the adipose tissue plays a major role in the pathophysiology of disease [404,425]. As ACSL1 accounts for 80 % of the ACSL activity in adipocytes [290], ablation of ACSL1 could potentially have a membrane fluidizing effect in scenarios of increased rigidity, e.g. as was observed in the ADIPOR2 KO models. Somewhat confusingly, increased saturation of membrane phospholipids in ADIPOR2 KO adipocytes showed unperturbed insulin signaling and thus might not after all be a relevant target for ACSL1-silencing. Clearly, more research is required to understand this better.

ACSL1 has recently been implicated in prostate cancer progression [435], where accumulation of end products of ACSL1 activity, namely long-chain fatty acid CoAs, was strongly correlated with tumor growth. Conversely, ACSL1-induced activation of complex long-chain PUFAs in the diet has been shown to suppress breast cancer growth in rodents by promoting a ferroptotic phenotype [436]. Membrane composition is clearly linked to cancer; cancer cells rely on a steady output from de novo lipogenesis to supply with new membranes required for rapid proliferation [177,178]. In addition, cancer cells display membrane composition-specific vulnerabilities since increased SFA-containing membrane lipids lead to exacerbated membrane rigidity [180], while excess PUFA-containing membrane lipid leads to lipid peroxidation and ferroptosis [184] with both scenarios leading to inhibited cancer growth. Together, this vulnerability opens up possible therapeutic avenues for cancer treatments. ACSL1 silencing might be one such venue, with an anticipated side effect being defects in mitochondria morphology, which seems to be well tolerated [359].

## TLCD1/TLCD2

This work has not only discovered and implicated FLD-1 in *C. elegans* and the mammalian TLCD1/2 in the regulation of phospholipid composition, but increased our understanding of their molecular mechanism of action, namely regulating MUFA incorporation at the sn-1 position of PEs. As of now, our observations suggest that TLCD1/2 act downstream of cellular MUFA uptake and upstream from the LPLAT enzymes. Further studies are warranted to discover whether the TLCDs act by CoA esterification onto MUFAs or if they govern intracellular lipid trafficking.

Similar to ACSL1, TLCD1 has been linked to cancer, namely hepatocellular carcinoma [394]. The native regulation of MUFA composition in PEs by TLCD1/2 is important since UFAs are sensitive to peroxidation, leading potentially to ferroptotic cell death [362,383]. Furthermore, we have implicated TLCD1/2 with NASH progression via pro-inflammatory eicosanoid signaling, i.e. promoting a favorable pro-inflammatory PE composition. Ablation of either or of both proteins simultaneously causes no obvious adverse side effects in rodents, suggesting that their modulation can be well tolerated. This opens up potential NASH treatment avenues by modulating these proteins, which would be immensely important to investigate since NASH patients have poor disease outcomes [376].

## ADIPOR2

More than a decade has passed since PAQR-2 in *C. elegans* was identified as a crucial protein that regulates membrane homeostasis [203]. Since then much has been discovered about this ancient protein and its mammalian homologs ADIPOR1 and ADIPOR2 [205–208,344]. This thesis has presented the most recent advancements in our understanding of ADIPOR2, namely that its presence is crucial for a proper cellular response to exogenous SFAs [345]. On a molecular level, ADIPOR2 is a key mediator in membrane phospholipid composition, especially PA-containing PCs and PEs. Lack of ADIPOR2 leads to dramatic increases specifically in 16:0/16:0 PCs and PEs. As such, ADIPOR2 modification can be used as a genetic model system with increased membrane phospholipid saturation, which is notoriously difficult to achieve through dietary interventions [43,405,406]. To our surprise, massive elevations of SFA-containing membrane phospholipids did not affect insulin signaling *in vitro* nor *in vivo*, highlighting the plasticity of the adipocytes to massive challenges to lipid metabolism.

Adipocytes and adipose tissues were the main focus for paper V, being the main cell type in handling lipids. However, it would be very interesting to study the other metabolic tissues more closely, namely liver and muscle. SFA-induced elevation of DAGs is a known response to mitigate the lipotoxicity of SFAs, and in muscle this leads to decreased insulin sensitivity in muscle cells [430,431]. This was also observed in our mouse models irrespective of diet. A closer look into muscle insulin signaling could reveal whether impairments due to the loss of ADIPOR2 were present. In addition, it would be interesting to study the testis and brain given that these organs do show defects in the ADIPOR2 KO mice. Work is indeed in progress regarding this. A preliminary conclusion is that while ADIPOR1 and ADIPOR2 likely act redundantly in most tissues, they have individual specific functions in some tissues: ADIPOR1 is essential in the eye [330], while ADIPOR2 is essential in testis and brain [228]. Indeed, it would be interesting to study to what degree ADIPOR1 compensates functionally in most cells/tissue of ADIPOR2 KO mice.

Despite our results pointing to the resistance of adipocytes to SFA-induced membrane insults in terms of insulin signaling, the total body of evidence is clear and points to SFAs being detrimental for human health [437]. Type 2 diabetics show elevation in SFA-containing membrane phospholipids [153] correlating with circulating red blood cells being enriched in SFA-containing phospholipids, potentially serving as a diagnostic tool for diabetes progression [163,164,303]. Therefore, it would be interesting to study ADIPOR2-induced changes in other cell types known to be vulnerable to SFAs. In particular, Type 2 diabetes is characterized by loss of pancreatic β-cells responsible for insulin synthesis and these cells are notoriously sensitive to SFA-induced lipotoxicity [438]. In β-cells, pre-proinsulin translocate to the ER lumen for native folding into insulin [439] which, in scenarios of increased protein demand, relies on the ER-UPR for proper homeostasis, reducing ER stress, ensuring proper insulin production in β-cells [440]. SFAs induce the ER-UPR in β-cells [411], with PA shown to be the most potent fatty acid

in activating the ER-UPR in β-cells [441]. Additionally, ADIPOR2 KO mice fed a HFD for long periods of time (20 weeks), showed increased fasting glucose levels, correlated with dysfunctional β-cell replication and neogenesis [302]. Given this context, it would be interesting to study the effect of ADIPOR2-induced ER-UPR stress on β-cell functionality and diabetes progression.

Further studies are needed to elucidate the precise molecular mechanism as to how ADIPOR2 asserts its membrane homeostatic effects. On that note, the results presented in this thesis strengthen our confidence in believing that the primary function of ADIPOR2 is to regulate membrane homeostasis as opposed to acting as an adiponectin receptor. More studies are naturally required to further cement this hypothesis.

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All figures in this thesis were created using Bio Render.

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