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SOFIA WOO-HEE DE MARÉ
FACULTY OF MEDICINE | LUND UNIVERSITY

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Sofia Woo-hee de Maré

LUND
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Faculty opponent
Dr. Joan Cerdà

Institut de Recerca i Tecnologia Agroalimentàries, Spain

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MADE IN SWEDEN

The scoopers become the scoopees

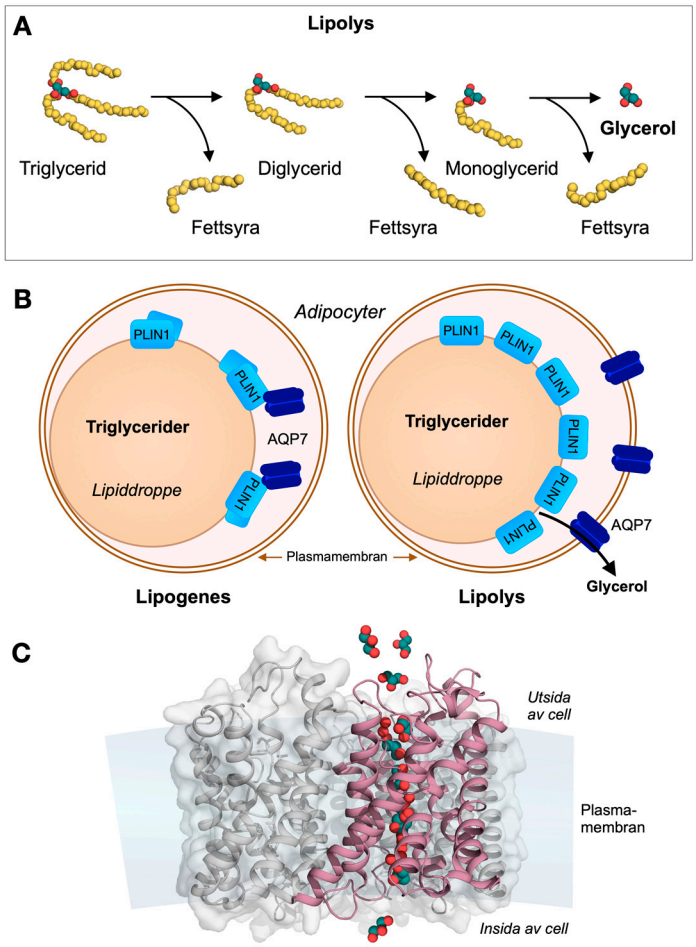
Table of Contents

Populärvetenskaplig sammanfattning

Forskningen i denna avhandling har dedikerats till att i detalj studera hur glycerolnivåer regleras i fettceller, så kallade adipocyter. Omsättningen av glycerol, eller glycerolmetabolism, har stor betydelse för förmågan att upprätthålla energibalansen i kroppen mellan måltider och vid träning. Adipocyter är en typ av celler som är specialiserade att lagra fett, och majoriteten av cellens volym tas upp av en enda stor lipiddroppe av triglycerider. Triglycerider är energirika fettmolekyler som består av glycerol och fettsyror, och som bildas av överskottsenergi från måltider. Lipiddroppen är täckt av ett membran samt olika proteiner som är viktiga för generering (lipogenes) och nedbrytning (lipolys) av triglyceriderna, och själva adipocyten är omsluten av ett plasmamembran. När energibehovet i kroppen ökar utsöndras specifika hormoner, katekolaminer, vilket leder till att lipolysen aktiveras och triglyceriderna bryts ned till glycerol och fettsyror som därefter lämnar cellen för vidare nedbrytning och energiutvinning. Glycerol diffunderar ut från adipocyten genom en glycerolkanal som sitter i plasmamembranet, och transporteras vidare till levern där den kan omvandlas till sockermolekylen glukos. Det finns olika typer av glycerolkanaler som är nära besläktade, och den vanligaste i adipocyter är aquaporin 7 (AQP7). Glycerolmetabolism är också en bidragande faktor i utvecklingen av metabola sjukdomar, och möss som saknar AQP7 utvecklar fetma och typ 2-diabetes. Dessutom ökar lipolysaktiviteten i adipocyter i samband med vissa typer av cancer för att kunna försörja tumörer med energi för snabb tillväxt. De underliggande mekanismerna är ännu inte helt klarlagda, och syftet med detta arbete har varit att i detalj undersöka både hur glycerol frigörs i lipolysen och hur glycerol lämnar cellen genom APQ7.

Lipolysen aktiveras då katekolaminer binder till specifika receptorer på ytan av adipocyten, vilket triggar en kaskad av reaktioner inuti cellen och leder till nedbrytning av triglycerider. Ett vanligt förekommande protein på lipiddroppen är perilipin 1 (PLIN1), som har en viktig roll i regleringen av lipolysen genom att binda proteiner som bryter ned triglyceriderna. Vi har visat att PLIN1 har ett dynamiskt beteende på lipiddroppen vilket styrs av samspelet mellan PLIN1 och specifika komponenter på lipiddroppen. PLIN1 existerar som isolerade domäner under lipogena förhållanden, och dessa domäner upphör efter katekolaminstimulering, vilket utgör en förutsättning för att lipolysen ska aktiveras fullt ut. Vi har även visat att PLIN1 reglerar glycerolnivåer genom att specifikt binda till glycerolkanalen AQP7. Katekolaminstimulering resulterar i en

modifikation av AQP7 vilket försvagar interaktionen med PLIN1. AQP7 kan då förflyttas från PLIN på lipiddroppen ut till plasmamembranet och därmed släppa ut glycerol från cellen.



Figur 1. **A.** I lipolysen bryts triglycerider stegvis ned till diglycerider, monoglycerider och slutligen glycerol, och frigör en fettsyra i varje steg. Reaktionen är i princip omvänd i lipogenesen. **B**. Adipocyter är specialiserade fettceller som till stor del består av en lipiddroppe som innehåller triglycerider. Perilipin 1 (PLIN1) är ett protein som sitter på ytan av lipiddroppen och reglerar glycerolnivåerna i adipocyten. PLIN1 bildar isolerade domäner under lipogenesen, som sedan upplöses under lipolysen. Detta är en förutsättning för att lipolysen ska aktiveras fullt ut. Under lipogenesen binder aquaporin 7 (AQP7) till PLIN1 och är därmed inaktiv. Interaktionen försvagas under lipolysen och AQP7 förflyttas till plasmamembranet och kan släppa ut glycerol som bildas i lipolysen. **C**. Modell av den tredimensionella strukturen av AQP7 som bestämdes som en del av denna avhandling. Fyra identiska molekyler av AQP7 bildar det kompletta proteinet i plasmamembranet. En av dessa molekyler är representerad i rosa, och de övriga tre i grått.

Det är känt att AQP7 är en proteinkanal som tillåter glycerol och vatten passera över plasmamembranet genom passiv diffusion. Detaljerna om hur AQP7 är permeabel för vissa molekyler men exkluderar andra, och hur glycerol förflyttas

genom kanalen var däremot tidigare okänt, eftersom den molekylära tredimensionella strukturen av AQP7 inte funnits tillgänglig. Proteiner är uppbyggda av långa kedjor av aminosyror och funktionen av ett protein beror på den tredimensionella veckningen av aminosyrakedjan. Det är inte möjligt att i detalj förutspå strukturen av ett protein endast med hjälp av aminosyrasekvensen och strukturen måste bestämmas experimentellt. Här har vi med hjälp av metoden röntgenkristallografi bestämt en högupplöst struktur av AQP7 med glycerol- och vattenmolekyler närvarande i poren. Med hjälp av den strukturella information visade vi att glycerolmolekylen delvis roterar genom AQP7 vilket underlättar förflyttningen mellan energetisk fördelaktiga positioner. AQP7 har hög affinitet för glycerol, och närvaro av glycerol hindrar vatten från att effektivt diffundera genom AQP7.

Lipogenes och lipolys i adipocyter är essentiella för vår förmåga att anpassa oss efter omväxlande energitillgång och behov, men har även implikationer i sjukdomstillstånd. Forskningen i denna avhandling har lett till en djupare förståelse om hur glycerolnivåer är reglerade av PLIN1, både i lipolysen och via reglering av AQP7. Strukturen av AQP7 har gett oss insikt i hur glycerolen diffunderar genom kanalen, och utgör grunden för framtida utveckling av läkemedel som är specifikt riktade mot AQP7.

Abstract

Glycerol levels in adipocytes depend on the lipolysis, the hydrolysis of triglycerides into glycerol and free fatty acids, and the efflux of glycerol across the plasma membrane through glycerol channels. The aims of this work were to investigate how glycerol levels are regulated on a molecular level by PLIN1, a major lipid droplet-associated protein and a key regulator of the lipolysis by scaffolding for lipolytic proteins on the lipid droplet. Furthermore to elucidate the selectivity and conducting mechanisms of aquaporin 7 (AQP7), the major glycerol channel in adipocytes.

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List of publications

Paper I

Hansen, J. S., De Maré, S., Jones, H. A., Göransson, O., & Lindkvist-Petersson, K. (2017). **Visualization of lipid directed dynamics of perilipin 1 in human primary adipocytes.** *Scientific reports*, *7*(1), 15011.

Paper II

Hansen, J. S., Krintel, C., Hernebring, M., Haataja, T. J., de Marè, S., Wasserstrom, S., ... & Jones, H. A. (2016). **Perilipin 1 binds to aquaporin 7 in human adipocytes and controls its mobility via protein kinase A mediated phosphorylation.** *Metabolism*, *65*(12), 1731-1742.

Paper III

de Maré, S. W., Venskutonytė, R., Eltschkner, S., de Groot, B. L., & Lindkvist-Petersson, K. (2019). **Structural Basis for Glycerol Efflux and Selectivity of Human Aquaporin 7**. *Structure,* https:// doi.org/10.1016/j.str.2019.11.011.

Paper IV

de Maré, S. W., Fisher, Z., Gourdon, P., Lindkvist-Petersson, K. **Crystallization of human aquaglyceroporin 7 for neutron diffraction studies.** *Manuscript.*

Abbreviations

ATGL	Adipose triglyceride lipase
AQP	Aquaporin
CE	Cholesteryl ester
DHAP	Dihydroxyacetone phosphate
EM	Electron microscopy
G3P	Glycerol-3-phosphate
HSL	Hormone sensitive lipase
KO	Knock-out (mice)
MD	Molecular dynamics (simulations)
MGL	Monoacylglycerol lipase
NMX	Neutron macromolecular crystallography
PKA	Protein kinase A
PLA	Proximity ligation assay
PLIN1	Human perilipin 1 protein
TG	Triglyceride
Å	Ångström, $1 \cdot 10^{-10}$ m

Introduction

Maintaining the energy homeostasis with fluctuating energy supplies and demands is solved by lipogenesis and lipolysis. Lipogenesis and lipolysis are two metabolic processes where surplus energy from food is converted into lipid molecules and then broken down in states of negative energy balance. The lipid molecules, triglycerides, are stored in the adipose tissue in adipocytes, cells that are specialized in storage and mobilization of triglycerides. Regulation of these processes is tightly controlled by several mechanisms and at different levels.

Triglycerides consist of glycerol and fatty acids. Glycerol levels within cells are regulated by the rate of lipolysis, as well as the export of glycerol from the adipocytes through glycerol channels to other tissues for further catabolism. Impaired regulation of glycerol levels has consequences related to metabolic disorders and tumor growth that requires energy for rapid growth.

The aims of this work have been to obtain detailed knowledge of how glycerol levels are regulated in adipocytes, both through activation of the lipolysis by protein-lipid interactions via perilipin 1 (PLIN1), and by glycerol efflux through the glycerol channel aquaporin 7 (AQP7). More specifically:

- What is the role of PLIN1-lipid interactions in the activation of lipolysis?
- What is the role of PLIN1 in the regulation of glycerol efflux mediated by AQP7 in adipocytes?
- Where in the adipocyte is AQP7 localized upon insulin and catecholamine stimulation?
- What is the molecular selectivity mechanism of AQP7?

My thesis work has been described in three publications and one manuscript to be published. In Paper I, a newly discovered dynamic localization pattern of PLIN1 was further investigated on the basis of PLIN1 and lipid droplet interactions, and the implications in regulation of the lipolysis. In Paper II, PLIN1 was identified as a binding partner to AQP7 in adipocytes, and the AQP7-PLIN1 interaction was investigated in terms of AQP7 translocation and glycerol regulation. The high-resolution structure of AQP7 was determined by x-ray crystallography in Paper III, and the selectivity and conductance mechanisms of AQP7 were analyzed. In Paper IV, we aimed to study AQP7 by neutron diffraction, in order to obtain structural details of hydrogen atoms for deeper analysis of the molecular mechanisms of AQP7.

Lipogenesis and lipolysis in the adipose tissue

The adipose tissue consists mainly of specialized fat cells, adipocytes, that are supported by other cell types such as endothelial cells and fibroblasts (1). Adipocytes are the main storage compartments of energy in the body, where fat is stored as triglycerides. Triglycerides consist of three fatty acid chains bound to a glycerol molecule and are generated in during lipogenesis in the absorptive state in the human body. During fasting conditions or exercise, lipolysis is activated and the triglycerides are broken down into glycerol and free fatty acids. The glycerol is released from the adipocyte through aquaglyceroporins, and free fatty acids by diffusion over the plasma membrane, possibly facilitated by fatty acid binding proteins (2, 3). The glycerol can be used as a precursor in the gluconeogenesis or glycolysis in the liver and free fatty acids are further catabolized in the β-oxidation in mitochondria (Figure 4). Understanding adipose biology has become very relevant as obesity and obesity-associated medical conditions have become a large-scale public health issue (1). In addition, adipose lipolysis has been shown to increase during cancer tumor development (4).

Adipose tissue

There are three types of adipose tissue: white, brown, and beige. The main function of white adipose tissue is to store energy as triglycerides, whereas brown adipose tissue generates heat and beige adipocytes have highly inducible heat generation capacity. White adipose tissue is the most abundant kind, with major subcutaneous depots, under the skin, and visceral, between the organs in the abdominal cavity (5-7). White adipose tissue is not just a passive storage unit of triglycerides, as previously considered, but rather has an orchestrating role in endocrine signaling for whole-body metabolism, by secreting a wide range of signaling and mediator proteins termed adipokines, including factors secreted by other glands as well as adipocyte-specific ones such as leptin (8)

The adipocyte

Adipocytes have evolved to efficiently store energy surplus from our food intake and to mobilize it at negative energy balance (9). A single, large lipid droplet occupies the majority of the human adipocyte cell volume and the remaining constituents of the cytoplasm are restricted to the perimeter of the cell (Figure 2). The flexibility of the adipocyte is demonstrated by its ability to fluctuate in overall size depending on the lipid content (10).

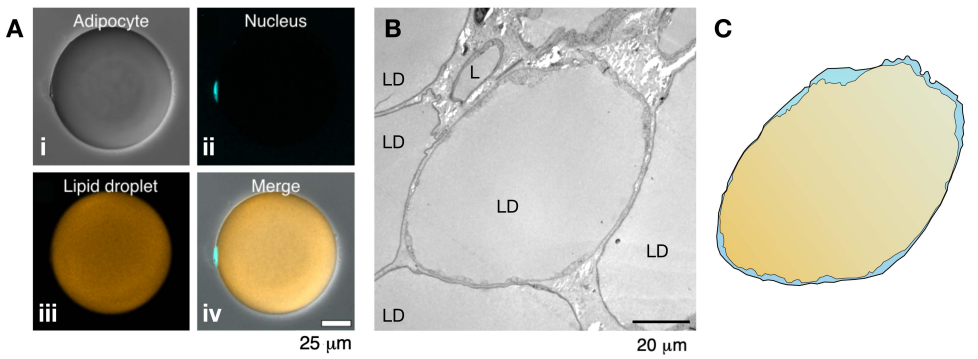

Figure 2. Morphology of human subcutaneous adipocytes. A. Brightfield and fluoresence micrographs of isolated adipocyte. i) Brightfield image, ii) DAPI nucleus stain, iii) Nile Red lipophilic dye, iv) merged microgrpahs. Supplementary figure 1, Paper I. **B**. Transmission electron micrsocopy micrograph of adipose tissue negatively stained with uranyl acetate. LD - lipid droplet, L - lumen of blood vessel. Image credit Peng Huang. **C**. Trace of adipocyte in B, showing lipid drolet in yellow and cytoplams in blue.

The lipid droplet

Lipid droplets are dynamic organelles found in most mammalian cell types, however adipocytes are specialized for storing lipids in the form of a unilocular droplet occupying up to 90 % of the entire cell volume (11). The lipid droplet is a pool of neutral lipids, primarily triglycerides and cholesteryl esters surrounded by a monolayer of phospholipids (12)

A wide range of proteins has been associated with the lipid droplet monolayer. Out of all identified proteins, 100-150 are considered to make up the lipid droplet proteome in mammalian cells (13). The proteome mainly contains enzymes and proteins required for the lipid metabolism, such as perilipin proteins. Some proteins are involved in membrane trafficking and protein degradation (13, 14) as well as proteins controlling the surface properties of the lipid droplet (15). The proteins are divided into two classes depending on the manner of recruitment to, and interaction with the monolayer (16). The lipid droplet is formed from the endoplasmic reticulum (ER) and class I proteins are initially inserted in the ER membrane and

diffuse to the nascent lipid droplet before it buds off from the ER. Transition from ER to the lipid droplet can also occur by lipid droplet-ER interactions after complete formation of the lipid droplet (16). Class I proteins are typically inserted in the membrane by two α-helical domains arranged in a hairpin motif with both terminal ends in the cytoplasm. In contrast, class II proteins are recruited to the lipid droplet directly from the cytoplasm. The hairpin interaction mode is also found among class II proteins, although class II proteins generally have amphipathic helices interacting with the lipid droplet, as in the case for perilipin proteins (13).

Perilipin 1

Perilipins are lipid droplet-associated proteins with five isoforms (PLIN1-5) in mammals. Perilipin 1 (PLIN1) has tissue-enriched expression, localizing primarily to adipose tissue, where it exclusively associates with the lipid droplet (17). Other PLINs have wider tissue distribution and can also localize to the cytoplasm (18). PLIN1 has been studied extensively since its role in lipid metabolism was recognized (19).

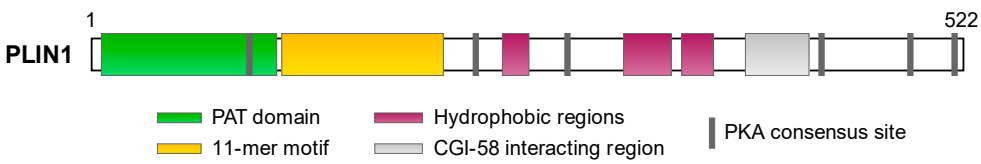

Figure 3. Schematic figure of the PLIN1 amino acid sequence. PLIN1 consists of 522 amino acid containing a conserved PAT domain (green) and 11-mer motif (yellow) of approximately 100 amino acids. Three hydrophobic regions (dark red) are present, as well as several PKA consensus sites (gray lines). Adapted from (18, 20, 21).

The detailed mechanisms how PLIN1 target and enrich on the lipid droplet are still unclear (13). PLIN1 has proven to be difficult to purify, possibly due to its amphipathic nature and the fact that PLIN1 is rapidly degraded when not associated with the monolayer (18). Consequently, biochemical assays with PLIN1 become challenging and the structure of PLIN1 is unknown. Nonetheless, sequence alignment and structure predictions indicate that the perilipin proteins share two conserved N-terminal motifs, the PAT domain[1] involved in binding HSL upon PKA phosphorylation events (22) and 11-mer repeats, varying in length depending on the isoform, forming an amphipathic helical motif (23) (Figure 3). The 11-mer motif is also found in other proteins associated with membranes, for example synuclein, a Parkinson's disease related protein, where the 11-mer helix

[1] Perilipin 1, 2 and 3 were named perilipin A, ADRP (adipose differentiation-related protein) and TIP47 (tail-interacting protein of 47 kDa), respectively, at the time of their discoveries, all of them containing a conserved domain which was named PAT after the three proteins (26).

interacts with phospholipids (24). PLIN1 has three additional hydrophobic sequences involved in lipid droplet anchoring (25) and it has been hypothesized that the amphipathic helix is responsible for initial targeting the monolayer (23).

Lipogenesis

Lipogenesis is the process when triglycerides are generated upon excessive caloric intake. Triglycerides are reduced and mostly anhydrous, and thereby contain large amounts of energy that are used to maintain energy homeostasis. The fatty acids originate either from the ingested fat or *de novo* synthesis from carbohydrates (27). The glycerol in adipocytes originates from glucose via glycolysis or from non-carbohydrate substrates via glyceroneogenesis (28).

The majority of the ingested fat is in the form of triglycerides, however storage of excess amount of fat in adipocytes requires processing in order to be transported from the gastrointestinal tract to the adipose tissue. In the small intestine, lipases hydrolyze the triglycerides into monoglyceride and two free fatty acids and the components diffuse to intestinal epithelial cells where they are reesterified into triglycerides. The triglycerides are transported as chylomicrons, droplets of triglycerides and other components such as apolipoproteins, in lymphatic capillaries to adipose tissue (29). Lipoprotein lipase on adipose tissue blood capillaries release fatty acids once again, which enter the adipocytes mediated by fatty acid transporters (Figure 4) (30, 31).

In contrast, *de novo* fatty acid synthesis involves conversion of glucose digested from carbohydrates into citrate in glycolysis and citric acid cycle. The citrate is converted to acetyl Co-A, malonyl Co-A, palmitate and finally fatty acids in a series of enzymatic reactions (27, 32). *De novo* fatty acid synthesis mainly takes place in the cytoplasm of the liver and adipocytes (33). Fatty acids synthesized in the liver are esterified with glycerol, and the triglycerides are transported to the adipose by the blood as very-low-density lipoproteins, VLDLs. VLDLs reach adipose tissue capillaries where the triglycerides are hydrolyzed, and fatty acids enter the adipocytes (Figure 4).

The glycerol backbone of the triglyceride in adipocytes derives mainly from dihydroxyacetone phosphate (DHAP), an intermediate in both the glycolysis and glyceroneogenesis. DHAP is reduced by glycerol-3-phosphate dehydrogenase resulting in glycerol 3-phosphate (G3P) (28) (34). Glycerol kinase phosphorylates glycerol into G3P, however the activity of glycerol kinase in white adipocytes is low under basal, unstimulated conditions (35). Stepwise esterification of G3P with free fatty acids in the form of fatty acyl-CoA, as well as a hydrolysis step by diacylglycerol diphosphate phosphatase, results in the triglyceride (Figure 4) (32).

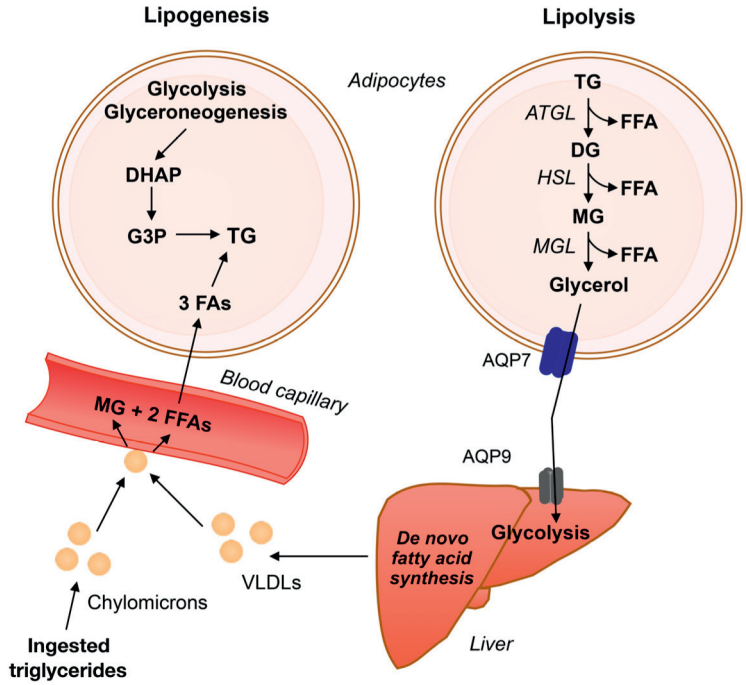

Figure 4. Overview of lipogenesis and lipolysis in adipocytes. Triglycerides (TG) are generated during lipolysis. The glycerol backbone is mainly derived in glycolysis and glyceroneogenesis. The fatty acids are either ingested or synthesized in the liver, and transported to the adipocyte as TG in chylomicrons and very-low-density lipoproteins (VLDLs), respectively. TG are hydrolyzed in adipose tissue capillaries before entering adipocytes. TG are hydrolyzed in lipogenesis mainly adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL). Glycerol exits the adipocyte through AQP7, and can enter the liver through AQP9 to be used in the glycolysis.

Lipolysis

Lipolysis is the process when triglycerides are hydrolyzed into glycerol and free fatty acids. The lipolytic machinery consists of multiple components including adrenergic receptors, kinases, lipases and other proteins such as perilipin proteins, and in particular PLIN1. Three lipases are mainly involved in triglyceride hydrolysis; adipose triglyceride lipase (ATGL) has high affinity to triglycerides and generates diglycerides which are further hydrolyzes by hormone sensitive lipase (HSL) into monoglycerides. In the final step, monoacylglycerol lipase (MGL) cleaves off the last remaining fatty acid freeing the glycerol molecule (Figure 4) (36). Hormonal signaling tightly controls the lipolysis; the catecholamines norepinephrine and epinephrine stimulate, and insulin suppresses (32). Several

factors influence the lipolysis rate such as age, gender, physical activity in addition to endocrine, paracrine and autrocrine signaling (Fruhbeck at al 2014).

The lipolytic activation cascade is primarily initiated upon binding of catecholamines to β-adrenergic receptors (β-ARs) (37). β-ARs are G protein-coupled receptors (GPCRs) with stimulatory $G_s\alpha$ subunits, and catecholamine binding leads to activation of adenylyl cyclase by interaction with the $G_s\alpha$ subunit. Adenylyl cyclase uses ATP to generate cyclic adenosine monophosphate (cAMP), and the increased intracellular cAMP levels in turn activate protein kinase A (PKA). PKA phosphorylates PLIN1 and HSL, which leads to activation of ATGL and translocation of HSL from the cytoplasm to PLIN1 on the lipid droplet, giving HSL direct access to cholesteryl esters and the newly generated diglycerides. The lipolysis can be activated through other pathways, including hormones acting through other GPCRs, growth hormone and natriuretic peptides (38).

Regulation of lipolysis by perilipin 1 and PKA phosphorylation

PLIN1 has an important role in both cellular and whole-body energy homeostasis by controlling triglyceride accumulation and hydrolysis (39). The importance of PLIN1 has been observed in PLIN1 loss-of-function mutations in humans. Lacking functional PLIN1 results in dysregulated lipolysis and insulin-resistant diabetes, as well as severe hyperlipidemia, i.e. elevated amounts of lipids in the blood. The ability to store triglycerides is lost and basal lipolysis is increased. Adipose tissue samples from these patients display macrophage infiltration and fibrosis, typical features of patients with obesity and insulin resistance (40, 41). The implications of PLIN1 have also been studied in mouse models. PLIN1-KO mice are lean, resistant to diet-induced obesity with elevated basal lipolysis (42, 43).

PLIN1 is localized to the lipid droplet monolayer where it displays a dynamic, hormonally dependent segregation pattern. Under lipolytic conditions, PLIN1 micro domains are formed, while under lipogenic conditions these micro domains disperse (**Paper I**). In order to gain a deeper understanding of how PLIN1 binding properties to lipids regulate the dynamics of the lipolysis, the interactions between PLIN1 and specific lipids were investigated in **Paper I**.

Perilipin 1 interacts specifically with cholesteryl esters (Paper I)

PLIN1 was heterologously overexpressed in *E. coli* BL21 (DE3) cells and the lysate containing PLIN1 was incubated with a range of adipocyte-specific lipids spotted onto a nitrocellulose membrane in a protein lipid overlay assay (44). Any PLIN1-lipid interactions were detected using a primary α-PLIN1 antibody and secondary fluorescent antibody (Figure 5A).

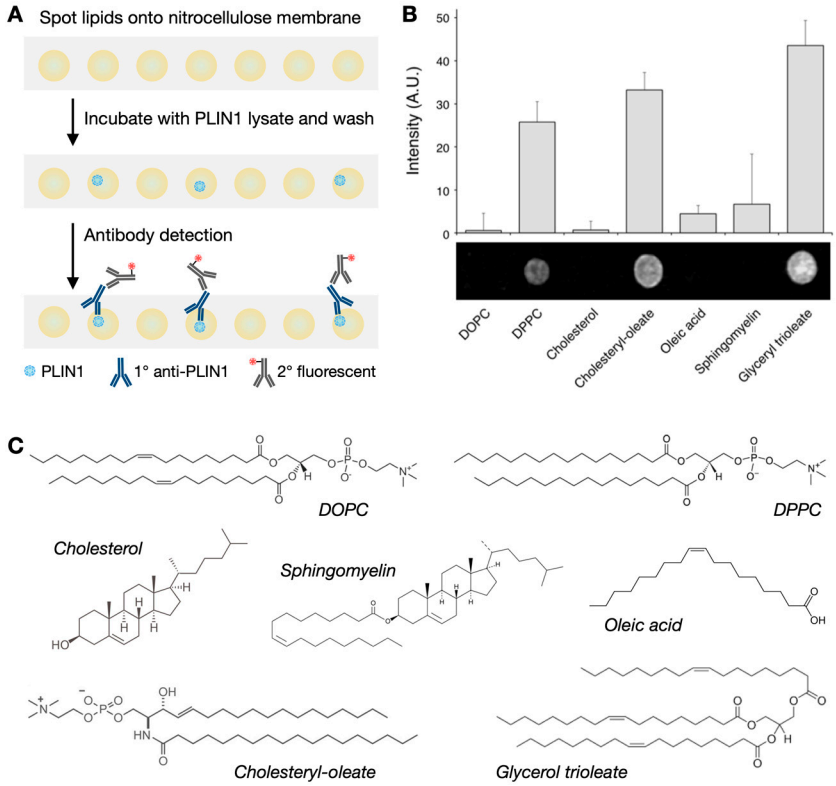

Figure 5. Detection of PLIN1 binding to lipids by a protein lipid overlay assay. A. Schematic figure of the overlay assay. A variety of lipids were spotted onto a nitrocellulose membrane and incubated with PLIN1 lysate. PLIN1 interactions to specific lipids were detected by a primary anti-PLIN1 and secondary **B**. PLIN1 interacts specifically with DPPC, cholesteryl-oleate and glycerol trioleate, but not with DOPC, cholesterol, oleic acis or sphingomyelin. Figure 4, Paper I. **C**. Lipids tested in the protein lipid overlay assay.

PLIN1 has a highly specific lipid-binding profile, interacting with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), the cholesteryl esters (CE) cholesteryl-oleate and triglycerides (glyceryl trioleate). In contrast, no interaction was detected for DOPC, cholesterol, oleic acid or sphingomyelin (Figure 5B-C).

Cholesteryl esters play an important role in the dynamic phase behavior of the lipid droplet monolayer and PLIN1 micro domains, and are hydrolyzed by HSL (45). In a lipid mixture, CE-enriched domains are formed at higher CE concentrations, while at lower concentrations, CE the form micro domains disperse (Paper I, Figure 5). During the basal or insulin-stimulated state, the CE concentration is relatively high, and PLIN1 micro domains are present. PLIN1 suppresses lipolysis by sequestering coactivator comparative gene identification-58, CGI-58, an activator of ATGL (46). During the lipolytic state, PKA-phosphorylated HSL translocates from the cytoplasm to the lipid droplet, binding

to PLIN1. HSL hydrolyzes CE in addition to diglycerides, which lowers the local CE concentration resulting in abolished PLIN1 domains (Paper I, Figure 6). PKA phosphorylation of PLIN1 results in the release of CGI-58 and subsequent activation of ATGL and hydrolysis of triglycerides into diglycerides. PLIN1 acts a scaffold for lipolytic proteins on the lipid droplet interface and the formation of dynamic PLIN1 micro domains based on the interaction with CE is proposed to regulate the rate of lipolysis. Dispersion of CE upon HSL hydrolysis results in a fully activated lipolysis seen in human primary adipocytes (Figure 7).

The overlay assay provides efficient identification of specific interactions of a native protein and lipids. The method can be used for studying proteins that cannot easily be purified, as incubation with a lysate from cells expressing sufficient amounts of the protein can be used. The method is applicable not only for protein-lipid interactions, but also protein-protein interactions (far dot Western Blot), and it is a fairly simple and straightforward protocol. However, as with all methods relying on antibodies, proper antibody validation is crucial. In the case of PLIN1, which was heterologously expressed, lysed cells transformed with the same plasmid lacking the gene insert function as a solid negative control. In addition, quantifying a secondary fluorescent antibody signal needs to be done with precaution in mind, ensuring that the signal does not become oversaturated and that the background noise is subtracted cohesively.

Glycerol efflux in adipocytes through aquaglyceroporins

Glycerol generated in the lipolysis in adipocytes exits the cell through aquaglyceroporins, glycerol channels present in the plasma membrane, to be used as a precursor in the gluconeogenesis or glycolysis in the liver, and the FFA are further catabolized in the β-oxidation in mitochondria. The efflux of glycerol is required to prevent reesterification with the fatty acids (47, 48).

Aquaporin family of proteins

Aquaglyceroporins belong to a subclass of the aquaporin family. Aquaporins are transmembrane proteins that facilitate transport of water and other solutes by diffusion over the plasma membrane (49). It was long suspected that highly efficient water channels existed in the plasma membrane of red blood cells as the measured water diffusion rate was too fast for simple diffusion over the lipid bilayer. Until the 1990s, these channels were still elusive. Peter Agre was the first in definitively isolating and characterizing a water channel named CHIP28 (channel-forming integral protein of 28 kDa) (50), now known as AQP1, for which he was later awarded the Nobel Prize in Chemistry. Aquaporins are found in essentially all organisms and 13 aquaporins in human have been identified (AQP0-AQP12) varying in specificity and tissue distribution. Aquaporins are divided into subclasses based on their solute permeability. Orthodox aquaporins are strict water channels with high flux rates, including AQP1. Superaquaporins have a broader range of solute specificity, although this class is not yet as well-characterized (49). Aquaglyceroporins are permeable to glycerol and other small, uncharged solutes such as urea (36, 51, 52). Protons are strictly excluded (53, 54), which is crucial in order to maintain the proton gradient across membranes. Aquaporin activity is known to be regulated by several different mechanisms such as translocation and gating coupled to pH, phosphorylation, binding of divalent cations and protein-protein interaction in response to physiological circumstances (55).

Aquaglyceroporins in adipose tissue

There are four human aquaglyceroporins; AQP3, AQP7, AQP9 and AQP10, which are permeable to glycerol as well as water (36). Moreover, AQP7 and AQP9 are permeable to urea (51, 52). More specific solutes include hydrogen peroxide for AQP3 (56) and arsenite for AQP7 and AQP9 (57).

AQP7 was the first aquaglyceroporin found in adipose tissue (52, 58), and for some time AQP7 was considered to be the sole aquaglyceroporin in adipose tissue (59). However, studies of AQP7-KO mice, revealed that the glycerol efflux was not completely abolished in adipocytes, suggesting that exit pathways other than AQP7 exist (47). Indeed, all human aquaglyceroporins have been detected in white adipose tissue (60, 61). However, there are divergent results with regards to the presence of AQP3, 9 and 10, and relative mRNA levels of all four aquaglyceroporins are not found to be completely consistent between different studies. It has been suggested that the aquaglyceroporin expression vary depending on source and depot of the adipose tissue as well as the body mass index of the human subject and gender (58-60, 62-64).

Aquaporin 3

AQP3 is expressed in a wide variety of tissues (65), most notably in the kidney, from which it was first cloned (66, 67), and in epidermis, the surface layer of the skin (68, 69). AQP3-KO mice display highly increased diuresis, suggesting that AQP3 functions as a water channel in the kidney (70). In the epidermis, however, both the glycerol and water permeability is essential in skin hydration and elasticity (71). Increasing evidence indicate the important role of AQP3 in cancer tumor formation (72). Notably, AQP3-KO mice were found to be resistant to skin tumorigenesis, as ATP generation and cell proliferation were correlated with AQP3-dependent glycerol uptake (73).

Human AQP3 was identified in adipose tissue, although mainly in the stromal vascular fraction, and to a lesser extent in adipocytes. AQP3 had higher expression in human visceral fat compared to AQP7 and AQP9, and an increased expression in obese type-2 diabetes patients (60). The precise role of AQP3 in adipose tissue is yet to be clarified.

Aquaporin 9

AQP9 is abundantly expressed in the liver and is mainly thought to be responsible for the uptake of glycerol generated in the adipocyte lipolysis (65). The glycerol can be used as a precursor in gluconeogenesis and glycolysis, or it can be

reesterified with free fatty acids into triglycerides (36). Thus, APQ9 has an important role in glycerol metabolism and overall energy homeostasis (47). A coordinated regulation of adipocyte AQP7 and hepatic AQP9 has been suggested; during the feeding state, insulin represses both AQP7 and AQP9 expression, and during the fasting state AQP9 levels are increased. This regulation of AQP9 is however only observed in male rodents (36). In adipose tissue, AQP9 was identified and found to be constitutively expressed in the adipocyte plasma membrane, displaying an increased gene expression associated with obesity (60).

Aquaporin 10

AQP10 was initially cloned from the small intestine, with particularly high expression in duodenum and jejunum. Although lacking glycerol and urea permeability, AQP10 was classified as an aquaglyceroporin based on amino acid sequence identity with the other aquaglyceroporins (74). Another splicing variant of AQP10 was later identified, also in the small intestine, which was found to be permeable to both glycerol and urea (75). In a more recent study, AQP10 was identified in adipose tissue, appearing to shuttle between the cytoplasm and surface of lipid droplet and the plasma membrane of adipocytes, similarly to AQP7 (61). Later AQP10 was reported to be gated in a pH-dependent manner, physiologically relevant in the adipocyte (76).

Aquaporin 7

Since AQP7 was identified as a glycerol channel in adipocytes (52, 58), the details of how AQP7 is involved in the glycerol metabolism have been investigated extensively. AQP7 is abundantly expressed in adipose tissue, but is also found in other tissues, such kidney, muscle, liver and endocrine pancreas (2). AQP7-mediated glycerol efflux in mouse cardiomyocytes is involved in heart energy production (77) and triglyceride accumulation and insulin secretion in pancreatic β cells (78).

Studies on AQP7-KO mice

Several independent AQP7-KO mouse models have been generated and although the conclusions from these studies are not completely agreeing, they confirm the importance of AQP7 in the adipose tissue (47, 48, 79, 80) and adipocyte morphology (47, 48, 80). The initial KO-study showed that plasma glycerol levels did not increase in response to lipolytic stimulation or fasting, concluded as a consequence of lacking AQP7. (47). In a follow-up study, the mice were followed for a longer period and after 12 weeks of age, the body weight and fat mass increased, and young mice developed diet-induced obesity as well as whole-body

insulin resistance (48). These findings were in line with another independent AQP7-KO model, in which the mice increased in fat mass, however the body weight did not differ from the wild-type mice, but KO mice were shorter in length (79). Both studies showed that AQP7-deficiency leads to adipocyte hypertrophy, as a result of impaired glycerol efflux (48, 80). The glycerol kinase enzymatic activity in adipocytes was increased as a result of higher glycerol concentration, ultimately leading to reesterification of glycerol and fatty acids into triglycerides (48).

AQP7-KO mice of different genetic background have displayed divergent phenotypes. So far, all studies have been conducted on whole-body AQP7-KO mice. Additional implications in the proximal tubule of the kidney (81) as well as in β cells (82) have been suggested due to the AQP7-deficiency. AQP7 expressed in adipocytes may thus not be exclusively involved in the overall energy metabolism, and tissue-specific AQP7-KO mice could help clarify the underlying mechanisms of the different phenotypes observed (2, 65).

AQP7 loss-of function mutation in humans

There are reported cases of AQP7 missense mutations in humans. One of these mutations is a single residue substitution, G264V, that leads to loss of water and glycerol permeability in functional assays using *Xenopus laevis* oocytes (83). Only a few patients homozygous for the G264V mutation have been identified, but no clear correlation to obesity or diabetes has been established. However, all patients but one had lowered plasma or urine glycerol levels (62, 83, 84).

Regulation of glycerol levels by aquaporin 7

Transcriptional regulation of aquaporin 7

The promoter regions of both human and mouse *AQP7* gene contain an insulin repression element (IRE) and a peroxisome proliferator response element (PPRE) that are involved in the transcriptional regulation of AQP7 (2, 83). *AQP7* mRNA levels are suppressed by insulin in a dose-dependent manner in 3T3-L1 adipocytes, and both AQP7 mRNA and plasma glycerol levels decrease in fasted mice, while refeeding leads to an increase (58). The peroxisome proliferator activated receptor gamma (PPARγ) regulates adipocyte differentiation and other adipose-related genes. PPARγ forms a heterodimer with retionoid X receptor alpha and binds to the PPRE, which also increases *AQP7* mRNA levels (2, 85).

Translocation of aquaporin 7 in the adipocyte

It has previously been suggested that AQP7 is regulated by translocation in adipocytes in response to hormonal stimulation. AQP7 localized to the lipid droplet after insulin stimulation, while translocated to the plasma membrane after catecholamine stimulation (60, 61). In order to clarify the underlying mechanisms of the AQP7 translocation events, a pull-down assay using His-tagged AQP7 and human adipocyte lysate identified PLIN1 as an interacting partner to AQP7 (**Paper II**). The interaction between AQP7 and PLIN1 was further characterized in **Paper II**.

Translocation of aquaporin 7 by interaction with perilipin 1 (Paper II)

In order to identify the regions of AQP7 important for interaction with PLIN1, five variants of human AQP7 with a C-terminal His-tag were heterologously overexpressed in a *Komagataella pastoris* strain (previously known as *Pichia pastoris*) without endogenous aquaporins. The variants of AQP7 were wild type (wt), truncated N-terminus (ΔN), truncated C-terminus (ΔC), both termini truncated (ΔNΔC) and with a removed putative N-terminal PKA phosphorylation site (S10A/T11A) (Figure 6A). All the variants were purified by nickel affinity chromatography and size exclusion chromatography with the detergent DM to keep the protein solubilized, generating highly pure protein (Figure 6B). In addition, PLIN1 lysate was prepared from heterologous overexpression in *E. coli*. Interaction between the AQP7 variants and PLIN1 was detected by the far dot Western Blot method with an α-PLIN1 antibody.

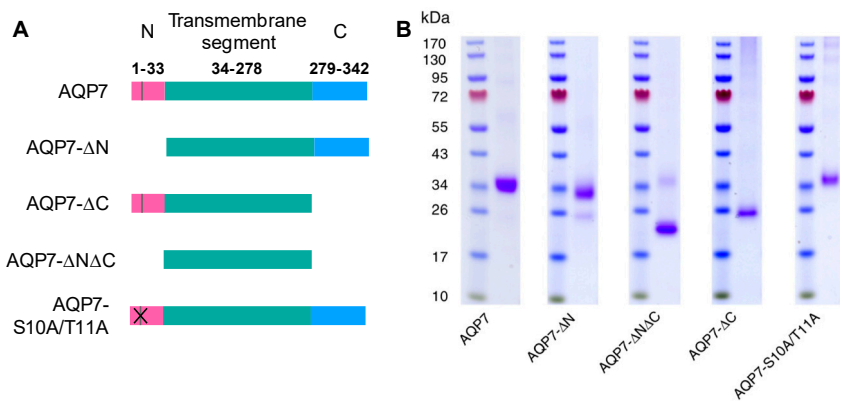

Figure 6. AQP7 variants and heterologous purification. A. Schematic figure of AQP7 variants: AQP7 - wildtype, AQP7-ΔN - N-terminus truncated, AQP7-ΔC - C-terminus truncated, AQP7-ΔNΔC - both termini truncated, AQP7-S10A/T11A - mutated putitative PKA phosphorylation site. Putatative PKA phosphorylation site marked by gray line. Adapted from Figure 1A, Paper II. **B**. Purification of AQP7 variants. Coomassie Blue staining of SDS-PAGE after size exclusion chromatography. Figure 1B, Paper II.

Removing either one of the AQP7 termini, or both at the same time, reduced the interaction to PLIN1 (Paper II, Figure 4). Further, wt-AQP7 was phosphorylated by PKA *in vitro*, which also resulted in a reduced binding affinity to PLIN1. The AQP7 variant S10A/T11A was however not PKA-phosphorylated, and binding to PLIN1 was not affected compared to unphosphorylated wt-AQP7 (Paper II, Figure 5).

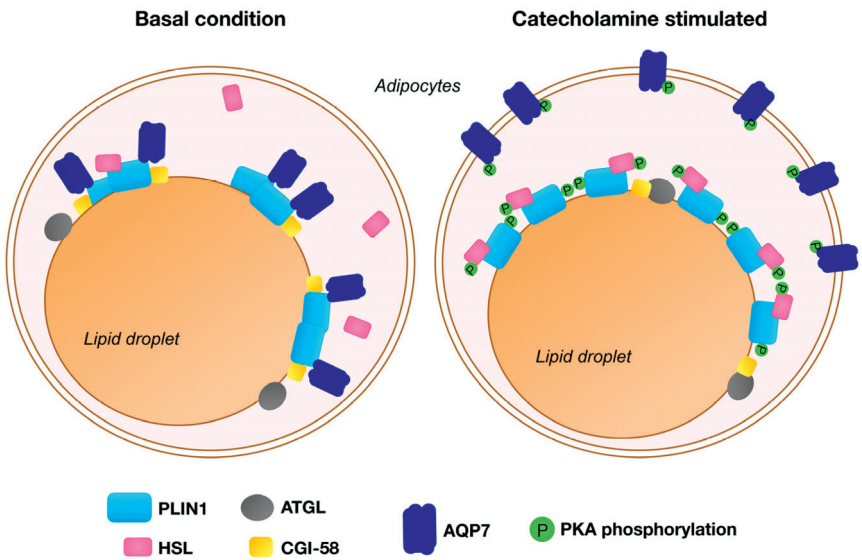

Figure 7. Schematic figure of glycerol regulation in adipocytes. Under basal conditions, perilipin 1 (PLIN1) forms micro domains on the lipid droplet by cholesteryl ester (CE) interaction, and binds aquaporin 7 (AQP7), coactivator comparative gene identification-58 (CGI-58) and to some extent unphosphorylated hormone sensitive lipase (HSL). Catecholamine stimulation leads to activation of lipolysis, by a cascade of reactions including cateholamine binding to β-adrenergic receptors, increased cyclic adenosine monophosphate (cAMP) levels and activation of protein kinase A (PKA). PKA phosphorylates PLIN1, which recruits PKA-phosphorylated HSL to the lipid droplet. Further, CGI-58 is released from phosphorylated PLIN1, which activates ATGL. Phosphorylated HSL hydrolyses cholestryl esters, in addition to diglycerides, and PLIN1 micro domains disperse on the lipid droplet as a result of decreased CE concentrations. As the N-terminus of AQP7 becomes phosphorylated by PKA, the AQP7-PLIN1 interaction diminishes and AQP7 translocates to the plasma membrane for glycerol efflux.

PLIN1 is central for regulating glycerol levels in adipocytes. Catecholamine stimulation results in PKA-phosphorylation of PLIN1 which activates the lipolysis by releasing the ATGL activator CGI-58 (46). Further, PLIN1 disperses on the lipid droplet and binds activated HSL which fully activate the lipolysis and increase intracellular glycerol levels (**Paper I**). Adding to this, PLIN1 is also involved in the regulation of AQP7 as PKA-phosphorylation of the AQP7 N-terminus decreases the binding affinity for PLIN1, resulting in translocation of AQP7 from the lipid droplet presumably to the plasma membrane for release of

glycerol generated in the lipolysis (Paper II). Structural information about PLIN1 is not available, however, the amino acid sequence contains an HSL-binding PAT domain, and the 11-mer amphipathic helical motif and hydrophobic regions involved in the lipid droplet binding. Another segment located on the C-terminal side of the hydrophobic regions has been suggested to be involved in binding CGI-58 (Bickel 2009). The three hydrophobic sequences are located next to the 11-mer motif, interspersed with PKA consensus sites (Figure 3). AQP7 could possibly bind to the regions between the hydrophobic regions or the C-terminal region. A structure of the AQP7-PLIN1 complex ideally, or functional studies with truncated versions of PLIN1 would clarify the details of the interaction.

Localization of aquaporin 7 in adipocytes

Distinguishing the location of a protein between intracellular compartments and the plasma membrane in adipocytes is challenging as the lipid droplet takes up most of the volume of the adipocyte. This leaves only a narrow space between the lipid droplet monolayer and the plasma membrane, around 250 nm (Figure 2B). The resolution of light microscopy does not allow distinguishing between the possible locations by direct immunofluorescence methods (Figure 2A-B). However, there are other methods to study protein localization in the narrow intracellular space between the lipid droplet and plasma membrane.

Proximity ligation assay. In **Paper II**, the proximity ligation assay (PLA) was applied on isolated human primary adipocytes to study the interaction between AQP7 and PLIN1. PLA is based on using primary antibodies against the two proteins of interest and secondary antibodies coupled to oligonucleotide proximity probes. If the two proteins are close in space, within 30 nm, the probes can guide the formation of circular DNA, which remains hybridized to the probe, and is used for rolling-circle amplification. The product is detected by complementary fluorescently labeled oligonucleotides (86).

With this method, the interaction between APQ7-PLIN1 was confirmed *ex vivo* in human primary adipocytes (Paper II, Figure 2). Applying the method on insulin- and catecholamine-stimulated adipocytes clearly showed a reduced AQP7-PLIN1 complex formation under lipogenic condition (Paper II, Figure 7). These results supported the previous finding of hormone-dependent translocation of AQP7 (60, 61), however, the precise localization of AQP7 after catecholamine stimulation was not determined.

Immunogold labeling for transmission electron microscopy. The high-resolution power of electron microscopy allows to clearly visualizing the cytosol present between the lipid droplet and plasma membrane of adipocytes (Figure 2B). Immunogold labeling is, as the name suggests, an antibody-based technique in which a colloidal, electron dense gold particle is attached to the secondary

antibody (Figure 8A). This method could thus pinpoint the localization of AQP7 in human primary adipocytes.

The immunogold labeling method allows for precise localization of the protein and by using gold particles of different sizes it is possible to stain for more than one protein at the time. If the staining is performed on postembedded tissue, the ultrastructure will be well-preserved and the particles can be quantified (87). However, given the radius of the gold particle and secondary antibody, nearby epitopes within this shadow range will not be recognized (Figure 8A). The method is thus not directly quantitative, and as for all techniques utilizing antibodies, the specificity of the antibody will affect the outcome. Further, the postembedding procedure and section preparation might alter antibody recognition, and comparing to a suitable a negative control is difficult when working with human tissue samples, compared to mice and rats samples where the possibility of knockout animals is straightforward.

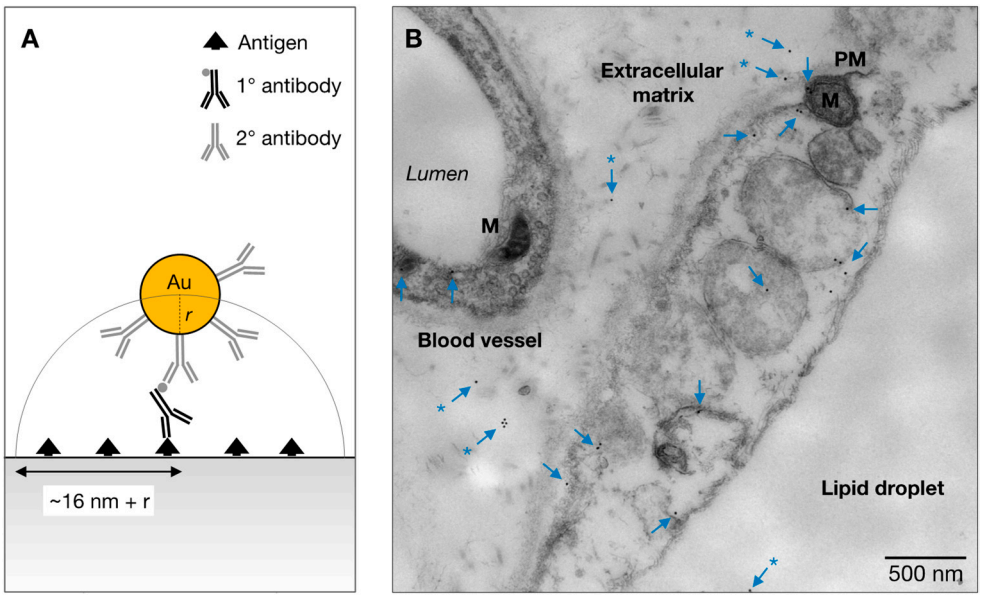

Figure 8. Morphology of human subcutaneous adipocytes. A. Bright-field and fluorescence micrographs of isolated adipocyte. i) Bright-field image, ii) DAPI nucleus stain, iii) Nile Red lipophilic dye, iv) merged micrographs. Supplementary figure 1, Paper I. **B**. Transmission electron microscopy micrograph of adipose tissue negatively stained with uranyl acetate. LD - lipid droplet, L - lumen of blood vessel. Image credit Peng Huang. **C**. Trace of adipocyte in B, showing lipid droplet in yellow and cytoplasm in blue.

Preliminary experiments of immunogold labeling of AQP7 in postembedded human adipose tissue revealed specific AQP7 staining. Tissue samples were either stimulated with insulin or isoprenaline, a catecholamine analog, or left unstimulated before fixation and embedding in resin. Sections around 80 nm in thickness were incubated with a primary α-AQP7 antibody and secondary gold-labeled antibody, and negatively stained with uranyl acetate and lead citrate. The ultrastructure was preserved and in unstimulated tissue, AQP7 localized in cytosol and in the vicinity of the plasma membrane and lipid droplet monolayer (Figure 8B). Unspecific staining in the extracellular matrix and in the lipid droplet was also detected. The results indicate that immunogold labeling of AQP7 in human adipose tissue is possible, and stimulated samples would need to be analyzed as well in order to further study the translocation events of AQP7.

Structural characteristics of aquaporins

Aquaporins as a protein family are structurally well studied and characterized, and there are several high-resolution aquaporin structures from bacteria to human available in the Protein Data Bank (PDB). The overall architecture of aquaporins is similar, but significant differences in the amino acid sequence form the basis for the varying specificity present among aquaporins.

Human aquaglyceroporin monomers consist of around 300 residues, AQP3 is the smallest and AQP7 the largest with 292 and 342 residues, respectively, and the molecular mass is around 30 kDa with a sequence similarity of approximately 50 % for all human variants. Two regions that are important for the selectivity are particularly conserved among all members of aquaporins, the aromatic/arginine region (ar/R) and two asparagine-proline-alanine (NPA) motifs (Figure 9A).

Aquaporin common fold

Aquaporins exist as homotetramers in the plasma membrane and each monomer functions as an individual channel (88) (Figure 9B). The topology of the AQP1 monomer was predicted at the time of its discovery (50), and with high-resolution structures available today, we know that the predictions were accurate. All aquaporins structurally determined to this date display the common fold with six transmembrane helices connected by five loops (loops A-E) with both the N-terminus and C-terminus on the cytoplasmic side. Loops B and E form shorter membrane-spanning helices that meet in the center of the membrane, creating a seventh pseudo-helix. The conserved NPA motifs are located on loops B and E, connecting the two half-helices (Figure 9C). The ar/R region is located towards the extracellular side of the channel and forms a constrictive selectivity filter (Figure 9C). The orthodox aquaporins have more narrow selectivity filters, which consequently exclude solutes larger than water, while the selectivity filters of aquaglyceroporins have wider selectivity filters, thus allowing passage of glycerol and other solutes as well (89). Protons are excluded by an electrostatic barrier created by the dipole moments of the two shorter helices (53). Further, a coordinated movement of water molecules moving through the selectivity filter

breaks the hydrogen bond network to neighboring water molecules, thus preventing proton jumping via the Grotthuss mechanism (54).

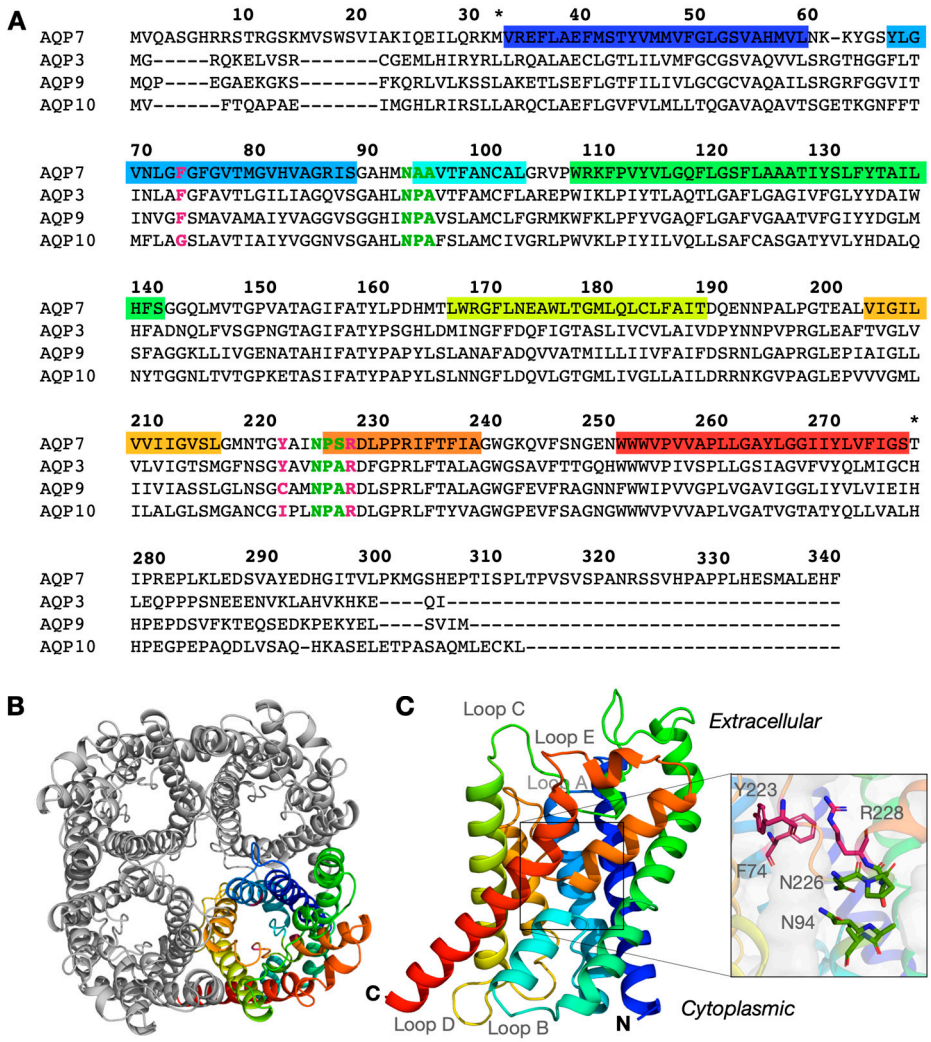

Figure 9. **A**. Sequence alignment of the human aquaglyceroporins AQP3, AQP7, AQP9 and AQP10. Colored segments indicate transmembrane alpha helix-forming regions, as shown in B and C. Residue numbering according to the AQP7 sequence, aR/R selectivity filter residues are marked in pink and NPA motif-residues in green. Asterisks indicate start and end of the crystallized truncated AQP7 variant. **B and C**. Architecture of aquaporins, illustrated by the structure of AQP7 (PDB: 6QZI) in gray and rainbow cartoon representation. **B**. Aquaporins form homotetramers in the plasma membrane, viewed from the extracellular side. **C**. Aquaporin monomer with 6 transmembrane helices connected by loops (A-E), with loops B and E forming shorter helical segments. The box shows the selectivity filter and NPA region with residues as sticks in pink and green, respectively, and the pore indicated by surface representation.

Structurally determined aquaglyceroporins

To date, there are two structurally determined human aquaglyceroporin, AQP7 (**Paper III**) and AQP10 (76). In addition, the bacterium *Escherichia coli* and the malaria parasite *Plasmodium falciparum* each have one aquaglyceroporin called GlpF and PfAQP, respectively, and both structures have been determined (90, 91). Structurally, AQP7 is most similar to GlpF and PfAQP, while AQP10 has distinctive features, such as a much wider selectivity filter and a restrictive passage located close to the intracellular vestibule (76).

Channel selectivity of aquaporin 7

Functional studies have established the importance of AQP7 as a glycerol channel in adipocytes. However, AQP7 is permeable to water and other solutes too, and the detailed mechanisms underlying the selectivity of AQP7 have not been entirely clarified, in part due to the lack of a three-dimensional structure. Although aquaporins are structurally conserved and homology models easily can be generated, experimentally determining the structure is still necessary for a detailed molecular analysis. To further clarify the selectivity mechanisms of AQP7 and to understand how glycerol is conducted, the x-ray structure of human AQP7 was determined at high resolution (Figure 9B-C, Figure 10, Figure 11) as described in **Paper III**, and the findings are summarized below.

Structure of aquaporin 7

AQP7 was expressed heterologously in a *Komagataella pastoris* strain without endogenous aquaporins, and purified by nickel affinity chromatography and size exclusion chromatography in the presence of the octyl glucose neopentyl glycol (OGNG) detergent. Protein crystals were grown by hanging drop vapor diffusion at 4 °C. As wild-type AQP7 did not yield well-diffracting crystals, an AQP7 variant with both termini truncated (AQP7-ΔNΔC) was used for structure determination. Diffraction data were collected from two crystals grown in the same drop, generating two separate structures at 1.9 Å and 2.2 Å resolution. The crystals had been soaked stepwise in up to 20 % glycerol or in 5 % glycerol only.

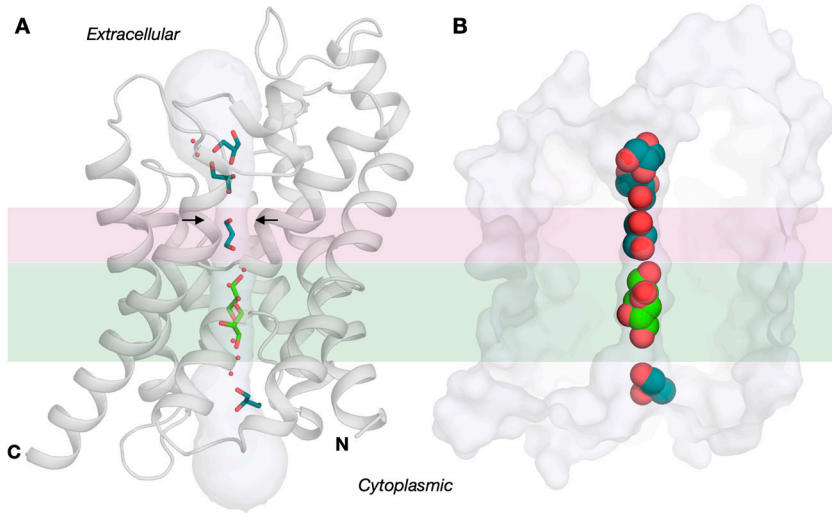

Figure 10. The structure of AQP7 with glycerol and water molecules lining the pore. The selectivity filter and NPA regions are highlighted in pink and green, respectively. **A**. AQP7 in gray cartoon with glycerol molecules shown as teal and light green sticks. Glycerol molecules in light green represent two alternatives of one glycerol molecule. The diameter of the pore was calculated by the HOLE program and represented as gray surface. Arrows indicate the most narrow part of the pore of 3.3 Å located in the selectivity filter. **B**. AQP7 represented as gray surface, with glycerol molecules shown as spheres.

Glycerol and water conducting pore

The structure of AQP7 follows the typical aquaporin fold with the selectivity filter creating the narrowest part of the channel of 3.3 Å (Figure 9C, Figure 10A). Both glycerol and water molecules were present in the conducting pore, bound through a network of hydrogen bonds (Figure 10, Figure 11). The asparagines of the unusual NPA motifs of AQP7, NAA (Asn94, Ala95, Ala96) and NPS (Asn226, Pro227, Ser228), were structurally conserved compared with the NPA motifs of GlpF, held in place by hydrogen bonding between Ser228 and the backbone nitrogen of Ala95 (Figure 11). Residue substitutions in the ar/R region of AQP7 compared to GlpF and PfAQP did not affect the structural integrity of the selectivity filter. Although, a phenylalanine in GlpF and PfAQP is substituted with a tyrosine in AQP7 (Tyr223), which offers additional hydrogen bonding possibilities (Figure 11, Figure S1, Paper III). Only minimal differences between the two AQP7 structures were observed, with a root-mean-square deviation of 0.83 Å, however the glycerol molecules located in the pore displayed a slight shift although located in similar positions. Two alternatives of a glycerol were modeled in the density in the NAA/NPS region and one glycerol was modeled in the selectivity filter (Figure 11), in addition to several glycerol molecules located in the intracellular and extracellular vestibules (Figure 10).

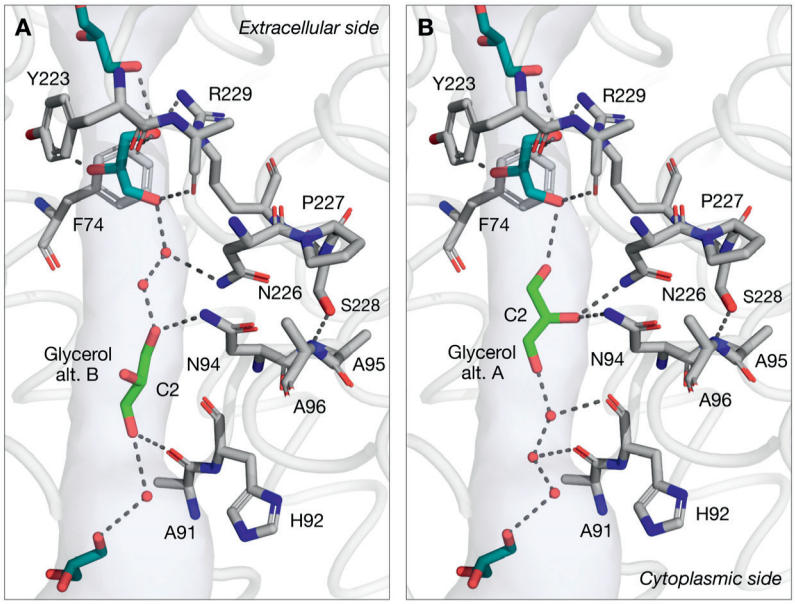

Figure 11. Glycerol and water molecules are bound in the AQP7 pore through a network of hydrogen bonds. AQP7 is shown in gray as cartoon tubes and residues in the ar/R selectivity filter and NPA motifs as sticks. Glycerol molecules are represented as teal and light green sticks, light green molecules are two alternatives of one glycerol molecule (alternatives A and B), and waters are represented by red spheres. Gray dashed lines indicate possible hydrogen bonds within 3.4 Å. **A**. Glycerol alternative B. **B**. Glycerol alternative A.

Glycerol conducting mechanism

The locations of the glycerol molecules in both structures provide an insight into the pathway of glycerol as it exits the cell through AQP7. As the glycerol enters the channel, the hydroxyl group of glycerol C2 is facing the polar side of the pore (Figure 11A). As it travels further along, the same hydroxyl group rotates forming hydrogen bonds directly to the asparagines of the NPA motifs (Figure 11B). In the selectivity filter, the glycerol C2 hydroxyl group is oriented in the opposite direction compared to GlpF and PfAQP, forming a hydrogen bond with Tyr223 (Figure 11). The extracellular vestibule is mainly hydrophobic and the lack of hydrogen bond possibilities might facilitate the release of the glycerol. The suggested glycerol conducting mechanism is based on the glycerol partly rotating through the pore (Figure 11), which could facilitate the transition by altering the hydrogen bond network and releasing the glycerol from more tightly bound positions.

Interestingly, the AQP7 loss-of-function mutation, G264V, found in human (83) is located on transmembrane helix 6, far from the conducting pore. Gly264 in AQP7 is situated in proximity to transmembrane helix 4 and this glycine is highly conserved in human aquaporins (92). An equivalent mutation to G264V was

identified an intragenic suppressor mutation screen of the yeast aquaglyceroporin Fps1. The mutation in Fps1, G519S, led to lower glycerol permeability, concluding that the mutation altered the positioning of transmembrane helix 4 which made the pore tighter and thus sterically hindered glycerol (93). It is possible that the glycine-to-valine substitution in AQP7 results in the same conformational change of transmembrane helix 4. AQP7-G264V has been characterized in the human subjects homozygous for the mutation and in *Xenopus* oocytes showing a decreased glycerol flux (62, 83, 84). It would be interesting to crystallize AQP7-G264V to reveal if there is a change in pore diameter, thus explaining the lost glycerol permeability.

Glycerol versus water permeability

Further, molecular dynamics (MD) simulations of the glycerol and water permeation through AQP7 were carried out. In a glycerol-free setup, AQP7 was highly permeable to water, while the osmotic water permeability (Pf) was reduced 2-4 fold when glycerol was present in the system (Figure 3, Paper III). The MD simulations indicated a high affinity and a rapid exchange of glycerol molecules. In addition, glycerol sterically blocks the pore, suggesting that glycerol hinders fast permeation of water (Figure 10B).

It has previously been shown that AQP7 is permeable to both glycerol and water, however it is debated whether the Pf is comparable to orthodox aquaporins or in fact much lower. The Pf was high when measured in *Xenopus* oocytes and liposome assays with the protein reconstituted in artificial membranes (52, 76, 83). In these methods, the Pf is measured indirectly by the change in volume after altering the osmolality. When Pf was measured by diffusion NMR on yeast cells recombinantly expressing human AQP7, the diffusion of water through AQP7 was similar to the rate as of the plasma membrane (57). This method measures the exchange rate of diffusion without applying an external osmotic pressure, providing a direct way of measuring the water exchange (94). The lower Pf was attributed to expression of the protein in a native plasma membrane. Based on our MD simulations, the discrepancy in Pf could further be explained by the absence of glycerol, as the shrinking and swelling assays are presumably glycerol-free.

Inhibition of aquaporin 7

The water permeability of AQP7 is inhibited by mercury chloride and can be reversed by β-mercaptoethanol (52) and several aquaporins share this feature (95). In the orthodox AQP1, mercury chloride binds to Cys189 that is located in the

pore and sterically blocks water passage (96). However, AQP7 only has two cysteine residues and both are located outside of the pore. These cysteine residues are conserved in rat AQP7 and human AQP3. Rat AQP7 has one additional cysteine, but was found to not be inhibited by mercury chloride (97). Human AQP3 has four additional cysteine residues, and Cys11 was identified as the mercury-sensitive residue (98). In order to clarify the mercury chloride inhibition of AQP7, more studies are required. The structure of AQP7 cannot explain the mercury inhibition of AQP7.

More interestingly, the glycerol permeability of AQP7 has been shown to be competitively inhibited by acyl glycerol derivatives and diglycerol, and the inhibitory effect decreased with decreasing number of hydroxyl groups. Monoacetin, monobutyrin and diacetin inhibited glycerol uptake, while triacetin had no inhibitory effect (Figure 12). Further, the glycerol uptake was inhibited completely by high concentrations of the compounds, suggesting that the recognition site is the same as for glycerol (99). In the structure of AQP7, two alternatives of a glycerol molecule were modeled close to the NPA motifs in elongated electron density, with several opportunities for establishing hydrogen bonds to the pore side chains. This supports the binding of diglycerol and the acyl glycerol derivatives, and also explains why the extension of the acyl group on monobutyrin had no impact on the affinity, compared to monoacetin. The structure could also explain the non-inhibitory effect of triacetin, as the third acetyl group of triacetin makes this compound more bulky, in addition to the lack of hydroxyl groups.

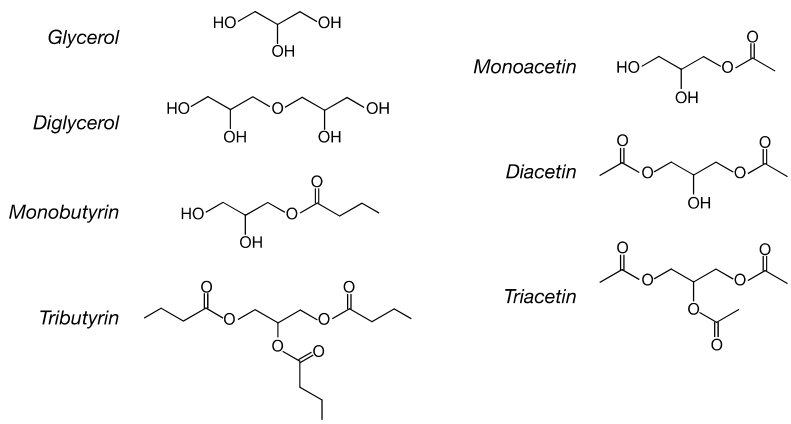

Figure 12. Chemical structures of glycerol, diglycerol and acyl glycerol derivatives.

Methods for protein structure determination

The structure of AQP7 in Paper III was determined by x-ray crystallography. X-ray crystallography has so far been the major tool for structure determination of macromolecules at atomic resolution. Well-ordered crystals of homogenous protein molecules diffract x-rays. The collected diffraction data together with phase information obtained either experimentally by anomalous diffraction or by molecular replacement are used to reconstruct electron density maps and the three-dimensional structure. The major bottleneck is to obtain the well-diffracting crystals (Figure 13), and for membrane proteins additional factors including detergents and low protein yields can further complicate the process. Other methods of structure determination include single particle cryo-electron microscopy (cryo-EM), NMR spectroscopy micro-electron diffraction and neutron diffraction. Cryo-EM has become increasingly more used, as technical improvements have pushed the resolution limits and made it possible to work with relatively small molecules. Major benefits include smaller protein samples are required and that the protein is vitrified from solution thus representing a more native state of the protein and with the possibility to provide different states, compared to a crystal structure (100). However, cryo-EM is not yet replacing x-ray crystallography. Rather, the two techniques could beneficially be used as complementary tools for revealing the mechanisms of a protein or protein complex. Cryo-EM can provide 3D maps of large complexes that have not been successfully crystallized, and crystal structures of separate domains can be docked within the cryo-EM map.

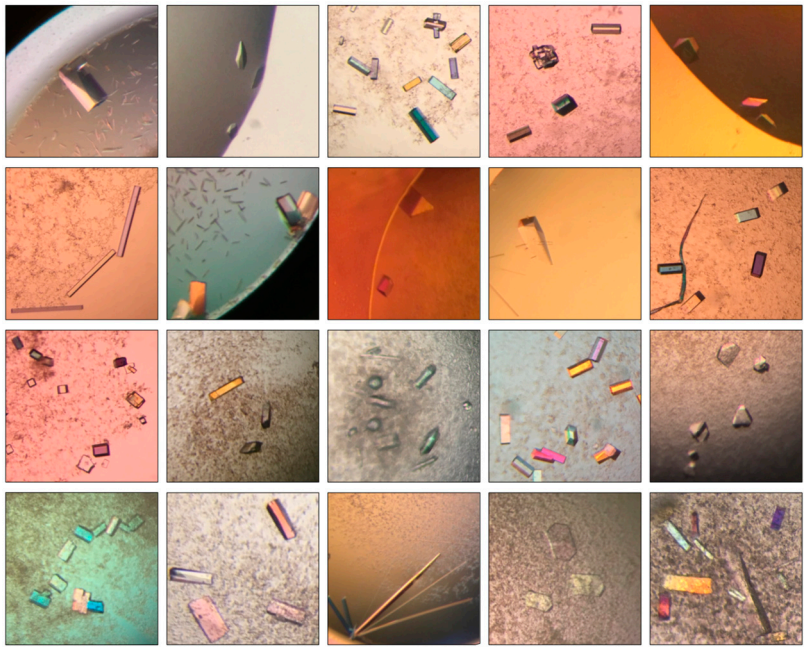

Figure 13. Beautiful yet poor diffracting crystals of AQP7 in various detergents and crystallization conditions.

Although crystallization of membrane proteins remains a challenge, the higher resolution possible to obtain using x-ray crystallography compared to cryo-EM and the relatively small size of AQP7 rationalize the approach to structure determination of AQP7. However, it was not possible to obtain a crystal structure of AQP7 with the termini present. In order to evaluate if it is possible to resolve the termini by using cryo-EM, full-length AQP7 was purified in the DM detergent and grids were prepared for single particle analysis. Evenly distributed protein particles in the vitreous ice layer were only achieved on graphene oxide grids and the particles appeared to have preferred orientation (Figure 14). Changing the detergent, grid preparation and type could not resolve these issues. Both AQP7 termini are cytosolic and are thought to be important for the interaction with PLIN1 and AQP7 translocation in the adipocyte. Therefore, focusing on a cryo-EM structure of the AQP7-PLIN1 complex may provide important insights into the stoichiometry and which parts of the proteins that are involved in the complex formation, especially regarding PLIN1.

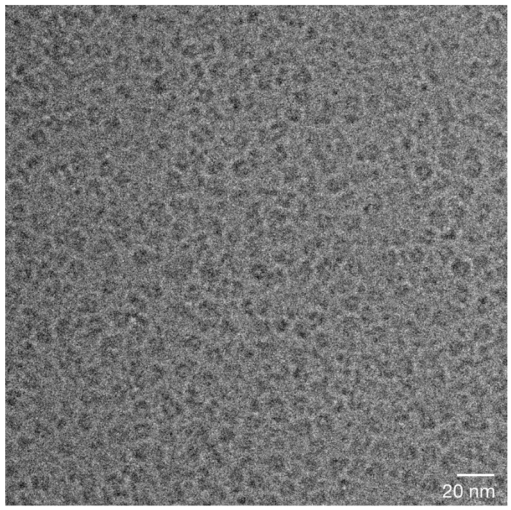

Figure 14. Micrograph of graphene oxide grid with AQP7 particles purified in the DM detergent evenly distributed in vitrified ice.

The need for a neutron structure of aquaporin 7

The x-ray structure of AQP7 at high resolution has provided information regarding the glycerol and water permeation. Precisely locating hydrogen atoms would elucidate the glycerol selectivity and conducting mechanisms further. The information about hydrogen atoms in x-ray structures is usually missing but can be obtained by neutron macromolecular crystallography (NMX), making NMX a useful complement to x-ray structures. However, large crystals are required for NMX, as the diffracted intensity depends on the incident beam in a directly proportional manner and the neutron beam fluxes inherently are several orders of magnitude lower than synchrotron x-ray beams. Further, the coherent scattering length of deuterium (D) is similar to those of oxygen, carbon and nitrogen and the incoherent neutron scattering of hydrogen is of greater magnitude compared to D. Thus, expression and crystallization of perdeuterated protein, or H/D exchange of the protein crystal is required (101). Initial NMX studies of AQP7 were carried out in **Paper IV**.

Neutron macromolecular crystallization of aquaporin 7 (Paper IV)

In order to grow large crystals of AQP7 suitable for neutron diffraction data collection, several parameters were investigated and evaluated on their ability to crystallize. Two different AQP7 variants, AQP7-ΔC and AQP7-ΔNΔC, were

expressed and purified in a range of detergents. Detergents that kept the protein homogenous in solution, as judged by the size exclusion chromatography elution profile, were chosen for vapor diffusion hanging drop crystallization (Figure 15). Extensive optimization of crystallization conditions to grow larger and well-diffracting crystals was carried out, including additive, detergent and grid screening, temperature, protein concentration, ratio of protein and precipitant in the crystallization drop and macro seeding.

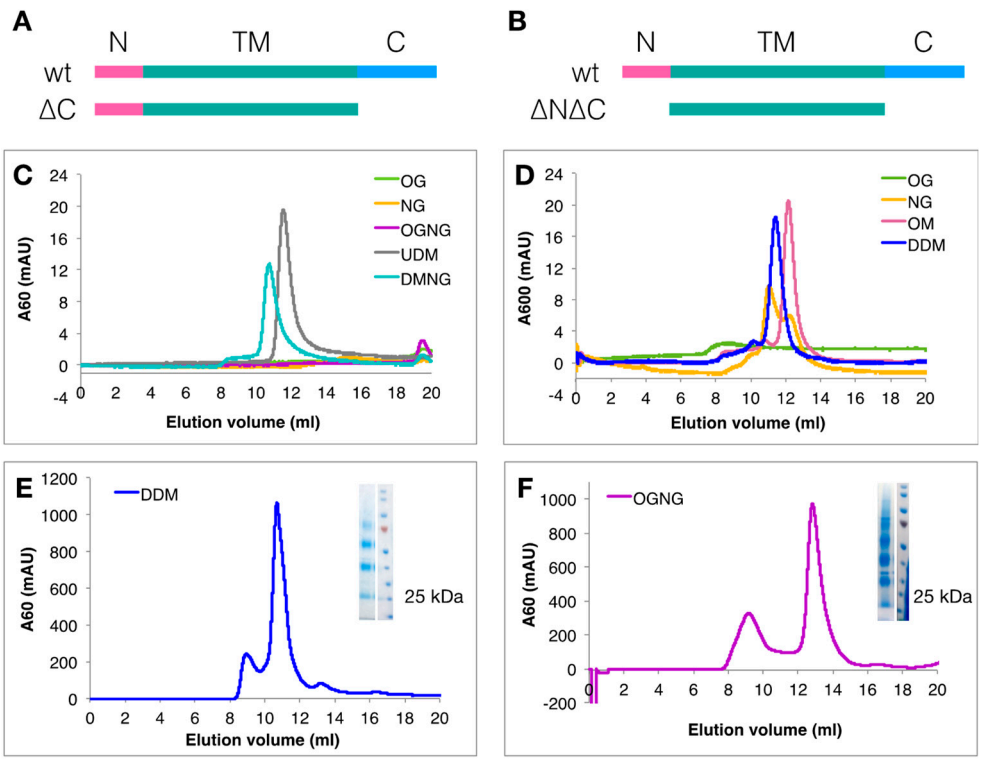

Figure 15. Size exclusion chromatography purification of AQP7-ΔC and AQP7-ΔNΔC in various detergents. A and B. Schematic picture of AQP7 variants AQP7-ΔC and AQP7-ΔNΔC. Wild type AQP7 shown above each schematic sequence for reference. Elution profiles of detergent screening for **C**. AQP7-ΔC and **D**. AQP7-ΔNΔC. **E**. Large-scale purification elution profile of AQP7-ΔC in DDM. **F**. Large-scale purification elution profile of AQP7-ΔNΔC in OGNG. Inserts in E and F show SimplyBlue-stained SDS-PAGE of the maximum peak fractions, indicated by asterisk. AQP7-ΔC and AQP7-ΔNΔC monomers migrate to around 25 kDa. Figure 4, Paper IV.

The two AQP7 variants were both kept soluble in several detergents, however none of the glucoside detergents were suitable for AQP7-ΔC. Larger crystals of AQP7-ΔC were obtained in the DDM detergent, in particularly when grown at high temperature (30 °C) where crystals grew up to 1 mm in the longest dimension

(Figure 5F-G, Paper IV). However, the crystal packing was poor, judged by weak x-ray diffraction (>7 Å), potentially due to the long alkyl chain of DDM preventing adequate crystal contact formation or flexibility of the AQP7 N-terminus. The majority of AQP structures deposited in PDB have been obtained with the glucoside detergent OG, and AQP7-ΔNΔC was successfully purified in NG, OTG and OGNG. Crystals were prone to grow in NG, and although crystals grew slightly larger in one of the dimensions, the crystal packing did not improve after optimization. Protein purified in OGNG crystallized in the presence of OG at a protein to precipitant ratio of 1:2. The crystals were small, approximately 0.05 mm in at least two dimension, but diffracted to up to 1.9 Å. Macroseeding of small crystals grown in this condition improved crystal size up to 0.3 mm (Figure 16), and plate-shaped crystals grew in the vapor diffusion sitting drop well setup.

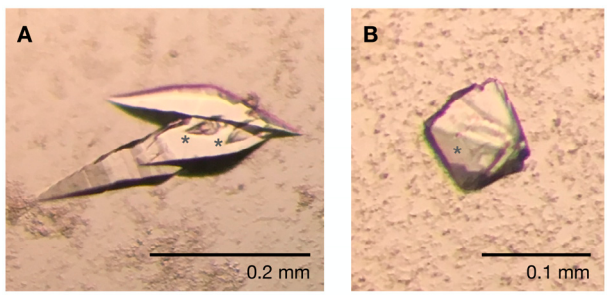

Figure 16. Macroseeded AQP7-ΔNΔC crystals continued to grow up to **A**. ~0.3 mm (Figure 2C, Paper IV) and **B**. ~0.1 mm in the longest dimension. Asterisks indicate original seeded crystal(s).

Growing crystals of a membrane protein large enough for neutron diffraction remains a huge obstacle (102). The absence of membrane protein neutron structures in PDB is a testament to this. Crystallization of AQP7 in maltoside detergents, in particular DDM, resulted in larger crystals however with poor crystal packing. Crystallization in glucoside detergents dramatically improved the diffraction, but the crystals were smaller. An optimized crystallization condition was successfully transferred from hanging to sitting drop format, and macroseeding improved crystal size. Although further optimization is required for determine a neutron structure of AQP7, including H/D exchange of titratable hydrogen atoms, this work lays the foundation for continued neutron crystallization.

Summary

Lipogenesis and lipolysis in adipose tissue are essential for meeting the changing energy demands in the human body. Glycerol generated during the lipolysis exit the cell through AQP7, the main aquaglyceroporin in adipocytes. Hence, glycerol levels are regulated both by triglyceride hydrolysis and glycerol efflux. However, the detailed mechanisms governing glycerol levels in adipocytes have not yet been fully described.

The regulatory function of PLIN1 in the lipid metabolism in adipose tissue is well studied. PLIN1 forms a scaffold for lipolytic protein on the lipid droplet and acts both by suppressing basal lipolysis as well as accelerating lipolysis upon β-adrenergic activation. Further, PLIN1 segregates to distinct micro domains in insulin-stimulated human adipocytes, which are abolished after catecholamine stimulation. This phenomenon was investigated in **Paper I** by biochemical and biophysical assays, showing that PLIN1 specifically interacts with cholesteryl esters (CE) that are present at the lipid droplet. The phase behavior of CE in a lipid mixture forms the basis for the dynamics of PLIN1 micro domains and regulation of the lipolysis.

In **Paper II** we identified PLIN1 as a novel interacting partner of AQP7. AQP7 is known to be regulated by translocation, shuttling in and out of the plasma membrane. The N-terminus of AQP7 was shown to be phosphorylated by PKA under lipolytic conditions, which diminished the APQ7-PLIN1 interaction. Thus, AQP7 could translocate from PLIN1 on the lipid droplet to the plasma membrane, providing a regulation mechanism of glycerol efflux in adipocytes.

In **Paper III** we reported the first crystal structure of AQP7. The structure was solved by x-ray crystallography to 1.9 Å with glycerol and water molecules present in the pore. The high resolution revealed partial rotation of the glycerol hydroxyl groups along the pore, suggesting that the rotating movement is the basis for how glycerol is conducted through AQP7. The structural information combined with MD simulations suggest that glycerol hinders an unrestricted flow of water through the channel.

A neutron diffraction structure of AQP7 would contribute to our full understanding of the selectivity and conductance mechanisms of glycerol and water through AQP7. However, in addition to x-ray crystallography neutron

diffraction experiments require very large protein crystals (>1 mm^3). In **Paper IV** we focus our efforts to obtaining a neutron structure of AQP7, by extensive detergent screening during purification as well as identifying crystallization conditions supporting growth of well-diffracting crystals and initial steps for optimizing these crystals to fit the size requirements for neutron diffraction data collection.

Concluding remarks and future perspectives

This work has been dedicated to study the regulation of glycerol levels in adipocytes and has provided new insights into the activation of lipolysis and into the translocation of AQP7 in the cell as well as structural details of the glycerol selectivity in AQP7.

It is well established that PLIN1 targets and interacts with the lipid droplet monolayer, however the molecular mechanisms behind this are unknown. PLIN1 was shown to have an additional role in the regulation of the lipolysis by specifically interacting with cholesteryl esters, DPPC and triglycerides, forming dynamic micro domains on the lipid droplet that disperse during the lipolytic state resulting in a fully activated lipolysis. The structure of PLIN1 is not known and a PLIN1 purification protocol has not been established. A structure of PLIN1 would provide insights into the interactions to the lipid droplet, and how it specifically targets certain lipids.

Further, PLIN1 was shown to regulate glycerol efflux by interacting with AQP7, the major glycerol channel in adipocytes, under basal conditions. The interaction was diminished after catecholamine stimulation, resulting in translocation of AQP7 to the plasma membrane and glycerol efflux. The interaction is believed to depend on PKA phosphorylation of the AQP7 N-terminus, however it is still unknown which parts of PLIN1 are important for the interaction. With purified PLIN1 available it would be possible to study the AQP7-PLIN1 interaction in more detail. It would also make structure determination possible, both in complex with full length AQP7 and on its own. Alternative approaches in the lack of a PLIN1 purification protocol are constructing truncated versions of PLIN1 to map the interaction to AQP7, or co-purification of AQP7 and PLIN1. PLIN1 presumably binds strongly to AQP7, as it was captured to AQP7 from an adipocyte lysate.

The crystal structure of AQP7 was determined at high resolution and the glycerol conducting mechanism through AQP7 was suggested to involve a partial rotation of the glycerol molecule, which facilitates the transition by altering the hydrogen bond network. MD simulations indicated that AQP7 has high affinity of glycerol,

and that glycerol hinders a rapid water flux through the channel. The osmotic water permeability in AQP7 was found 2-4 times lower when glycerol was present in the system, compared to a glycerol-free setup. In accordance, the glycerol molecules present in the channel in the high-resolution structure create steric hindrance for water to flow freely through the pore. The osmotic water permeation experiments that measure shrinkage or swelling are likely to be performed in the absence of glycerol, which could be one explanation for the discrepancies in water permeability. AQP7 has enriched expression in adipocytes where glycerol is present, which makes it more physiologically relevant to study and draw conclusions about the water permeation in the presence of glycerol. However, our MD simulations do indicate high water fluxes in the absence of glycerol, in agreement with other studies.

To further characterize the selectivity and conducting though AQP7, optimization for growing larger crystals for a neutron diffraction structure of AQP7 was also outlined in this thesis. Although larger crystals were obtained by macroseeding and the protein crystallized in the sitting drop setup, usually a prerequisite for large crystal growth, further optimization efforts are required before we can determine a neutron structure of AQP7.

All aquaglyceroporins, AQP3, 7, 9 and 10, have been identified in the adipose tissue, however it has been debated to which degree AQP3, 9 and 10 are present. Different regulation mechanisms have been suggested; AQP10 was found to be activated by low pH and AQP9 to be constitutively located to the plasma membrane, while the glycerol permeability of AQP7 remains the same at both high and low pH (57, 103), and is regulated by trafficking. The presence of several aquaglyceroporins as well as different regulations mechanisms suggests the importance of efficient glycerol efflux in adipocytes.

Glycerol metabolism has implications in lipid accumulation, insulin sensitivity and glucose homeostasis and AQP7 has a central role as a major glycerol channel. (104). Further, increased expression of aquaglyceroporins has been found in tumors which makes them potential therapeutic targets. Tumors can also induce lipolysis in adipocytes, and increasing the glycerol metabolism can fuel the cancer cell proliferation (4, 105). Detailed knowledge of the mechanisms of glycerol metabolism and the high-resolution structure of AQP7 provide the basis for development of inhibitors targeting AQP7.

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Writing this I realize my communication depend heavily on emojis which don't seem to be compatible in Word □.

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