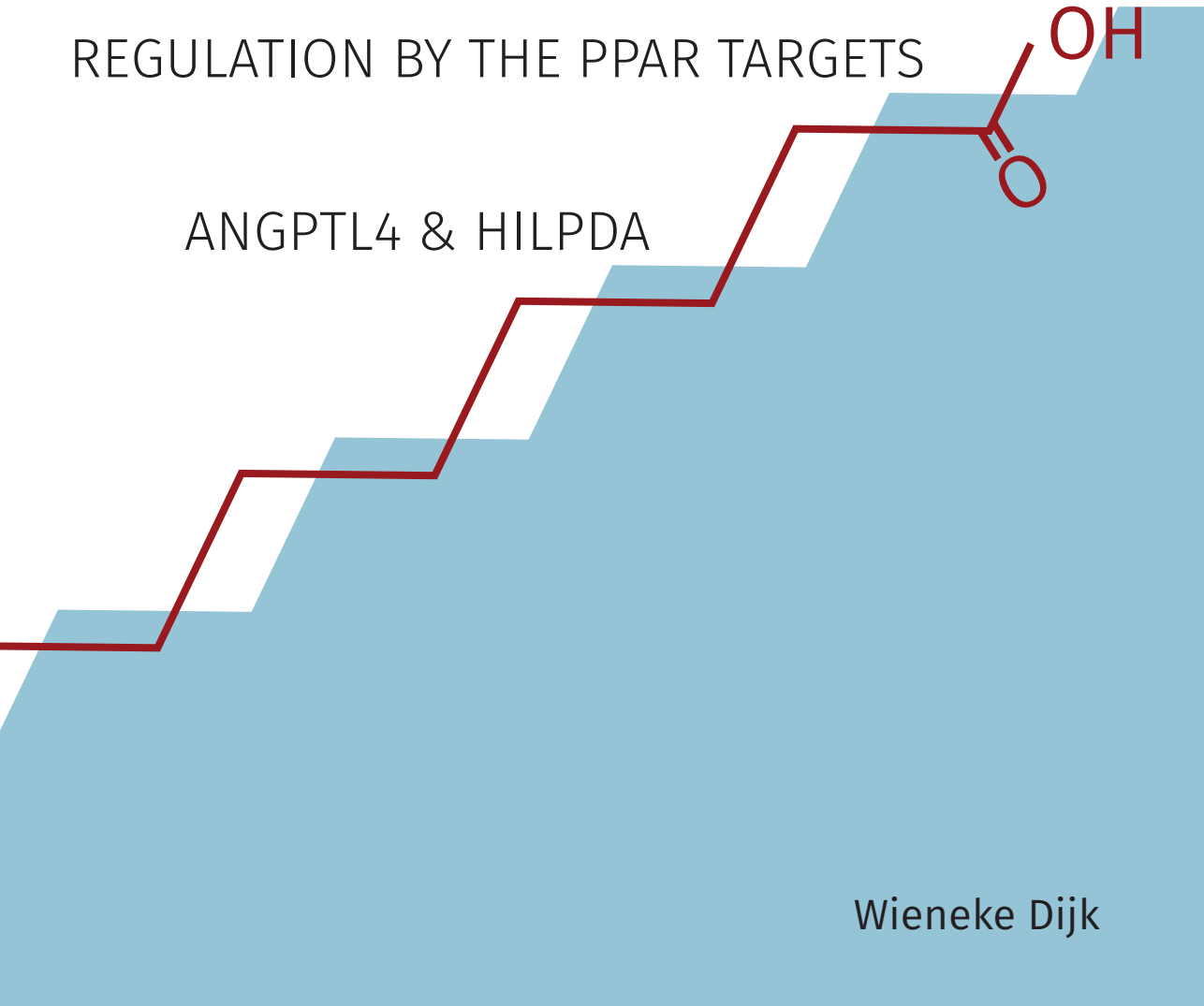
INTRACELLULAR & EXTRACELLULAR LIPOLYSIS

REGULATION BY THE PPAR TARGETS ANGPTL4 & HILPDA



Wieneke Dijk

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Wieneke Dijk

Thesis committee

Promotor

Prof. Dr. Ir. A.H. Kersten
Professor of Nutrition, Metabolism and Genomics
Wageningen University

Other Members

Prof. Dr. R.F. Witkamp, Wageningen University
Prof. Dr. F. Kuipers, University of Groningen
Dr J.A. Kuijvenhoven, University of Groningen
Dr G. Goossens, Maastricht University

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REGULATION BY THE PPAR TARGETS ANGPTL4 & HILPDA

Wieneke Dijk

Thesis

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Voor pap en mam

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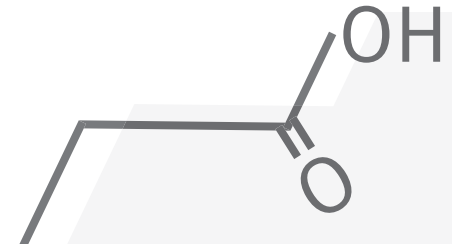
GENERAL INTRODUCTION



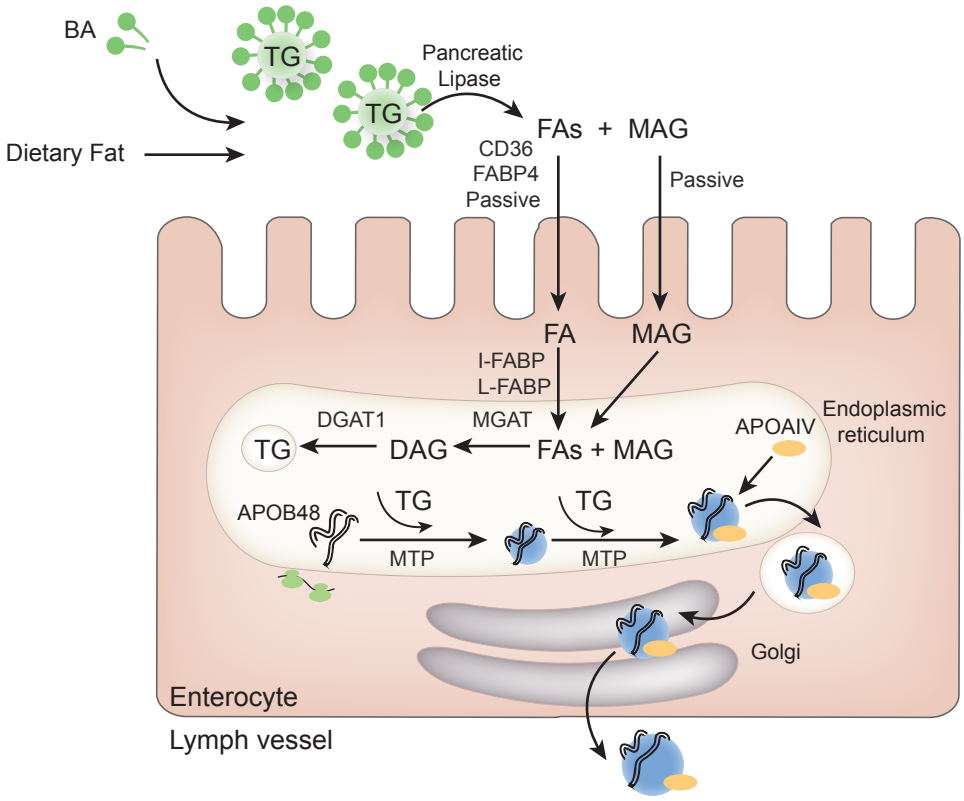


Figure 1: Triglyceride formation in the intestine.

Dietary triglycerides (TGs) are primarily digested in the small intestine, where bile and pancreatic juice are added. Bile is formed in and released from the gall bladder and primarily consists of bile acids, phospholipids, cholesterol and water (102). Bile acids emulsify dietary TGs to facilitate their digestion by enzymes present in the pancreatic juice, such as co-lipase and pancreatic lipase (11). Hydrolysis of TGs by pancreatic lipase results in the release of fatty acids and monoacylglycerol (MAG) (10). These fatty acids are taken up into enterocytes either by passive diffusion or by active transport *via* proteins such as fatty acid transport protein 4 (FATP4) and cluster of differentiation 36 (CD36) (17, 18). In contrast, MAG is primarily taken up by passive diffusion (19). After uptake, the fatty acids and MAGs are transported through the cytosol to the endoplasmic reticulum (ER) by intracellular fatty acid-binding proteins (FABPs) (20). Prior to re-esterification, the fatty acids are esterified to a CoA molecule to form a fatty acyl-CoA, a process that is catalysed by acyl-coA synthases (21). In the ER, the fatty acids and MAG are re-esterified into triglycerides by acyl-coA:monoacylglycerol acyltransferases (MGAT) to form diacylglycerol (DAG) and, subsequently, acyl-coA:diacylglycerol acyltransferases (DGAT) to form TGs (10, 22). The newly formed TGs accumulate between the ER membrane bilayer and can either partition into the cytosol to form cytosolic lipid droplets (LDs) or partition into the ER lumen to form luminal LDs used for short-term storage or chylomicron assembly (10, 15, 103). For transport in the circulation, the hydrophobic TGs are transferred onto apolipoprotein B48 (APOB48) in the inner leaflet of the ER by the enzyme microsomal triglyceride transfer protein (MTP) to form primordial, lipid-poor chylomicron particles (15, 16). APOB48, a protein encoded by the APOB gene, is the major nonexchangeable apolipoprotein present on the surface of chylomicrons (104). Primordial chylomicrons acquire more lipids and apolipoproteins such as APOA4 and are eventually secreted into the lymphatic system to enter the circulation at the level of the subclavian vein (10, 11, 15, 16).

EFFECTIVE PARTITIONING OF FATTY ACIDS

Fatty acids are indispensable for human physiology; they are integral components of our cell membranes, highly efficient energy substrates and potent signaling molecules. Due to their relatively high toxicity, fatty acids are mostly incorporated into triglycerides (TGs) to facilitate transport and storage (1, 2). Indeed, the majority of circulating fatty acids are found as TGs in so-called triglyceride-rich lipoproteins (TRLs), while most intracellular fatty acids are stored as TGs in cytoplasmic lipid droplets (3–5). The inability of TGs to move across cellular membranes necessitates that TGs are degraded to fatty acids *via* a process called lipolysis: the hydrolysis of TGs into three fatty acids and a glycerol molecule by lipases (6, 7). Lipolysis occurs at multiple locations: in the intestinal tract to degrade dietary TGs to fatty acids for uptake by the enterocytes, intravascularly to liberate fatty acids from TRLs for uptake by the underlying cells, and intracellularly to release fatty acids from lipid droplets for secretion into the circulation or usage as fuel (2, 8). To assure that fatty acid substrates are efficiently partitioned in response to metabolic demand, the rates of extracellular (gastro-intestinal and intravascular) lipolysis and intracellular lipolysis need to be tightly and dynamically controlled. Indeed, a mismatch between the rates of lipolysis and the usage of fatty acids by tissues can lead to ectopic lipid accumulation and dyslipidemia, important features of metabolic disorders such as obesity and cardiovascular disease (2, 9). However, despite the significance of proper regulation of lipolysis for metabolic health, the underlying molecular mechanisms are far from being completely understood.

EXTRACELLULAR LIPOLYSIS

Gastro-intestinal lipolysis and TRL formation

The majority of the fatty acids in our body are derived from TGs that are present in our diet. Degradation of dietary TGs to fatty acids and monoacylglycerol (MAG) starts in the mouth and stomach, but primarily takes place in the small intestine, where pre-digested food is combined with bile and pancreatic juice (8). Bile acids present in the bile emulsify dietary TGs to facilitate their hydrolysis by pancreatic lipase (10, 11). The activity of pancreatic lipase is extensively regulated by bile acids, calcium and the amount and type of dietary fat, but also by the protein Angiopoietin-like 4 (ANGPTL4) that is secreted by the intestine (12–14). The exact nature of the crosstalk between intestinal proteins and pancreatic lipase activity remains, however, ill-defined. Upon absorption into enterocytes, the fatty acids and MAGs are transported to the endoplasmic reticulum (ER), re-esterified to form TGs and transferred onto apolipoprotein B48 (APOB48) to form so-called chylomicrons, intestine-derived TRL particles (10, 15–22) (see **Figure 1** for further details). Chylomicrons mature by further lipidation and by the incorporation of exchangeable apolipoproteins such as APOA4. They subsequently exit the enterocyte and enter the circulation *via* the lymphatic system (10, 11, 15, 16) (**Figure 1**).

Whereas the intestine is responsible for the incorporation of exogenous fatty acids into TRLs, the liver incorporates endogenous TGs into TRLs called very low-density lipoproteins (VLDLs). Similar to chylomicron formation in the intestine,

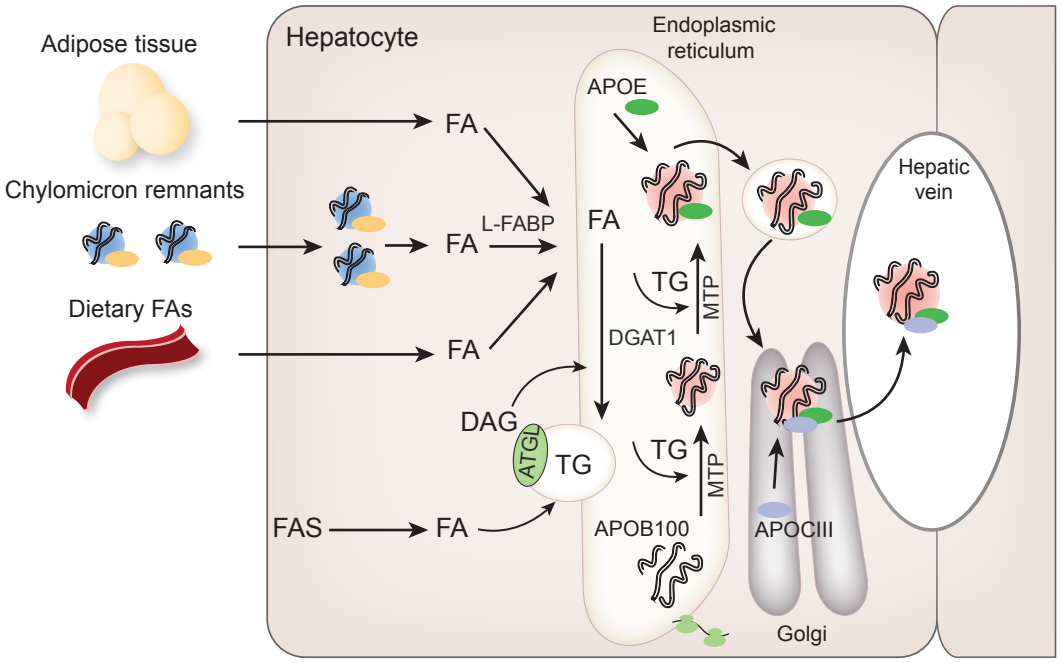


Figure 2: Triglyceride formation in the liver

For the formation of very low-density lipoproteins (VLDLs), the liver synthesizes triglycerides (TGs) by re-esterification of free fatty acids that are derived from multiple sources: 1) the circulation; albumin-bound fatty acids released from adipocytes or chylomicron remnants taken up by the liver, 2) the diet; dietary fatty acids absorbed in the intestine and transported through the portal vein to the liver, and 3) the liver; from storage or endogenous fatty acid synthesis (FAS) (3, 23). Exogenously derived fatty acids are preferentially incorporated into VLDLs as opposed to *de novo* synthesized fatty acids (23). The pathways of TG re-esterification in liver mimic the pathways in the intestine, as described in **Figure 1** (23, 25). However, exogenously derived fatty acids can also be directly esterified by the enzyme acyl-coA:diacylglycerol acyltransferase 1 (DGAT1) onto diacylglycerols (DAG) liberated from cytosolic lipid droplets (23, 105). Liver TGs are transferred onto APOB100 in the endoplasmic reticulum (ER) by microsomal triglyceride transfer protein (MTP) to form a primordial, lipid-poor VLDL particle (26). Maturing VLDLs continue to acquire TGs and exchangeable apolipoproteins such as APOE and APOC3 in the ER and Golgi, after which the mature VLDLs are secreted into the hepatic vein (3, 27).

the formation of VLDLs heavily relies on the availability of hepatic TGs. The liver synthesizes TGs by re-esterification of fatty acids from multiple sources: 1) the circulation; albumin-bound fatty acids released from adipocytes by intracellular lipolysis or chylomicron remnants, 2) the diet; dietary fatty acids transported through the portal vein to the liver, and 3) the liver; stored or endogenously synthesized fatty acids (3, 23, 24) (**Figure 2**). In general, the processes of VLDL formation in the liver closely resemble those of chylomicron formation in the intestine, except that in the liver the full-length APOB (APOB100) protein and not the truncated APOB48 is lipidated (23, 25, 26) (see **Figure 2** for further details). VLDLs mature by acquiring additional TGs and exchangeable apolipoproteins such as APOE and APOC3 in the ER and Golgi before being secreted into the hepatic vein (3, 27) (**Figure 2**). Upon arrival in the

circulation, mature chylomicrons and VLDLs gain further exchangeable apolipoproteins such as APOC2, APOC3 and APOE (11). Acquisition of APOC2 promotes the intravascular lipolysis of TRLs by the enzyme lipoprotein lipase (LPL) that is present on the surface of the capillary endothelium in multiple organs, including adipose tissue and heart (28, 29).

Intravascular lipolysis by LPL

Similar to the importance of pancreatic lipase for gastro-intestinal lipolysis, LPL is pivotal for the intravascular lipolysis of TRLs, as underscored by the severe chylomicronaemia observed in patients with complete loss-of-function mutations in the *LPL* gene (30). Similarly, common loss-of-function and gain-of-function variants in the *LPL* gene are responsible for modest increases or decreases in plasma TG levels, respectively, in the general population (31). Furthermore, the perturbed activity of LPL has been repeatedly associated with a dysregulation of insulin action and energy homeostasis and with an increased risk for cardiovascular disease (31–33).

I. From translation to endothelium

LPL hydrolyses circulating TGs on the surface of the capillary endothelium, but is primarily expressed by the underlying parenchymal cells (28, 34). Following transcription, two critical steps in the ER permit LPL to attain catalytic activity: asparagine-linked glycosylation and LPL dimer assembly (35–39) (**Figure 3**). Asparagine-linked glycosylation of LPL *via* co-translational transfer of oligosaccharide chains high in mannose residues promotes the proper folding of LPL and targets LPL for secretion (35–37, 40). Assembly of LPL into a homodimer of two head-to-tail connected 55 kDa subunits is chaperoned by lipase maturation factor 1 (LMF1), a multi-pass transmembrane protein that in complex with Sel-1 suppressor of lin-12-like protein (Sel1L) stabilizes the LPL homodimers and controls their ER exit (38, 39, 41). Upon translocation from the ER to the Golgi, the high-mannose oligosaccharides on LPL are trimmed and sequentially replaced by more complex oligosaccharides to form mature LPL (35–37) (**Figure 3**). From the *trans*-Golgi, mature LPL is either transported to the cell surface and secreted or targeted for degradation (**Figure 3**). Pulse-chase experiments have indicated that up to 80% of newly synthesized LPL is directed towards intracellular degradation, although the stimuli that target LPL for degradation remain largely unknown (42, 43). Proteins that have been proposed to play a role in the intracellular trafficking of LPL include ANGPTL4, sortilin and sortilin-related receptor (SORLA), but their exact functions have remained elusive (44–46). After secretion, LPL is bound to heparan sulphate proteoglycans (HSPGs) on the cell surface, from where LPL can be internalized for degradation or transported to the endothelium by glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) (47–49). Endothelial GPIHBP1 avidly binds to LPL in the sub-endothelial spaces and transports LPL to the capillary lumen, where it continues to bind and stabilize LPL (48, 49). Indeed, unless stabilized by factors such as LMF1, SEL1L, HSPGs or GPIHBP1, the LPL homodimer rapidly dissociates and loses its catalytic activity (39, 50).

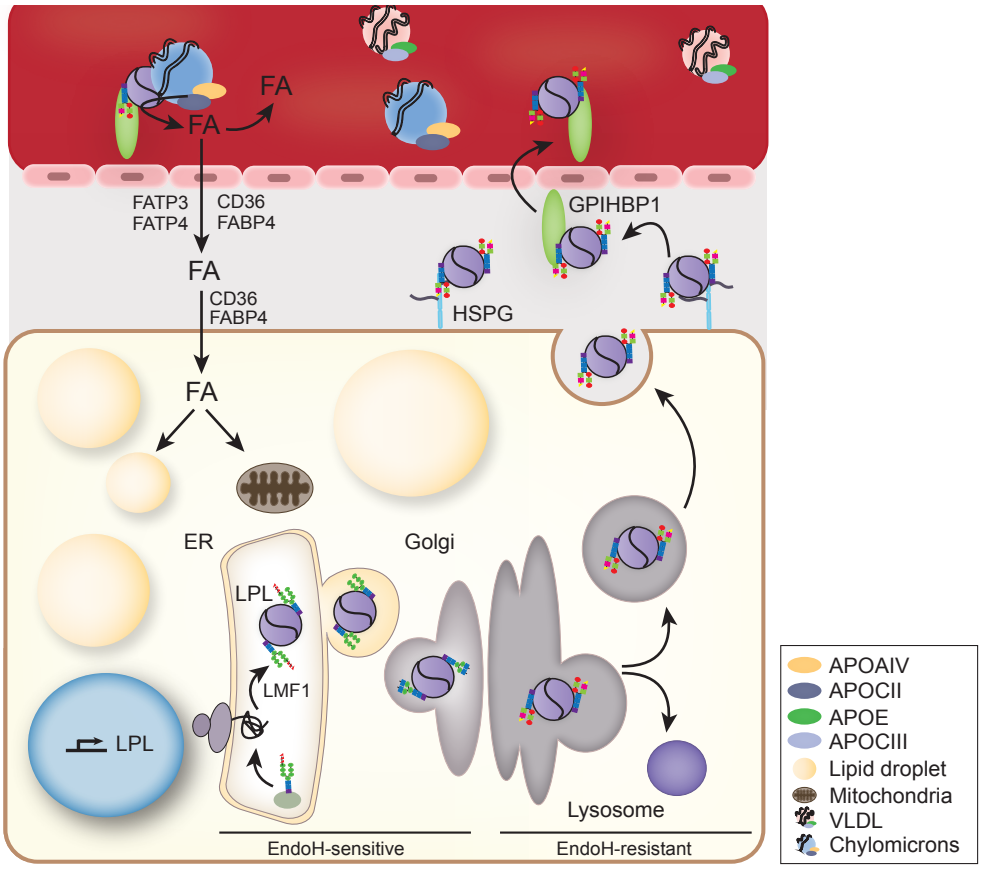


Figure 3: LPL-mediated hydrolysis of circulating TG.

In the endoplasmic reticulum (ER) of parenchymal cells, lipoprotein lipase (LPL) attains catalytic activity by asparagine-linked glycosylation and dimer assembly, a process chaperoned by lipase maturation factor 1 (LMF1) (35–39). Upon translocation from the ER to the Golgi, the high-mannose oligosaccharides on LPL are trimmed and sequentially replaced by more complex oligosaccharides (35–37). Oligosaccharides high in mannose residues are susceptible for cleavage by the enzyme EndoH (EndoH-sensitive), while more complex oligosaccharides cannot be cleaved by EndoH (EndoH-resistant). Upon acquisition of complex oligosaccharides, LPL is transported either to the cell surface for secretion or targeted for degradation. Secreted LPL is bound to heparan sulphate proteoglycans (HSPGs) on the cellular surface (47). Endothelial-expressed glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) avidly binds LPL in the subendothelial spaces and transports LPL to the capillary lumen, where it continues to bind and stabilize LPL (48, 49). Upon hydrolysis of chylomicrons or very low-density lipoproteins (VLDLs) by LPL, the resultant fatty acids have multiple fates. Dependent on the tissue, the fatty acids are transported to the underlying parenchymal cells for either storage in lipid droplets or oxidation in mitochondria or, alternatively, lost in the venous blood in a process called 'fatty acid spillover' (74). Endothelial fatty acid transport is mediated by FATP3 and FATP4, as well as CD36 and FABP4 (69, 75–77).

II. Regulation of LPL activity

Continuous changes in metabolic demand require that the partitioning of circulating TGs and, accordingly, the levels of tissue LPL activity are highly dynamic. For example, during prolonged deprivation of food, fuel utilization shifts towards the exclusive utilization of TGs. As a result, the activity of LPL in heart significantly increases to assure that an adequate amount of fuel is delivered to the heart, while the activity of LPL in white adipose tissue (WAT) decreases (51–53). Consequently, the activity of LPL is tightly regulated at the transcriptional, translational and post-translational levels in a tissue-specific manner. At the mRNA level, LPL is controlled by the peroxisome proliferator-activated receptor (PPAR) family of transcription factors (54). PPARs are a family of nuclear hormone transcription factors that are primarily activated by fatty acids and of which three isoforms have been identified: PPARα, PPARδ and PPARγ (55). Other physiologically relevant factors that impact LPL transcription include insulin, glucocorticoids and β-adrenergic stimulation (28, 56). However, despite minor regulation at the transcriptional and translational level, LPL activity is primarily regulated at the post-translational level.

Proteins that regulate LPL activity at the post-translational level can be roughly divided into two groups: 1) the exchangeable apolipoproteins APOC1, APOC2, APOC3, APOA5 and APOE and 2) members of the angiopoietin-like protein family, specifically ANGPTL3, ANGPTL4 and ANGPTL8 (29, 57–60). Of the APOC proteins, APOC1, APOC3, and APOE appear to negatively regulate LPL activity, while APOC2 and APOA5 potently stimulate endothelial LPL activity at physiological levels (29, 57). Indeed, loss-of-function mutations in the APOC2 and APOA5 genes result in a pronounced hyperchylomicronaemia (29, 57). Despite their impact on LPL activity and TRL clearance, however, it is unlikely that the circulating apolipoproteins are responsible for the tissue-specific changes in LPL activity that are observed during different physiological conditions.

Members of the angiopoietin-like protein family are secreted glycoproteins that share a common architecture (60). ANGPTL3, ANGPTL4 and ANGPTL8 potently impact plasma lipid levels by inhibiting the activity of LPL and other related extracellular lipases, such as pancreatic lipase, endothelial lipase and hepatic lipase (13, 60–65). In contrast to the apolipoproteins, the expression of several ANGPTL proteins is highly responsive to changes in metabolic demand. Indeed, the upregulation of ANGPTL4 (originally called fasting-induced adipose factor) expression in WAT is responsible for the reduction of LPL activity levels in WAT upon fasting (52, 66). In contrast, the expression of ANGPTL8 in WAT is increased upon refeeding, which has led to the suggestion that ANGPTL8, in collaboration with ANGPTL3, assures the replenishment of adipose tissue stores during refeeding by inhibiting LPL activity specifically in oxidative tissues (67). In **Chapter 2** & **Chapter 3** of this thesis, the mechanisms by which the ANGPTL proteins influence plasma lipid levels and LPL activity are more elaborately described.

A specific physiological condition that dramatically impacts tissue energy demands and lipid distribution is cold exposure. During cold exposure, brown adipose tissue (BAT), the main metabolic organ dedicated to the generation of heat, is activated to assure the maintenance of core body temperature. Activation of BAT by

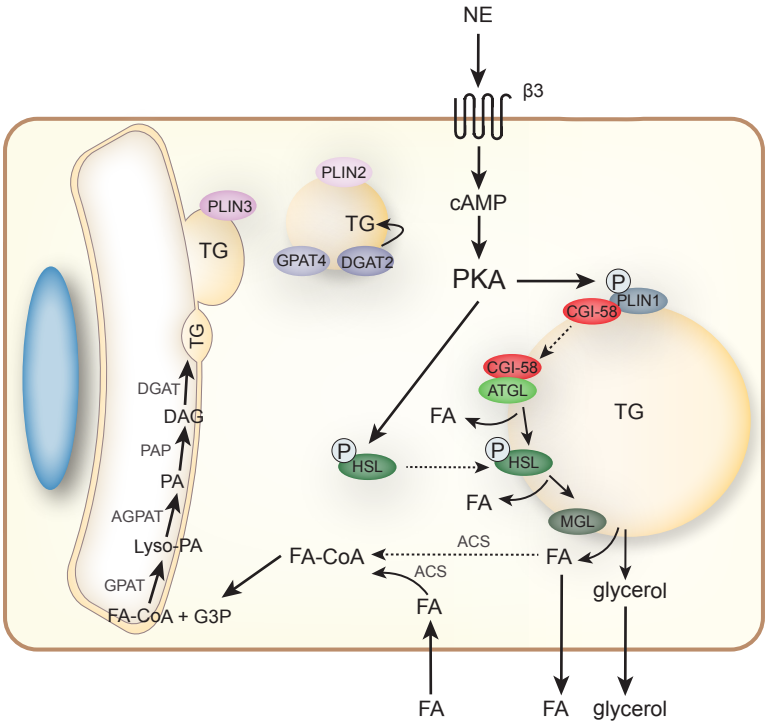


Figure 4: Pathways of lipid droplet biogenesis & intracellular lipolysis in adipocytes.

To generate the triglycerides (TGs) required for lipid droplet formation, exogenous or endogenous fatty acids need to be activated by esterification to a CoA molecule to form fatty acyl-CoAs, a process catalysed by acyl-coA synthases (ACS) (21). Upon activation, *de novo* TG synthesis occurs by re-esterification of fatty acyl-CoAs to glycerol-3-phosphate (G3P) via the G3P-pathway. First, G3P acyltransferase (GPAT) combines a fatty acyl-CoA to G3P to form lysophosphatidic acid (lyso-PA), where after 1-acyl-G3P acyltransferase (AGPAT) adds another fatty acyl-CoA to form phosphatidic acid (PA). Next, phosphatidic acid phosphatase (PAP) removes a phosphate group and acyl-coA:diacylglycerol acyltransferase (DGAT) adds another fatty acyl-CoA to form TGs (80, 81). Alternatively, TGs may be synthesized from fatty acyl-CoAs and DAGs by DGATs or from re-esterification of fatty acyl-CoAs to MAG by MGATs and DGATs (10, 23, 79, 83). Condensation of fatty acyl-CoAs with cholesterol, a step catalysed by acyl-CoA:cholesterol acyltransferases (ACATs), yields cholesterylesters, the major neutral lipid component of lipid droplets besides TGs (79). Newly formed TGs and cholesterylesters accumulate in the inner leaflet of the ER bilayer to form blisters that eventually bud off to form small cytosolic lipid droplets (79, 82). Nascent lipid droplets may become larger *via* fusion, diffusion-mediated transfer of core TGs, or local synthesis of additional TGs on the lipid droplet surface by the acquisition of enzymes involved in TG synthesis from the ER such as GPAT4 and DGAT2 (85, 86). To release fatty acids from lipid droplets, catecholamines such as norepinephrine bind to the β3-adrenergic receptors on the cell surface of adipocytes and activate protein kinase A (PKA) *via* an intracellular signalling cascade that involves cyclic AMP (cAMP) (91). PKA promotes lipolysis by phosphorylating multiple proteins involved in lipolysis, including hormone-sensitive lipase (HSL) and perilipin 1 (PLIN1) (90, 91). PLIN1 normally binds comparative gene identification-58 (CGI-58), a potent stimulator of adipose triglyceride lipase (ATGL) activity, but upon phosporylation PLIN1 releases CGI-58 to allow for ATGL activation (92). Similarly, phosphorylation of HSL results in translocation of HSL to the lipid droplet. Next, ATGL, HSL and monoglyceride lipase (MGL) act in consecutive steps to release one fatty acid each (90). The resultant fatty acids can either be secreted into the circulation or re-esterified by ACS to form fatty acyl-CoAs that are reincorporated into lipid droplets.

β-adrenergic signaling results in the combustion of fatty acids and subsequent heat generation *via* the uncoupling of ATP production from mitochondrial respiration by the protein uncoupling-protein 1 (UCP1) (68). To replenish intracellular energy stores, the uptake of glucose, fatty acids and TG-derived fatty acids by BAT is dramatically increased upon cold exposure. Indeed, the activity of LPL in BAT significantly increases during cold exposure, although it is currently unclear what mechanisms promote the increase in LPL activity during cold exposure and whether the ANGPTL proteins are implicated (68–71).

From intravascular lipolysis to fatty acid storage or oxidation

For intravascular lipolysis to occur, chylomicrons and VLDLs must first dock to the capillary endothelium *via* a process called margination. Multiple lines of evidence suggest that the combined actions of LPL and GPIHBP1 are essential for intravascular TRL margination. Studies in mice with a tissue-specific expression of LPL indicated that the degree of margination reflects the amount of LPL present on the endothelium (72). Similarly, the absence of TRL margination in *Gpihbp1*$^{-/-}$ mice overexpressing endothelial LPL indicated an essential role for GPIHBP1 in margination (73). Upon TRL margination and intravascular hydrolysis of TGs by LPL, the resultant fatty acids have multiple fates. Dependent on the tissue, the fatty acids are transported through the endothelium to the underlying parenchymal cells for either storage or oxidation, or lost in the venous blood in a process called 'fatty acid spillover' (74) (**Figure 3**). While it is clear that fatty acid spillover significantly contributes to circulating fatty acid levels, the mechanisms underlying fatty acid spillover have remained enigmatic (74). Part of the ambiguousness stems from the lack of insight into the mechanisms responsible for the endothelial transport of fatty acids. Only recently, the fatty acid transport proteins 3 and 4 (FATP3 and FATP4), as well as cluster of differentiation 36 (CD36) and fatty acid binding protein 4 (FABP4) were identified as mediators of endothelial fatty acid transport (69, 75–77). Furthermore, a recent study identified two transcription factors, mesenchyme homeobox 2 and transcription factor 15, that drive the expression of CD36 and, surprisingly, LPL in endothelial cells to promote and facilitate the uptake of circulating and hydrolyzed fatty acids (78).

INTRACELLULAR LIPOLYSIS

Lipid droplet biogenesis

Upon arrival in the parenchymal cells, part of the fatty acids is re-esterified and incorporated into cytoplasmic lipid droplets. In eukaryotes, lipid droplet formation occurs in the ER, the location of TG and cholesterylester synthesis, in a process that closely resembles chylomicron and VLDL formation. First, fatty acids are re-esterified either to glycerol-3-phosphate (G3P) to form TGs *via* the G3P-pathway or to cholesterol to form cholesterylesters, the major neutral lipid component of lipid droplets besides TGs (79–81) (see **Figure 4** for further details). Newly formed TGs and cholesterylesters accumulate at low concentrations in the inner leaflet of the ER bilayer. With increasing concentrations, 'blisters' that contain higher concentrations of TGs and cholesterylesters form in the bilayer and subsequently bud off to form small cy-

tosolic lipid droplets (79, 82) (**Figure 4**). It has been suggested that various proteins, such as Seipin, the fat storage–inducing transmembrane protein (FITM/FIT) and members of the perilipin (PLIN) family facilitate, but are not essential for the budding and the subsequent formation of lipid droplets (79, 83). In fact, PLINs may have specific spatial and temporal functions during lipid droplet formation, with PLIN3 primarily coating nascent lipid droplets, PLIN2 coating intermediate-sized lipid droplets and PLIN1, in adipocytes, coating large cytosolic lipid droplets (84). Nascent lipid droplets may enlarge *via* two specific mechanisms; 1) by the local synthesis of TGs on the lipid droplet surface *via* the acquisition of enzymes involved in TG synthesis, such as G3P acyltransferase (GPAT4) and acyl-coA:diacylglycerol acyltransferase 2 (DGAT2), from the ER (85, 86) or 2) by fusion with another lipid droplet or the diffusion-mediated transfer of TGs from one lipid droplet to another (87). For example, cell death–inducing DFF45-like effector C (CIDEC/FSP27) has been suggested to promote lipid droplet growth by stimulating the transfer of TGs from smaller to larger lipid droplets (88).

From intracellular lipolysis to fatty acid release or oxidation

Cytoplasmic lipid droplets are found in all metabolically active tissues, including heart, skeletal muscle, liver, BAT and WAT, but fulfil different functions dependent on the tissue. For example, fatty acids generated from intracellular lipolysis of WAT lipid droplets are released into the circulation during fasting conditions to directly fuel tissues such as the heart or to be incorporated into VLDLs in the liver. In contrast, fatty acids generated from lipolysis in other tissues are not released into the circulation, but are used by these tissues for lipid synthesis or fatty acid oxidation. It is of interest that accumulating evidence suggests that in oxidative tissues such as muscle and heart most exogenous fatty acids cycle through the process of re-esterification at least once before entering the mitochondria for oxidation (81, 89).

Despite the differences in lipid droplet function, the main steps of intracellular lipolysis are similar between all tissues and include three lipases that act in consecutive steps to release one fatty acid each: adipose triglyceride lipase (ATGL), the rate-limiting enzyme, hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) (90). In the adipose tissue, lipolysis is initiated by a reduction in insulin levels and an increase in catecholamine levels (91). Catecholamines bind to β-adrenergic receptors on the cell surface of adipocytes and activate protein kinase A (PKA) *via* an intracellular signalling cascade that involves cyclic AMP (91). PKA promotes lipolysis by phosphorylating multiple proteins, including HSL and PLIN1 (90, 91) (**Figure 4**). PLIN1 phosphorylation releases comparative gene identification-58 (CGI-58/ABHD5), a protein that binds and potently stimulates ATGL activity (92). Other recently identified regulators of adipocyte lipolysis include members of the PLIN family, the CIDE protein family and G0/G1 switch gene 2 (G0S2), although their exact functions in lipolysis are far from being understood (6, 93–98).

The importance of the proper regulation of intracellular lipolysis is illustrated by the numerous human and mouse studies in which key regulatory enzymes are disrupted. For example, loss-of-function mutations in the ATGL stimulating *ABHD5* (*CGI-58*)result in dysfunctional lipolysis, cellular lipid accumulation and neutral lipid storage disease (99, 100). However, dysregulation of intracellular lipolysis is also

present in the general population, as exemplified by the increased basal rates of intracellular lipolysis in obese subjects (101). Overall, intracellular lipolysis is remarkably complex and substantial further investigation is required to identify the specific functions of the increasing number of regulatory proteins.

OUTLINE OF THIS THESIS

During changing metabolic demand, the dynamic regulation of extracellular and intracellular lipolysis assures the effective partitioning of lipid fuel. The main aim of this thesis was **to increase our understanding of the molecular mechanisms that underlie the regulation of extracellular and intracellular lipolysis.** In the first part, the focus lies on the regulation of extracellular lipolysis by the ANGPTL proteins, in particular. As briefly noted above, the ANGPTL proteins regulate extracellular lipolysis by inhibiting LPL and other members of the extracellular lipase family. A contemporary overview of the role of the ANGPTL proteins in lipid metabolism is provided in **Chapter 2**. In **Chapter 3**, ANGPTL4 and ANGPTL4-mediated regulation of LPL activity are discussed in further detail. In **Chapter 4**, we extend our understanding of the function of ANGPTL4 by demonstrating that ANGPTL4 is indispensable for the proper distribution of TRL-derived fatty acids during cold exposure. By employing *Angptl4*$^{-/-}$, wild-type, and *Angptl4*-Tg mice, we elegantly show that the reduction in ANGPTL4 expression in BAT facilitates the increase in BAT LPL activity that is observed during sustained cold exposure.

Although ANGPTL4 has been known to inhibit LPL for nearly 15 years, the location and the mechanisms by which ANGPTL4 inhibits LPL have remained largely elusive. In **Chapter 5**, we show that ANGPTL4 inhibits LPL intracellularly in adipocytes, by demonstrating that LPL is more secreted from adipocytes of mice without *Angptl4* than from wild-type adipocytes. In **Chapter 6**, we further extend these observations and propose that ANGPTL4 might inhibit intracellular LPL by promoting the cleavage of LPL by proprotein subtilisin/kexins (PCSKs) in the adipocytes.Besides regulating LPL activity, ANGPTL4 also potently impacts the activity of pancreatic lipase in the gastro-intestinal tract. Therefore, in **Chapter 7**, we switch to gastro-intestinal lipolysis and investigate the role of intestinal ANGPTL4. We show that intestinal ANGPTL4, possibly *via* its impact on pancreatic lipase, regulates intestinal bile acid uptake and is associated with an altered gut microbial composition.

In the second part of this thesis, we focus on the regulation of intracellular lipolysis and on the newly-identified PPAR-target gene hypoxia-inducible lipid droplet-associated (HILPDA), in particular. In **Chapter 8**, we show that overexpression of HILPDA in adipocytes modestly inhibits intracellular lipolysis, whereas knockdown of HILPDA in adipocytes does not affect lipolytic rates. Furthermore, we demonstrate that the adipocyte-specific deletion of *Hilpda* in mice does not impact plasma non-esterified fatty acid levels or other relevant metabolic parameters upon fasting, cold exposure or pharmacologic β-adrenergic stimulation. Our data thus not support a major role for HILPDA in the regulation of adipocyte lipolysis, in contrast to a previous study that identified a role for HILPDA in the regulation of intracellular lipolysis in the liver. Finally, in **Chapter 9**, the data presented in this thesis are integrated with current literature and elaborately discussed.

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REGULATION OF LIPID METABOLISM BY ANGIOPOIETIN-LIKE PROTEINS

Wieneke Dijk
Sander Kersten

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ABSTRACT

Purpose of Review

The angiopoietin-like proteins (ANGPTLs) 3, 4 and 8 have emerged as key regulators of plasma lipid metabolism by serving as potent inhibitors of the enzyme lipoprotein lipase. In this review, we provide an integrated picture of the role of ANGPTL3, ANGPTL4 and ANGPTL8 in lipid metabolism by focusing on their impact on lipoprotein lipase activity and plasma triglyceride clearance during physiological conditions such as fasting, refeeding, exercise and cold exposure.

Recent findings

Upon refeeding, circulating ANGPTL3 and ANGPTL8 promote the replenishment of white adipose tissue depots by specifically inhibiting lipoprotein lipase activity in oxidative tissues. During exercise and cold exposure, ANGPTL4 represses local lipoprotein lipase activity to assure that plasma triglycerides are specifically shuttled to exercising muscle and BAT, respectively. Overall, ANGPTL4 is the central component of a fatty acid-driven feedback mechanism that regulates plasma triglyceride hydrolysis and subsequent tissue fatty acid uptake in response to changes in lipid availability and cellular fuel demand.

Summary

ANGPTL3, ANGPTL4 and ANGPTL8 together ensure that triglycerides from triglyceride-rich lipoproteins are adequately distributed during different physiological conditions. The impact of the ANGPTLs on plasma lipid levels has led to scrutiny of ANGPTLs as therapeutic targets for dyslipidemia.

INTRODUCTION

The angiopoietin-like (ANGPTL) proteins (ANGPTL1-8) are secreted glycoproteins composed of an N-terminal coiled-coil domain and a fibrinogen-like C-terminal domain. Despite their common architecture, the eight family members carry distinct physiological functions, with suggested roles in angiogenesis, expansion of stem cells, inflammation, tissue remodeling and lipid metabolism (1, 2). A shared feature of ANGTPL3, ANGPTL4 and ANGPTL8 is their involvement in lipoprotein metabolism. Indeed, genetic studies have revealed pronounced effects of common variants in the *ANGPTL3*, *ANGPTL4* and *ANGPTL8* genes on plasma lipid levels (1, 3, 4). Functional studies have indicated that ANGPTL3, ANGPTL4 and ANGPTL8 influence plasma lipid levels by inhibiting the activity of extracellular lipases, including the structurally related lipoprotein lipase (LPL), hepatic lipase, endothelial lipase and pancreatic lipase (4–9). In this review, we aim to provide an integrated picture of the impact of the angiopoietin-like proteins on lipid metabolism, with special emphasis on the functional roles of ANGPTL3, ANGPTL4 and ANGPTL8 in governing the activity of LPL during physiological conditions such as fasting, refeeding, exercise and cold exposure.

ANGPTL3

ANGPTL3 was linked to lipid metabolism *via* identification of an *Angptl3*-mutant KK/San mouse strain characterized by abnormally low plasma triglycerides (10). Since then, biochemical and animal studies have convincingly established that ANGPTL3 serves as an inhibitor of LPL and thereby reduces plasma triglyceride clearance, partially explaining the hypolipidemic phenotype of the KK/San mice (6, 10–12). Consistent with these findings, genome wide association studies as well as dedicated studies of common variants in and near the *ANGPTL3* gene locus have repeatedly associated *ANGPTL3* with plasma triglyceride and plasma HDL cholesterol levels (13–19). Furthermore, exome sequencing in patients with severe familial combined hypolipidemia—a condition characterized by low plasma LDL cholesterol, HDL cholesterol and triglyceride levels—revealed rare loss-of-function mutations in the *ANGPTL3* gene (20, 21). Affected individuals exhibit markedly elevated plasma LPL activity and LPL mass (20).

Besides inhibiting LPL, there is some evidence that ANGPTL3 also interferes with the activity of endothelial lipase (9, 22). Endothelial lipase mainly carries phospholipase activity and lowers plasma HDL cholesterol levels (23–25). Treatment of mice with a monoclonal antibody against ANGPTL3 significantly reduced plasma HDL cholesterol levels in wild-type but not endothelial lipase-deficient mice (22). In line with human genetic data and consistent with inhibition of endothelial lipase by ANGPTL3, several studies have reported a positive association between plasma ANGPTL3 levels and plasma HDL cholesterol levels in humans (9, 20, 23, 26, 27).

Even though ANGPTL3 potently inhibits LPL, the level of ANGPTL3 in plasma does not seem to correlate with plasma triglyceride concentrations (9, 26–28). A possible explanation for the apparent discrepancy is that the human plasma samples were collected in the fasted state (26, 27). It has been shown that differences in tissue LPL activity levels and VLDL-derived triglyceride uptake between wild-type and *Angptl3*$^{-/-}$ mice are most pronounced in the postprandial state, suggesting that

ANGPTL3 mainly affects LPL activity and plasma triglyceride levels shortly after feeding. Upon feeding, loss of *Angptl3* led to a significant increase in LPL activity in post-heparin plasma and in several tissues (12, 29). Intriguingly, however, the increase in LPL activity was accompanied by decreased post-prandial uptake of VLDL-derived triglycerides into WAT and increased uptake into heart, muscle and BAT (29). Based on these data, it was proposed that ANGPTL3 promotes the replenishment of WAT triglyceride stores upon refeeding by primarily inhibiting the uptake of circulating triglycerides into heart and BAT (29). These data underscore that measurement of LPL activity in post-heparin plasma or in tissue biopsies may not always give a proper impression of functional LPL activity at the endothelium (30). For now, the underlying mechanism for this tissue-specific action of ANGPTL3 remains unclear. One possibility is that it somehow has to do with the ill-defined interaction between ANGPTL3 and ANGPTL8. ANGPTL3 and ANGPTL8 have been suggested to interact to effectively inhibit LPL (4, 29). Clearly, additional studies are required to better understand the tissue-specific action of ANGPTL3.

As indicated above, carriers of *ANGPTL3* loss-of-function mutations have reduced LDL cholesterol levels (20, 21). In addition, a positive association was found between plasma ANGPTL3 and plasma LDL cholesterol (26). The findings have led to exploration of the potential mechanism underlying the relation between ANGPTL3 and LDL (20, 21, 26). Carriers of *ANGPTL3* mutations and mice treated with ANGPTL3-inactivating human monoclonal antibody display reduced VLDL secretion rates (20, 31). Lower VLDL secretion is likely caused by a reduced hepatic influx of fatty acids from the plasma due to absence of ANGTPL3-stimulated adipose tissue lipolysis (31, 32). However, inactivation of *Angptl3* specifically affected secretion of triglycerides and not of ApoB100 lipoproteins, ruling out decreased VLDL secretion as an explanation for reduced plasma LDL (20, 21, 31). Since the reduction in plasma LDL cholesterol levels in mice treated with anti-ANGPTL3 antibody was independent of LDLR, LRP1, APOE and Syndecan-1, it was suggested that ANGPTL3 may inhibit clearance of APOB-containing lipoproteins *via* inhibition of LPL (31). Indeed, LPL has been suggested to act as a molecular bridge between lipoproteins and the hepatocyte cell surface, which could promote clearance of VLDL remnants and reduce the fraction of VLDL that is converted to LDL (31, 33, 34). While the impact of ANGPTL3 on LDL cholesterol levels *via* LPL complements the established function of ANGPTL3 as an inhibitor of plasma triglyceride clearance, further studies should clarify how inhibition of LPL by ANGPTL3 impacts VLDL remnant clearance and plasma levels of LDL cholesterol. Furthermore, it remains to be determined how the role of ANGPTL3 in triglyceride distribution upon refeeding can be linked to its effect on LDL and HDL cholesterol levels, which are both not substantially altered upon refeeding (35). A final point of interest is the discrepancy between the stimulatory effect of ANGPTL3 on intracellular WAT lipolysis described in a number of studies and the purported inhibitory effect of ANGPTL3 on WAT lipolysis observed during the replenishment of WAT triglyceride stores upon refeeding (20, 29, 31, 32). Answers to these questions may provide further insight into the functioning of ANGPTL3.

ANGPTL4

ANGPTL4, originally referred to as fasting-induced adipose factor, is a glycosylated protein that is highly expressed in liver and adipose tissue and to a lesser extent in heart, skeletal muscle, intestine, and several other tissues (7, 36, 37). Expression of ANGPTL4 is sensitively regulated by the fatty acid-activated peroxisome proliferator-activated receptors (PPARs) (36, 38–40). Human and mouse genetic studies strongly support a role for ANGPTL4 in lipid metabolism *via* regulation of LPL activity (3, 12, 41–45). The E40K gene variant has repeatedly been associated with decreased plasma triglyceride and increased HDL cholesterol levels (3, 41–43). Genome-wide association studies have linked common variants near the *ANGPTL4* gene to HDL cholesterol levels, but so far no studies have linked common *ANGPTL4* gene variants to plasma triglyceride levels (14, 16).

The inhibitory effect of ANGPTL4 on LPL activity has been extensively documented in numerous *in vivo* and *in vitro* studies (5, 12). Several recent studies suggest that ANGPTL4 is the master regulator of plasma triglyceride metabolism during physiological conditions such as fasting, cold exposure and exercise (35, 37, 46). The data point to a scenario where ANGPTL4 is the central component of a feedback mechanism that regulates plasma triglyceride hydrolysis and subsequent tissue fatty acid uptake in response to changes in lipid availability and cellular fuel demand (35, 37, 40, 46, 47). According to this mechanism, *Angptl4* expression is induced by elevated plasma free fatty acid levels *via* PPARs, subsequently leading to inhibition of LPL and decreased plasma triglyceride-derived fatty acid uptake (40, 46). In WAT, induction of ANGPTL4 during fasting leads to repression of LPL activity, thereby preventing lipid storage and ensuring adequate provision of lipid fuels to other tissues (35). In skeletal muscle, *ANGPTL4* is induced by exercise *via* elevated free fatty acids specifically in the non-exercising muscle, leading to reduced local uptake of plasma triglyceride-derived fatty acids to spare for use by the exercising muscle (46). In exercising muscle, the free fatty acid-induced upregulation of ANGPTL4 is countered by AMPK-mediated downregulation, promoting the use of plasma triglycerides as fuel for active muscles (46). In BAT, ANGPTL4 is markedly suppressed by cold exposure *via* activation of AMPK, enhancing LPL activity and uptake of plasma triglyceride-derived fatty acids (37). By contrast, ANGPTL4 is induced by cold in WAT, thereby limiting LPL activity and prioritizing fuel provision to BAT to generate heat (37). In addition to being important for physiological regulation of plasma triglyceride metabolism, there is evidence that ANGPTL4 is also implicated in certain clinical forms of hypertriglyceridemia. Specifically, ANGPTL4 may mediate the hypertriglyceridemia of nephrotic syndrome (48) .

Despite the strong genetic and experimental evidence for a key role of ANGPTL4 in plasma triglyceride metabolism, most studies have failed to find a significant correlation between plasma ANGPTL4 and plasma triglyceride levels (26, 27, 49–53). This result is somewhat surprising but does not refute an important function of ANGPTL4 in human lipid metabolism. One possibility is that the lack of association of plasma ANGPTL4 levels with plasma triglycerides is explained by the inability of the employed enzyme-linked immunosorbent assays (ELISAs) to detect N-terminal ANGPTL4 (53, 54). An alternative explanation is that circulating ANGPTL4 is not

exclusively responsible for regulation of LPL activity (47, 55, 56). Indeed, recent studies are suggestive of a model in which ANGPTL4 may regulate LPL intracellularly and in the sub-endothelial space rather than along the capillary lumen (55, 57, 58). Both circulating triglyceride-rich lipoproteins and glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1)—the protein responsible for translocation of LPL to the endothelial cell surface—prevent inhibition of LPL activity by ANGPTL4 (56, 59, 60). Further studies should elucidate the exact molecular mechanism and location of inhibition of LPL by ANGPTL4.

In contrast to the clear inhibition of LPL, ANGPTL4 has not been reported to inhibit endothelial lipase, while the effects of ANGPTL4 on hepatic lipase have been contradictory (8, 12, 16). Remarkably, a recent study provided evidence that ANGPTL4 inhibits pancreatic lipase in the intestinal tract and suggested that ANGPTL4 acts as a gatekeeper in the regulation of intestinal lipid uptake to protect against enterocyte lipid overload (7). Expression of ANGPTL4 in the intestinal tract is sensitively stimulated by specific probiotic bacteria and short-chain fatty acids, while conventionalization of germ-free mice allegedly suppressed the expression of *Angptl4* in the intestine (61–67). Exactly how the microbial community affects intestinal *Angptl4* expression and how changes in intestinal *Angptl4* expression in turn affect intestinal lipid metabolism and the microbial community remains to be investigated.

ANGPTL8

In 2012, three research groups independently identified ANGPTL8—also referred to as RIFL, lipasin and betatrophin—as a novel lipid-modulating member of the ANGPTL family with a high homology to ANGPTL4 and ANGPTL3 (4, 68, 69). ANGPTL8 has the characteristic N-terminal coiled-coil domain required for the inhibition of lipase activity but lacks the C-terminal fibrinogen-like domain (4, 68, 69). *In vitro*, a clear dose-dependent inhibitory effect of recombinant ANGPTL8 on LPL activity was found (68). *ANGPTL8* and *ANGPTL3* likely have a shared origin, as both genes are located in introns of genes from the *DOCK* family (4, 68, 69). However, the tissue distribution of ANGPTL8 is more similar to ANGPTL4, with relatively high expression levels in liver and adipose tissue (4, 68, 69). In contrast to ANGPTL4, expression of *Angptl8* in liver and adipose tissue is highest in the (re)fed state (4, 69).

Similar to *ANGPTL3* and *ANGPTL4*, *ANGPTL8* was found to associate with plasma HDL cholesterol and triglyceride levels in a study that focused on low-frequency DNA sequence variants and rare alleles (3). In the Dallas Heart Study, the R59W gene variant was associated with reduced plasma LDL and HDL cholesterol levels but not triglycerides (4). Following these findings, multiple studies have tried to link plasma ANGPTL8 levels to various lipid and metabolic parameters. So far, the results have been contradictory. One serious concern is that the specificities of the ELISAs used to measure ANGPTL8 have been insufficiently established, casting doubt on the validity of the data (70–75).

Several studies in mice have demonstrated a pronounced effect of ANGPTL8 on LPL activity and plasma triglyceride levels, which is mainly evident in the fed state. Adenoviral-mediated overexpression of ANGPTL8 in liver significantly raised plasma triglyceride and free fatty acid levels (4, 68). Conversely, whole body knock-

out of *Angptl8* and antibody-mediated inactivation of ANGPTL8 in mice increased post-heparin LPL activity and lowered plasma triglyceride levels (76, 77). As briefly noted previously, ANGPTL8 and ANGPTL3 likely cooperate to regulate plasma triglyceride levels, which is largely based on the observation that adenoviral overexpression of ANGPTL8 increased plasma triglyceride levels in wild-type but not *Angptl3*$^{-/-}$ mice (4, 77). In line with a predominant role of ANGPTL8 in the fed state, loss of *Angptl8* significantly lowered the increase in VLDL-derived triglyceride uptake in adipose tissue upon refeeding, suggesting that ANGPTL8 promotes the postprandial flux of triglycerides into adipose tissue (77). Even though *Angptl8* expression is much higher in adipose tissue than in heart and muscle, loss of *Angptl8* or antibody-mediated inactivation of ANGPTL8 increased LPL activity in heart and muscle but not in WAT (4, 69, 76, 77). Another intriguing observation is that both feeding and cold exposure lead to a parallel increase in *Angptl8* expression and LPL activity in WAT and BAT, respectively (4, 35, 37, 69, 78). These data suggest that ANGPTL8 serves to fine-tune LPL activity and may be unable to overcome the dominant inhibitory effect on LPL activity by ANGPTL4. Alternatively, ANGPTL8 may mainly have an endocrine function. In agreement with that notion, ANGPTL8 has been reported to circulate as part of high molecular weight complexes in the blood together with ANGPTL3 (4, 77).

Besides regulating triglyceride metabolism, ANGPTL8 has also been linked to pancreatic β-cell function. Specifically, it was suggested that ANGPTL8 stimulates β-cell expansion, generating considerable excitement and turning ANGPTL8 into a promising therapeutic target for the treatment of diabetes (79). Several subsequent studies have failed to detect an effect of ANGPTL8 on glucose metabolism and/or β-cell expansion (77, 80–83). Indeed, faced with the overwhelming contradictory evidence, the initial authors acknowledged that the consequences of *Angptl8* deletion do not support the idea that ANGPTL8 is capable of inducing pancreatic β-cell proliferation (84).

INTEGRATED VIEW OF THE ROLE OF ANGPTL3, ANGPTL4 AND ANGPTL8 IN LIPID METABOLISM

The recent literature leads to the emergence of a collective picture in which ANGPTL3, ANGPTL4 and ANGPTL8 together ensure that circulating triglycerides are adequately distributed during different physiological conditions. Circulating ANGPTL3 and ANGPTL8 together promote the replenishment of WAT in the fed state by inhibiting LPL activity and subsequent uptake of circulating triglycerides into oxidative tissues (4, 29, 77). Conversely, ANGPTL4 prevents uptake of plasma triglycerides into WAT and other tissues in the fasted state, while at the same time stimulating adipose tissue lipolysis, thereby favoring the use of free fatty acids as fuel (35, 85) (**Figure 1**). During exercise and cold exposure, alterations in local expression of *Angptl4* and concomitant changes in LPL activity ensure that muscle and BAT receive an adequate supply of triglycerides, reflecting an overall role of ANGPTL4 to specifically shuttle plasma triglycerides to the tissues in need (35, 37, 46). Whether ANGPTL3 and ANGPTL8 impact the distribution of plasma triglycerides during exercise and cold exposure remains an open question. Nevertheless, it is likely that LPL activity levels in different tissues and during different physiological conditions reflect a balance

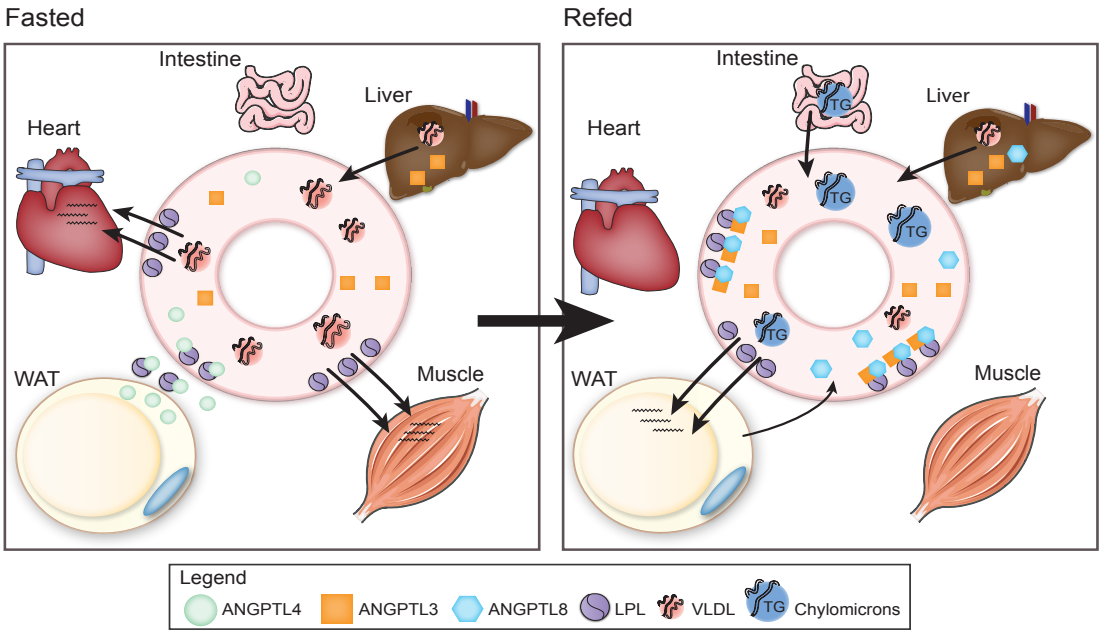

Figure 1: Hypothetical model of how angiopoietin-like 3 (ANGPTL3), angiopoietin-like 4 (ANGPTL4) and angiopoietin-like 8 (ANGPTL8) may ensure the adequate distribution of circulating triglycerides during fasting and refeeding conditions.

During fasting (*left panel*), the expression of *Angptl4* is increased in the white adipose tissue (WAT) to inhibit the activity of lipoprotein lipase (LPL) in WAT. This inhibition ensures that circulating triglycerides are hydrolyzed by LPL in muscle and heart and that the resultant fatty acids are subsequently taken up into heart and muscle. Upon refeeding (*right panel*), the expression of *Angptl4* is decreased in WAT, whereas the expression of the circulating factor *Angptl8* is increased in both liver and WAT. Together with circulating ANGPTL3, ANGPTL8 specifically inhibits the activity of LPL in oxidative tissues such as heart and muscle. This inhibition permits for the hydrolysis of circulating triglycerides by LPL in WAT and promotes the replenishment of WAT depots upon refeeding.

between the systemic effects of circulating ANGPTL3 and ANGPTL8 and the local effects of ANGPTL4. To what extent the fluctuations in LPL activity during different physiological conditions are primarily dictated by regulation of ANGPTLs or are also partially achieved *via* variation in the production of LPL-inhibiting or LPL-activating apolipoproteins such as APOCs, APOA5 or APOE, remains to be determined (86).

CONCLUSION

In conclusion, ANGPTL3, ANGPTL4 and ANGPTL8 play a crucial role in the regulation of lipid metabolism. The significant effects of the ANGPTL proteins on plasma lipid levels in combination with the emerging genetic evidence indicating that elevated plasma triglyceride levels represent a causal risk factor for coronary heart disease have sparked interest of pharmaceutical companies in ANGPTLs as drug targets (87). Recently, a human monoclonal antibody (REGN1500) was demonstrated to potently

bind and inhibit ANGPTL3 *in vivo*, lowering plasma lipid levels in dyslipidemic mice and cynomolgous monkeys (22). Furthermore, a conjugated anti-sense oligonucleotide against ANGPTL3 from IONIS pharmaceuticals successfully lowered plasma triglyceride levels in healthy individuals in phase I clinical trials and will be evaluated in a phase 1/2 clinical study (88). The position of ANGPTL4 as a pharmacological target remains uncertain, as loss of *Angptl4* and treatment of mice with anti-ANGPTL4 antibodies—despite effectively lowering plasma triglycerides—causes a pro-inflammatory state characterized by the appearance of giant cells in enlarged mesenteric lymph nodes and progressive illness of the mice involving chylous ascites, cachexia and death (45, 89). The current status of ANGPTLs as therapeutic targets for dyslipidemia warrants further investigation into the biology of ANGPTL3, ANGPTL4, and ANGPTL8. An important outstanding question relates to the nature of the cooperation between ANGPTL3 and ANGPTL8. In addition, the role of local versus systemic ANGPTL4 and ANGPTL8 in the regulation of extracellular lipase activity deserves further study. Resolving these questions and other questions postulated in this review would greatly benefit from the generation of double knock-out mice and tissue-specific knock-out mice of the three ANGPTLs. Together, these efforts should further elucidate the specific role of ANGPTL3, ANGPTL4 and ANGPTL8 in lipid metabolism and provide a solid foundation for further clinical research on ANGPTL-targeting therapeutics as treatment for dyslipidemia.

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REGULATION OF LIPOPROTEIN LIPASE BY ANGPTL4

Wieneke Dijk
Sander Kersten

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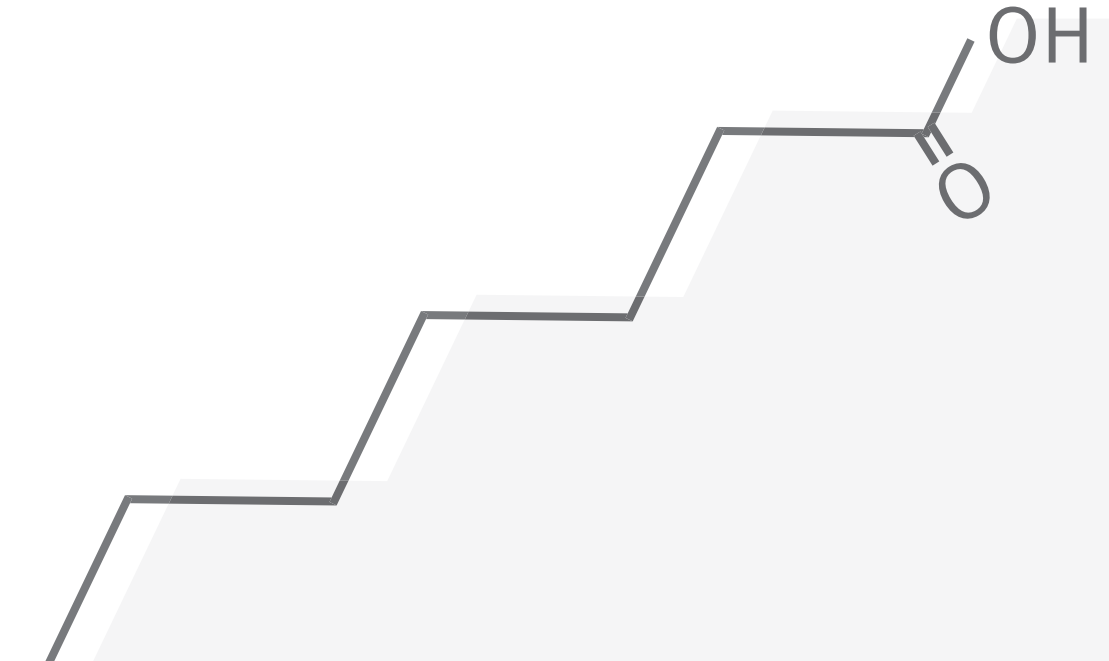

ABSTRACT

Triglyceride-rich chylomicrons and very-low density lipoproteins distribute fatty acids to various tissues by interacting with the enzyme lipoprotein lipase (LPL). The protein angiopoietin-like 4 (ANGPTL4) is under sensitive transcriptional control of fatty acids and the fatty acid-activated peroxisome proliferator activated receptors and its tissue expression largely overlaps with that of LPL. Growing evidence indicates that ANGPTL4 mediates the physiological fluctuations in LPL activity, including the decrease in adipose tissue LPL activity during fasting. This review focuses on the major ambiguities concerning the mechanism of LPL inhibition by ANGPTL4, as well as the physiological role of ANGPTL4 in lipid metabolism by highlighting its function in a variety of tissues, and uses this information to make suggestions for further research.

REGULATION OF PLASMA TRIGLYCERIDE CLEARANCE

Fatty acids (FA) are a principal fuel for numerous cell types and serve as substrate for energy storage in fat tissue. A major portion of FA taken up by cells is transported through the blood in the form of triglycerides (TG) as part of chylomicrons and very low-density lipoproteins (VLDL). Chylomicrons are formed from dietary TG and cholesterol in enterocytes and reach the bloodstream after passing through the splanchnic lymphatic system. VLDL are assembled from TG synthesized in liver and are secreted directly into the blood. In the circulation, chylomicrons and VLDL distribute TG to various tissues such as heart, adipose tissue and skeletal muscle by interacting with the enzyme lipoprotein lipase (LPL), which catalyzes the hydrolysis of TG in the capillary bed (1). The resulting free fatty acids (FFA) are taken up by adipocytes and (cardio) myocytes to be either stored or oxidized. In order to properly match uptake of plasma TG-derived FA to the needs of the underlying tissue, the activity of LPL is subject to multiple regulatory influences. A mismatch in regulation of TG uptake and usage by tissues can lead to cellular lipid overload and is an important feature of metabolic disorders such as obesity, diabetes type II and cardiovascular disease (2, 3).

Although LPL mRNA levels within a certain tissue do vary, the prevailing notion is that LPL activity is primarily governed *via* post-translational mechanisms. Three proteins that are able to inhibit LPL activity and thereby influence plasma TG values are angiopoietin-like 3 (ANGPTL3), angiopoietin-like 4 (ANGPTL4, FIAF), and angiopoietin-like 8 (ANGPTL8, betatrophin), all part of the family of angiopoietin-like proteins. Overexpression of all three proteins profoundly increases plasma TG levels in mice, whereas antibody-mediated inactivation or genetic deletion of these proteins lowers plasma TG levels (4–9). Interestingly, ANGPTL3, ANGPTL4 and ANGPTL8 share a common structure yet differ in their tissue expression pattern and regulation by various stimuli. Whereas ANGPTL3 is mainly produced by the liver, the expression of ANGPTL4 and ANGPTL8 is more ubiquitous and is regulated by stimuli such as fasting (10–12). Consequently, it has been suggested that the three angiopoietin-like proteins are important during different metabolic states. For example, whereas deletion of *ANGPTL3* has more pronounced effects in the fed state, the effects of *ANGPTL4* deletion are more evident in the fasted state (4). Likewise, the expression levels of *ANGPTL4* and *ANGPTL8* in adipose tissue show opposite changes in response to fasting (12). The interrelationship between ANGPTL3, ANGPTL4 and ANGPTL8 requires, however, further investigation.

Of the three angiopoietin-like proteins, ANGPTL4 has been the most extensively studied and is the subject of the present manuscript. Although a wealth of knowledge on ANGPTL4 has been collected since its discovery in 2000 (13–15), various aspects of its inhibitory action on LPL activity have remained unclear and/or are controversial. This review is aimed at highlighting the major ambiguities related to the mechanism of action of ANGPTL4 by reviewing the current knowledge on ANGPTL4 structure, cleavage and biochemical action, as well as the physiological role of ANGPTL4 in lipid metabolism, and uses this information to make suggestions for further research. The review will focus entirely on the role of ANGPTL4 in lipid metabolism, and will not discuss evidence linking ANGPTL4 to endothelial function, tumor biology, wound healing and the extra-cellular matrix (16). Complementary

background information on LPL can be found in **Box 1**, whereas an overview of the role of ANGPTL3 in lipid metabolism can be found elsewhere (17, 18).

BIOCHEMICAL ASPECTS OF ANGPTL4

Structure of ANGPTL4

Similar to other family members, ANGPTL4 can be divided into distinct regions: a N-terminal signal peptide, a region containing two separate coiled-coil domains, a linker region and a large C-terminal fibrinogen-like domain (19, 20). During evolution, the C-terminal and N-terminal regions were present separately in different organisms, suggesting that the different domains of *ANGPTL4* may have distinct physiological functions (20). In agreement with that notion, full-length ANGPTL4 (flANGPTL4, 50 kDa) is proteolytically cleaved by proprotein convertases (PCs) to yield an N-terminal (nANGPTL4) and a C-terminal (cANGPTL4) fragment. Combined *in vivo* and *in vitro* data now indicate that only nANGPTL4 and flANGPTL4 inhibit LPL, whereas cANGPTL4 has entirely different biological functions and does not appear to play a role in lipid metabolism (17, 21–25). The inhibition of LPL by flANGPTL4 and nANGPTL4 is mediated by a relatively short amino acid sequence close to the N-terminus (6, 21, 26).

Several PCs including PACE4, furin, PC5/6, and PC7 are capable of cleaving ANGPTL4 at the PC recognition motif RRKR *in vitro*, but which enzyme is predominantly responsible for proteolytic processing *in vivo* is unclear (19, 20, 23, 25). The proteolytic cleavage of flANGPTL4 has been suggested to increase its functionality by stimulating release of ANGPTL4 from the cell surface (20). Based on data from liver and adipose tissue, it can be hypothesized that ANGPTL4 is differentially processed between tissues and even within a single tissue, presumably due to differential expression of PCs (20, 27, 28).

In vivo, both nANGPTL4 and flANGPTL4 form high molecular weight structures (19, 23, 26), which is at least partially mediated by two disulfide bond-forming cysteine residues in nANGPTL4 (25, 26). Mutations of these cysteine residues limit the extracellular accumulation of both nANGPTL4 and flANGPTL4, thereby reducing LPL inhibition by ANGPTL4, possibly by destabilizing ANGPTL4 and rendering it a better substrate for degradation by proteases (23). Following cleavage, nANGPTL4 remains oligomerized, whereas cANGPTL4 dissociates into monomers (19) and this oligomerization is required for the inhibition of LPL activity by ANGPTL4 (26). Whether ANGPTL4 oligomerizes inside the cell or late in the secretory pathway on the cellular surface and whether this process is chaperoned by another yet unidentified protein remains debatable (23, 29, 30).

One of the key questions is whether (patho)physiological variations in LPL activity are mediated *via* regulation of ANGPTL4 cleavage and/or oligomerization and which factors are involved in modulating ANGPTL4 *in vivo*. Recent biochemical evidence suggests that FA may be able to promote dissociation of oligomers, which by destabilizing the protein would impair its ability to inhibit LPL (29). Destabilization of ANGPTL4 by FA is, however, seemingly at odds with the marked stimulatory effect of FA on ANGPTL4 production observed *in vitro* and *in vivo* (31–35).

BOX 1: LIPOPROTEIN LIPASE & TRIGLYCERIDE METABOLISM

LPL belongs to a family of lipases that also includes hepatic lipase, pancreatic lipase and endothelial lipase. Since LPL is essential in the lipolytic processing of chylomicrons and VLDL, LPL is primarily expressed in tissues that either require large amounts of FA as fuel or are responsible for TG storage, including heart, skeletal muscle and adipose tissue. Upon production by the underlying parenchymal cells, LPL is released into the subendothelial space and transported to the luminal side of the capillary endothelium by the protein GPIHBP1 (glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein), which after transport continues to anchor LPL to the capillary endothelium (39, 40). The essential role for LPL in the clearance of plasma TG is well-demonstrated by the severe hypertriglyceridemia of patients carrying homozygous mutations in the *LPL* gene (2). Similarly, the generalized deletion of *LPL* in mice results in severe hypertriglyceridemia, resulting in the premature death of pups within 24 hours after birth (93). Analogous to the deletion of *LPL*, the mislocalization of LPL to the subendothelial spaces due the absence or misfolding of GPIHBP1 also results in severe chylomicronemia and hypertriglyceridemia (39, 40).

The LPL enzyme is catalytically active as a non-covalent head-to-tail dimer with a catalytic amino-terminal domain and a non-catalytic carboxyl-terminal domain (94, 95). Folding of LPL into its dimer conformation occurs in the endoplasmic reticulum, chaperoned by lipase maturation factor 1, calreticulin and calnexin (96–98). In its active three-dimensional conformation, the catalytic site of LPL is postulated to be covered by a lid, which can be opened by the binding of chylomicrons and VLDL to the carboxyl-terminus (95). The active LPL dimers rapidly exchange subunits, indicating that a dynamic equilibrium exists between LPL dimers and dimerization-competent monomers. Dimerization-competent monomers have, however, not yet been isolated and it is unclear whether this monomer is catalytically active (2, 24, 94). The enzymatic activity of LPL is lost when the LPL dimer is converted into inactive, folded monomers (94). This conversion to inactive monomers is mainly regulated *via* post-translational mechanisms and is dependent on nutritional state (99–101). Enzymatic activity of inactive monomers can be regained *in vitro* by the addition of calcium, which indicates that inactivation of LPL is a reversible process (102).

LPL inhibition by ANGPTL4

Multiple studies, both *in vitro* and *in vivo,* have now convincingly shown that ANGPTL4 inhibits LPL activity (4, 5, 17, 21–24, 26, 36). Indeed, overexpression of *Angptl4* as well as injection of recombinant ANGPTL4 significantly increase plasma TG levels, in concert with a decrease in post-heparin plasma and tissue LPL activity, in mice (4, 5, 22, 36). Conversely, the complete knockout of *Angptl4* results in pronounced hypotriglyceridemia with a concomitant elevated tissue LPL activity and post-heparin plasma LPL activity (4). The exact mechanism by which ANGPTL4 inhibits LPL is, however, still debated. In many tissues, including heart, skeletal muscle and adipose tissue, ANGPTL4 is co-expressed and produced in the same cell with LPL

(11), raising the possibility for direct interaction between the two proteins inside the cell and/or upon secretion into the subendothelial space. According to that scenario, ANGPTL4 may inactivate LPL before it reaches the endothelial surface. In support of this notion, it was found that ANGPTL4 and LPL co-localize inside the cell when overexpressed in cultured muscle cells (31). A recent study indicates, however, that in 3T3-L1 adipocytes inactivation of LPL by ANGPTL4 occurs after both proteins have arrived at the cell surface, suggesting that inhibition of LPL activity by ANGPTL4 takes place in the subendothelial space or at the endothelium (37). Interestingly, intravenous injection of ANGPTL4 as well as overexpression of *Angptl4* in liver in mice, a tissue that does not produce LPL, raises plasma TG levels and lowers post-heparin plasma LPL activity (4), clearly indicating that the inhibitory action of ANGPTL4 on LPL is not limited to the subendothelial space. Currently, the full scope of interaction, location of interaction and possible co-secretion of ANGPTL4 and LPL is unclear and requires further investigation, possibly by employing novel methodologies such as Fluorescence Resonance Energy Transfer (FRET) and Proximity Ligation Assay.

The currently accepted molecular model for the inhibition of LPL by ANGPTL4 is that ANGPTL4 stimulates the conversion of catalytically active LPL dimers into inactive monomers, following *in vitro* studies showing that coincubation of LPL and ANGPTL4 increase the abundance of LPL monomers (21, 24). Subsequent studies revealed that the proportion of LPL dimers is reduced in post-heparin plasma of mice that overexpress *Angptl4* in favor of LPL monomers, providing *in vivo* support for the dimer to monomer conversion (22). The elucidation of the purported biochemical mechanism has strengthened the status of ANGPTL4 as LPL inhibitor, yet several questions related to the *in vivo* mechanism remain unanswered. Whereas the original *in vitro* experiments favored the hypothesis that ANGPTL4 enzymatically and irreversibly catalyzes the LPL dimer-to-monomer conversion (21, 24), an *in vivo* study of *Angptl4* transgenic mice suggested that ANGPTL4 is physically bound to LPL monomers, thereby driving the LPL dimer-monomer equilibrium towards inactive monomers (22). The latter study also revealed that the relative decrease in post-heparin plasma LPL activity upon *Angptl4* overexpression is much more pronounced than the relative decrease in heparin-releasable LPL dimers, pointing to an additional or alternative mechanism (22). In support, a recently published study suggests that ANGPTL4, instead of acting as a catalyst, functions as a conventional, non-competitive inhibitor that binds to LPL to prevent the hydrolysis of substrate (38). Evidence was provided indicating that inhibition of LPL by ANGPTL4 is of a reversible nature that may not necessarily require a dimer-to-monomer conversion, even though the exact mechanism of inhibition remains to be determined (38). Altogether, the available data suggests that the *in vivo* mechanism of action of ANGPTL4 is perhaps more complex that currently envisaged and requires further study.

One complicating factor *in vivo* is glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1), a small glycoprotein required for the lipolytic processing of TG-rich lipoproteins. GPIHBP1 is responsible for trans-endothelial transport of LPL from the underlying parenchymal cells towards the capillary lumen and has a high binding affinity for LPL (39–41). It has been demonstrated that the inhibitory action of ANGPTL4 on LPL *in vitro* is diminished when LPL is bound by GPIHBP1, which may be mediated by stabilization of LPL in the active

dimer conformation or, alternatively, by competition of ANGPTL4 and GPIHBP1 for binding to LPL (42). One possible scenario is that by binding LPL in the subendothelial space, GPIHBP1 displaces ANGPTL4 from LPL, thereby neutralizing its inhibitory action. Such an action of ANGPTL4 in the subendothelial space has been previously proposed by Nilsson and colleagues (43). Since flANGPTL4 binds the extracellular matrix (ECM) through interactions with heparan and dermatan sulfate chains as well as with fibronectin and vitronectin (25, 44, 45), it is conceivable that by physically interacting with LPL, ANGPTL4 retains LPL in the subendothelial space bound to the ECM. Intriguingly, Sonnenburg and colleagues observed that knockout of *Angptl4* almost completely abolished the severe hypertriglyceridemia in *Gpihbp1*$^{-/-}$ mice, indicating that in the absence of *Gpihbp1* the loss of *Angptl4* allows substantial amounts of LPL to reach the endothelial surface and catalyze TG hydrolysis (42). This indicates that there may be another transport mechanism for LPL to the endothelial surface, which is suppressed by ANGPTL4. Such a mechanism is also compatible with reversible non-competitive inhibition of LPL by ANGPTL4, as proposed by Lafferty and colleagues (38). In summary (see **Figure 1**), we postulate that GBIHBP1 and ANGPTL4 compete for binding to LPL, with ANGPTL4 trying to keep LPL in the subendothelial space *via* its binding to the ECM, and GPIHBP1 opposing that action by drawing LPL across the endothelium. To further elucidate possible interactions between GPIHBP1, ANGPTL4 and LPL, immunofluorescence co-localization studies on human or mouse tissues should be performed. Further characterization of the molecular mechanism of action of ANGPTL4 would also benefit immensely from elucidation of the molecular structure of LPL and ANGPTL4 *via* X-ray crystallography. Despite considerable effort, no structures are available for either protein as yet.

ROLE OF ANGPTL4 IN PHYSIOLOGICAL REGULATION OF LIPID METABOLISM

LPL and ANGPTL4 are regulated by changes in nutritional state in a tissue-specific manner, reflecting the different functions of these tissues and their corresponding differences in physiological requirements for lipids (13, 14, 46). Below, we discuss current knowledge on the regulation of ANGPTL4 and LPL in response to various physiological stimuli and address the importance of ANGPTL4 in lipid uptake. An overview of the role of ANGPTL4 in physiological regulation of lipid metabolism is presented in **Figure 2**.

White Adipose Tissue

White adipose tissue (WAT) continuously shifts between net storage of FA in the fed state, and net release of FA after fasting, which is mediated *via* fluctuations in intravascular and intracellular lipolysis (2). In contrast to the marked oscillations in LPL activity, WAT LPL mRNA and protein mass vary only slightly, suggesting that the activity of LPL in WAT is primarily regulated at the post-translational level (47). Recent studies have indicated that the decrease in adipose LPL activity during fasting is mediated by induction of *Angptl4* (47, 48). In contrast to wild-type mice, mice lacking *Angptl4* do not show any decrease in WAT LPL activity during fasting and have almost undetectable plasma TG levels, whereas mice overexpressing *Angptl4* in WAT

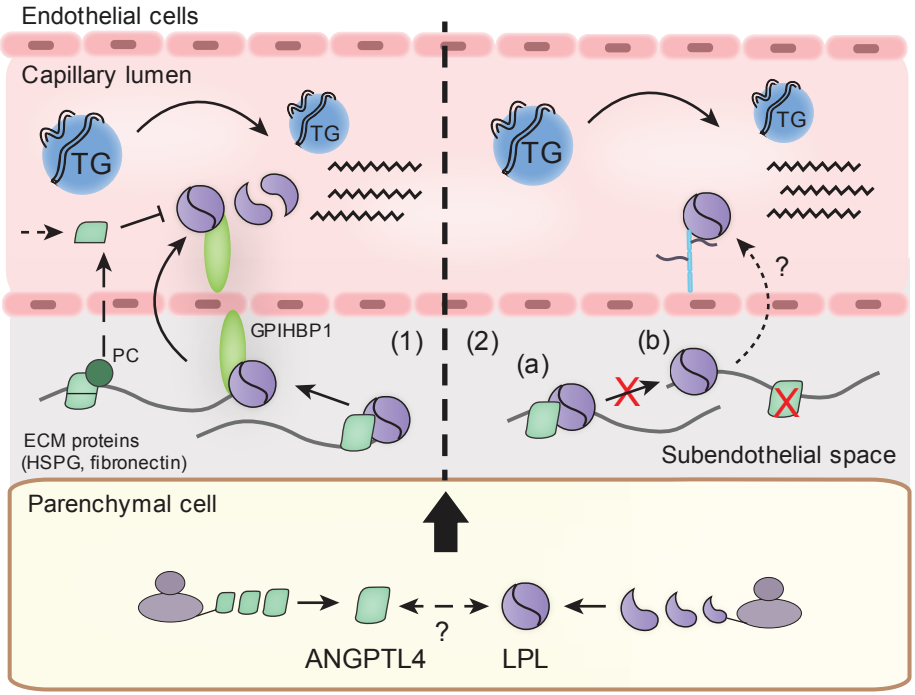

Figure 1: Hypothetical model for mechanisms of LPL inhibition by ANGPTL4.

Angiopoietin-like 4 (ANGPTL4) and lipoprotein lipase (LPL) are expressed in the parenchymal cells of muscle, heart and adipose tissue. Following secretion of LPL and ANGPTL4 into the subendothelial space, transport of LPL to the capillary lumen is mediated by two mechanisms. The principal transport mechanism (a) relies on GPIHBP1 (glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein) picking up LPL from the subendothelial space and transporting it to the capillary lumen. This action by GPIHBP1 is opposed by ANGPTL4, which is bound to extracellular matrix proteins and retains and inhibits LPL. In the presence of GPIHBP1, high expression levels of ANGPTL4 are needed to overcome the competition with GPIHBP1. ANGPTL4 secreted into the capillary lumen, primarily as N-terminal truncation fragment generated by cleavage by proprotein convertases (PCs), inhibits LPL activity on the endothelium by promoting the irreversible conversion of LPL dimers into inactive monomers and/or *via* a reversible mechanism that requires binding of ANGPTL4 to LPL. The second transport mechanism (b) involves a yet unidentified carrier and can be disrupted by ANGPTL4. In the absence of GPIHBP1, ANGPTL4 fully retains LPL in the subendothelial space. The additional loss of ANGPTL4 liberates LPL and allows it to be transported to the endothelial surface *via* the unidentified carrier. This model suggests that ANGPTL4 and LPL start interacting before arrival in the capillary lumen, either in the parenchymal cells or in the subendothelial space.

and other tissues exhibit reduced uptake of plasma TG-derived FA into adipose tissue and elevated plasma TG levels (22, 47). Whether *Angptl4* expressed in WAT primarily functions locally or also has systemic effects has yet to be resolved.

Induction of WAT *Angptl4* expression during fasting is probably mediated *via* multiple transcriptional mechanisms. As FAs serve as peroxisome proliferator-activated receptor gamma (PPARγ) agonists and are potent activators of *ANGPTL4* gene expression in adipocytes, the increased plasma FFA levels during fasting may stim-

ulate transcription of ANGPTL4 *via* activation of PPARγ (34, 35). Also, the reduction in plasma insulin levels may relieve the suppressive effect of insulin on adipose *ANGPTL4* mRNA (49). Finally, elevated levels of plasma glucocorticoids may stimulate *ANGPTL4* transcription *via* the Glucocorticoid Receptor (GR) (28). Interestingly, ANGPTL4 has also been linked to the stimulation of lipolysis in adipocytes during fasting (5, 50–52). The combined action of stimulating intracellular lipolysis and inhibiting LPL-mediated lipolysis points to a key role of ANGPTL4 in promoting fat liberation and reducing fat storage.

Liver

The liver lies at the crossroad of TG metabolism as it actively takes up lipids associated with remnant particles as well as FFA originating from adipose tissue lipolysis, while secreting TG-rich VLDL into the blood. The TG-containing remnant particles are not metabolized by LPL, which is expressed very weakly in liver, but are taken up *via* receptor-mediated endocytosis after some of its lipid cargo is hydrolyzed by hepatic lipase. Whereas LPL expression is low, *ANGPTL4* mRNA levels are high in liver (13). Studies using mice that specifically overexpress *Angptl4* in liver revealed that liver-derived ANGPTL4 is capable of inhibiting LPL in peripheral tissues (4, 27). Thus, whereas ANGPTL4 produced in adipose tissue, heart and skeletal muscle may primarily target local lipid uptake, liver-derived ANGPTL4 likely serves as an endocrine factor. The implication of an endocrine role of ANGPTL4 is that any regulation of hepatic *Angptl4* expression presumably impacts LPL activity universally and not selectively targets plasma TG clearance in certain tissues. Whether liver-expressed *ANGPTL4* may have an inhibitory effect on hepatic lipase remains controversial, as divergent results have been obtained (4, 22).

The regulation of *ANGPTL4* in liver has not yet been extensively studied, but no profound changes in hepatic *Angptl4* expression have been observed on either a high fat diet (13) or during chronic intermittent hypoxia (53). Similar to WAT, however, *Angptl4* expression in liver is rapidly increased during fasting (13, 54). Currently, the physiological relevance of the induction of liver *Angptl4* during fasting is not fully clear, but it can be envisaged that secreted ANGPTL4 may suppress LPL activity in peripheral tissues to favor tissue uptake of plasma FFA at the expense of plasma TG-derived FA, which would save energy by circumventing hepatic re-synthesis of TG from plasma FFA. Whereas PPARα is a key determinant of basal *Angptl4* expression in liver, the up-regulation of *Angptl4* in liver during fasting is mediated independently of PPARα *via* activation of GR by circulating glucocorticoids, activation of PPARδ by plasma FFA, and/or the drop in plasma insulin levels during fasting (27, 28, 52, 55).

Heart

The energy requirements of the heart are primarily met by oxidation of FA, with the remainder of the energy coming from glucose and lactate. Most of these FA are derived from LPL-dependent hydrolysis of circulating TG (56). Whereas an insufficient FA supply may impair cardiac function, excessive uptake of FA may result in lipid overload and lipotoxicity of the heart (57). Hence, it is imperative that the activity of LPL in heart is carefully regulated. As opposed to WAT, a high proportion of LPL in

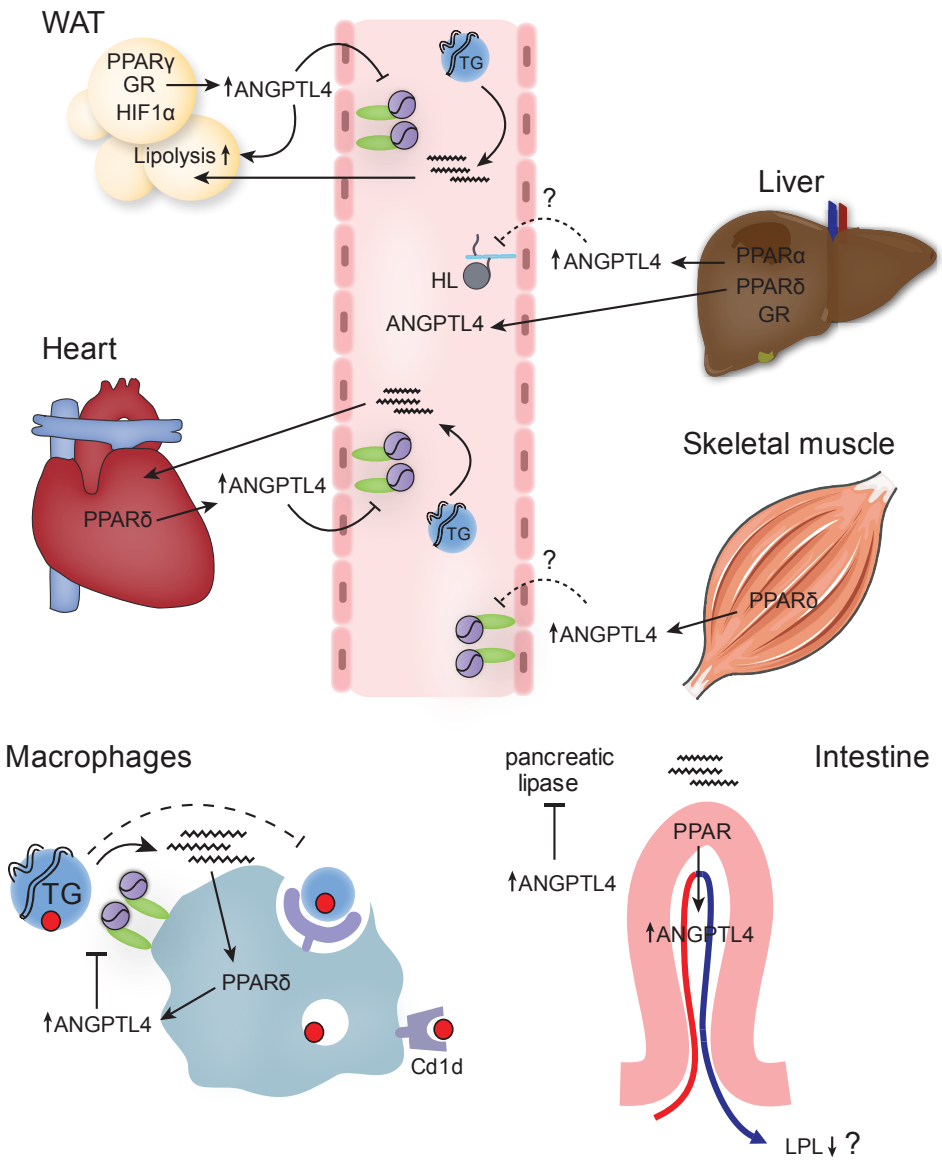

Figure 2: Regulation and role of ANGPTL4 in lipid metabolism.

Angiopoietin-like 4 (ANGPTL4) is expressed in parenchymal cells of white adipose tissue (WAT), liver, heart and muscle, as well as in macrophages, where it is subject to cell- and tissue-specific regulation. ANGPTL4 is a sensitive target of peroxisome proliferator-activated receptor (PPAR) transcription factors in several tissues. In WAT, the expression of ANGPTL4 is induced during fasting and by the transcription factors PPARγ, glucocorticoid receptor (GR) and hypoxia-inducible factor 1α (HIF1α). In WAT, ANGPTL4 stimulates lipolysis of stored triglycerides and inhibits lipoprotein lipase (LPL) activity. Expression of ANGPTL4 in liver is stimulated by PPARα, PPARδ and GR. Since the liver does not express LPL, ANGPTL4 is mainly released into the blood, affecting LPL activity in peripheral tissues. ANGPTL4 may also impact hepatic lipase activity in liver. Expression of ANGPTL4 in heart and skeletal muscle is potently induced by fatty acids via PPARδ activation. Car-

the heart is present intracellularly, with the result that changes in total tissue LPL activity mostly reflect changes in total LPL mass rather than functional LPL. A role for ANGPTL4 in the regulation of LPL activity in heart was demonstrated in mice with cardiac-specific overexpression of *Angptl4*. These mice exhibit reduced heparin-releasable LPL activity in the heart and elevated circulating TG levels (57). Similarly, following an oral fat load, transgenic mice that overexpress *Angptl4* in various tissues including heart had markedly lower cardiac FA uptake from [^{3}H]triolein-labeled VLDL-like particles compared to wild-type mice (32). The expression of *Angptl4* in heart is induced by dietary FA *via* the transcription factor PPARδ as part of a possible feedback mechanism aimed at protecting the heart against lipid overload and thereby averting FA-induced toxicity (31, 32, 58). Interestingly, both *Angptl4* expression and heparin-releasable LPL activity are increased by fasting in the heart, concomitant with enhanced uptake of plasma TG-derived FA (22, 59, 60). These data suggest that a factor different from ANGPTL4 is responsible for the increase in cardiac LPL activity during fasting. The increase in *Angptl4* expression may serve to limit the increase in LPL activity during fasting in order to prevent lipid overload that may be driven by elevated plasma FFA levels (60). Detailed study of cardiac heparin-releasable LPL activity in *Angptl4* knockout mice during fasting and refeeding is expected to further clarify the mechanism responsible for up-regulation of LPL activity during fasting.

Skeletal Muscle

A key feature of the skeletal muscle is its metabolic flexibility, enabling the muscle to employ a variety of carbohydrate and lipid fuels. Important sources of energy for skeletal muscle are FA derived from plasma FFA and circulating TG-rich lipoproteins (56, 61). Expression of *ANGPTL4* in muscle is markedly increased during fasting in mouse and humans, probably *via* elevation of plasma FFA levels (22, 62). Similar to heart, induction of *ANGPTL4* expression is likely regulated *via* the PPARδ/RXR axis, as agonists for both PPARδ and RXR enhance ANGPTL4 mRNA levels in cultured myocytes, concomitant with a reduction in secreted LPL activity (31, 63). In as much as there is no evidence that functional LPL activity in muscle is altered by fasting, the functional implications of the fasting-induced increase in *ANGPTL4* expression remain unclear (64–67). Besides fasting, a primary modulator of LPL activity in skeletal muscle is exercise. Both acute exercise and chronic exercise training markedly induce LPL activity, at least partly *via* changes in LPL mRNA and protein expression (68, 69). A role of ANGPTL4 in modulation of LPL activity in muscle during exercise is likely but remains unsubstantiated. Existing data indicate that exercise increases

diac and likely skeletal muscle LPL activities are inhibited by ANGPTL4. Fatty acids also stimulate ANGPTL4 expression in macrophages *via* PPARδ, leading to local inhibition of LPL activity. We hypothesize that macrophage LPL enables uptake of remnant particles containing lipid antigens, which are subsequently presented to Natural Killer T cells. In the intestine, fatty acids stimulate ANGPTL4 expression *via* one of the PPARs. ANGPTL4 produced by enterocytes may be released towards the lumen and inhibit pancreatic lipase activity. ANGPTL4 produced by enteroendocrine cells is released towards the blood and may inhibit LPL in distant tissues.

plasma ANGPTL4 levels in humans, an elevation that was entirely abolished when subjects were given oral glucose, which suppresses plasma FFA levels (35).

Intestine

ANGPTL4 is relatively well expressed in the intestine and is upregulated upon high-fat feeding (70). In contrast, LPL is only weakly expressed in the intestine and is not known to play a role in intestinal lipid metabolism. Accordingly, it is possible that intestinal ANGPTL4 may serve an endocrine function by inhibiting LPL in distant tissues (71). Alternatively, ANGPTL4 may target another lipase in the small intestine. Indeed, ANGPTL4 was recently proposed to inhibit pancreatic lipase, a family member of LPL, thereby reducing fat absorption (70). Pancreatic lipase is not known to form a dimer, excluding a mechanism involving dimer dissociation. Instead, ANGPTL4 may reversibly inhibit pancreatic lipase by forming an inhibitory complex, in analogy with a mechanism that was recently postulated for LPL (38). ANGPTL4 has received considerable attention in connection with the gut microbiota. Intestinal ANGPTL4 expression was found to be markedly reduced in germ-free mice compared to conventionally raised mice and upon conventionalization of germ-free mice or zebra fish (72–75). Evidence was provided that suppression of ANGPTL4 accounts for enhanced fat gain upon conventionalization *via* increased adipose tissue LPL activity (72). However, a direct link between intestinal-derived ANGPTL4 and adipose LPL activity has not been established (73). In apparent conflict with reduced ANGPTL4 expression following conventionalization, multiple bacterial strains stimulate ANGPTL4 expression in colonic cell lines (76, 77). Likewise, recent data show that the microbial metabolites propionate and butyrate but not acetate potently induce ANGPTL4 expression in intestinal cells (71, 78). A possible explanation is that regulation of intestinal ANGPTL4 *in vivo* depends on gut microbiota composition, which together with substrate provision determines the mixture of microbial metabolites formed. Whereas colonization of germ-free mice with *Bacteroides thetaiotaomicron* or *Methanobrevibacter smithii,* which produce acetate and methane, respectively, reduces intestinal ANGPTL4 expression, colonization with *Clostridium tyrobutyricum*, a butyrate-producing bacteria, markedly elevated intestinal ANGPTL4 expression (78, 79). Hence, the ability of gut microbial strains to produce butyrate may be a key determinant of induction of intestinal ANGPTL4. Altogether, intestinal ANGPTL4 expression is modulated by the gut microbiota *via* generation of specific fermentation products, including butyrate. It can be speculated that induction of ANGPTL4 may play a role in mediating effect of dietary fibers on a number of parameters, including plasma lipoproteins and lipid absorption.

Macrophages

Expression of *ANGPTL4* in macrophages is relatively high and stimulated by FA and PPAR agonists, likely *via* PPARδ (30, 80). Similar to muscle, WAT and heart, macrophages are capable of hydrolyzing TG-rich lipoproteins *via* a LPL-dependent mechanism, but unlike the other organs macrophages can also take up the resultant TG-depleted and cholesterol-enriched remnant molecules (81). The reason for the expression of *LPL* by macrophages and not by other leukocytes is puzzling, but it has

been suggested that TG-derived FA serve as an important fuel for macrophages during low glucose availability and during periods of intense metabolic activity such as phagocytosis (82). However, except in the subscapular sinus of the mesenteric lymph nodes, tissue macrophages may hardly get in contact with circulating TG-rich lipoproteins, as they cannot easily cross the endothelium.

Macrophages have been extensively studied for their role in foam cell formation in the vascular wall and associated atherogenesis. Consistent with the well-established pro-atherogenic effect of LPL (83), ANGPTL4 was found to protect against atherosclerotic plague development in mice (80). Specifically, *in vivo* overexpression of *Angptl4* significantly reduced lesion size and area in atherosclerosis-prone *ApoE3*-Leiden transgenic mice, while addition of recombinant ANGPTL4 *in vitro* limited the uptake of VLDL and oxidized LDL by macrophages (80). Intriguingly, reduced atherosclerosis was observed in *Angptl4* knockout mice on a *ApoE* background, which was likely due to a decrease in circulating LDL-C and TG levels and probably unrelated to a direct effect of ANGPTL4 on foam cell formation (84).

Theoretically, foam cell formation may occur whenever macrophages take up excess lipid. A unique location where macrophages are exposed to extremely high concentrations of TG-rich lipoproteins is the subscapular sinus of the mesenteric lymph nodes, which are part of the drainage route for freshly synthesized chylomicrons. It was shown that ANGPTL4 inhibits lipid uptake by macrophages present in the mesenteric lymph nodes (85). In the absence of *Angptl4*, feeding mice a diet high in saturated fat causes a complex and ultimately lethal phenotype originating from excess chylomicron-derived lipid uptake by mesenteric lymph node macrophages (85). These findings have led to the proposition that macrophages in mesenteric lymph nodes and elsewhere survey the chyle for so-called lipid antigens *via* LPL-mediated lipolysis. Lipid antigens encompass a broad scope of lipids carried by pathogenic microbes and gut microbiota, which are presented to Natural Killer T-cells (NKT cells) by antigen presenting macrophages and dendritic cells. The purported role of macrophage ANGPTL4 is to control LPL activity and to balance the need to sample chylomicrons for lipid antigens yet prevent excessive lipid uptake, especially when chylomicron levels are elevated. Additional studies are clearly needed to validate this model but for now it provides a reasonable explanation for the coexpression of *LPL* and *ANGPTL4* by macrophages.

Physiological role of LPL inhibition by ANGPTL4

From the available data on ANGPTL4 in different tissues, several common denominators are apparent, allowing for construction of a more integrative picture of ANGPTL4 function (**Figure 2**). The present view on the physiological role of ANGPTL4 may perhaps be best described as ensuring the proper distribution of plasma TG over various tissues during different nutritional states, which requires on the one hand that FA are taken up by tissues for rapid oxidation or are effectively stored for later use, and on the other hand that excess lipid uptake is avoided and cellular lipotoxicity is averted. ANGPTL4 executes this function by being the primary physiological regulator of LPL activity.

CLINICAL PERSPECTIVES OF ANGPTL4

In support of the mice studies, genetic studies have provided support for a similar function of ANGPTL4 in humans. Specifically, a genetic loss-of-function variant of *ANGPTL4* (E40K) that is found in 3% of European Americans is associated with reduced plasma TG levels (86). Remarkably, none of the genome wide association studies performed so far has linked SNPs in or near the *ANGPTL4* gene to plasma TG levels. Likewise, so far no positive correlation has been found between plasma ANGPTL4 and plasma TG levels (35, 87–90). A possible explanation for the lack of correlation between plasma ANGPTL4 and TG levels may be the inability of the employed enzyme-linked immunosorbent assays (ELISAs) to detect nANGPTL4, the cleavage form that potently inhibits LPL activity (88). It has been shown that the ELISA used in all studies published so far exclusively detects flANGPTL4 and cANGPTL4 (88, 91). Hence, further studies utilizing newly developed ELISAs that specifically recognize nANGPTL4 and/or flANGPTL4 should help to clarify the relation between plasma ANGPTL4 and plasma TG levels. Alternatively, it is possible that ANGPTL4 in blood may not represent fully functional ANGPTL4, meaning that plasma ANGPTL4 concentrations may not reflect the ANGPTL4 level in the vicinity of LPL, as proposed in this paper (43).

Since elevated plasma TGs are considered a (minor) risk factor for atherosclerosis, ANGPTL4 has been extensively scrutinized as a putative therapeutic target for cardiovascular disease. It appears, however, that the progressive development of a severe clinical phenotype in mice lacking *Angptl4* and in mice injected with anti-ANGPTL4 antibodies has led to permanent dismissal of ANGPTL4 as pharmacological target (85, 7). Besides being a potential therapeutic target, plasma ANGPTL4 levels could perhaps also serve as biomarker for a specific disease. Currently, the potential of ANGPTL4 as biomarker has mainly been examined in the context of cancer and to a lesser extent in the context of lipid-related diseases. In contrast to the lack of correlation between plasma TG and plasma ANGPTL4 levels, clear associations have been found between plasma ANGPTL4 levels and plasma FFA levels in humans in the fasted state (88, 89, 92). Likewise, obese subjects generally have higher levels of plasma ANGPTL4 (87, 92). The scarcity and inconsistency of the available data render it difficult to attach a clear value to plasma ANGPTL4 levels in the context of lipid-related disorders and studies with ELISAs specifically detecting one of the three (truncated) forms of the ANGPTL4 protein are necessary. An interesting observation with the current ELISA is that a very small fraction of the subjects studied have plasma ANGPTL4 levels that far exceed the average level (up to ~50 fold) without having any other metabolic abnormalities.

CONCLUDING REMARKS & FUTURE PERSPECTIVES

In the past decade, angiopoietin-like proteins have been demonstrated to powerfully regulate plasma TG levels in mice and humans. The elucidation of these proteins as inhibitors of LPL activity has led to a paradigm shift of how clearance of circulating TG and thereby tissue uptake of FA is regulated. Most of our understanding on angiopoietin-like proteins has resulted from detailed study of ANGPTL4. From the data described in this review, it is becoming increasingly clear that a major portion of the

BOX 2: OUTSTANDING QUESTIONS

- What is the importance of ANGPTL4 cleavage and oligomerization for ANGPTL4 function in vivo?
- What is the precise biochemical mechanism behind the inhibition of LPL activity by ANGPTL4?
- At which cellular location(s) does the inhibition of LPL by ANGPTL4 occur and, if at multiple locations, what is the relative contribution of both tissue-produced ANGPTL4 compared to circulating ANGPTL4 with respect to inhibition of tissue LPL activity.
- What is the interplay between GPIHBP1 and ANGPTL4 in the regulation of LPL activity?

physiological variation in LPL activity in various tissues can be attributed to regulation of ANGPTL4 production. We predict that ANGPTL4 will turn out to be equally important for governing LPL activity in muscle during exercise, in brown adipose tissue during cold, and in a number of tissues during fasting. Whether an additional layer of regulation is conferred by tissue-specific cleavage of the ANGPTL4 protein and/or formation of oligomers remains to be determined.

Besides the increasing recognition of the pivotal role of ANGPTL4 in lipid metabolism as an inhibitor of LPL, major insight has been gained into the molecular mechanism of action of ANGPTL4 over the past years. Key questions remain, however, especially related to the interaction between LPL, GPIHBP1 and ANGPTL4 on the endothelium and in the subendothelial space. Several other points of interest have been highlighted throughout the text and include the elucidation of the molecular structure for LPL and ANGPTL4 by X-ray crystallography and the clarification of *in vivo* ANGPTL4 cleavage and oligomerization (for an overview see **Box 2: Outstanding Questions**). A final subject that needs to be addressed more extensively is the interrelationship between ANGPTL4, ANGPTL3 and ANGPTL8, which all have the capacity to inhibit LPL *in vivo* and modulate plasma TG levels. Overall, it is fascinating to witness how a small set of proteins, principally GPIHBP1 and ANGPTL4, have reinvigorated the LPL field over the past years, more than five decades after LPL was first identified as the clearing factor lipase. Let us hope that an equally fruitful time is lying ahead of us.

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ANGPTL4 MEDIATES SHUTTLING OF LIPID FUEL TO BROWN ADIPOSE TISSUE DURING SUSTAINED COLD EXPOSURE

Wieneke Dijk
Markus Heine
Laurent Vergnes
Mariëtte R. Boon
Gert Schaart
Matthijs K.C. Hesselink
Karen Reue
Wouter D. van Marken Lichtenbelt
Gunilla Olivecrona
Patrick C.N. Rensen
Joerg Heeren
Sander Kersten

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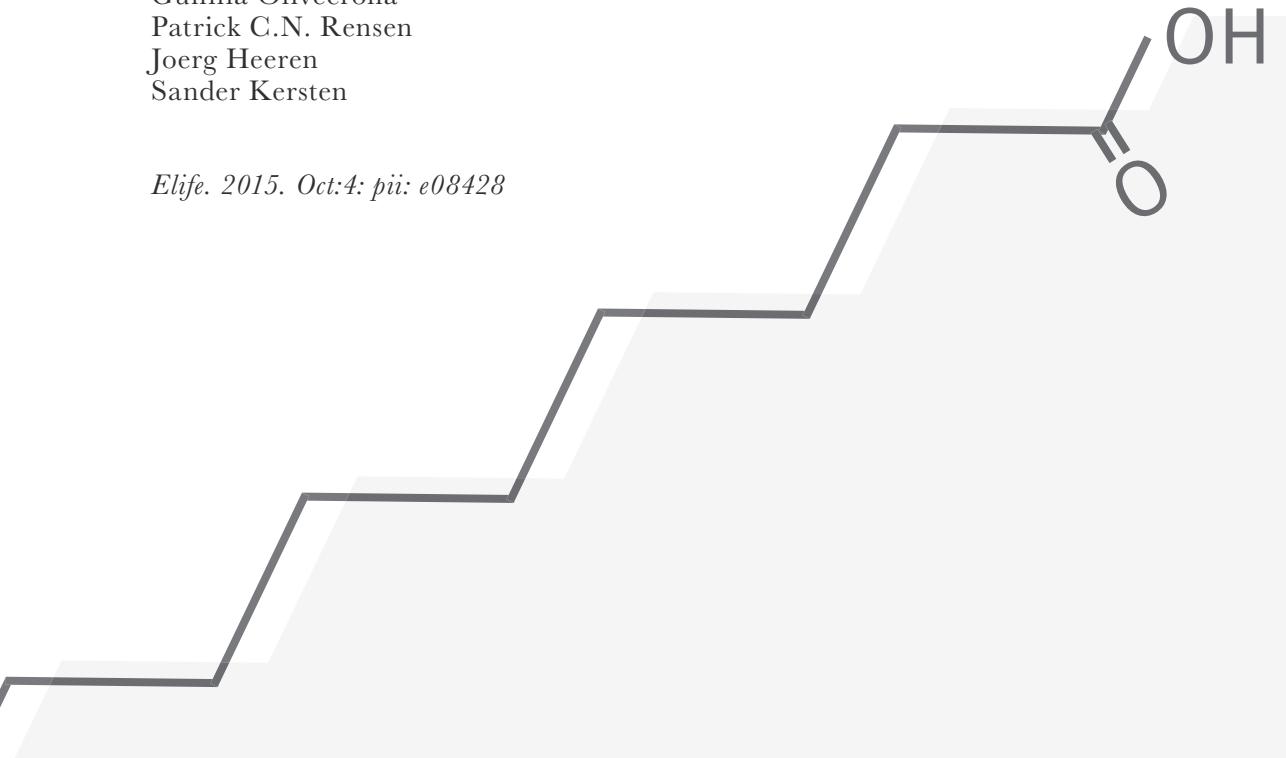

ABSTRACT

Brown adipose tissue (BAT) activation *via* cold exposure is increasingly scrutinized as a potential approach to ameliorate cardio-metabolic risk. Transition to cold temperatures requires changes in the partitioning of energy substrates, re-routing fatty acids to BAT to fuel non-shivering thermogenesis. However, the mechanisms behind the redistribution of energy substrates to BAT remain largely unknown. Angiopoietin-like 4 (ANGPTL4), a protein that inhibits lipoprotein lipase (LPL) activity, is highly expressed in BAT. Here, we demonstrate that ANGPTL4 is part of a shuttling mechanism that directs fatty acids derived from circulating triglyceride-rich lipoproteins to BAT during cold. Specifically, we show that cold markedly down-regulates ANGPTL4 in BAT, likely *via* activation of AMPK, enhancing LPL activity and uptake of plasma triglyceride-derived fatty acids. In contrast, cold up-regulates ANGPTL4 in WAT, abolishing a cold-induced increase in LPL activity. Together, our data indicate that ANGPTL4 is an important regulator of plasma lipid partitioning during sustained cold.

INTRODUCTION

Adipose tissue can be classified into white adipose tissue (WAT) and brown adipose tissue (BAT). Whereas WAT represents the main energy storage organ in the body, BAT is dedicated to the generation of heat *via* the burning of lipids. BAT is activated during cold exposure, when additional heat production is needed to maintain core body temperature. Heat production by BAT is stimulated *via* release of norepinephrine by the sympathetic nervous system, causing activation of β-adrenergic signalling and subsequent uncoupling of ATP production from mitochondrial respiration (1). Uncoupling in BAT is mediated by the uncoupling protein UCP1, which is highly abundant specifically in BAT (1). Studies in the last decade have shown the presence of BAT in humans and have provided preliminary evidence for an inverse relationship between BAT activity and parameters of obesity (2–4). As a consequence, interest in BAT function and the possible targeting of BAT for treatment or prevention of metabolic diseases has surged.

Upon cold exposure, oxidation of fuels by BAT is dramatically increased. In addition to circulating glucose and free fatty acids, fatty acids derived from circulating triglyceride-rich lipoproteins (TRLs) represent a major fuel source for BAT (1). The liberation of fatty acids from TRLs is catalyzed by the enzyme lipoprotein lipase (LPL), which is highly abundant in BAT (5, 6). Cold exposure markedly stimulates LPL activity in BAT, causing a concomitant increase in TRL-derived fatty acid uptake and even uptake of whole lipoprotein particles (6–8). The increase in fatty acid uptake upon cold exposure, which can be mimicked by pharmacological ß3-adrenergic receptor activation, is highly specific for BAT, suggesting that the body may specifically re-direct lipid fuels to BAT during cold exposure (6, 7, 9). Both transcriptional and (post-)translational regulation has been implicated in the increased LPL activity in BAT upon cold exposure. However, the specific underlying mechanisms have remained elusive (10–12).

Angiopoietin-like 4 (ANGPTL4) has previously been identified as an inhibitor of LPL activity in muscle and WAT. Alterations of *Angptl4* expression in these tissues mediate the changes in LPL activity observed during exercise and fasting, respectively (13, 14). In the initial paper describing the cloning of ANGPTL4, we had seen high expression of *Angptl4* mRNA in BAT (15). Since the exact mechanisms behind regulation of LPL activity in BAT upon cold exposure are currently unclear, we hypothesized that ANGPTL4 may act as an important regulator of LPL-mediated fatty acid uptake into BAT. Accordingly, in the present paper we studied the role of ANGPTL4 in lipid metabolism during cold exposure, taking advantage of *Angptl4*-deficient (*Angptl4*$^{-/-}$) mice and *Angptl4*-overexpressing (*Angptl4*-Tg) mice.

MATERIALS & METHODS

Mice

Three- to four-month old *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice were either placed at a thermo-neutral temperature (~28˚C) (n=7/8, as indicated in figure legends) or at a cold temperature (~4˚C) (n=7-10, as indicated in figure legends) for a period of 10 days. All animals are backcrossed on a pure C57Bl/6J background for multiple generations (>10). Wild-type and *Angptl4*-Tg mice are littermates. *Angptl4*$^{-/-}$ mice have been obtained via homologous recombination of embryonic stem cells and lack part of the *Angptl4* gene, resulting in a non-functional ANGPTL4 protein (16, 17). *Angptl4*-Tg mice over-express the *Angptl4* gene in various tissues under its own promoter (18). Food intake, body weight and body temperature were monitored daily. Body temperature of cold-exposed mice was monitored *via* read-out of transponders (IPTT-300) that were injected subcutaneously prior to the experiment (Bio Medic Data Systems, Seaford, USA). The Animal Ethics Committees of Wageningen University and University Medical Center Hamburg-Eppendorf approved all experiments.

TRL labeling

Radiolabeled VLDL-like emulsion particles were essentially prepared as described previously (19). Briefly, 100 mg of lipids (triolein, egg yolk phosphatidylcholine, lysophosphatidylcholine, cholesteryl-oleate and cholesterol) were mixed with glycerol tri[^{3}H]oleate and [^{14}C]cholesteryl-oleate (GE Healthcare, Little Chalfont, UK) and sonicated (Soniprep 150, MSE Scientific Instruments, UK). The emulsion was fractionated by consecutive density gradient ultracentrifugation (Beckman, California, USA) to yield VLDL-like particles with a diameter of ~80 nm.

Radiolabeled chylomicron-like particles with a diameter of ~250 nm were prepared from lipids derived from human TRLs of apoCII-deficient subjects (approved by Ärtzekammer Hamburg, Germany) as described previously (20). Briefly, 10 mg of isolated lipid was mixed with glycerol tri[^{14}C]oleate and [^{3}H]cholesteryl-oleyloleate (Perkin Elmer, Rodgau, Germany) in chloroform, after which solvent was removed and TRL particles were formed by sonication in 1 mL of PBS for 10 minutes at 60˚C. Aggregates were removed by filtration through a 450 nm filter (Millipore). TRL particles used for intravital microscopy were prepared similarly, but radiolabels were replaced by hydrophobic fluorescent nanocrystals (QD-TRLS).

TG clearance experiments

To study the clearance of radiolabeled TRL-like particles (80 & 250 nm), *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice were exposed to cold or thermo-neutral temperature for 10 days (see **Supplemental Figure 1** for an overview). Prior to the experiment, animals were fasted for 4h. To asses TG clearance, mice were injected intravenously with 200 µL radiolabeled TRL-like particles (0,2 mg TG for VLDL-like particles, 2 mg TG for chylomicron-like particles). Lipid turnover was determined for VLDL-like particles from plasma taken at 2,5,10 and 15 min following injection. Total plasma volumes were calculated as 0.04706 x body weight (g) (21). 15 minutes after injection,

mice were sacrificed and perfused *via* the heart with ice-cold PBS containing 50 IU/mL heparin. Multiple organs were collected, weighed and solubilized in Tissue Solubilizer (Amersham Biosciences, Roosendaal, the Netherlands; for VLDL-like particles) or Solvable (Perkin Elmer; for chylomicron-like particles) overnight. ^{3}H and ^{14}C radioactivity was determined *via* liquid scintillation counting. Uptake of radioactivity derived from TRL-like particles was calculated as % uptake of the injected radiolabel per gram tissue.

Intravital Microscopy

For intravital microscopy, interscapular BAT was exposed in anesthetized *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice and visualized by a confocal microscope with resonant scanner (Nikon A1R). QD-TRLs were injected *via* a tail vein catheter in anesthetized mice, where after 30 confocal images per second of the interscapular BAT were recorded for a period of 15 minutes. The acquired data were edited in Nikon NIS Elements. After recording, mice were perfused with PBS containing 50 IU/mL heparin. Then, BAT, gonadal WAT and inguinal WAT were taken out for subsequent cryosectioning, to assess the uptake of QD-TRLs *via* confocal microscopy.

Plasma measurements

Plasma concentrations of glucose (Sopachem, Ochten, the Netherlands), triglycerides, cholesterol (Instruchemie, Delfzijl, the Netherlands), glycerol (Sigma-Aldrich, Houten, the Netherlands) and free fatty acids (Wako Chemicals, Neuss, Germany; HR(2) Kit) were determined following the manufacturers' instructions.

LPL activity measurements

LPL activity in whole tissue homogenates from BAT, gonadal WAT and inguinal WAT was measured as described previously (22). Briefly, extracts of frozen tissue samples were prepared in 9 ml lysis buffer / g tissue (0.025 M NH_3, 5 mM Na_2EDTA, and per ml: 1 mg bovine serum albumin, 10 mg Triton X-100, 1 mg SDS, 5 IU heparin, and Complete protease inhibitors (Roche)) by homogenization with a Polytron homogenizer. Homogenates were spun down for 15 min at 3000 x g to obtain the supernatant used to measure LPL activity. 2 µl of supernatant was assayed, in a total volume of 200 µL, with a ^{3}H-oleic acid-labeled triolein containing substrate emulsion having 100 mg soybean triglycerides and 10 mg egg yolk phospholipids per mL. Incubation was at 25˚C for 30-100 min, dependent on the expected level of LPL activity. One milliunit (mU) of enzyme activity corresponds to 1 nmol of fatty acid released per min.

RNA isolation & qPCR

Total RNA was isolated using TRIzol reagent (Life Technologies Europe BV, Bleiswijk, the Netherlands). RNA from WAT depots was purified using the Qiagen RNeasy Micro kit (Qiagen, Venlo, the Netherlands). RNA was reverse transcribed using a

First-Strand cDNA Synthesis Kit (Thermo Scientific, Landsmeer, the Netherlands) (for cells) or iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, the Netherlands) (for tissues). Real-time PCR was carried out using SensiMiX (Bioline, GC Biotech, Alphen aan de Rijn, the Netherlands) on a CFX 384 Bio-Rad thermal cycler (Bio-Rad). TBP and 36B4 were used as housekeeping genes. Primer sequences can be found in **Table 1**.

Tissue H&E staining

Fresh tissues (WAT and BAT) were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. H&E staining was performed using standard protocols.

Tissue immunofluorescence

Frozen human BAT sections obtained during surgery (5 µm thick) were fixated during 15 min in 3.7% formaldehyde in PBS, followed by incubation for 45 min at room temperature with a primary antibody (polyclonal rabbit hANGPTL4 or a polyclonal rabbit UCP1-antibody (kind gift of Dr. B. Cannon, Stockholm University)) (23, 24) diluted in 0.05% Tween20 in PBS. After three washing steps with PBS, sections were incubated for 45 min at room temperature with the appropriate fluorescently labelled secondary antibodies. The specificity of the antibody for ANGPTL4 was demonstrated previously *via* immunoblot of human plasma using appropriate peptide controls and was validated by staining of ANGPTL4 in human heart, intestine and muscle (14, 25, 26).

Human cold exposure experiment & hANGPTL4 ELISA

Plasma ANGPTL4 levels were measured in plasma samples from a published study in which lean and obese human subjects were exposed to a mild cold (16˚C) for 48h (27). Plasma hANGPTL4 levels were measured as described previously (28). Briefly, 96-well plates were coated with anti-human ANGPTL4 polyclonal goat IgG antibody (AF3485; R&D Systems) and were incubated overnight at 4˚C. After blocking, 100 µL of 20-fold diluted human plasma was applied to each well, followed by incubation at room temperature for 2 h. Next, 100 µL of diluted biotinylated anti-human ANGPTL4 polyclonal goat IgG antibody (BAF3485; R&D Systems) was added and incubated for 2 h. Subsequently, streptavidin-conjugated HRP was added for 20 min, followed by tetramethylbenzidine substrate reagent for 6 min. The reaction was stopped by adding 50 µL of 10% H_2SO_4. The absorbance was measured at 450 nm.

Western Blot

Tissues were lysed in a mild RIPA-like lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol; Thermo Scientific) with protease and phosphatase inhibitors (Roche). Cells were lysed directly in 2x laemmli sample buffer (LSB) with DTT. Protein lysates (20–30 µg protein per lane) were loaded on a denaturing gel (Bio-Rad) and separated by SDS gel electrophoresis. Proteins were

transferred to a PVDF membrane by means of a Transblot Turbo System (Bio-Rad). The primary antibody [rabbit anti–phospho-AMPK antibody (Thr172), rabbit anti-AMPKα1,α2 antibody (Cell Signaling Technology, #2535 and #2532), rabbit anti-mouse/human AMPKα1 (Cell Signaling Technology, #2795), rabbit anti-mouse/human AMPKβ1 and AMPKβ2 (Cell Signaling Technology, #4148 and #4178), rabbit anti-mouse AMPKα2 antibody (Abcam, #ab3760), rat anti-mouse ANGPTL4 antibody (Adipogen, #Kairos 142–2), goat anti-mouse LPL antibody (kind gift from André Bensadoun), rabbit anti-mouse HSP90 antibody (Cell Signaling Technology, #4874S), or mouse anti-mouse β-tubulin antibody (Santa-Cruz Biotechnology, #sc23949)] was used at a ratio of 1:1,000 (AMPKα1,α2, AMPKα2, AMPKα1, AMPKβ1, AMPKβ2, ANGPTL4, HSP90, β-tubulin), 1:2,000 (phospho–AMPK) or 1:5,000 (LPL). Corresponding secondary antibodies (HRP-conjugated) (Sigma-Aldrich) were used at 1:5,000 dilutions. All incubations were performed in Tris-buffered saline, pH 7.5, with 0.1% Tween-20 (TBS-T) and 5% dry milk, except for anti-AMPKα1,2 and anti-phospho-AMPK Thr172 antibody, where 5% bovine serum albumin (BSA) was used instead of milk. All washing steps were in TBS-T without dry milk or BSA. Blots were visualized using the ChemiDoc MP system (Bio-Rad) and Clarity ECL substrate (Bio-Rad).

Cell culture

3T3-F442a cells (P8-P14, Sigma) were maintained in DMEM (Lonza), supplemented with 10% newborn calf serum and 1% penicillin/streptomycin (P/S) under 5% CO_2 at 37˚C. At confluency, cells were switched to DMEM (Lonza, Verviers, Belgium), supplemented with 10% fetal bovine serum (FBS), 1% P/S and 5 µg/mL insulin (Sigma-Aldrich) to stimulate differentiation. During differentiation, medium was changed every 2/3 days. After 10 days of differentiation, cells were switched back to regular medium for 2/3 days, after which experiments were performed.

T37i cells (P31-36; kind gift of Marc Lombès) were cultured in DMEM/F-12 (Gibco, Life Technologies, Blijswijk, the Netherlands), supplemented with 10% FBS and 1% P/S. Two days post-confluency, cell culture medium was supplemented with 112 ng/mL insulin and 2 nM T3 (Sigma-Aldrich) to induce differentiation. After 7 days of differentiation, cells were switched back to regular medium and used for experiments 2/3 days after (29).

HepG2 cells (passage unknown) were maintained in DMEM (Lonza) supplemented with 10% FBS and 1% P/S. At each passage, the cell pellet was filtered through a 40 µm filter to reduce cell clumping.

Culture of BA adipocytes was performed as described previously (30). Briefly, immortalized brown adipocytes are grown to confluence with differentiation medium (DMEM, 10% FBS, 20 nM insulin, 1 nM T3). Upon confluence, cells were treated with induction medium (differentiation medium supplemented with 0.5 mM IBMX, 0.5 µM dexamethasone, 0.125 mM indomethacin) for two days. After washing, cells were incubated in differentiation medium for another 5 to 7 days.

Isolation of primary adipocytes

WAT was dissected from C57Bl/6J mice and put in DMEM supplemented with 1% P/S and 1% BSA. Tissues of 3-4 mice were pooled, minced with scissors and digested for 1h at 37˚C in collagenase-containing medium (DMEM with 3.2 mM $CaCl_2$, 1.5 mg/mL Collagenase type II (Sigma-Aldrich), 10% FBS, 0.5% Bovine Serum Albumin (Sigma-Aldrich) and 15 mM HEPES, filtered). After digestion, cell mixture was passed over a 100 µm cell strainer and centrifuged at 1600 rpm for 10 minutes. Supernatant was removed and the pellet containing the stromal vascular fraction was re-suspended in erythrocyte lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) and incubated for 2-3 minutes at room temperature. Following neutralization, cells were centrifuged at 1200 rpm for 5 minutes. Cells were re-suspended in DMEM containing 10% FBS and 1% P/S and plated. Upon confluence, cells were differentiated following standard protocol of 3T3-L1 cells, as described previously (31).

For isolation of primary brown adipocytes, BAT from 1-month-old pups was used. Tissues of 5-10 pups were pooled, minced with scissors and digested for 30 min in collagenase-containing medium at 37˚C (DMEM w/o serum, 2 mg/mL Collagenase type II, 2% BSA, 25 mM HEPES). After digestion, cells were passed through a 70 or 100 µM filter, mature adipocytes were discarded and cells were centrifuged at 800*g for 5 min. Cells were re-suspended in differentiation medium (DMEM, 10% FBS, 20 nM insulin, 1 nM T3) and plated. Upon confluence, cells were treated with induction medium (differentiation medium supplemented with 0.5 mM IBMX, 0.5 µM dexamethasone, 0.125 mM indomethacin) for two days. After washing, cells were incubated in differentiation medium for another 5 to 7 days.

siRNA experiments

T37i cells were cultured and differentiated as described above. At day 10 of differentiation, mature adipocytes were trypsinized and replated at 70% density. 2 hours post-plating, siRNAs against AMPKα1 and AMPKα2, or non-target control (Dharmacon, *via* Thermo Fisher) complexed to Lipofectamine RNAimax reagent (Life Technologies) were added, according to the manufacturer's protocol. 48h post-transfection, cells were washed and treated as indicated before harvesting for RNA analyses.

BAT and WAT explants

BAT and WAT tissues were dissected from male C57Bl/6J mice and transferred to DMEM supplemented with 1% P/S and 1% BSA. Tissues of 4 mice were pooled and minced finely with scissors. Approximately 50 mg of tissue was transferred to a well of a 24-wells tissue culture plate and equilibrated for 1h in DMEM, supplemented with 1% P/S. After 1h, explants were treated as indicated before being harvested for RNA and protein analyses.

Chemicals

Isoproterenol, AICAR, metformin, rosiglitazone, Wy14,643, GW510516, GW742, dexamethasone, insulin, 3-isobutyl-1-1methylxanthine (IBMX), actinomycin D were purchased from Sigma-Aldrich. A769662 was purchased from Abcam (Cambridge, United Kingdom), phenformin hydrochloride was purchased from Cayman Chemicals (*via* SanBio, Uden, the Netherlands).

mAngptl4 PPRE construct

A fragment of 1517 bp containing intron 3 of the mouse *Angptl4* gene was amplified from DNA of the mouse *Angptl4* gene, using the primers: Fwd 5'-TTGCTGTCATCTGGCAACTC-3' and Rev 3'-CACCTAAAGCCTACCCCACA-5'. The resulting fragment was gel-purified using QIAquick Gel Extraction Kit (Qiagen) and subjected to a second PCR to specifically amplify the three functional PPREs of *Angptl4* and to introduce *Xho1* and *Kpn1* restriction sites. Amplification of the 565 bp fragment was done with the following primers: mouse Fwd 5'-atggtaccTTCACACCCTAAGGCTGC-3', and mouse Rev 3'-atctcgagGGGGGAAGAGGAAGAAAA-5'. Following purification from gel, the fragment was subjected to restriction with *Xho1* and *Kpn1* enzymes and cloned into the *Xho1* and *Kpn1* sites of the pGL3 promoter vector (Promega, Leiden, the Netherlands). Presence of the correct insert was validated by sequencing (EZ-Seq, Macrogen, Amsterdam, the Netherlands) using a RV3 primer: '5-CTAGCAAAATAGGCTGTCCC-3'.

Transactivation assays

m*Angptl4* PPRE pGL3 reporter vector was transfected into the human hepatocellular cell line HepG2 (ATCC, Manassas, USA) in the presence or absence of pSG5 vectors expressing *Pparγ* and *Rxr* and pcDNA vector expressing HA-*P300* (kind gift of Eric Kalkhoven, University Medical Centre Utrecht, the Netherlands). A vector expressing renilla luciferase under a SV40 promoter was co-transfected with all samples to determine transfection efficiency. Transfections were performed using polyethylenimine (PEI) (Polysciences Inc. *via* Qiagen). 16h post-transfection cells were incubated with rosiglitazone (5μM) and/or AICAR (1 mM) for 9h. Firefly and renilla luciferase activity were determined using the Dual Glo Luciferase Assay system (Promega), according to manufacturer's instructions, on a Fluoroskan Ascent apparatus (Thermo Scientific).

Statistics

Data are expressed as mean ± SEM, unless otherwise indicated. Differences were evaluated for statistical significance by student t-test or two-way ANOVA, followed by a post-hoc Tukey HSD test, and considered statistically significant when $p < 0.05$.

RESULTS

ANGPTL4 expression in BAT is down-regulated upon sustained cold exposure

ANGPTL4 and LPL protein are co-expressed in BAT (**Figure 1A**), suggesting a possible role for ANGPTL4 in regulating LPL in BAT. The specificity of the antibody used in the immunoblotting for ANGPTL4 was demonstrated by the lack of observable signal in BAT of *Angptl4*$^{-/-}$ mice (**Figure 1B - left panel**). Interestingly, we detected bands for ANGPTL4 at a molecular weight of ~52 kDa and ~45 kDa. The expected molecular weight of ANGPTL4 protein is 45 kDa, with a predicted glycosylation site at amino acid Asparagine-177 (in humans) or Asparagine 181 (in mice) (32–35). Consistent with the notion that these bands represent glycosylated (~52 kDa) and non-glycosylated forms (~45 kDa) of ANGPTL4, the 52 kDa band disappeared following treatment of BAT lysates with the endoglycosidase PGNase, while the 45 kDa band became more intense (**Figure 1B – right panel**). These data indicate that ANGPTL4 protein is present in BAT in glycosylated and non-glycosylated forms. Immunofluorescent staining on sections from human BAT obtained during surgery (as qualified based on UCP1 staining) showed that ANGPTL4 is also expressed in human BAT (**Figure 1C**). In agreement with high ANGPTL4 expression levels in BAT, *Angptl4* mRNA increased markedly upon differentiation of a mouse brown adipocyte cell line (**Figure 1D**).

We next explored the possible impact of cold on ANGPTL4 expression in BAT. Intriguingly, whereas 1 day of cold exposure did not affect *Angptl4* expression, 10 days of cold exposure led to a marked reduction in *Angptl4* mRNA (**Figure 1E**). The reduction in *Angptl4* mRNA was paralleled by a marked decrease in ANGPTL4 protein, particularly the glycosylated form of ANGPTL4 **(Figure 1F).** Expression of *Lpl*, on the other hand, was mildly elevated after both 1 and 10 days of cold exposure (**Figure 1G**).

Down-regulation of ANGPTL4 expression promotes BAT LPL activity upon sustained cold exposure

To investigate a possible role for ANGPTL4 in BAT function, we exposed *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice to a cold (4˚C) or thermo-neutral (28˚C) environment for 10 days in order to activate and recruit BAT **(Figure 2A)**. The *Angptl4*-Tg mice overexpress *Angptl4* under its own promoter and show elevated *Angptl4* expression in a variety of tissues, including BAT (18). The sustained cold exposure resulted in pronounced changes in BAT morphology, food intake, body weight, weights of BAT and WAT and body temperature, but no clear differences between the genotypes could be observed **(Figure 2B-F)**. Likewise, expression of the key thermogenic genes *Ucp1* and *Elovl3* was significantly increased upon cold exposure but not affected by *Angptl4* genotype **(Figure 2G)**.

Following cold exposure, the energy requirements of BAT increase dramatically. Two major fuel sources for BAT are plasma glucose and free fatty acids, both of which were unaltered by cold and *Angptl4* genotype (**Figure 2H&2I**). Also, plasma glycerol, which is perhaps a better indicator of adipose tissue lipolysis than free fatty

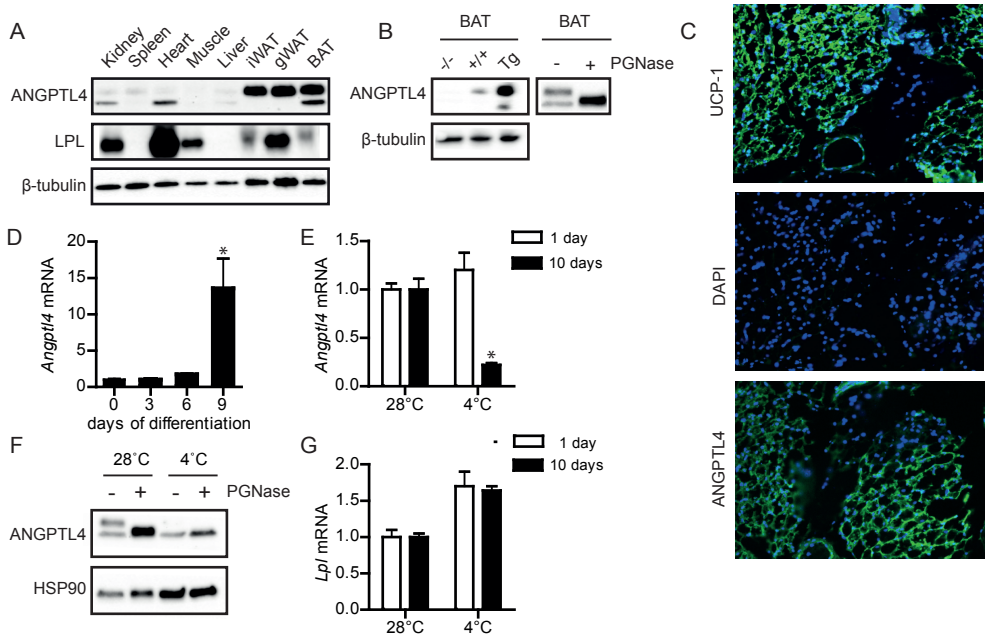

Figure 1: ANGPTL4 expression in BAT is down-regulated upon sustained cold exposure.

(A) Immunoblot for mouse ANGPTL4 and mouse LPL in lysates of kidney, spleen, heart, muscle, gonodal WAT, inguinal WAT and BAT of a C57BL/6J wild-type mouse. (B) Validation of anti-mANGPTL4 antibody in BAT lysates of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice (*left panel*). Detection of glycosylated and non-glycosylated mANGPTL4 following treatment of BAT homogenate of a wild-type mouse with PGNase (*right panel*). (C) Immunofluorescent staining of UCP1 (*upper panel*; UCP1=green, DAPI=blue), DAPI only (*middle panel*) and hANGPTL4 (*lower panel*; hANGPTL4 = green, DAPI=blue) in frozen sections (5 µm) of human BAT. (D) *Angptl4* mRNA in T37i cells after 0, 3, 6 or 9 days of differentiation. (E) *Angptl4* mRNA in BAT lysates of wild-type mice exposed to 4˚C or 28˚C for 1 or 10 days. (F) Immunoblot for ANGPTL4 in BAT homogenates of wild-type mice exposed to 4˚C or 28˚C for 10 days, following treatment with or without PGNase. (G) *Lpl* mRNA in BAT lysates of wild-type mice exposed to 4˚C or 28˚C for 1 or 10 days. * Statistically significant compared to control wells or compared to mice exposed to 28˚C according to Student's t-test ($p<0.05$). Error bars represent ± SEM. n= 8-10 mice per group.

acids, was not different between the three genotypes (**Figure 2J**). Additionally, *ex vivo* treatment of differentiated primary white adipocytes with the non-selective β-adrenergic receptor agonist isoproterenol indicated a lack of effect of *Angptl4* genotype on adipose tissue lipolysis (**Figure 2K**). Besides glucose and free fatty acids, circulating triglycerides (TG) represent a major fuel for BAT during cold (1). Based on the marked decrease in *Angptl4* mRNA levels in BAT upon prolonged cold exposure, we hypothesized that ANGPTL4 might play a role in the metabolism of circulating TG by BAT during cold. In line with this notion, the reduction in plasma TG visible in wild-type and *Angptl4*$^{-/-}$ mice in response to cold was greatly attenuated in *Angptl4*-Tg mice **(Figure 2L).** Lipoprotein profiling by fast protein liquid chromatography (FPLC) supported markedly augmented plasma TRL levels in cold-exposed *Angptl4*-Tg mice (**Figure 2M**).

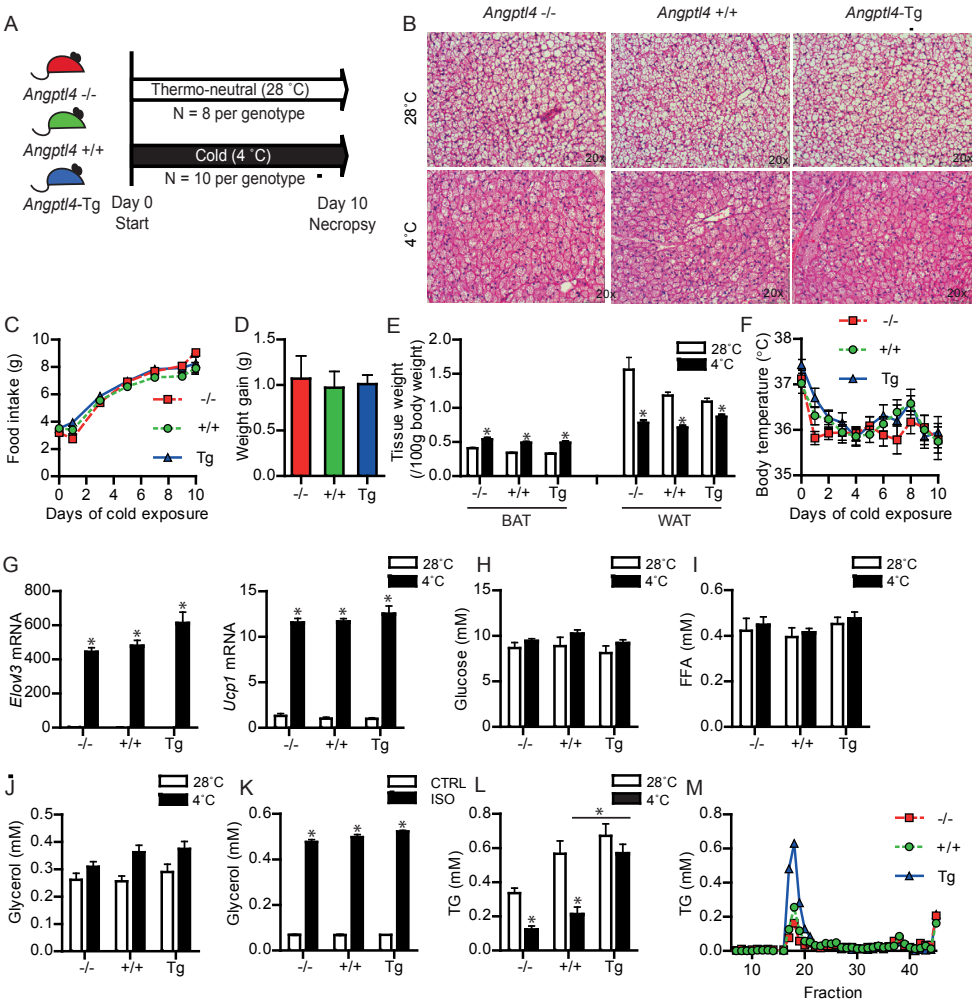

Figure 2: Down-regulation of ANGPTL4 in BAT upon sustained cold exposure affects plasma TG levels.

(A) Schematic representation of cold exposure experiment with *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice. (B) Haematoxylin & Eosin staining on BAT sections (5 µm) of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days. (C) Food intake of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C for 10 days. (D) Weight gain of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C during 10 days. (E) BAT and WAT tissue weights and (F) body temperature of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days. (G) *Elovl3* and *Ucp1* mRNA expression levels of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days. (H) Plasma glucose, (I) plasma free fatty acids, and (J) plasma glycerol levels of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days. (K) Glycerol levels in medium of differentiated primary white adipocytes from *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice, serum-starved and treated with 10 µM isoproterenol for 3h. (J) plasma TG levels of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days. (K) Fast protein liquid chromatography (FPLC) on pooled plasma samples of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C for 10 days, followed by analysis of TG levels in all fractions. * Statistically significant compared to mice of equal genotype at 28˚C or between groups as indicated by bars according to two-way ANOVA followed by a post-hoc Tukey HSD test ($p<0.05$). Error bars represent ± SEM. n = 8-10 mice per group.

It is well-established that cold-induced reductions in plasma TG levels are mediated by increased LPL activity in BAT (5, 6, 8). Accordingly, we tested whether ANGPTL4 levels influence changes in BAT LPL activity upon prolonged cold exposure. Whereas wild-type mice respond to cold with a reduction in BAT ANGPTL4 mRNA and protein levels, *Angptl4*-Tg mice maintain higher ANGPTL4 mRNA and protein levels (**Figure 3A & 3B**). Mirroring the levels of ANGPTL4, the cold-induced changes in LPL activity in BAT exhibited a gradient across the three *Angptl4* genotypes, with highest LPL activity observed in *Angptl4*$^{-/-}$ mice and lowest LPL activity in *Angptl4*-Tg mice (**Figure 3C**). Furthermore, the marked increase in LPL activity during cold observed in the wild-type mice was significantly blunted in *Angptl4*-Tg mice (**Figure 3C)**. Interestingly, the two-fold increase in LPL activity by cold in *Angptl4*$^{-/-}$ mice indicates that part of the induction of LPL activity in BAT is independent of ANGPTL4 (**Figure 3C)**, possibly *via* an increase in *Lpl* mRNA, which was observed in all three genotypes **(Figure 3D)** (6, 8). Overall, the marked gradient in LPL activity between *Angptl4*$^{-/-}$, wild-type, and *Angptl4*-Tg mice strongly suggests that ANGPTL4 acts as an inhibitor of LPL activity in BAT.

Down-regulation of ANGPTL4 expression promotes uptake of TRL-derived fatty acids by BAT upon sustained cold exposure

To investigate the role of ANGPTL4 in plasma TG clearance by BAT, we injected cold-exposed *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice with radiolabeled VLDL-like emulsion particles containing glycerol tri[^{3}H]oleate (hydrolysable by LPL; TRL-derived fatty acids) and [^{14}C]cholesteryl-oleate (not hydrolysable by LPL; TRL-Chol) (see **Supplemental Figure 1** for experimental set-up) (19). After 15 minutes, the mice were sacrificed and the tissue distribution of ^{3}H and ^{14}C activity was determined. As expected, cold exposure markedly increased the rate of clearance of the injected VLDL-like particles from the plasma **(Figure 4A & 4B)**. However, after cold exposure, plasma clearance of glycerol tri[^{3}H]oleate (TRL FA), but not [^{14}C] cholesteryl-oleate (TRL Chol), was significantly slower in *Angptl4*-Tg mice, indicating that *Angptl4*-overexpression inhibits LPL-mediated plasma TG clearance in the cold **(Figure 4C & 4D)**. Cold exposure caused a marked increase in TRL-derived fatty acid uptake into BAT in all three genotypes, indicating that part of the increase in fatty acid uptake is independent of ANGPTL4 (**Figure 4E**). However, similar to LPL activity, a clear gradient in TRL-derived fatty acid uptake into BAT was observed between the three genotypes, with lowest uptake in *Angptl4*-Tg mice (**Figure 4E**). Similar results were obtained for [^{14}C]cholesteryl-oleate, showing a marked decrease in BAT uptake in the *Angptl4*-Tg mice (**Figure 4F**). We repeated the plasma TG clearance studies using radiolabeled lipoprotein particles with a larger diameter, resembling postprandial chylomicrons. Again, *Angptl4*-overexpression markedly reduced uptake into BAT of the TRL-derived fatty acids and the core label cholesteryl-oleyloleate (**Figure 4G & 4H)**. To visualize the TRL uptake process, we injected hydrophobic fluorescent nanocrystals embedded in lipoprotein particles (QD-TRLs) into cold-exposed *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice (20). In agreement with a role for ANGPTL4 in TRL processing in BAT, increased accumulation of QD-TRLs was observed after cold exposure of wild-type mice, but not of *Angptl4*-Tg mice (**Fig-**

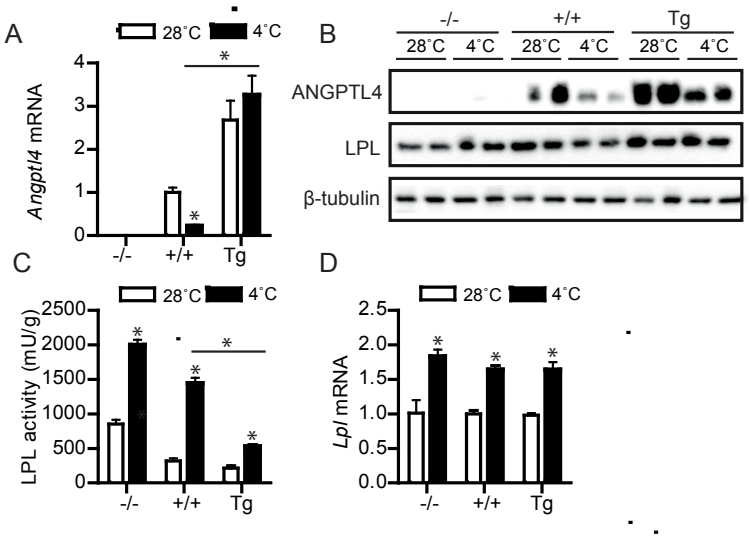

Figure 3: Down-regulation of ANGPTL4 in BAT upon sustained cold exposure promotes an increase in BAT LPL activity.

(A) *Angptl4* mRNA in BAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4°C or 28°C for 10 days. (B) Immunoblot for ANGPTL4 and LPL in BAT homogenates from *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4°C or 28°C for 10 days. (C) Total LPL activity and (D) *Lpl* mRNA in BAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4°C or 28°C for 10 days. * Statistically significant compared to mice of equal genotype at 28°C or between groups as indicated by bars, according to two-way ANOVA followed by a post-hoc Tukey HSD test ($p<0.05$). Error bars represent ± SEM. n = 8-10 mice per group.

ure 4I). Furthermore, *Angptl4*$^{-/-}$ mice show an accumulation of QD-TRLs in BAT even when maintained at 28°C (**Figure 4I**). Together, these data are supportive of a major role for ANGPTL4 as a regulator of LPL activity and concomitant uptake of fatty acids into BAT upon prolonged cold exposure.

ANGPTL4 expression and LPL activity are oppositely regulated in WAT and BAT upon sustained cold exposure

Whereas BAT utilizes lipids to fuel thermogenesis, WAT provides lipid fuels to be used by BAT. Accordingly, lipid uptake and LPL activity are expected to be regulated differently in BAT as compared to WAT. Indeed, whereas in wild-type mice LPL activity in BAT increased dramatically during cold, LPL activity in WAT remained unchanged, at least in wild-type and *Angptl4*-Tg mice (**Figure 5A**). However, LPL activity was increased by cold in *Angptl4*$^{-/-}$ mice, indicating that ANGPTL4 prevents LPL activity in WAT from going up during cold (**Figure 5A**). In support of this function for ANGPTL4, cold exposure caused a marked increase in *Angptl4* mRNA and protein levels in WAT (**Figure 5B & 5C**). In contrast to *Angptl4*, *Lpl* mRNA levels were not significantly altered in WAT upon sustained cold exposure (**Figure 5D**). Interestingly, exposure of human subjects to mild cold (16°C) for 48h significantly

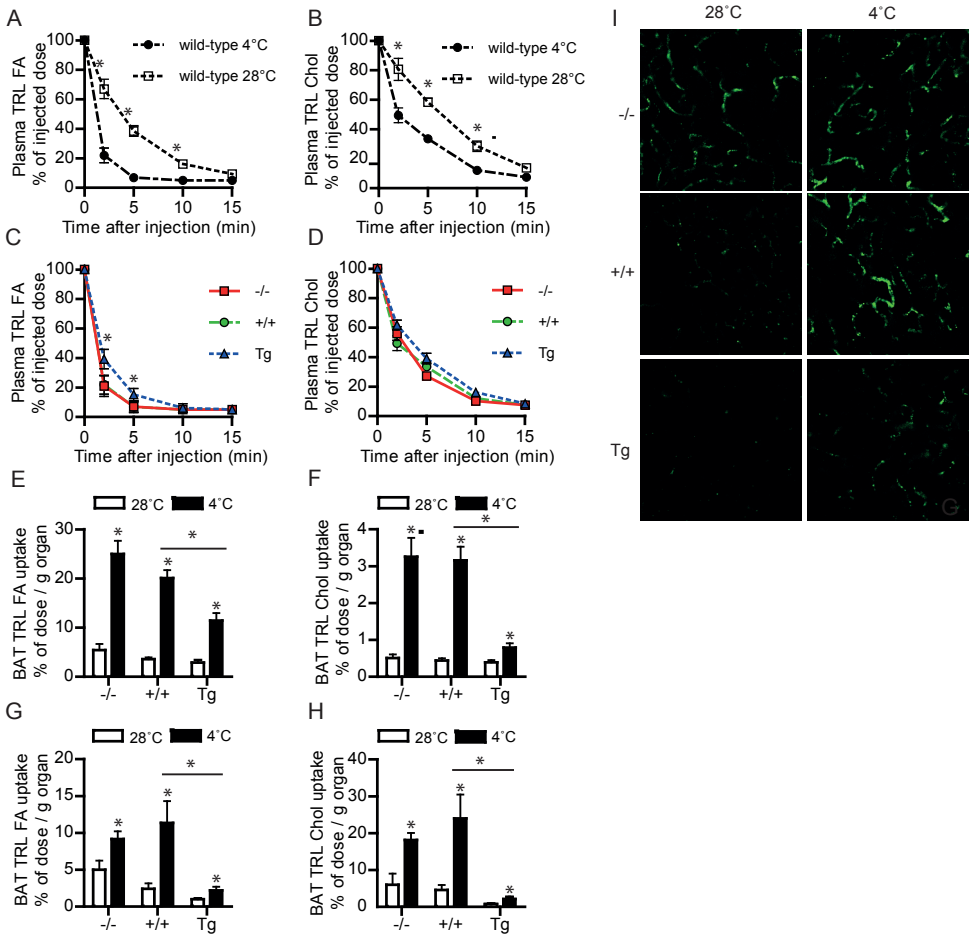

Figure 4: Down-regulation of ANGPTL4 in BAT upon sustained cold exposure promotes an increase in TRL-derived fatty acid uptake by BAT.

(A&B) Plasma ^{3}H (A) and ^{14}C (B) activity in wild-type mice exposed to 4˚C or 28˚C for 10 days levels, intravenously injected with VLDL-like particles, labelled with glycerol tri[^{3}H]oleate (TRL FA) and [^{14}C]cholesteryl-oleate (TRL Chol). (C & D) Plasma ^{3}H (C) and ^{14}C (D) activity in *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice intravenously injected with VLDL-like emulsion particles, labelled with glycerol tri[^{3}H]oleate (TRL FA) and [^{14}C]cholesteryl-oleate (TRL Chol), following exposure to 4˚C for 10 days. (E & F) ^{3}H activity (E) and ^{14}C activity (F) in BAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days and intravenously injected VLDL-like particles, labelled with glycerol tri[^{3}H]oleate (TRL FA) and [^{14}C] cholesteryl-oleate (TRL Chol). (G&H) ^{14}C and ^{3}H activity in BAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days, intravenously injected with chylomicron-like particles, labelled with glycerol tri[^{14}C]oleate (TRL FA) (G) and [^{3}H]cholesteryl-oleyloleate (TRL Chol). (I) Fluorescent image of uptake of intravenously injected QD-TRLs into BAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 9 days. Image was taken 12 minutes post-injection. n=2 mice per group* Statistically significant compared to mice of equal genotype at 28˚C or between groups as indicated by bars, according to two-way ANOVA followed by a post-hoc Tukey HSD test ($p<0.05$). Error bars represent ± SEM. n = 7 mice per group, unless otherwise indicated.

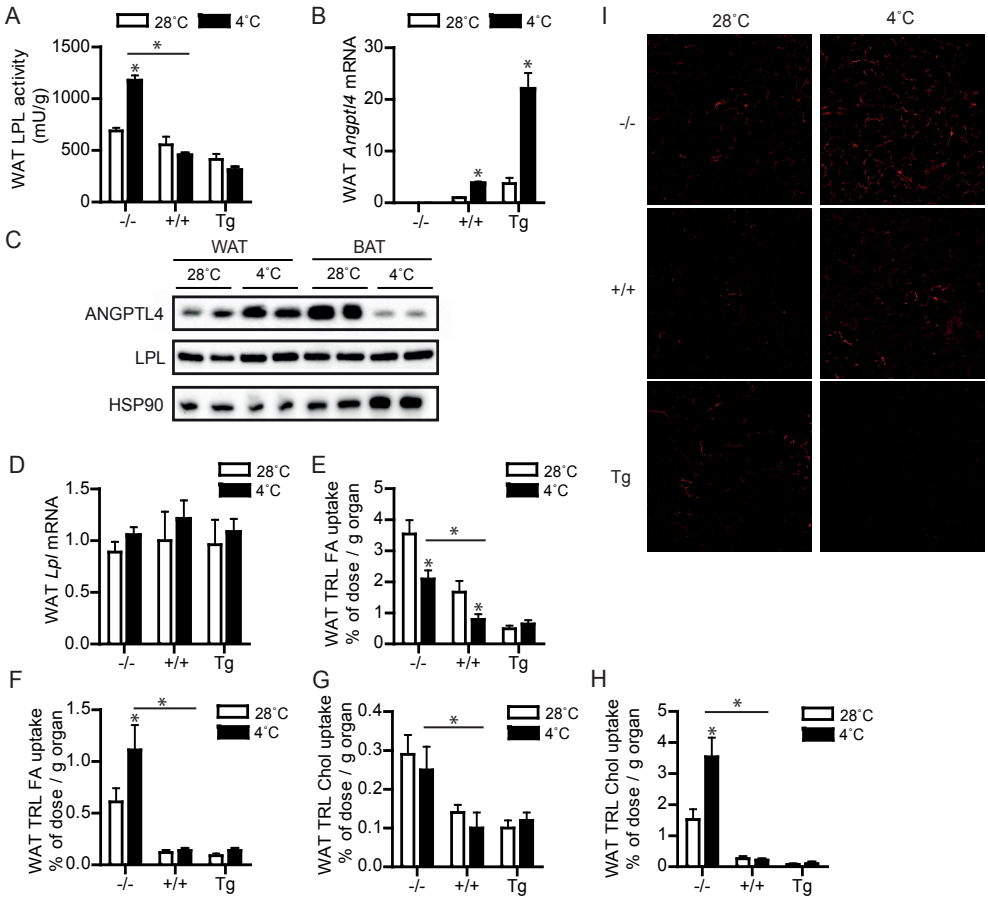

Figure 5: Up-regulation of ANGPTL4 in WAT upon sustained cold exposure suppresses WAT LPL activity and TRL-derived fatty acid uptake.

(A) Total LPL activity levels and (B) *Angptl4* mRNA in WAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days. (C) Immunoblot for ANGPTL4 and LPL in WAT homogenates of wild-type mice exposed to 4˚C or 28˚C for 10 days. (D) *Lpl* mRNA in WAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days. (E-H) Activity of ^{3}H and ^{14}C radiolabels in WAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days, intravenously injected with radiolabelled VLDL-like and chylomicron-like particles. TRL FA uptake (E&F) reflects uptake of glycerol tri[^{3}H/^{14}C]oleate, whereas TRL Chol uptake (G&H) reflects uptake of the core labels [^{14}C]cholesteryl-oleate or [^{3}H]cholesteryl-oleyloleate. (I) Fluorescent image of uptake of intravenously injected QD-TRLs into WAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 9 days. Image was taken after perfusion of mice with PBS containing 50 IU/mL heparin and upon cryosectioning of tissues. n = 2 mice per group. * Statistically significant compared to mice of equal genotype at 28˚C or between groups as indicated by bars, according to two-way ANOVA followed by a post-hoc Tukey HSD test ($p<0.05$). Error bars represent ± SEM. n = 7-10 mice per group, unless otherwise indicated.

increased plasma ANGPTL4 levels in obese subjects, but not in lean subjects. Considering the higher adipose tissue mass in obese individuals, these data suggest that the increase in ANGPTL4 production in WAT dominates ANGPTL4 levels in human plasma (**Supplemental Figure 2**).

To determine if the suppressive effect of ANGPTL4 on LPL activity had any impact on uptake of TRL-derived fatty acids in WAT during cold, we measured fatty acid uptake following injection of radiolabeled VLDL-like and chylomicron-like emulsion particles. Even though the uptake behaviour of the two types of TRL-particles was somewhat different, ANGPTL4 expression caused a clear dose-dependent reduction in uptake of TRL-derived fatty acids and the core labels cholesteryl-oleate/cholesteryl-oleyloleate **(Figure 5E-H)**. In addition, inhibition of TRL processing by ANGPTL4 in WAT was visually confirmed by injection of QD-TRLs, showing increased accumulation of QD-TRLs in WAT of *Angptl4*$^{-/-}$ mice, as compared to wild-type and *Angptl4*-Tg mice **(Figure 5I)**.

It is noteworthy that in the inguinal fat depot, cold-induced changes in *Lpl* mRNA, *Angptl4* mRNA and LPL activity were similar to the changes observed in the gonadal fat depot described above, whereas changes in uptake of TRL-derived fatty acids upon cold exposure were quite distinct, most likely due to marked activation of browning in inguinal fat (**Figure 6A&6B; Supplemental Figure 3**). In contrast to BAT and WAT, uptake of TRL-derived fatty acids or cholesteryl-oleate/cholesteryl-oleyloleate from VLDL-like particles and chylomicron-like particles was minimally different between *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice in liver, skeletal muscle and spleen **(Figure 6A & 6B)**. Taken together, our data indicate that in BAT down-regulation of ANGPTL4 promotes uptake of plasma TRL-derived fatty acids *via* enhanced LPL activity, whereas in WAT ANGPTL4 suppresses uptake of plasma TRL-derived fatty acids *via* inhibition of LPL activity, thereby directing plasma TG to BAT to be used as fuel.

The opposite regulation of ANGPTL4 expression in BAT and WAT may be mediated by differential activation of AMPK

We next explored the mechanism accounting for the inverse regulation of ANGPTL4 expression in BAT and WAT. Since many of the cold-induced adaptations are triggered by β-adrenergic signaling, we examined the effect of β-adrenergic activation on ANGPTL4 expression in BAT and WAT using murine BAT (T37i cells) and WAT (3T3-F442a) cell lines and primary cells (29). In both BAT and WAT cells, treatment with isoproterenol, a non-selective agonist of β-receptors, consistently resulted in a marked increase in expression of *Angptl4* mRNA **(Figure 7A & 7B)**. Induction of ANGPTL4 by β-adrenergic stimulation was confirmed at the protein level (**Figure 7C**). From these data, it is evident that activation of the β-adrenergic signaling pathway may contribute to the cold-induced up-regulation of ANGPTL4 in WAT, but cannot explain the down-regulation of ANGPTL4 observed in BAT. Levels of *Lpl* mRNA and protein in white and brown adipocytes were only mildly affected by treatment with β-adrenergic agonists (**Figure 7C & 7D**).

We, therefore, considered mechanisms that are clearly different between BAT and WAT. Previously, it had been shown that 5'AMP-activated protein kinase

(AMPK) is progressively activated with prolonged cold exposure and that AMPK expression is significantly higher in BAT compared to WAT (36). Consistent with this finding, we found that the catalytic α-subunits of AMPK, as well as phosphorylation of AMPK at Threonine-172—indicative of AMPK activity—are barely detectable in WAT in the basal state **(Figure 7E)** (37, 38). Previously, differential tissue expression of isoforms of the α, β, and γ subunits of the AMPK heterotrimer was suggested to determine cellular and systemic responses to different metabolic stressors (37, 38). Intriguingly, we detected large basal differences in AMPK α- and β-subunit isoform distribution between BAT and WAT and, more specifically, found a high expression of the AMPKα2 catalytic subunit and the AMPKβ1 regulatory subunit in BAT compared to WAT (**Figure 7E**). The differences in total AMPK expression and subunit distribution may explain why WAT AMPK levels remain weak following sustained cold exposure, whereas a strong increase in AMPK and phospho-AMPK is observed in BAT **(Figure 7F).**

In muscle cells, we found that AMPK activation strongly down-regulates ANGPTL4 mRNA and protein levels (14). To examine the consequences of AMPK activation in BAT, we treated differentiated T37i brown adipocytes with multiple AMPK activators, including AICAR, A769662, metformin and phenformin hydrochloride. Without exception, AMPK activation markedly down-regulated *Angptl4* mRNA expression (**Figure 8A**). To further confirm the down-regulation of ANGPTL4 by AMPK, we treated different *in vitro* model systems for BAT with the AMPK activator AICAR. AICAR treatment of differentiated T37i adipocytes, BA adipocytes (30) or murine primary brown adipocytes resulted in a marked decrease in ANGPTL4 mRNA and protein levels (**Figure 8B & 8C**). Part of the inhibitory effect of AMPK activation on *Angptl4* expression could be rescued by siRNA-mediated knock-down of AMPKα1 and AMPKα2 in differentiated T37i adipocytes, corroborating the suppressive effect of AMPK on *Angptl4* expression (**Figure 8D-8F**). Consistent with the notion that the negative regulation of ANGPTL4 by AMPK is specific for BAT, AICAR treatment markedly reduced ANGPTL4 mRNA and protein levels in BAT explants, but not WAT explants (**Figure 8G & 8H**). Based on these data, we propose that the different amount and activation of AMPK between BAT and WAT may be the critical factor in the differential regulation of ANGPTL4 between the two tissues during sustained cold.

AMPK may act through PPARγ to regulate ANGPTL4 levels

We further pursued the mechanism behind the down-regulation of ANGPTL4 by AMPK. Treatment of brown adipocytes with AICAR and the transcriptional inhibitor actinomycin D showed that both compounds reduced *Angptl4* gene expression by nearly the same extent. Co-treatment of actinomycin D and AICAR did not result in an additive effect, suggesting that AMPK activation almost completely inhibited *Angptl4* gene transcription (**Figure 9A**).

Expression of ANGPTL4 is under sensitive control of peroxisome proliferator-activated receptors (PPARs) in many tissues, with PPARγ being the dominant regulator of ANGPTL4 in adipose tissue (15, 35, 39). We found that in BA and T37i brown adipocytes ANGPTL4 mRNA and protein are highly induced by PPARγ ago-

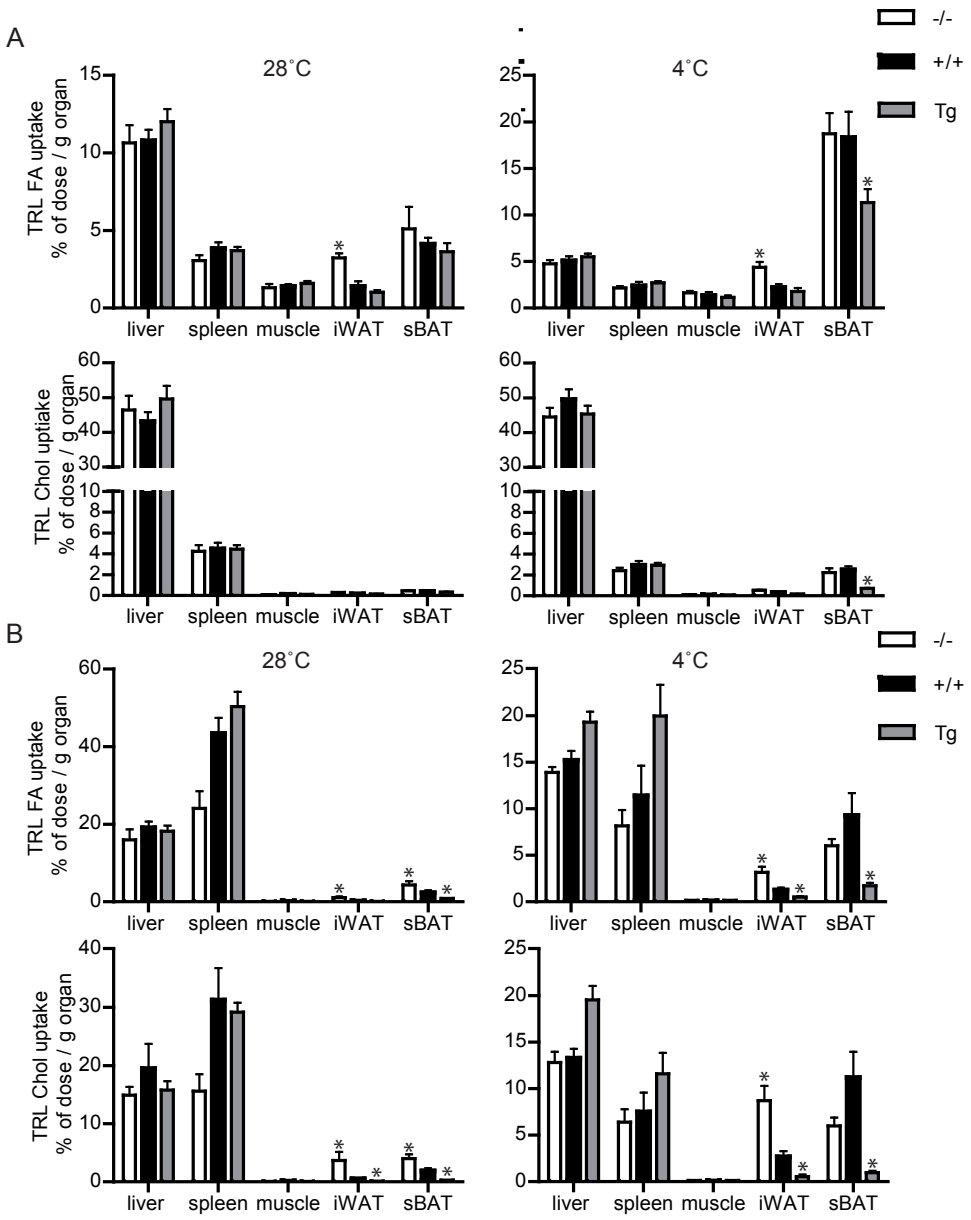

Figure 6: Uptake of TRL-like particles in liver, spleen and muscle is not affected by *Angptl4* genotype.

(A) ^{3}H and ^{14}C activity in liver, spleen, muscle, inguinal WAT (iWAT) and subscapular BAT (sBAT) of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days, intravenously injected with VLDL-like emulsion particles, labelled with glycerol tri[^{3}H]oleate (TRL FA) and [^{14}C]cholesteryloleate (TRL Chol). (B) ^{3}H and ^{14}C activity in liver, spleen, muscle, iWAT and sBAT of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days, intravenously injected with chylomicron-like particles, labelled with glycerol tri[^{14}C]oleate (TRL FA) and [^{3}H]cholesteryl-oleyloleate (TRL Chol). *Statistically significant compared to values of wild-type mice, for each tissue, and 4˚C and 28˚C separately, according to Student's t-test ($p<0.05$). Error bars represent ± SEM. n = 7 mice per group.

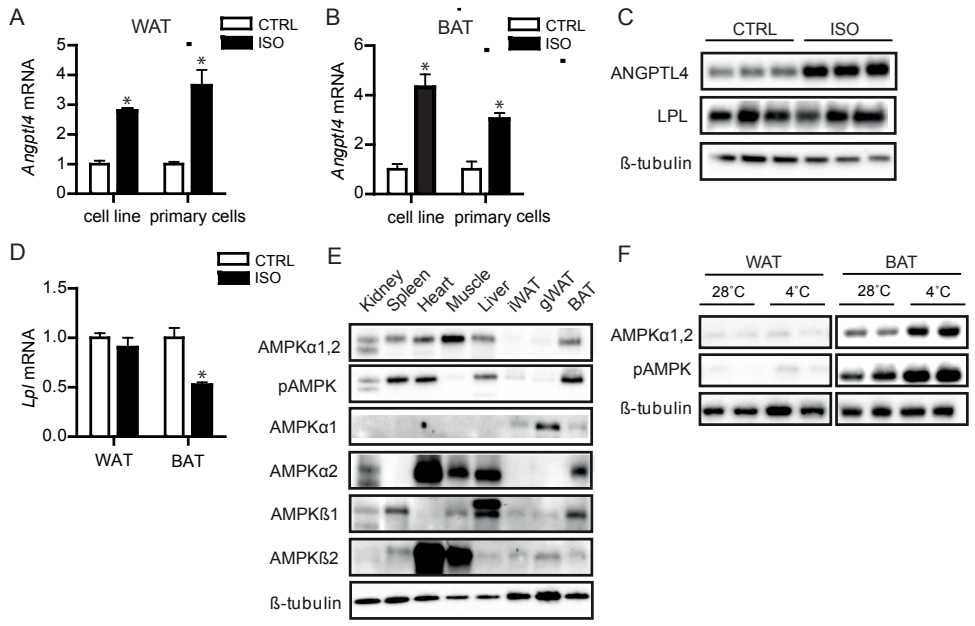

Figure 7: AMPK is activated in BAT, but not WAT, upon sustained cold exposure.

(A) *Angptl4* mRNA in differentiated mouse white adipocytes (primary adipocytes and 3T3F442a adipocytes) upon treatment with 10 μM isoproterenol (ISO) or H_2O control medium (CTRL) for 3h. (B) *Angptl4* mRNA in differentiated brown adipocytes (primary adipocytes and T37i adipocytes) treated with 10 μM isoproterenol (ISO) or H_2O control medium (CTRL) for 2.5h. (C) Immunoblot for ANGPTL4 and LPL protein in differentiated 3T3F422a cells treated with 10 μM isoproterenol (ISO) or H_2O control medium (CTRL) for 3h. (D) *Lpl* mRNA in differentiated mouse primary white or brown adipocytes upon treatment with 10 μM isoproterenol (ISO) or H_2O control medium (CTRL) for 2.5h (BAT) or 3h (WAT). (E) Immunoblot for AMPKα1,2 and phospho-AMPK Thr172, AMPKα1, AMPKα2, AMPKβ1 and AMPKβ2 in tissue lysates of kidney, spleen, heart, muscle, liver, inguinal WAT, gonodal WAT and BAT. Homogenates are identical to the homogenates presented in Figure 1A. (F) Immunoblot for AMPKα1,2 and phospho-AMPK Thr172 in BAT and WAT lysates of wild-type mice exposed to 4˚C or 28˚C for 10 days. *Statistically significant compared to control samples or between indicated treatments according to Student's t-test ($p<0.05$). Error bars represent ± SEM.

nists and to a lesser extent by agonists for PPARα and PPARδ (**Figure 9B & 9C**).

Previous studies have shown that AMPK may inhibit PPARα and PPARγ transcriptional activity (40, 41). Indeed, we observed that AMPK activation almost completely blocked the induction of *Angptl4* mRNA following treatment with the PPARγ agonist rosiglitazone (**Figure 9D**). Accordingly, we hypothesized that activation of AMPK following prolonged cold exposure may inhibit PPARγ-mediated transcription of the *Angptl4* gene. To examine this possibility, a luciferase construct was prepared containing the three conserved PPAR response elements (PPREs) of intron 3 of the murine *Angptl4* gene. These three PPREs have previously been shown to be responsible for PPAR-mediated up-regulation of *Angptl4* (39, 42). Upon transfection of the *Angptl4* PPRE construct into HepG2 cells, rosiglitazone treatment sig-

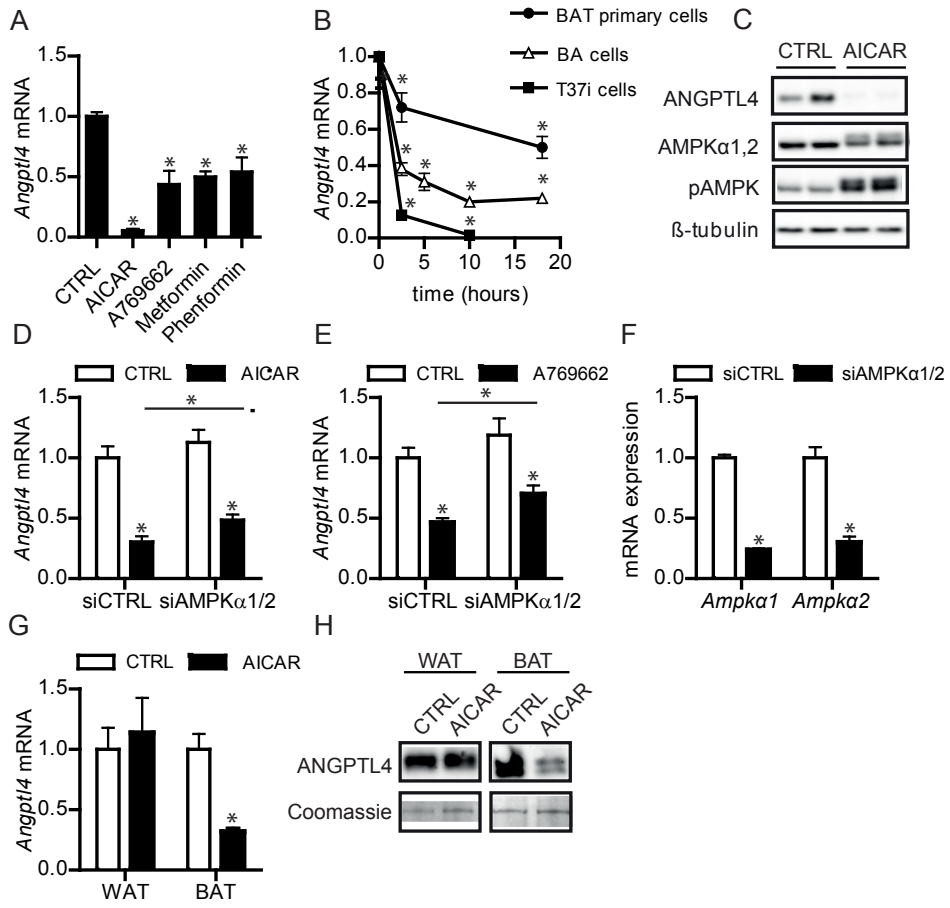

Figure 8: Activation of AMPK down-regulates ANGPTL4 expression specifically in brown adipocytes.

(A) *Angptl4* mRNA in differentiated T37i adipocytes treated for 6h with 1 mM AICAR, 100 µM A769662, 1 mM metformin or 250 µM phenformin hydrochloride. (B) *Angptl4* mRNA in differentiated primary brown adipocytes, BA adipocytes, or T37i adipocytes treated for indicated times with 1 mM AICAR. (C) Immunoblot for ANGPTL4, LPL, AMPKα1,2 and phospho-AMPK Thr172 in differentiated T37i cells treated with H_2O control medium (CTRL) or 1 mM AICAR for 3h. (D) *Angptl4* mRNA in differentiated T37i adipocytes treated with CTRL siRNA or siRNA against AMPKα1 and AMPKα2 for 48h, followed by incubation with H_2O control medium (CTRL) or 1 mM AICAR for 3h. (E) *Angptl4* mRNA in differentiated T37i adipocytes treated with CTRL siRNA or siRNA against AMPKα1 and AMPKα2 for 48h, followed by incubation with H_2O control medium (CTRL) or 100 µM A769662 for 6h. (E) *Ampka1* and *Ampka2* mRNA in differentiated T37i adipocytes treated with CTRL siRNA or siRNA against AMPKα1 and AMPKα2 for 48h. (F) *Angptl4* mRNA levels in BAT and WAT explants from C57BL/6J wild-type mice (~50 µg) treated with H_2O control medium (CTRL) or 1 mM AICAR for 3h. (G) Immunoblot for ANGPTL4 in BAT and WAT explants from C57BL/6J wild-type mice (~50 mg) treated with H_2O control medium (CTRL) or 1 mM AICAR for 3h. *Statistically significant compared to control samples or between indicated treatments, according to Student's t-test ($p<0.05$). Error bars represent ± SEM.

nificantly induced luciferase activity, which was further increased upon co-transfection with *Pparγ*/Retinoid X receptor (*Rxr*). Co-treatment with AICAR, on the other hand, blunted the increase in luciferase activity (**Figure 9E**). Interestingly, whereas co-transfection of the PPARγ co-activator P300 (43) further stimulated luciferase activity in the absence of AICAR, it failed to do so in the presence of AICAR, suggesting that regulation of P300 may be part of the mechanism of inhibition of ANGPTL4 by AMPK (**Figure 9E**). In support of this possibility, treatment of brown adipocytes with AICAR resulted in phosphorylation of P300 at serine residue 89, which is known to reduce the capacity of P300 to co-activate nuclear transcription factors, whereas no change in total P300 protein levels was observed (**Figure 9F**). Collectively, these data suggest that the negative regulation of ANGPTL4 in BAT upon cold exposure may be mediated by the inhibition of PPARγ transcriptional activity by AMPK.

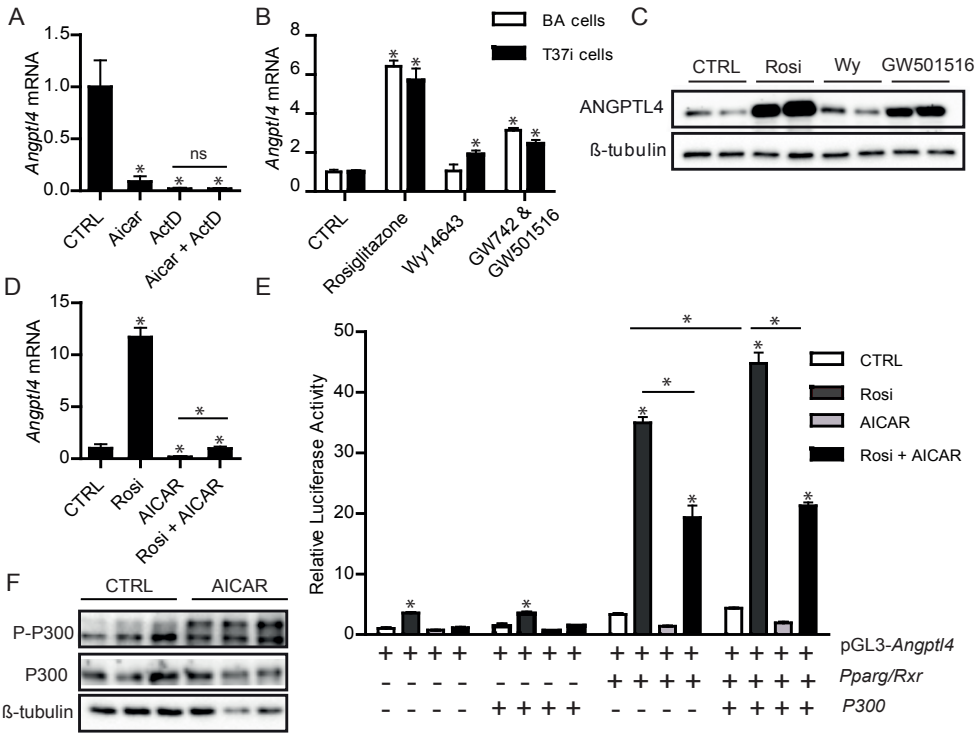

Figure 9: Down-regulation of *Angptl4* expression by AMPK is likely mediated *via* inhibition of PPARγ-mediated transcription of *Angptl4*.

(A) *Angptl4* mRNA in differentiated T37i adipocytes pre-incubated with 0.5 µg/mL actinomycin D (ActD) or DMSO-containing control medium for 1h and treated with 1 mM AICAR or H_2O control medium for 3h. (B) *Angptl4* mRNA in differentiated T37i or BA adipocytes treated with DMSO-containing control medium, 5 µM rosiglitazone, 10 µM Wy14643, and 5 µM GW742 (BA adipocytes) or 5 µM GW501516 (T37i adipocytes) for 6h. (C) Immunoblot for ANGPTL4 in differentiated T37i adipocytes treated with 5 µM rosiglitazone (Rosi), 10 µM Wy14643 (Wy), and 5 µM GW501516 for 24h. (D) *Angptl4* mRNA in differentiated T37i cells treated with DMSO-containing control medium, 5 µM rosiglitazone (Rosi), 1 mM AICAR or both rosiglitazone and AICAR for 6h. (E) Relative luciferase activity of HepG2 cells transfected with pGL3-*Angptl4*, pSG5-*Pparγ*, pSG5-*Rxr*, pcDNA-P300HA vectors, as indicated and treated 16h post-transfection with 5 µM rosiglitazone, 1 mM AICAR or both compounds for 9h. Data are represented as ± SD. (F) Immunoblot for P300 and phospho-P300 (Ser-89) in differentiated T37i brown adipocytes treated with H_2O control medium or 1 mM AICAR for 3h. *Statistically significant compared to control samples or between indicated samples according to Student's t-test ($p<0.05$). Error bars represent ±SEM, unless otherwise specified.

DISCUSSION

The energy requirements of BAT increase manifold during cold exposure. The increased energy demands coincide with a marked increase in LPL activity, stimulating uptake of TRL-derived fatty acids (6–8, 44, 45). Increased LPL activity has been shown to be essential for the lipid-lowering effect of cold exposure, as injection of heparin or tetrahydrolipstatin compromises LPL-dependent uptake of TRLs and TRL-derived fatty acids (6). Since *Lpl* mRNA in BAT is only moderately increased upon prolonged cold exposure, it has been suggested that the pronounced increase in LPL activity in BAT occurs at the post-translational level (8, 12). Our data demonstrate that a substantial part of the increase in LPL activity in BAT during prolonged cold exposure is mediated by down-regulation of ANGPTL4.

Overall, our findings reveal a major role for ANGPTL4 in the regulation of lipid partitioning during sustained cold. Specifically, the data implicate ANGPTL4 as an important mediator of preferential shuttling of TRL-derived fatty acids to BAT during cold exposure. *Via* direct effects on local LPL activity and subsequent fatty acid uptake, the reciprocal regulation of ANGPTL4 in BAT and WAT assures an adequate fuel delivery to BAT during cold exposure. The differential regulation of ANGPTL4 and LPL between BAT and WAT leads to corresponding changes in fatty acid uptake from TRLs, with our data showing a clear dose-dependent and causal relationship between ANGPTL4 expression and TRL-derived fatty acid uptake into both tissues. The importance of ANGPTL4 in the regulation of LPL activity during cold complements the already established role of ANGPTL4 in regulation of LPL during fasting and exercise in WAT and skeletal muscle, respectively (13, 14). ANGPTL4 can thus be viewed as the master regulator of tissue LPL activity and fatty acid uptake during physiological conditions such as fasting, exercise and cold exposure.

Our data suggest that the opposite regulation of ANGPTL4 expression during prolonged cold between BAT and WAT may be explained by the differential expression and activation of AMPK between the two tissues. Heterotrimeric AMPK has one catalytic (α), and two regulatory (β and γ) subunits, each having distinctive isoforms with a tissue-specific distribution (37, 38). Tissue-specific combinations of different subunit isoforms may confer tissue-specific properties to AMPK by determining subcellular localization and substrate targeting, thereby controlling cellular and systemic responses to metabolic stressors, including sustained cold (37, 38). Indeed, with prolonged cold exposure, AMPK becomes progressively activated in BAT and only to a minor extent in WAT (36, 46).

Systemic activation of AMPK has been previously shown to increase the activity of LPL in both heart and muscle and to lower plasma TG levels (14, 47–51). Furthermore, AMPK activation was found to cause a pronounced reduction in ANGPTL4 expression in muscle cells (14). Similar to muscle and heart, we found the AMPKα2 catalytic subunit to be abundantly present in BAT, but not WAT (37). Together, the increased AMPK activation and different AMPK subunit expression in BAT as compared to WAT may explain why the repressive effect of cold-induced AMPK activation on ANGPTL4 expression is much more pronounced in BAT than in WAT. We suggest that regulation of ANGPTL4 in WAT during cold may be dominated by activation of β-adrenergic signaling, which may explain the increase in

ANGPTL4 expression observed in WAT during sustained cold exposure.

We provide evidence that regulation of ANGPTL4 by AMPK occurs at the transcriptional level, affecting PPARγ-mediated transcription of the *Angptl4* gene. ANGPTL4 has been repeatedly shown to be a highly sensitive target of all PPAR transcription factors in a variety of tissues and cells and following a variety of physiological stimuli (15, 25, 39, 52). Previously, PPARγ-mediated transcription has been shown to be inhibited by activation of AMPK (40, 53, 54). A potential link between PPARγ and AMPK may be the modulation of co-activator recruitment to PPARγ by AMPK. A well-established co-activator of PPARγ that has been shown to be regulated by AMPK is P300 (41, 43, 55). P300 is a key regulator of the assembly and mobilization of the transcriptional machinery by connecting transcription factors to the transcriptional machinery and enhancing DNA accessibility (56). AMPK activation enhances P300 degradation and causes phosphorylation of P300 at serine residue 89, thereby blocking the interaction of P300 with PPARγ and reducing PPARγ transcriptional activity (41, 55, 57). Although our *in vitro* data suggest an involvement of P300 and PPARγ in the suppression of ANGPTL4 by AMPK activation, whether P300 is also involved in *in vivo* regulation of ANGPTL4 during cold exposure remains to be determined.

Despite the unmistakable dependency of BAT LPL activity on ANGPTL4 expression, a modest cold-induced increase in LPL activity and TRL-derived fatty acid uptake is observed in BAT of *Angptl4*$^{-/-}$ mice, which may be (partially) explained by the moderate increase in *Lpl* mRNA in BAT in the cold. Alternatively, there may be a role for another, yet to be identified, cold-induced post-translational modulator of LPL. It may be hypothesized that this post-translational modulator is also involved in the rapid increase in LPL activity during acute cold exposure (8).

No overt abnormalities in cold-tolerance were observed in the *Angptl4*$^{-/-}$ or *Angptl4*-Tg mice as compared to wild-type mice. This observation, however, does not refute the importance of ANGPTL4 in fuel delivery to BAT during cold exposure. BAT is a well-conserved organ, that is postulated to have conferred to mammals the evolutionary advantage to survive cold stressors such as birth or low environmental temperatures (1). It is likely that differential uptake of TG in other organs and altered uptake of other available fuels (free fatty acids, glucose) compensate for the reduced uptake of TRL-derived fatty acids to BAT in *Angptl4*-Tg mice. Strikingly, adipose tissue-specific deletion of LPL in mice does not result in an overt phenotype (58, 59). While plasma TG levels are elevated in these mice, no other parameters were altered, indicating that even in mice completely lacking LPL in adipose tissue, alternative mechanisms exist to fuel BAT and WAT (58, 59).

In conclusion, our data show that regulation of ANGPTL4 is an important factor in directing lipid fuels towards BAT and away from WAT during prolonged cold exposure. Better understanding of the mechanisms underlying fuel re-distribution may pave the way for new strategies to combat metabolic diseases, such as cardiovascular disease and diabetes type 2, in which a mismatch in regulation of lipid uptake and usage by tissues is an important feature (60, 61).

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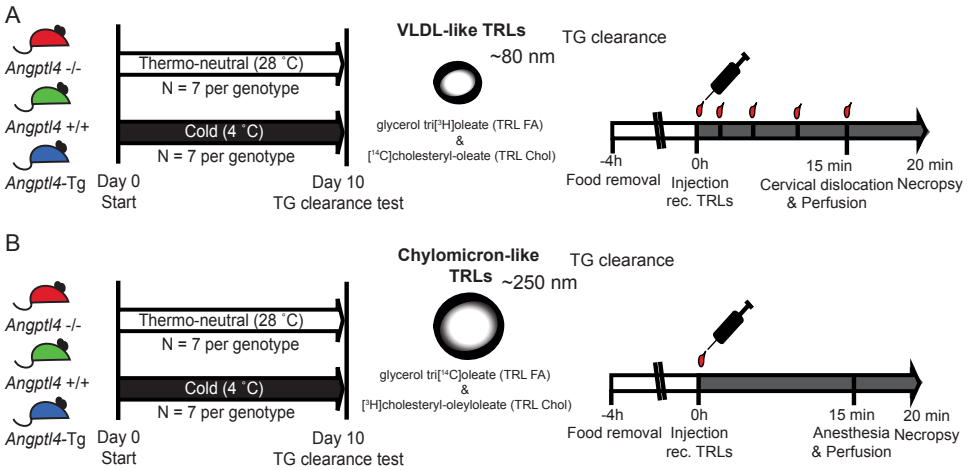
SUPPLEMENTAL FIGURES

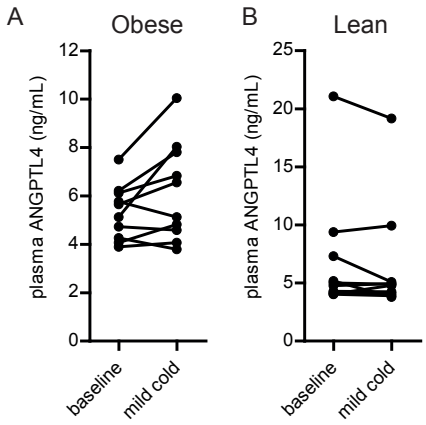
Table 1. Primer sequences.

Gene	Forward primer	Reverse Primer
m36b4	ATGGGTACAAGCGCGTCCTG	GCCTTGACCTTTTCAGTAAG
mAngptl4	GTTTGCAGACTCAGCTCAAGG	CCAAGAGGTCTATCTGGCTCTG
mLpl	GGGAGTTTGGCTCCAGAGTTT	GGGAGTTTGGCTCCAGAGTTT
mUcp1	CCTGCCTCTCTCGGAAACAA	TGTAGGCTGCCCAATGAACA
mPgc1α	AGTCCCATACACAACCGCAGTCGCAACATG	CCCTTTCTTGGTGGAGTGGCTGCCTTGG
mCidea	TGACATTCATGGGATTGCAGAC	GGCCAGTTGTGATGACTAAGAC
mElovl3	TTCTCACGCGGGTTAAAAATGG	GAGCAACAGATAGACGACCAC
mPrdm16	CCACCAGCGAGGACTTCAC	GGAGGACTCTCGTAGCTCGAA



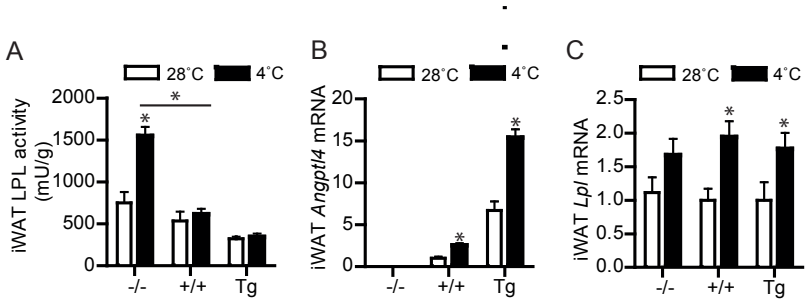
Supplemental Figure 1.

(A & B) Schematic representation of cold exposure experiment, followed by a triglyceride clearance experiment in which either radiolabelled VLDL-like particles or chylomicron-like particles were injected via the tail vein of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice. 20 min post-injection mice were sacrificed to analyze the distribution of the radioactive labels.



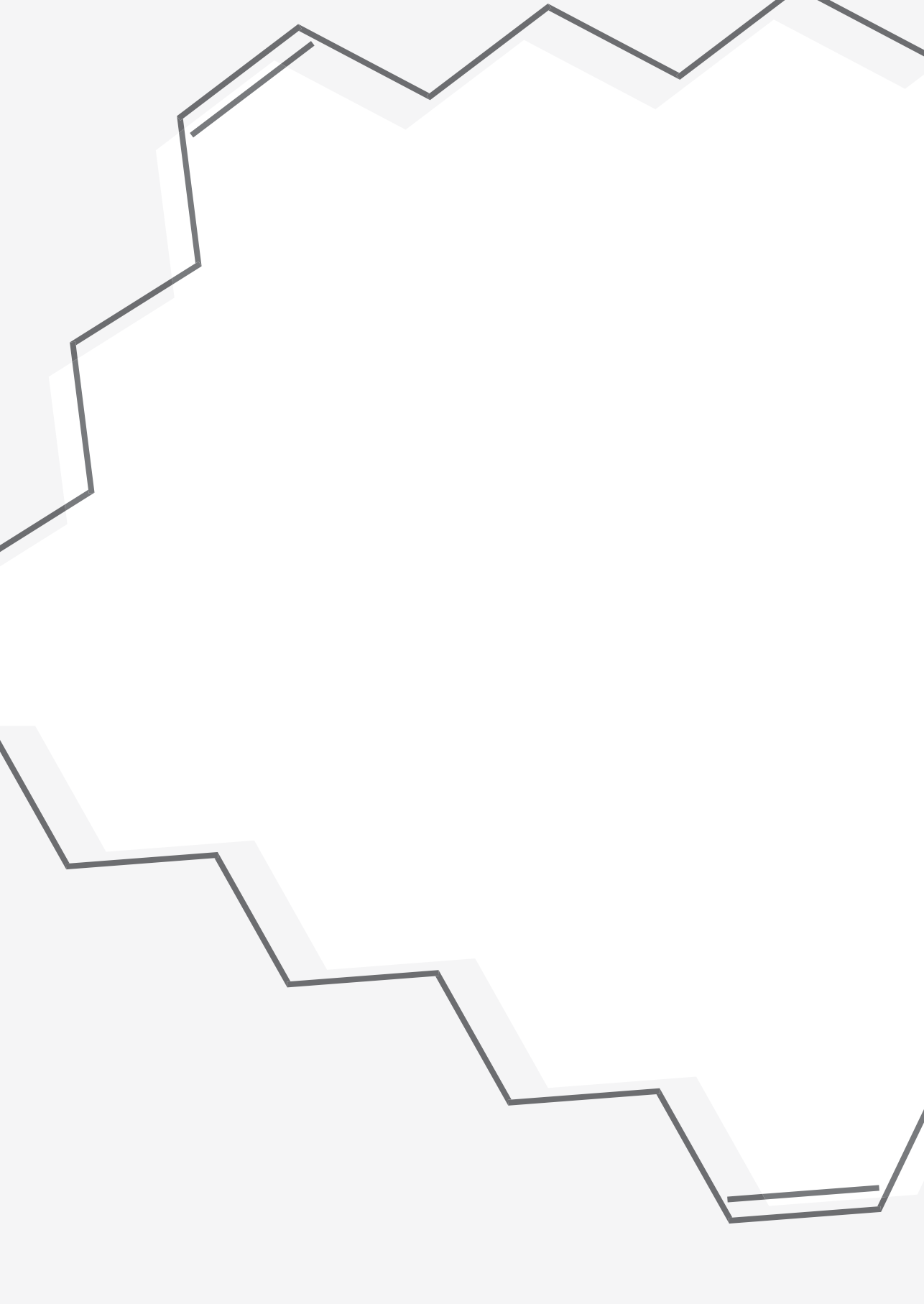
Supplemental Figure 2.

(A & B) Plasma ANGPTL4 levels in 10 obese (A) and 10 lean (B) individuals before and after exposure to a mild cold (16˚C) for 48h (27). Differences between mild cold and baseline were statistically significant in the obese group (paired Student's t-test ($p<0.05$)). n = 10 individuals per group.



Supplemental Figure 3.

(A) Total LPL activity levels, (B) *Angptl4* mRNA, and (C) *Lpl* mRNA in inguinal WAT (iWAT) of *Angptl4*$^{-/-}$, wild-type and *Angptl4*-Tg mice exposed to 4˚C or 28˚C for 10 days. *Statistically significant compared to mice of equal genotype at 28˚C or between groups as indicated by bars, according to two-way ANOVA followed by a post-hoc Tukey HSD test ($p<0.05$). Error bars represent ± SEM. n = 7-10 mice per group



5

ANGPTL4 PROMOTES INTRACELLULAR DEGRADATION OF LIPOPROTEIN LIPASE IN ADIPOCYTES

Wieneke Dijk
Anne P. Beigneux
Mikael Larsson
André Bensadoun
Stephen G. Young
Sander Kersten

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ABSTRACT

Lipoprotein lipase (LPL) hydrolyzes triglycerides in triglyceride-rich lipoproteins along the capillaries of heart, skeletal muscle, and adipose tissue. The activity of LPL is repressed by angiopoietin-like 4 (ANGPTL4) but the underlying mechanisms have not been fully elucidated. Our objective was to study the cellular location and mechanism for LPL inhibition by ANGPTL4. We performed studies in transfected cells, *ex vivo* studies and *in vivo* studies with *Angptl4*$^{-/-}$ mice. Co-transfection of CHO pgsA-745 cells with ANGPTL4 and LPL reduced intracellular LPL protein levels, suggesting that ANGPTL4 promotes LPL degradation. This conclusion was supported by studies of primary adipocytes and adipose tissue explants from wild-type and *Angptl4*$^{-/-}$ mice. Absence of ANGPTL4 resulted in accumulation of the mature-glycosylated form of LPL and increased secretion of LPL. Blocking ER–Golgi transport abolished differences in LPL abundance between wild-type and *Angptl4*$^{-/-}$ adipocytes, suggesting that ANGPTL4 acts upon LPL after LPL processing in the ER. Finally, physiological changes in adipose tissue ANGPTL4 expression during fasting and cold resulted in inverse changes in the amount of mature-glycosylated LPL in wild-type but not *Angptl4*$^{-/-}$ mice. We conclude that ANGPTL4 promotes loss of intracellular LPL by stimulating LPL degradation after LPL processing in the ER.

INTRODUCTION

Circulating triglyceride-rich lipoproteins, such as chylomicrons and very low-density lipoproteins (VLDL), supply tissues with lipid nutrients for storage or oxidation. Hydrolysis of circulating triglycerides is mediated by lipoprotein lipase (LPL), a glycoprotein found in multiple tissues, including adipose tissue, brain, heart and skeletal muscle (1–4). LPL is produced by parenchymal cells and then transported to the luminal surface of capillaries by an endothelial cell protein, GPIHBP1 (glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1) (5).

A critical step in the maturation of LPL is asparagine-linked glycosylation (6–8). LPL is glycosylated within the endoplasmic reticulum (ER) *via* co-translational transfer of oligosaccharide chains high in mannose residues (6–9). After being translocated to the Golgi apparatus, the high-mannose oligosaccharides are trimmed and replaced by more complex oligosaccharides (6–9). Endoglycosidase H (EndoH) cleaves high-mannose oligosaccharides from proteins but not complex oligosaccharides (6, 10), making it possible to distinguish LPL within the ER and Golgi compartments.

The catalytic activity of LPL along the capillary endothelium is considered rate-limiting for the lipolytic processing of plasma triglycerides and for the subsequent uptake of fatty acids by surrounding tissues (1, 4, 11). To ensure that supply of fatty acids to tissues matches metabolic demand, LPL activity is under tight regulatory control. Although it is possible to identify small fluctuations in *Lpl* mRNA levels, LPL activity appears to be regulated mainly at the post-translational level (1, 4). An important physiological regulator of LPL is angiopoietin-like 4 (ANGPTL4). ANGPTL4 potently inhibits LPL activity in multiple tissues and regulates LPL activity during a variety of physiological conditions, including fasting, cold and exercise (12–14). For example, changes in the expression of ANGPTL4 (originally called fasting-induced adipose factor) allow for swift changes in adipose tissue LPL activity during fasting (13, 15). Expression of ANGPTL4 is induced by fatty acids *via* Peroxisome Proliferator-Activated Receptors (PPARs) as part of a feedback mechanism aimed at preventing lipid overload within cells (16, 17).

Genetic studies strongly support a role for ANGPTL4 in determining plasma triglyceride levels in humans and have linked inactivating variants in the *ANGPTL4* gene to a reduced risk of coronary heart disease (18–20). However, cross-sectional studies have not revealed a clear correlation between the plasma levels of ANGPTL4 and triglycerides (21–24). These observations suggest that the plasma pool of ANGPTL4 may not be primarily responsible for regulating plasma triglyceride levels and that the inhibitory effects of ANGPTL4 on LPL activity may not occur exclusively on the surface of capillaries. Indeed, Robciuc and coworkers proposed that ANGPTL4 could inhibit LPL activity not only at the cell surface but also intracellularly, partly based on microscopy studies showing co-localization of LPL and ANGPTL4 within cells (25). At the same time, recent studies have raised the possibility that ANGPTL4 regulation of LPL might occur within the subendothelial spaces rather than along the capillary lumen (26–28). Studies of 3T3-L1 adipocytes suggested that inhibition of LPL activity by ANGPTL4 begins only after these proteins arrive at the cell surface (29). However, the extent to which the cultured cell studies are relevant to LPL activity in adipose tissue *in vivo* is uncertain. Accordingly, our objective in the current

studies was to investigate the cellular location and mechanism for LPL inhibition by ANGPTL4 in adipose tissue. To address this objective, we used a combination of cell culture studies, and *ex vivo* and *in vivo* studies of adipocytes and adipose tissue from wild-type and *Angptl4*$^{-/-}$ mice.

MATERIALS AND METHODS

All animal experiments were performed in accordance with Directive 2010/63/EU from the European Union. All animal studies were reviewed and approved by the Animal Ethics Committee of Wageningen University.

Cell culture

CHO pgsA-745 cells were cultured as described previously (30, 31). CHO pgsA-745 cells were electroporated with an expression vector for Flag-tagged human ANGPTL4 (either the full-length protein hANGPTL4[1–406] or the amino-terminal domain of hANGPTL4 [1–160]) or a vector for V5-tagged human LPL (or empty vector). After the cell transfections, the cells were mixed and co-plated in the same well of a 24-well plate. After 24 h, the cell culture medium was collected for a LPL activity assay. After 48 h, the cell culture medium was collected and cell lysates were prepared for SDS-PAGE and western blotting. ANGPTL4 and LPL expression vectors were also co-transfected into CHO pgsA-745 cells. For those studies, the cells were co-electroporated with a vector for Flag-tagged human ANGPTL4 (either hANGPTL4[1–406] or hANGPTL4 [1–160]) and a vector for V5-tagged human LPL (or empty vector). After 24 h, the cell culture medium was collected for a LPL activity assay. After 48 h, the cell culture medium was collected and cell lysates were prepared for SDS-PAGE and western blotting.

Isolation and differentiation of stromal vascular fraction

Inguinal or gonadal WAT was removed from *Angptl4*$^{-/-}$ and wild-type mice and placed in DMEM (Lonza) supplemented with 1% penicillin/streptomycin (P/S) and 1% bovine serum albumin (BSA) (Sigma-Aldrich, Houten, The Netherlands). Fat pads from 2–3 mice were pooled, minced with scissors, and digested for 1 h at 37°C in collagenase-containing medium [DMEM with 3.2 mM $CaCl_2$, 1.5 mg/mL collagenase type II (C6885, Sigma-Aldrich), 10% fetal calf serum (FCS), 0.5% BSA, and 15 mM HEPES]. Next, the cell suspension was filtered through a 100 µm cell strainer (Falcon) and centrifuged at 1600 rpm for 10 min. The supernatant fluid was removed, and the pellet containing the stromal vascular fraction was resuspended in erythrocyte lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) and incubated for 2–3 minutes at room temperature. After neutralization, cells were centrifuged at 1200 rpm for 5 min. Pelleted cells were resuspended in DMEM containing 10% FCS and 1% P/S and plated. After reaching confluency, cells were differentiated according to standard protocol for 3T3-L1 cells with addition of 1 µM rosiglitazone (32).

Mouse studies

Tissue samples from angiopoietin-like 4 knockout mice *(Angptl4*$^{-/-}$), wild-type mice, and *Angptl4*-transgenic (*Angptl4*-Tg) mice from previously published studies were used for analyses of LPL glycosylation and mass (13, 14, 33–35). *Angptl4*$^{-/-}$ mice, wild-type mice, and *Angptl4*-Tg mice have been on a C56BL/6J background for > 10 genera-

tions. Wild-type and *Angptl4*-Tg mice are littermates. In *Angptl4*$^{-/-}$ mice part of the *Angptl4* gene was deleted by homologous recombination in embryonic stem cells, resulting in a non-functional ANGPTL4 protein (33, 34). *Angptl4*-Tg mice over-express the *Angptl4* gene in various tissues under the endogenous promoter (35). Brown adipose tissue samples from *Angptl4*$^{-/-}$ and wild-type mice exposed to cold or thermoneutral temperature for 10 days were from a study by Dijk *et al.* (14). Gonadal white adipose tissue (WAT) samples from fed, fasted and refed *Angptl4*$^{-/-}$ and wild-type mice were from a study described by Kroupa *et al.* (13). Hearts from fed and overnight-fasted *Angptl4*$^{-/-}$ and wild-type mice were collected and snap-frozen in liquid nitrogen. For "post-heparin" tissues, wild-type and *Angptl4*$^{-/-}$ mice were fed or fasted for 16 h. The mice were then anesthetized with isoflurane and injected with heparin (100 IU/kg in 0.9% NaCl) through the jugular vein. After 5 min, the mice were killed by cervical dislocation. Gonadal WAT was dissected, frozen immediately in liquid nitrogen, and stored at −80°C for further analyses.

LPL activity measurements

LPL activity levels in culture medium and cell lysates of transfected cells was assessed in duplicate with a [^{3}H]triolein substrate (PerkinElmer) as previously described (36).

WAT explants

Gonadal WAT was harvested from *Angptl4*$^{-/-}$ and wild-type mice and placed in DMEM supplemented with 1% P/S and 1% BSA. Fat pads were minced into small pieces, which were further divided to make small mounds of WAT (~50–100 mg of tissue). WAT explants were placed into wells containing medium (DMEM with 1% P/S and 10% FCS) with or without heparin (50 IU/mL). Explants were incubated for different times (as indicated in the figure legends), after which the medium was harvested and explant weights were determined. Explants were immediately lysed to prepare protein extracts. Protein concentrations in explants were determined and equal amounts of protein were used for SDS-PAGE and western blotting studies.

NP40 solubilization of adipocytes

Adipocytes that had been differentiated from the stromal vascular fractions of *Angptl4*$^{-/-}$ and wild-type mice were prepared as described earlier. Upon differentiation, adipocytes were lysed in NP40 lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5% NP40, 150 mM NaCl, 5 mM MgCl2) supplemented with protease and phosphatase inhibitors (Roche). After centrifugation, the supernatant fluid (NP40S) was mixed with 2x Laemmli sample buffer (LSB) and heated at 65°C for 15 min. The pellet was resuspended in PBS and 2x LSB and heated at 95°C for 30 min (NP40P). NP40S and NP40P fractions were then loaded onto SDS-PAGE for further analyses by western blotting.

Western blots

Fat pads, explants and differentiated adipocytes were lysed in a mild RIPA-like lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol; Thermo Scientific, Landsmeer, The Netherlands) containing protease and phosphatase inhibitors (Roche). Lysates were centrifuged 2–3 times at 13,000 rpm for 10 min to remove fat droplets. Protein lysates (10–30 µg protein per lane), medium (10–15 µL), or plasma (0.75 µL) samples were diluted in Laemmli sample buffer, cooked for 5 minutes at 95˚C, and loaded onto pre-casted TGX gels (Bio-Rad, Veenendaal, The Netherlands). For Native PAGE, protein lysates were diluted in a SDS-free and DTT-free sample buffer, directly loaded onto pre-casted TGX gels, and run in a Tris-Glycine running buffer without SDS. The separated proteins were transferred onto a PVDF membrane with a Transblot Turbo System (Bio-Rad). Membranes were probed with a goat anti-mouse LPL antibody (37); a rabbit anti-mouse HSP90 antibody (Cell Signalling Technology, #4874); a rabbit anti-mouse H2A antibody (Abcam, #ab18255); a rat anti-mouse ANGPTL4 antibody (Adipogen, #Kairos 142-2); or a rabbit anti-mouse ADIPOQ antibody (ThermoScientific, #PAI-054) at 1:5000 (LPL), 1:2000 (HSP90, ANGPTL4) or 1:1000 (H2A, ADIPOQ) dilution. All incubations were performed in Tris-buffered saline, pH 7.5, 0.1% Tween-20 (TBS-T), and 5% nonfat dry milk. Membranes were washed in the Tris-buffered saline/Tween-20 solution. Antibody signals were detected with the ChemiDoc MP system (Bio-Rad) and Clarity ECL substrate (Bio-Rad). Equal loading of medium and plasma samples was verified by Coomassie blue staining, H2A or HSP90 expression.

Proteins in culture supernatants and cell lysates from transfected CHO pgsA-745 cells were separated on 12% Bis-Tris SDS-polyacrylamide gels (Life Technologies) and transferred to a nitrocellulose membrane for western blotting. The antibody dilutions were 1:500 for an IRdye800-conjugated mouse monoclonal against the V5-tag; 1:500 for an IRdye680-conjugated mouse monoclonal against the Myc-tag; 1:500 for IRdye680-conjugated mouse monoclonal 4-1a against human LPL (38); 1:500 for a mouse monoclonal against the Flag-tag (Sigma-Aldrich); 1:2000 for IRdye680-conjugated donkey anti-mouse IgG (LI-COR Biosciences); 2 µg/ml for a goat polyclonal against β-actin (Santa Cruz Biotechnology); 1:2000 for IRdye680-conjugated donkey anti-goat IgG (LI-COR); 2 µg/ml for a polyclonal antibody against human SLURP1 (Novus Biologicals); 1:2000 for IRdye800-conjugated donkey anti-mouse IgG (LI-COR).

EndoH and PNGase digestions

Glycosylation of proteins was analyzed by western blotting after digestion of 10–20 µg of protein with EndoH or PNGase (New England BioLabs) according to manufacturer's instructions.

RESULTS

Secreted ANGPTL4 inactivates LPL outside of the cell

To assess to what extent secreted angiopoietin-like 4 (ANGPTL4) affects the amount of lipoprotein lipase (LPL) and LPL activity in cells and cell culture medium, CHO pgsA-745 cells were transfected with either human ANGPTL4 (hANGPTL4) or human LPL (hLPL) expression vectors. The ANGPTL4-transfected cells were then mixed with the LPL-transfected cells and co-plated. Co-plating of hANGPTL4-expressing cells with hLPL-expressing cells did not change the amount of LPL in the cell extracts, but co-plating did reduce the amount of LPL in cell culture medium (**Figure 1A**). A similar reduction in the amount of LPL in cell culture medium was found when hLPL-transfected cells were co-plated with cells expressing the amino-terminal 160 residues of hANGPTL4 (**Figure 1A**). As expected, co-plating of hLPL-expressing cells with hANGPTL4-expressing cells resulted in a striking reduction in LPL activity levels in the cell culture medium (**Figure 1B**). Thus, the presence of ANGPTL4 in the cell culture medium reduces the amount of LPL protein in the medium and markedly inhibits LPL activity in the medium. However, co-plating of hLPL- and hANGPTL4-expressing cells had no effect on intracellular levels of LPL.

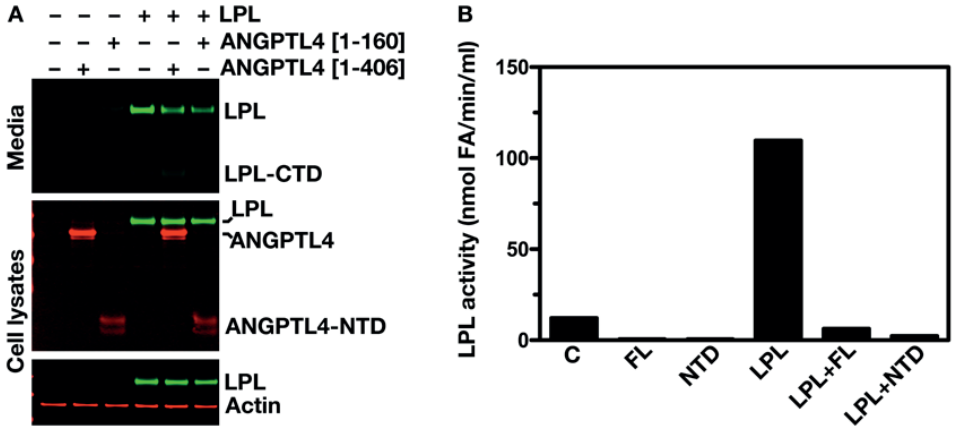

Figure 1. ANGPTL4 inactivates LPL outside of the cell.

(A) Western blots of cell culture media and cell lysates of CHO pgsA-745 cells. Cells were transfected with either a Flag-tagged ANGPTL4 expression vector or a vector for V5-tagged human LPL. The ANGPTL4- and LPL-transfected cells were then mixed and co-plated. A full-length ANGPTL4 expression vector (hANGPTL4[1–406]) or a vector encoding the amino-terminal domain of ANGPTL4 (hANGPTL4 [1–160]) was used. Western blots were probed with an anti-Flag antibody to detect ANGPTL4 (red); an anti-V5 antibody to detect LPL (green); and an anti-actin antibody (red) (as a loading control). LPL-CTD, carboxyl-terminal domain of LPL; ANGPTL4-NTD, amino-terminal domain of ANGPTL4. (B) Bar graph representing LPL activity levels in the media of CHO pgsA-745 cells that had been transfected with empty vector (C); full-length ANGPTL4 alone (FL); the amino-terminal domain of ANGPTL4 alone (NTD); human LPL alone (LPL); a mixture of ANGPTL4 (full-length)-transfected cells and LPL-transfected cells (LPL + FL); a mixture of ANGPTL4 (amino-terminal domain)-transfected cells and LPL-transfected cells (LPL + NTD).

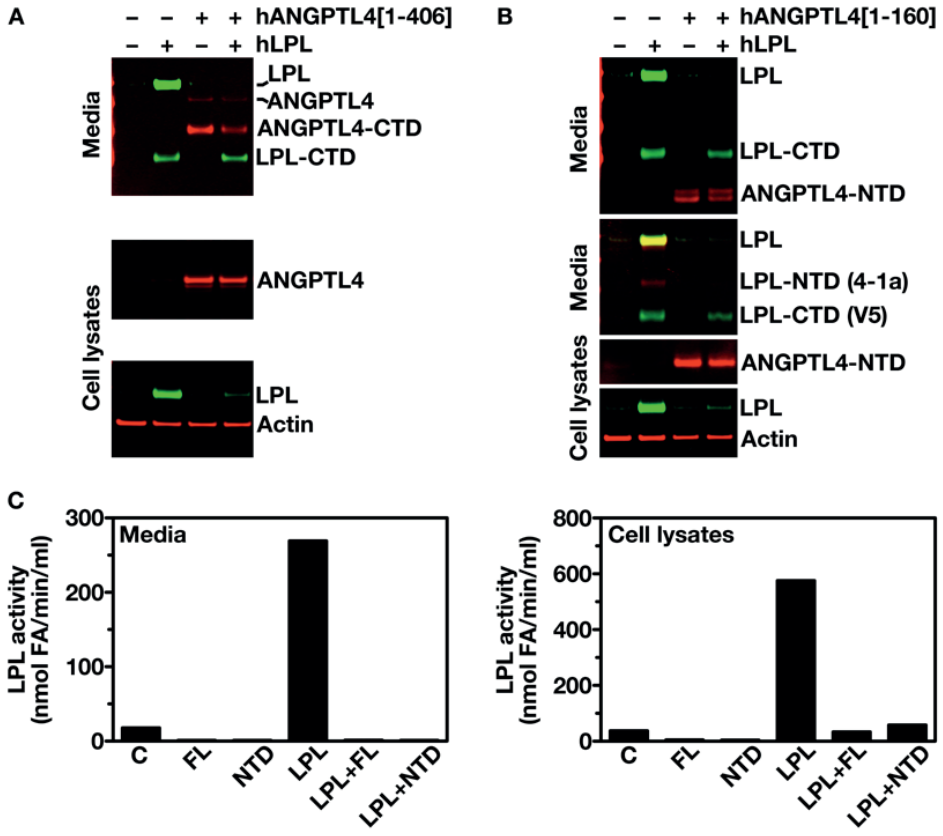

Figure 2. ANGPTL4 inactivates LPL inside cells.

(A) Western blots of cell culture media and cell lysates of CHO pgsA-745 cells that had been co-transfected with an expression vector for Flag-tagged human ANGPTL4 (hANGPTL4[1–406]) and a vector for V5-tagged human LPL (or empty vector). Western blots were probed with an anti-Flag antibody to detect ANGPTL4 (red); an anti-V5 antibody to detect LPL (green); and an anti-actin antibody (red) (as a loading control). LPL-CTD, carboxyl-terminal domain of LPL; ANGPTL4-CTD, carboxyl-terminal domain of ANGPTL4. (B) Western blots of cell culture media and cell lysates of CHO pgsA-745 cells co-transfected with an expression vector for myc-tagged human ANGPTL4 (hANGPTL4[1–160]) and a vector for V5-tagged human LPL (or empty vector). Western blots were probed with an anti-Myc antibody to detect ANGPTL4 (red); an anti-V5 tag antibody (green) or Mab 4-1a (red) to detect LPL; and an anti-actin antibody (red) (as a loading control). LPL-CTD, carboxyl-terminal domain of LPL; LPL-NTD, amino-terminal domain of LPL; ANGPTL4-NTD, amino-terminal domain of ANGPTL4. (C) LPL activity levels in the culture media and cell lysates of CHO pgsA-745 cells that had been transfected with an empty vector alone (C); full-length hANGPTL4 alone (FL), amino-terminal domain of hANGPTL4 alone (NTD); full-length ANGPTL4 and LPL (LPL + FL); the amino-terminal domain of ANGPTL4 and LPL (LPL + NTD).

Co-expression of ANGPTL4 and LPL inactivates LPL within cells

ANGPTL4 and LPL are both secreted glycoproteins, and are expected to follow a similar route to the cell surface (6, 8, 39). To determine if ANGPTL4 expression affects the levels of LPL protein inside cells, CHO pgsA-745 cells were simultaneously transfected with hLPL and hANGPTL4 expression vectors. Co-transfection of hLPL and hANGPTL4 markedly reduced the amount of LPL in both the cell extracts and the cell culture medium (**Figure 2A**). When we performed analogous studies with the amino-terminal portion of hANGPTL4 (residues 1–160), a similar reduction in the amounts of LPL in the medium and inside cells was found (**Figure 2B**). We also observed markedly reduced amounts of LPL catalytic activity, both within cells and in the cell culture medium (**Figure 2C**). Similar to hLPL, co-transfection of mLPL with hANGPTL4 reduced the amount and activity of mLPL within cells and in the cell culture medium (**Figure 3A & 3B**). The reduced amounts of intracellular LPL in the co-transfection studies could not be explained by lower levels of *LPL* transcripts (**Figure 4A**). Likewise, the reduced amounts of intracellular LPL in the ANGPTL4 co-expression studies could not be explained by a global decrease in secreted proteins

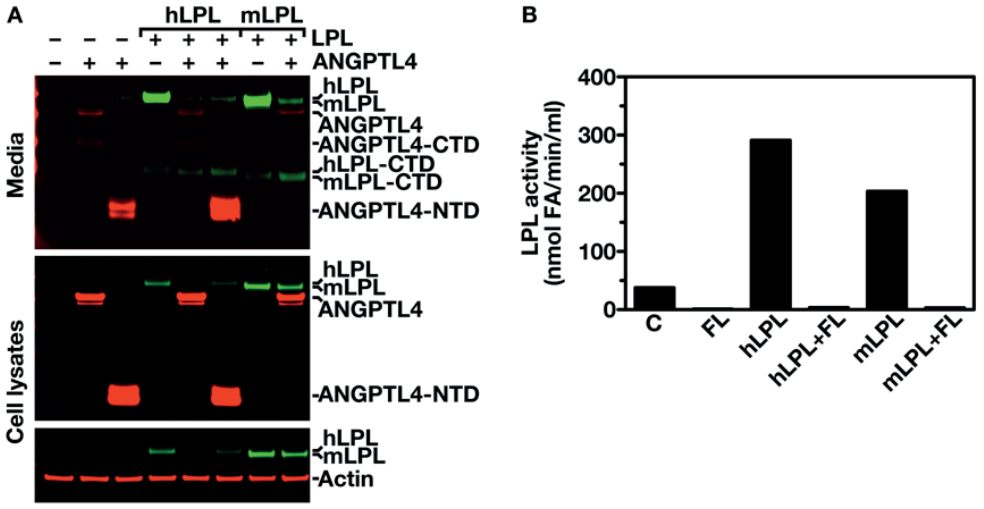

Figure 3. hANGPTL4 inactivates mouse LPL inside cells.

(A) CHO pgsA-745 cells were co-transfected with an expression vector for Flag-tagged human ANGPTL4 (hANGPTL4[1-406]), or the amino-terminus domain of hANGPTL4 (hANGPTL4[1-160] and a vector for V5-tagged human LPL (hLPL) or mouse LPL (mLPL), or empty vector. 48 h later, cell culture media and cell lysates were examined by SDS-PAGE and Western blotting. Western blots were probed with an anti-Flag antibody to detect ANGPTL4 (red); an anti-V5 tag antibody was used to detect hLPL and mLPL (green). Actin (red) was used as a loading control. LPL-CTD, carboxyl-terminal domain of LPL; ANGPTL4-CTD, carboxyl-terminal domain of ANGPTL4; ANGPTL4-NTD, amino-terminal domain of ANGPTL4. (B) Bar graph representing LPL activity levels in cell culture media of CHO pgsA-745 cells that were co-transfected with an expression vector for Flag-tagged human ANGPTL4 (hANGPTL4[1-406]) and a vector for V5-tagged human LPL (hLPL) or mouse LPL (mLPL), or empty vector. C; empty vector control; FL, full-length ANGPTL4.

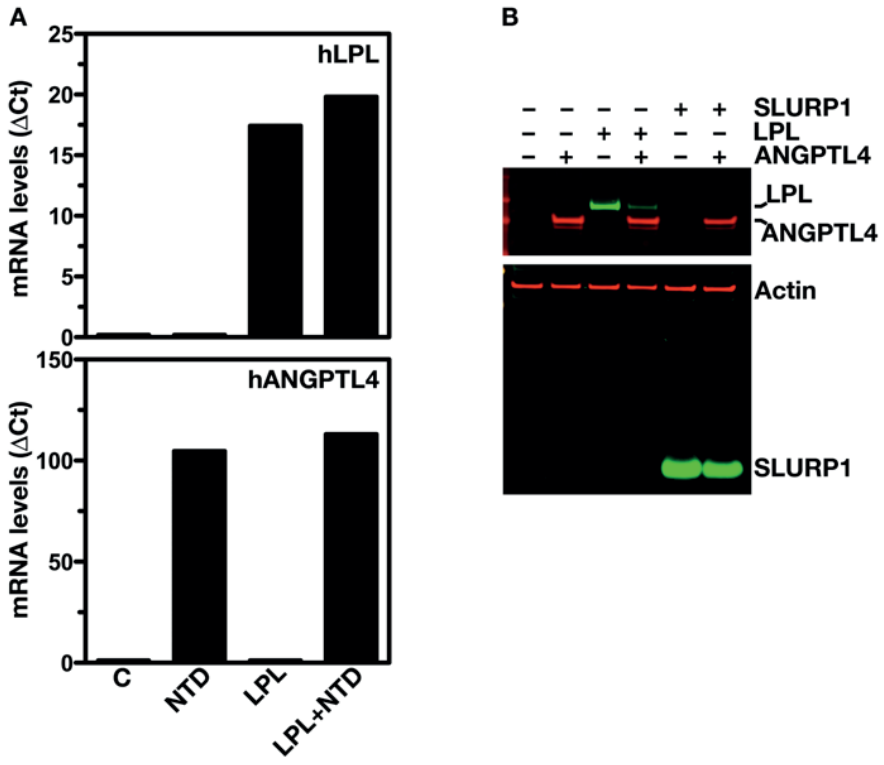

Figure 4. ANGPTL4 does not affect transcription of LPL or secretion of SLURP1.

(A) Transcript levels for LPL and ANGPTL4 in CHO pgsA-745 cells that had been transfected with empty control vector (C); an expression vector for the Flag-tagged amino-terminal domain of hANGPTL4 (NTD); a vector for V5-tagged hLPL (LPL); or amino-terminal hANGPTL4 and V5-tagged hLPL (LPL+NTD). (B) Western blots of cell lysates of CHO pgsA-745 cells that had been co-transfected with an expression vector for Flag-tagged full-length hANGPTL4 and either a vector for V5-tagged hLPL or a vector for hSLURP1. Western blots were probed with an anti-Flag antibody to detect ANGPTL4 (red); an anti-SLURP1 antiserum (green); an anti-V5 tag antibody to detect LPL (green); and an anti-Actin antibody (red) (as a loading control).

inside cells; co-expression of ANGPTL4 with SLURP1, another secreted protein, did not result in reduced amounts of intracellular SLURP1 (**Figure 4B**).

Deletion of ANGPTL4 causes accumulation of EndoH-resistant LPL in adipocytes

Adipose tissue displays robust expression of both ANGPTL4 and LPL (14). To determine if ANGPTL4 expression affects intracellular LPL levels in adipose tissue, we studied the effects of ANGPTL4 deficiency on LPL protein levels in mouse adipose tissue. A deficiency of ANGPTL4 resulted in a marked accumulation of full-length

LPL in adipose tissue explants and adipocytes from *Angptl4*$^{-/-}$ mice (**Figure 5A & 5B**). The accumulation of LPL could not be attributed to higher *Lpl* expression levels because *Lpl* mRNA levels were similar in the explants and adipocytes of wild-type and *Angptl4*$^{-/-}$ mice (**Figure 5C & 5D**).

To determine the location of LPL accumulation in adipose tissue from *Angptl4*$^{-/-}$ mice, adipose tissue lysates were treated with endoglycosidase H (EndoH). These studies revealed that the accumulation of LPL in *Angptl4*$^{-/-}$ explants and adipocytes was mainly EndoH-resistant LPL (LPL in the Golgi or on the cell surface) rather than EndoH-sensitive LPL (LPL within the ER) (**Figure 5A & 5B**). Further studies revealed that the EndoH-resistant LPL could be removed from cells by heparin, suggesting that the EndoH-resistant LPL in *Angptl4*$^{-/-}$ adipose tissue explants and *Angptl4*$^{-/-}$ adipocytes represented LPL attached to the cell surface by heparin-sulphate proteoglycans (HSPGs) or LPL present in rapidly-releasable secretory vesicles (40, 41) (**Figure 5A & 5B**).

The accumulation of heparin-releasable EndoH-resistant LPL in *Angptl4*$^{-/-}$ adipose tissue was accompanied by a constitutively higher release of LPL from gWAT explants and adipocytes (**Figure 6A & 6B**). Treatment with heparin magnified these differences; significantly higher amounts of heparin-releasable LPL were detected in the medium of gWAT explants and adipocytes from *Angptl4*$^{-/-}$ mice as compared to wild-type mice (**Figure 6A & 6B**). By contrast, secretion of ADIPOQ was not altered in *Angptl4*$^{-/-}$ adipocytes, implying that a deficiency of ANGPTL4 did not have a general effect on the secretion of adipocyte proteins (**Figure 6A & 6B**).

Mechanism of ANGPTL4-mediated loss of LPL

To assess whether ANGPTL4 affects LPL present on the cell surface and/or intracellularly, we treated differentiated adipocytes from *Angptl4*$^{-/-}$ and wild-type mice with heparin to remove cell-surface LPL. The cells were then washed and incubated for up to 180 min in the presence of heparin, followed by measurement of LPL levels in medium and cells. Heparin was included to prevent the sequestration of LPL on cell-surface HSPGs and to prevent its degradation in the medium (42). The accumulation of LPL in the medium was much greater in *Angptl4*$^{-/-}$ adipocytes than in wild-type adipocytes, implying increased LPL secretion from *Angptl4*$^{-/-}$ adipocytes (**Figure 6C**). These data suggest that ANGPTL4 promotes intracellular LPL degradation in adipocytes, leading to reduced LPL secretion.

We next explored the mechanism accounting for the effect of ANGPTL4 on specifically EndoH-resistant LPL in adipocytes. Earlier studies revealed an accumulation of EndoH-sensitive LPL in *cld/cld* (*Lmf1*$^{-/-}$) and *Sel1L*$^{-/-}$ mice, characterized by aggregated LPL in the ER that was targeted for endoplasmic reticulum–associated degradation (ERAD) (6, 10, 43–45). To determine if LPL in ANGPTL4-expressing cells is targeted for degradation in the ER, we incubated primary adipocytes with brefeldin A, a fungal lactone antibiotic that is known to block protein transport from the ER and induce translocation of Golgi proteins to the ER. Brefeldin A–mediated inhibition of ER–Golgi transport abolished differences in the amounts of total LPL and EndoH-resistant LPL between wild-type and *Angptl4*$^{-/-}$ adipocytes, suggesting that ANGPTL4-mediated LPL degradation occurs in a post-ER compartment (**Fig-**

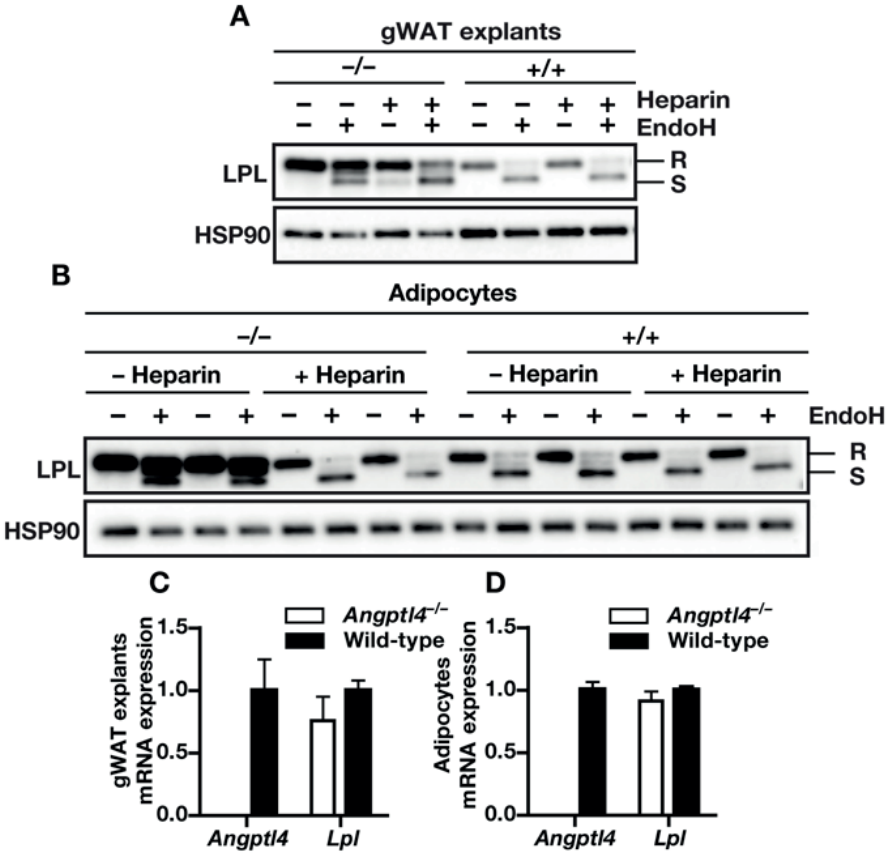

Figure 5. ANGPTL4 lowers the amount of LPL on the adipocyte cell surface.

(A) Western blot of cell lysates of gWAT explants from *Angptl4*$^{-/-}$ and wild-type mice incubated in the absence or presence of heparin (50 IU/mL) for 3 h. Western blots were probed with antibodies against LPL and HSP90 (as loading control). (B) Western blot of cell lysates of adipocytes that had been differentiated from stromal vascular fractions of WAT from *Angptl4*$^{-/-}$ and wild-type mice and incubated in the absence or presence of heparin (10 IU/mL) for 20 min. Western blots were probed with antibodies against LPL and HSP90 (as loading control). (C) *Angptl4* and *Lpl* mRNA levels in gWAT explants from *Angptl4*$^{-/-}$ and wild-type mice. (D) *Angptl4* and *Lpl* mRNA levels in adipocytes that had been differentiated from stromal vascular fractions of WAT from *Angptl4*$^{-/-}$ and wild-type mice. EndoH-resistant LPL (complex oligosaccharides; Golgi and cell surface LPL) is indicated with R; EndoH-sensitive LPL (high-mannose oligosaccharides, ER LPL) is indicated with S.

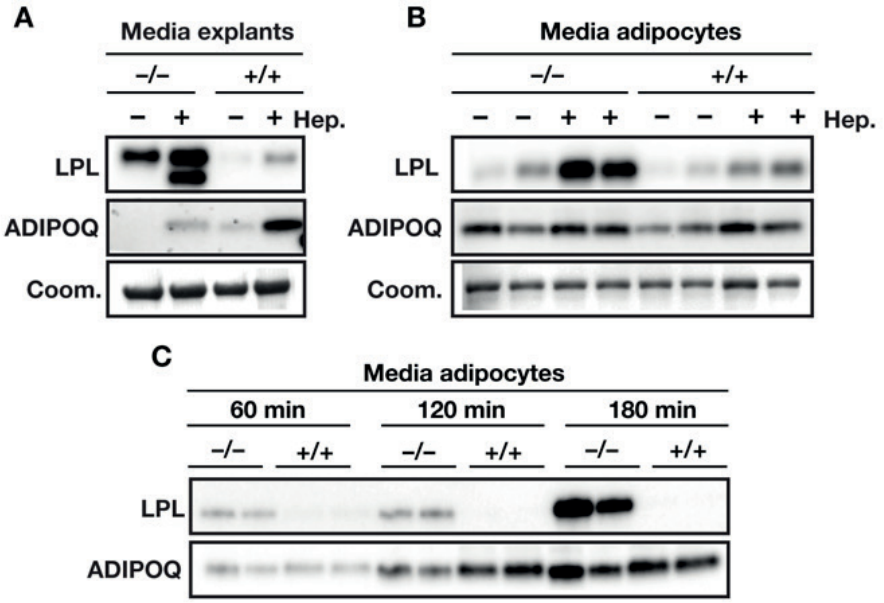

Figure 6. ANGPTL4 lowers LPL secretion.

(A) Western blot of media of gWAT explants from *Angptl4*$^{-/-}$ and wild-type mice treated in the absence or presence of heparin (50 IU/mL) for 3 h. Western blots were probed with antibodies against LPL and ADIPOQ. Coomassie blue staining was used to assess loading. (B) Western blot of media of adipocytes that had been differentiated from stromal vascular fractions of WAT from *Angptl4*$^{-/-}$ and wild-type mice and incubated in the absence or presence of heparin (10 IU/mL) for 20 min. Western blots were probed with antibodies against LPL and ADIPOQ. Coomassie blue staining was used to assess loading. (C) Western blot of media of adipocytes that had been differentiated from stromal vascular fractions of WAT from *Angptl4*$^{-/-}$ and wild-type mice. Cells were pretreated for 20 min with heparin (10 IU/mL), washed with PBS, and incubated with heparin (10 IU/mL) for the indicated times. Western blots were probed with antibodies against LPL and ADIPOQ. EndoH-resistant LPL (complex oligosaccharides; Golgi and cell surface LPL) is indicated with R; EndoH-sensitive LPL (high-mannose oligosaccharides, ER LPL) is indicated with S.

ure 7A). In support of this notion, no differences in ERAD-associated LPL aggregation (*NP40P*) were observed between adipocytes of wild-type and *Angptl4*$^{-/-}$ mice (**Figure 7B**). Also, incubation of adipocytes with monensin, an inhibitor of *medial* to the *trans*-Golgi protein transport, abolished differences between wild-type and *Angptl4*$^{-/-}$ adipocytes (**Figure 7C**).

A potential explanation for the effect of ANGPTL4 on EndoH-resistant LPL is that ANGPTL4 targets LPL from the *trans*-Golgi towards lysosomal degradation, which has been postulated to account for the removal of up to 80% of basal LPL production (41, 42). Accordingly, we treated differentiated adipocytes from *Angptl4*$^{-/-}$ and wild-type mice with different inhibitors of autophagosomal and lysosomal degradation. Treatment of cells with 3-methyladenine (3MA), a PI3K inhibitor that blocks autophagy, as well as with the inhibitors of lysosomal proteases leupeptin and e64D,

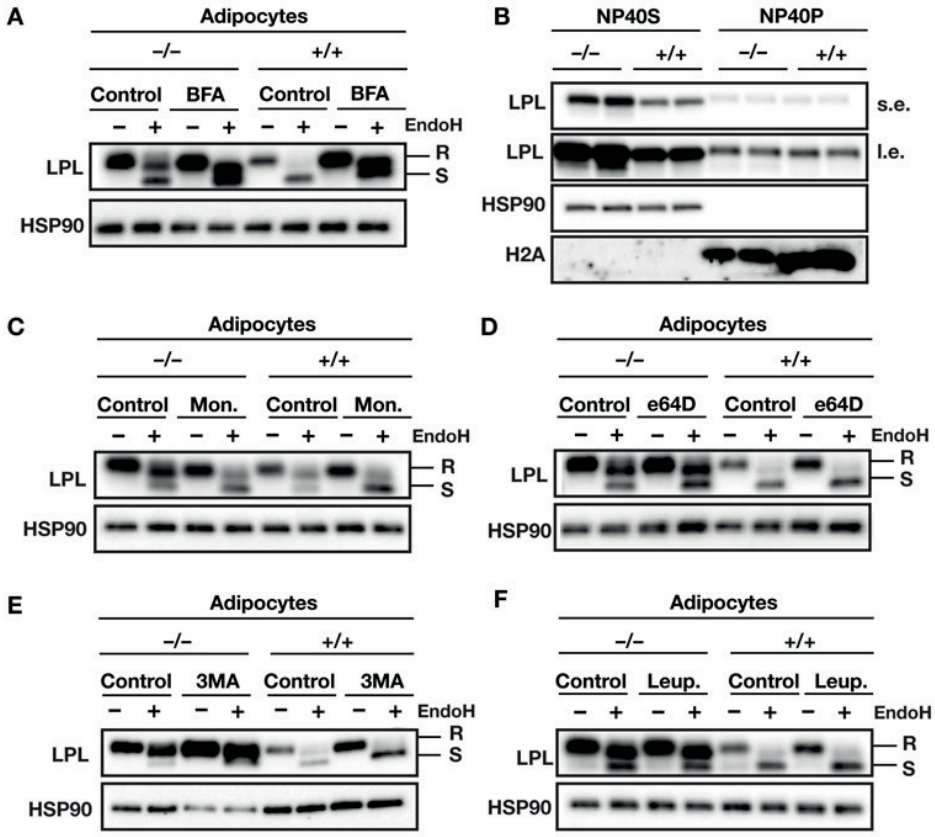

Figure 7. ANGPTL4-mediated loss of LPL protein occurs in a post-ER compartment.

(A) Western blot of EndoH-treated cell lysates of adipocytes that had been differentiated from stromal vascular fractions of WAT from *Angptl4*$^{-/-}$ and wild-type mice. Cells were treated with 5 μg /mL Brefeldin A for 2 h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (B) Western blot of cell lysates of adipocytes that had been differentiated from stromal vascular fractions from WAT of *Angptl4*$^{-/-}$ and wild-type mice. Cells were lysed in NP-40 lysis buffer. Western blots were probed with antibodies against LPL, HSP90 (as a loading control), and H2A (as a loading control). NP40S, NP40-soluble LPL; NP40P, NP40-precipitated LPL; s.e., short exposure; l.e., long exposure. (C) Western blot of EndoH-treated cell lysates of adipocytes that had been differentiated from the stromal vascular fractions of WAT from *Angptl4*$^{-/-}$ and wild-type mice. Cells were treated with 10 μM monensin (Mon.) for 3h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (D) Western blot of EndoH-treated cell lysates from adipocytes that had been differentiated from stromal vascular fractions of WAT from *Angptl4*$^{-/-}$ and wild-type mice. The cells were treated with 20 μM E64D for 24 h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (E) Western blot of EndoH-treated cell lysates of adipocytes that had been differentiated from stromal vascular fractions of WAT from *Angptl4*$^{-/-}$ and wild-type mice. Cells were treated with 5 mM 3-methylamine for 10 h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (F) Western blot of EndoH-treated cell lysates of adipocytes that had been differentiated from stromal vascular fractions from WAT of *Angptl4*$^{-/-}$ and wild-type mice. The cells had been treated with 10 mM leupeptin (leup.) for 10 h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). EndoH-resistant LPL (complex oligosaccharides; Golgi and cell surface LPL) is indicated with R; EndoH-sensitive LPL (high-mannose oligosaccharides, ER LPL) is indicated with S.

resulted in a pronounced accumulation of LPL but did not eliminate the differences in the amounts of EndoH-resistant LPL between *Angptl4*$^{-/-}$ and wild-type adipocytes (**Figure 7D-F**). Of note, 3MA consistently affected levels of the loading control HSP90 specifically in *Angptl4*$^{-/-}$ adipocytes, despite equal protein loading (data not shown). These data suggest that LPL is degraded by lysosomes, but that inhibition of lysosomal degradation with e64D, 3MA or leupeptin cannot prevent ANGPTL4-mediated reduction in EndoH-resistant LPL.

Physiological regulation of ANGPTL4 affects LPL quantity and glycosylation *in vivo*

To determine if the physiological regulation of *Angptl4* expression affects intracellular degradation of LPL *in vivo,* we examined LPL quantity and LPL glycosylation in adipose tissue of fed and fasted wild-type and *Angptl4*$^{-/-}$ mice. Consistent with the *ex vivo* experimental data, the total LPL protein levels and levels of EndoH-resistant LPL were noticeably higher in *Angptl4*$^{-/-}$ adipose tissue than in wild-type adipose tissue (**Figure 8A & 8B**). Accumulation of LPL in *Angptl4*$^{-/-}$ adipose tissue was observed for dimeric LPL (~110 kDa) and multimeric LPL (~220 kDa) as determined by Native PAGE (**Figure 8A**). Interestingly, while total LPL protein in WAT of wild-type mice mirrored previously published *Lpl* mRNA levels during fasting and refeeding (13), the amount of EndoH-resistant LPL decreased with fasting and increased with refeeding (**Figure 8C**). These changes are in accordance with increased ANGPTL4 mRNA and protein levels upon fasting and decreased ANGPTL4 mRNA and protein levels upon refeeding (**Figure 8D**) (13). By contrast, the amount of EndoH-resistant LPL in WAT of *Angptl4*$^{-/-}$ mice was not regulated by fasting and slightly increased by refeeding (**Figure 8C**). Together, these data imply that the reciprocal regulation of EndoH-resistant LPL levels upon fasting and feeding in wild-type mice is likely mediated by ANGPTL4**.**

To determine whether the increased amount of EndoH-resistant LPL in adipose tissue of *Angptl4*$^{-/-}$ mice reflects the absence of ANGPTL4-mediated inhibition of LPL in capillaries, we detached LPL from the endothelium by perfusing tissues with heparin and injecting heparin intravenously. As expected, heparin reduced amounts of EndoH-resistant LPL in WAT lysates but did not eliminate the differences in EndoH-resistant LPL between wild-type and *Angptl4*$^{-/-}$ mice (**Figure 8E & 8F**). Together, these data suggest that EndoH-resistant LPL was inside cells or was within the interstitial spaces (possibly on the surface of adipocytes). Interestingly, the levels of LPL in the pre-heparin plasma were higher in *Angptl4*$^{-/-}$ mice than in wild-type mice, suggesting that some of the LPL that accumulates in the setting of *Angptl4* deficiency ends up in plasma (**Figure 9A**).

To exclude the possibility that the regulation of LPL by ANGPTL4 in adipocytes is an artefact related to the use of *Angptl4*$^{-/-}$ adipocytes (where ANGPTL4 is completely absent), we assessed total and EndoH-resistant LPL levels in mouse adipose tissue from *Angptl4*$^{-/-}$, *Angptl4*$^{+/-}$, wild-type, and *Angptl4*-Tg mice. A clear dose-dependent reduction of EndoH-resistant LPL was observed with increasing ANGPTL4 levels (**Figure 9B & 9C**). In addition, we tested whether inducing ANGPTL4 expression in 3T3-F442a and 3T3-L1 adipocytes with rosiglitazone leads to reduced amounts of

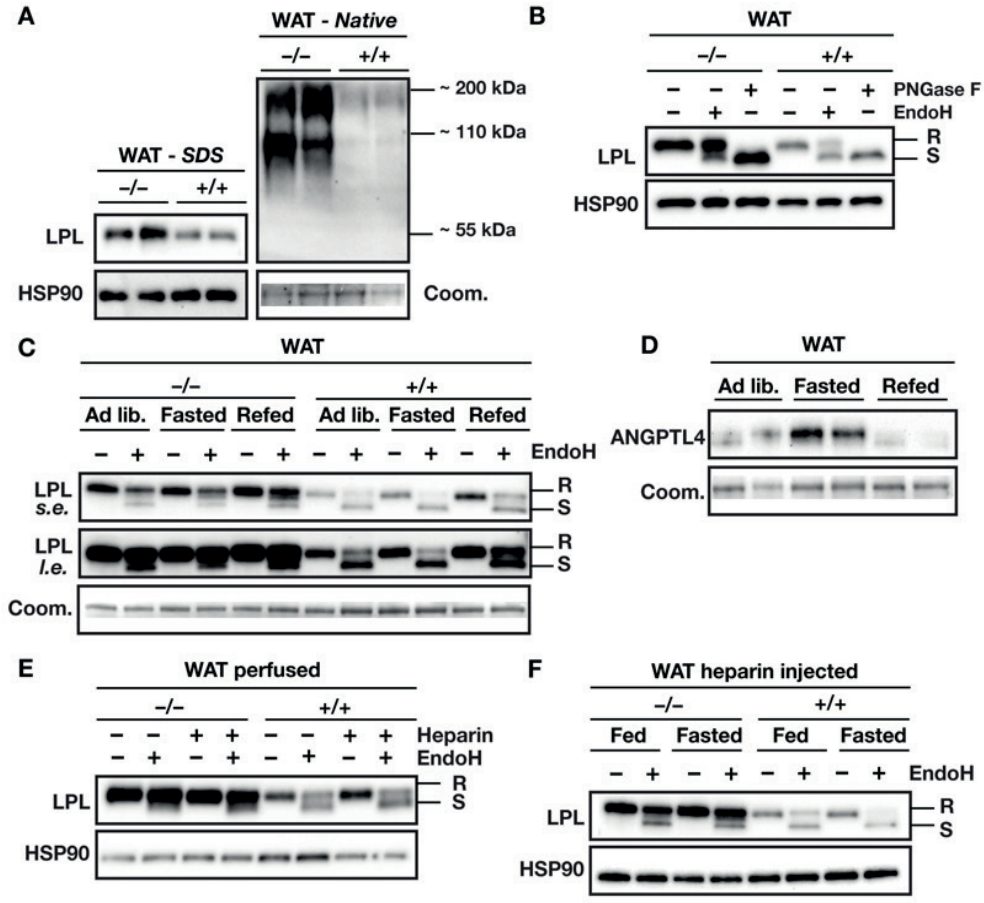

Figure 8. Physiological regulation of ANGPTL4 expression affects levels of EndoH-resistant LPL *in vivo*.
(A) Western blot of gWAT lysates of *Angptl4*$^{-/-}$ and wild-type mice as analysed by SDS-PAGE and Native PAGE. Western blots were probed with antibodies against LPL and HSP90. Coomassie blue staining was used to assess loading. (B) Western blot of EndoH-treated and PNGase-treated gWAT lysates of *Angptl4*$^{-/-}$ and wild-type mice. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (C) Western blot of EndoH-treated gWAT lysates prepared from ad libitum fed, overnight-fasted or refed *Angptl4*$^{-/-}$ and wild-type mice. The tissue samples were taken from an experiment described earlier (13). Western blots were probed with an antibody against LPL; Coomassie blue staining was used to assess loading. (D) Western blot of gWAT lysates prepared from ad libitum fed, overnight-fasted, or refed wild-type mice. The tissue samples were taken from an experiment described earlier (13). Western blots were probed with an antibody against ANGPTL4; Coomassie blue staining was used to assess loading. (E) Western blot of EndoH-treated gWAT lysates from *Angptl4*$^{-/-}$ and wild-type mice that were either not perfused or perfused with PBS containing heparin (50 IU/mL). Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (F) Western blot of EndoH-treated gWAT lysates prepared from fed and overnight-fasted *Angptl4*$^{-/-}$ and wild-type mice that had been given an intravenous injection of heparin (100 IU/kg). Western blots were probed with antibodies against LPL and HSP90 (as a loading control). EndoH-resistant LPL (complex oligosaccharides; Golgi and cell surface LPL) is indicated with R; EndoH-sensitive LPL (high-mannose oligosaccharides, ER LPL) is indicated with S.

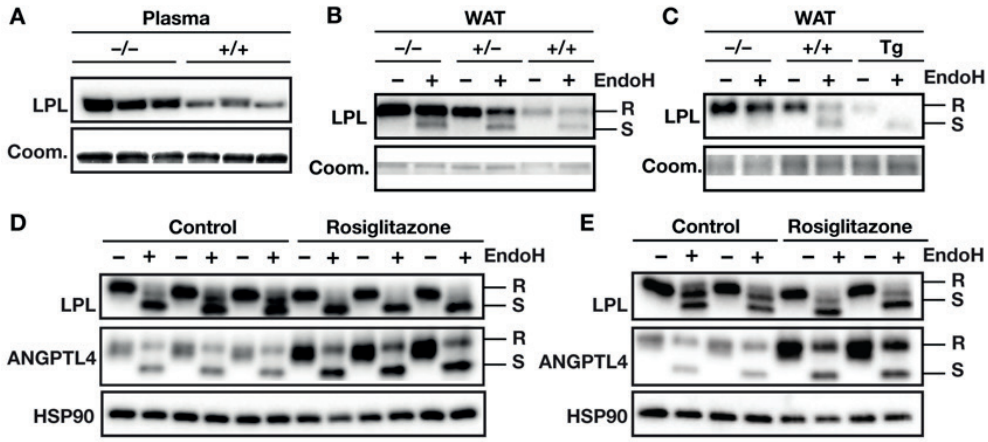

Figure 9. Levels of EndoH-resistant LPL are inversely related to Angptl4 expression.

(A) Western blot on 0.75 µl of plasma from *Angptl4*$^{-/-}$ and wild-type mice. Western blots were probed with an antibody against LPL; Coomassie blue staining was used to assess loading. (B) Western blot of EndoH-treated WAT lysates prepared from *Angptl4*$^{-/-}$, *Angptl4*$^{+/-}$, and wild-type mice. Western blots were probed with an antibody against LPL; Coomassie blue staining was used to assess loading. (C) Western blot of EndoH-treated WAT lysates prepared from *Angptl4*$^{-/-}$, wild-type, and Angptl4-*Tg* mice. Western blots were probed with an antibody against LPL; Coomassie blue staining was used to assess loading. (D) Western blot of EndoH-treated lysates of 3T3-F442a adipocytes that had been treated with rosiglitazone (10 µM) or DMSO control for 6 h. Western blots were probed with antibodies against LPL, ANGPTL4 and HSP90 (as a loading control). (E) Western blot of EndoH-treated lysates of 3T3-L1 adipocytes that had been treated with rosiglitazone (10 µM) or DMSO control for 6 h. Western blots were probed with antibodies against LPL, ANGPTL4 and HSP90 (as a loading control). EndoH-resistant LPL (complex oligosaccharides; Golgi and cell surface LPL) is indicated with R; EndoH-sensitive LPL (high-mannose oligosaccharides, ER LPL) is indicated with S.

EndoH-resistant LPL. In rosiglitazone-treated adipocytes, ANGPTL4 expression was increased concomitant with a reduction in levels of EndoH-resistant LPL (**Figure 9D & 9E**).

A final question is whether physiological regulation of ANGPTL4 expression affects LPL abundance and LPL glycosylation in other tissues. Prolonged cold exposure markedly increases LPL activity in brown adipose tissue (BAT) as a result of reduced ANGPTL4 expression (14). As in WAT, the amount of EndoH-resistant LPL in BAT was noticeably higher in *Angptl4*$^{-/-}$ mice than in wild-type mice both at thermoneutral and cold temperatures (**Figure 10A**). Cold exposure mildly affected levels of multiple loading controls, despite an equal loading of protein (**Figure 10A**; data not shown). Consistent with reduced ANGPTL4 expression after prolonged cold exposure, the amount of EndoH-resistant LPL in BAT was markedly increased in wild-type mice but less so in *Angptl4*$^{-/-}$ mice (**Figure 10B**). In contrast, the amounts of total and EndoH-sensitive LPL in hearts of *Angptl4*$^{-/-}$ and wild-type mice during basal and fasting conditions were not different (**Figure 10B**). Thus, it appears that

ANGPTL4-mediated loss of EndoH-resistant LPL in vivo requires a high basal level of ANGPTL4 expression, which is characteristic of BAT and WAT but not heart (14).

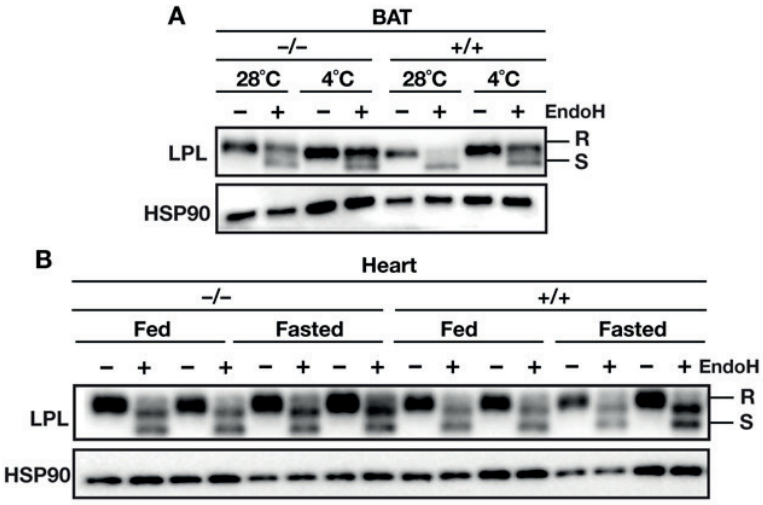

Figure 10. ANGPTL4 regulates levels of EndoH-resistant LPL in adipose tissue but not heart.

(A) Western blot of EndoH-treated BAT lysates from *Angptl4*$^{-/-}$ and wild-type mice exposed to cold (4°C) or thermoneutral temperature (28°C) for 10 days. The tissue samples were taken from an experiment described earlier (14). Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (B) Western blot on EndoH-treated heart lysates from *Angptl4*$^{-/-}$ and wild-type mice fed *ad libitum* or fasted overnight. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). EndoH-resistant LPL (complex oligosaccharides; Golgi and cell surface LPL) is indicated with R; EndoH-sensitive LPL (high-mannose oligosaccharides, ER LPL) is indicated with S.

DISCUSSION

ANGPTL4 is known to inhibit LPL catalytic activity in a variety of tissues, including skeletal muscle and adipose tissue (17, 46). Here we show that ANGPTL4 reduces LPL protein in adipocytes by promoting intracellular degradation. Co-transfecting CHO pgsA-745 cells with ANGPTL4 and LPL expression vectors—but not co-plating of independently transfected cells—markedly reduced the amount of LPL within cells. Conversely, the absence of ANGPTL4 expression in adipocytes led to an accumulation of heparin-releasable, EndoH-resistant LPL. The suppressive effect of ANGPTL4 on total and EndoH-resistant LPL in adipose tissue was confirmed in adipose tissue *in vivo*. Together, our findings demonstrate that ANGPTL4 reduces the amount of LPL protein within adipocytes by promoting LPL degradation after its post-translational processing in the ER (see **Figure 11** for a schematic model).

Inactivating variants in the human *ANGPTL4* gene have been repeatedly linked to low plasma triglycerides and elevated HDL cholesterol, and have been associated with a reduced risk of coronary heart disease (18–20). Given the marked impact of ANGPTL4-inactivating mutants on plasma triglycerides, it is somewhat surprising that no clear correlation between plasma ANGPTL4 and plasma triglyceride levels has been observed (21–24, 47–49). A possible explanation for this apparent discrepancy is that ANGPTL4 regulates LPL *via* a local mechanism rather than *via* an endocrine mechanism—so as to match the uptake of fatty acids to the metabolic needs of underlying tissue. Our data strongly suggest that ANGPTL4 impacts LPL locally by acting on LPL intracellularly along the secretory pathway (25). An important action of ANGPTL4 inside the cell and in the subendothelial space rather than intravascularly is consistent with the observation that both circulating triglyceride-rich lipoproteins and GPIHBP1 protect LPL from inhibition by ANGPTL4 (27, 28). It should be noted that our findings per se do not argue against the ability of ANGPTL4 to act on extracellular and intravascular LPL. Indeed, secreted ANGPTL4, recombinant ANGPTL4, and anti-ANGPTL4 antibodies have all been shown to be able to impact extracellular LPL activity (25, 28, 50). In addition, liver-specific *Angptl4* overexpression or injection of mice with recombinant ANGPTL4 effectively lowers plasma triglycerides and plasma LPL activity levels, implying that circulating ANGPTL4 is able to target and inhibit intravascular LPL (33, 51). Similarly, monoclonal antibodies against ANGPTL4—which are expected to primarily affect intravascular ANGPTL4 and to a lesser extent subendothelial ANGPTL4—have been shown to potently lower plasma triglyceride levels in hyperlipidaemic mice and monkeys (19, 52, 53). Future studies are necessary to assess the relative importance of intracellular, subendothelial, and intravascular inhibition of LPL by ANGPTL4. It can be speculated that the importance of the three routes may differ between tissues and may depend on the physiological state of the organism.

Our experiments show that EndoH-resistant LPL could be rapidly released by heparin, suggesting that the accumulated EndoH-resistant LPL in *Angptl4*$^{-/-}$ mice is either bound to HSPGs on the cell surface or located in rapidly-releasable secretory vesicles (40, 41). The precise location for the interaction of ANGPTL4 and LPL within cells remains to be determined. However, both ANGPTL4 and LPL are secreted proteins that consequently are expected to be found at similar locations within

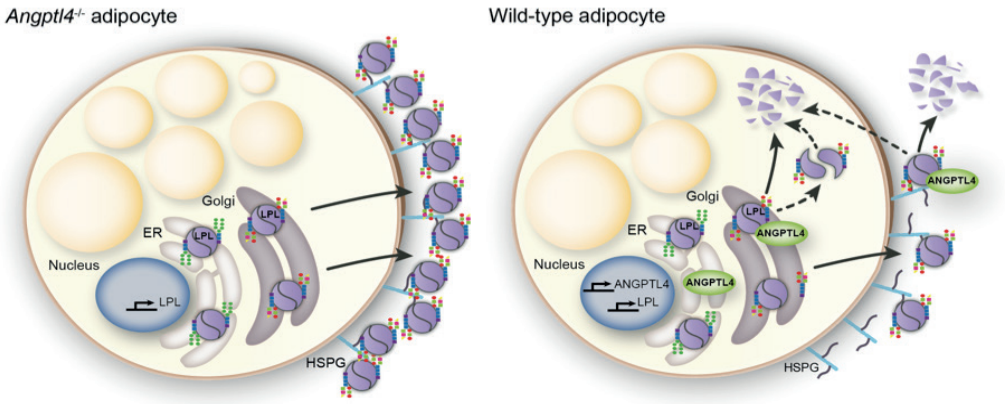

Figure 11. Schematic model.

Within the ER, LPL acquires oligosaccharides side chains via co-translational transfer of oligosaccharides high in mannose residues. Upon translocation to the Golgi apparatus, the high-mannose oligosaccharides are trimmed and replaced by more complex oligosaccharides. In the absence of ANGPTL4 (i.e., *Angptl4*$^{-/-}$ adipocytes), LPL with complex oligosaccharide side chains is packaged in secretory vesicles and secreted. Secreted LPL accumulates on the cell surface of adipocytes, mostly bound to heparin-sulphate proteoglycans (HSPGs). In ANGPTL4-expressing adipocytes (i.e. wild-type adipocytes), ANGPTL4 interacts with LPL in a post-ER compartment. This interaction leads, potentially via converting LPL homodimers to monomers, to degradation of LPL. This results in a specific reduction of LPL with complex oligosaccharide side chains in wild-type adipocytes versus *Angptl4*$^{-/-}$ adipocytes. Once secreted, ANGPTL4 also reduces levels and activity of secreted LPL.

the cell, which limits the applicability of immunofluorescence studies. Makoveichuk and co-workers found that ANGPTL4-producing cells have lower LPL activity in cell culture medium but concluded that the inhibition of LPL by ANGPTL4 occurred after both proteins arrived at the cell surface (29, 50). Our data suggest that ANGPTL4-mediated removal of LPL starts within cells—before LPL is secreted. In the presence of heparin, which is known to stabilize LPL (42), the secretion of LPL by *Angptl4*$^{-/-}$ adipocytes is higher than by wild-type adipocytes. Similarly, co-expression of ANGPTL4 and LPL in CHO pgsA-745 cells, which are deficient in cell-surface HSPGs (54), markedly reduced amounts of LPL within cells. Presently, we cannot fully exclude the possibility that the elimination of LPL occurs (partly) on the cell surface, as secreted LPL may be inactivated by ANGPTL4 on the cell surface and directly taken up by the cell for degradation. However, our data strongly suggest that ANGPTL4 eliminates the majority of LPL before the enzyme reaches the cell surface. ANGPTL4 could mediate intracellular degradation of LPL by stimulating endosomal transport of LPL from the (*trans*-)Golgi or cell surface to lysosomes (42, 55–58). Pulse-chase experiments have shown that ~80% of newly synthesized LPL in adipocytes is degraded intracellularly, primarily by lysosomes (41, 42). Our data confirmed that inhibition of the lysosomal pathway reduces intracellular degradation of LPL. However, inhibition of autophagosomal or lysosomal degradation did not abolish differences in the intracellular LPL levels between *Angptl4*$^{-/-}$ and wild-type adipocytes. It is possible that the use of chemical inhibitors may not permit us to detect the effect of ANGPTL4

on lysosomal degradation of LPL or that ANGPTL4-induced removal of EndoH-resistant LPL occurs, at least in part, in a compartment other than the lysosomes. One possibility is that ANGPTL4 alters LPL protein conformation and stability or interferes with the addition of complex oligosaccharides. Biochemical studies have indicated that ANGPTL4 inactivates LPL by converting the catalytically active LPL dimers into catalytically inactive monomers (59). Accordingly, the degradation of LPL could be secondary to an effect of ANGPTL4 on dimer–monomer conversion and/or LPL protein stability. The exact mechanism by which ANGPTL4 promotes intracellular LPL degradation will be the subject of further investigation.

The fact that LPL might be regulated before secretion is not surprising given that other key players in lipid metabolism, illustrated by apolipoprotein B and the LDL receptor, are regulated along the secretory pathway (60–65). We propose that a multilevel regulation of LPL by ANGPTL4, along with tight regulation of ANGPTL4 expression, permits for rapid adjustments in local LPL activity in accordance with the requirements of the tissue. To assure the appropriate distribution of lipid nutrients, ANGPTL4 may collaborate with its family members ANGPTL3 and ANGPTL8 to regulate LPL activity levels during different physiological conditions (66). While ANGPTL3 and ANGPTL8 have been proposed to mainly exert their actions *via* an endocrine mechanism, it would be of interest to study whether ANGPTL3 and ANGPTL8 may also regulate LPL intracellularly and promote LPL degradation (66). In this context, it is interesting to mention that homozygous carriers of an inactivating mutation in the *ANGPTL3* gene have markedly higher levels of post-heparin LPL mass and activity as compared to non-carriers (67).

In conclusion, our data indicate that ANGPTL4 interacts with LPL inside cells, both in CHO pgsA-745 cells and in adipocytes *ex vivo* and *in vivo*. ANGPTL4 promotes intracellular degradation of LPL within adipocytes after LPL processing within the ER. Together, our studies reveal a novel site and mechanism for ANGPTL4-mediated regulation of LPL activity.

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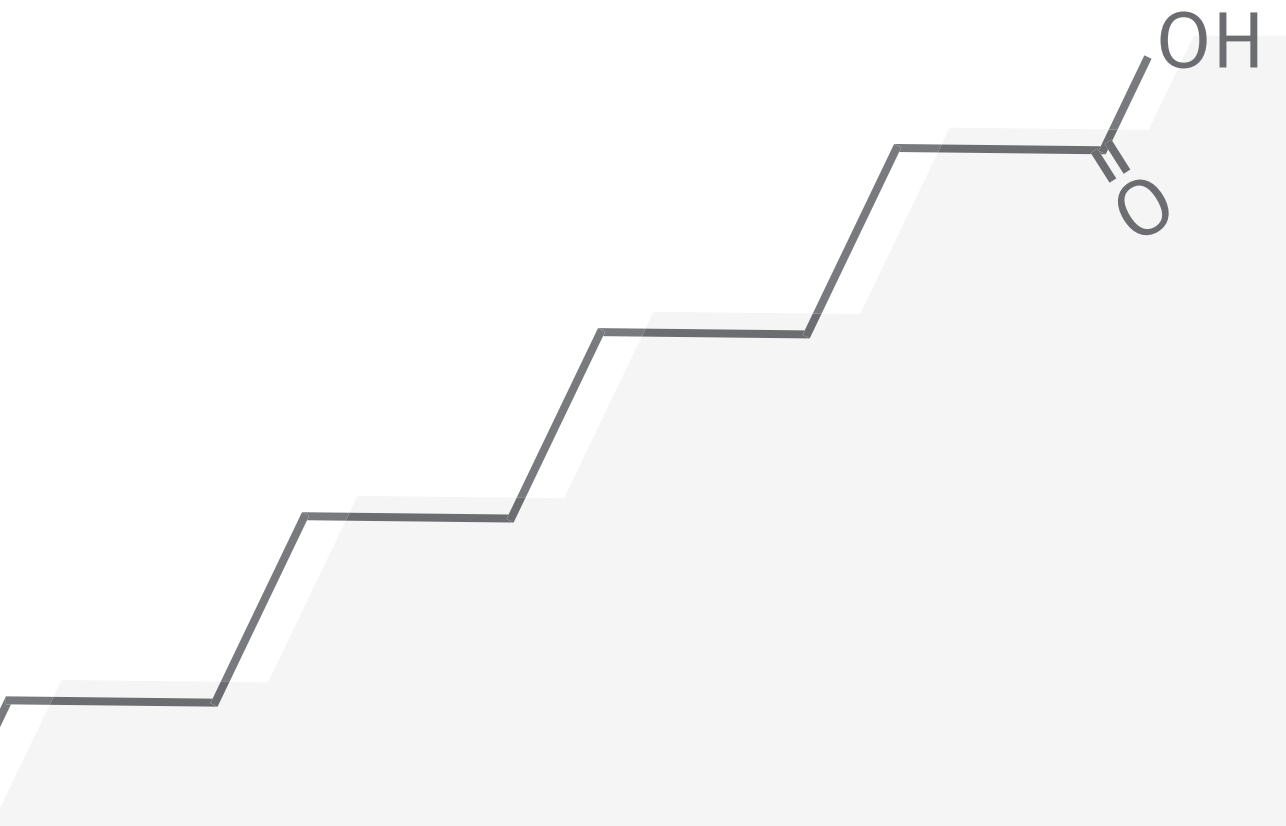
6

ANGPTL4 PROMOTES THE INTRACELLULAR CLEAVAGE OF LIPOPROTEIN LIPASE BY PCSK3 IN ADIPOCYTES

Wieneke Dijk
Lynette J. Oost
Sander Kersten

Manuscript in preparation



ABSTRACT

Lipoprotein lipase (LPL) hydrolyzes triglycerides from circulating triglyceride-rich lipoproteins at the capillary endothelium to yield fatty acids that are stored or oxidized in the underlying parenchymal cells. To assure the effective distribution of fatty acids, the activity of LPL is tightly regulated at the post-translational level. *In vitro* studies indicate that LPL is cleaved by members of the proprotein convertase subtilisin/kexin (PCSK) protein family. Our objective was to assess the relevance of PCSK-mediated cleavage of LPL *in vivo* and to clarify the underlying mechanisms. By treatment of different *in vitro* adipocyte models with the PCSK-inhibitor Dec-RVKR-CMK, we demonstrate that LPL is cleaved by PCSKs in the adipose tissue. Treatment of mature 3T3-L1 adipocytes with heparin revealed that (part of) the LPL cleavage occurs intracellularly, suggesting a role for the *trans*-Golgi located PCSK3. Indeed, siRNA-mediated knockdown of *Pcsk3* significantly reduced the amount of LPL cleavage in cell culture medium and cell lysates of 3T3-L1 adipocytes. Finally, we show that PCSK-mediated cleavage of LPL is diminished in adipocytes of mice lacking angiopoietin-like 4 (ANGPTL4), a well-established inhibitor of LPL. In conclusion, cleavage of LPL by PCSKs is a physiological process in adipocytes that may be promoted by ANGPTL4.

INTRODUCTION

At the capillary endothelium, lipoprotein lipase (LPL) hydrolyzes triglycerides from circulating triglyceride-rich lipoproteins such as chylomicrons or very low-density lipoproteins. LPL-mediated hydrolysis of circulating triglycerides yields free fatty acids and monoglycerides that are either stored or oxidized by the underlying parenchymal cells, including (cardio)myocytes and adipocytes (1). Expression of LPL is limited to the parenchymal cells and, accordingly, LPL requires active transport to the endothelial cell surface by the enzyme GPIHBP1 (glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1) (2). To assure an effective distribution of fatty acids, the activity of LPL is tightly regulated, primarily at the post-translational level (3).

Three members of the angiopoietin-like (ANGPTL) protein family potently inhibit LPL activity at the post-translational level; ANGPTL3, ANGPTL4 and ANGPTL8 (4). ANGPTL3 and ANGPTL8 circulate in the blood and cooperate to inhibit the activity of LPL in oxidative tissues, such as the heart, upon refeeding (5, 6). In contrast, tissue-specific changes in the expression of ANGPTL4 determine the changes in LPL activity that occur during physiological conditions such as exercise, fasting and cold exposure (7–9). ANGPTL4 inhibits LPL activity intracellularly, in the subendothelial spaces and at the endothelium (10–12). However, the exact mechanisms by which ANGPTL4 inhibits LPL remain debated. For example, ANGPTL4 has been proposed to convert the LPL dimer into catalytically inactive monomers or to function as a non-competitive, reversible inhibitor of LPL (13, 14).

Members of the proprotein convertase subtilisin/kexin (PCSK) protein family (PCSK1-7, SKI-1/S1P and PCSK9) are calcium-dependent serine endopeptidases that convert proproteins into their active forms by cleavage (15, 16). PCSK1-7 recognize and cleave substrates at specific lysine- and/or arginine-containing basic amino acid sequences (15). Despite overlap in substrate recognition, their functions are tissue-specific and dependent on their cellular localization. For example, PCSK3 (FURIN) is primarily found in the *trans*-Golgi network, in the endosomes and on the cell surface, while PCSK5 (PC5/6) and PCSK6 (PACE4) are primarily present on the cell surface (16). Interestingly, several PCSKs have been implicated in the cleavage of proteins involved in intravascular lipolysis. For example, ANGPTL3 and ANGPTL4 are potently cleaved by PCSKs *in vitro* and *in vivo*, whereas LPL is post-translationally cleaved by PCSKs *in vitro* (17–21).

Here, our objective was to assess whether PCSK-mediated cleavage of LPL also occurs *in vivo* in the adipose tissue, a tissue with a high expression of LPL. We show, for the first time, that cleavage of LPL by PCSKs takes place in the adipose tissue and that this cleavage is mediated by PCSK3. Furthermore, our data suggest that ANGPTL4 promotes PCSK-mediated cleavage of LPL in adipocytes, an observation that may help to clarify how ANGPTL4 inhibits adipose tissue LPL activity.

MATERIALS AND METHODS

All animal experiments were performed in accordance with Directive 2010/63/EU from the European Union. All animal studies were reviewed and approved by the Animal Ethics Committee of Wageningen University.

Chemicals

AICAR, dexamethasone, IBMX, insulin, rosiglitazone, and intralipid were from Sigma. Furin inhibitor I was from Cayman Chemicals (*via* Sanbio, Uden, The Netherlands). A769662 was from Tocris (Tocris Bioscience, Bristol, United Kingdom).

Cell culture

3T3-L1 fibroblasts (P7-P16) were maintained in DMEM (Lonza, Verviers, Belgium) supplemented with 10% newborn calf serum (NCS) (Lonza) and 1% penicillin/streptomycin (P/S) (Lonza). Two days post-confluency, cells were switched to DMEM, supplemented with 10% fetal bovine serum (FBS), 1% P/S, 0.5 mM IBMX, 10 µM dexamethasone, 5 µg/mL insulin for 2 days. Subsequently, cells were maintained in DMEM supplemented with 10% FBS, 1% P/S, and 5 µg/mL insulin for 6 days and switched to DMEM with 10% FBS and 1% P/S for 3 days, after which experiments were performed as indicated in figure legends (22). To examine the accumulation of amino-terminal LPL on the cell surface, mature 3T3-L1 adipocytes were incubated with 10 IU/mL heparin for 20 minutes.

For isolation and differentiation of primary adipocytes, inguinal WAT was removed from *Angptl4*$^{-/-}$ and wild-type mice and placed in DMEM supplemented with 1% P/S and 1% bovine serum albumin (BSA) (Sigma-Aldrich) (10). Material from 2–3 mice was pooled, minced with scissors, and digested in collagenase-containing medium [DMEM with 3.2 mM $CaCl_2$, 1.5 mg/mL collagenase type II (C6885, Sigma-Aldrich), 10% FBS, 0.5% BSA, and 15 mM HEPES] for 1 h at 37°C. Following digestion, the cells were filtered through a 100 µm cell strainer (Falcon) to remove remaining cell clumps. The cell suspension was centrifuged at 1600 rpm for 10 min, after which the supernatant was removed and the pellet was resuspended in erythrocyte lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA). Upon incubation for 2–3 minutes at room temperature, cells were centrifuged at 1200 rpm for 5 min and the pelleted cells were resuspended in DMEM supplemented with 10% FCS and 1% P/S, and plated. Upon confluency, the cells were differentiated according to the protocol as described above for 3T3-L1 cells, with the addition of 1 µM rosiglitazone during the initial differentiation step.

For WAT explants, gonadal WAT was taken from wild-type mice and *Angptl4*$^{-/-}$ mice and placed in DMEM supplemented with 1% P/S and 1% BSA. Fat pads were minced into small pieces and divided into small mounds of WAT (~50–100 mg of tissue). WAT explants were placed into wells containing medium (DMEM with 1% P/S and 10% FCS) and incubated as indicated in the figure legends. Next, the medium was harvested and explant weights were determined. Explants were immediately lysed to prepare protein extracts.

siRNA knockdown

Dharmacon ON-TARGETplus SMARTpool siRNAs against mouse *Pcsk3* were purchased from Thermo Fisher-Scientific. siRNAs were diluted in Dharmacon 1x siRNA buffer (final concentration 20 mM KCl, 6 mM HEPES pH 7.5, 0.2 mM $MgCL_2$). Transfections were performed with Lipofectamine RNAiMAX transfection reagent (Life Technologies, Bleiswijk, The Netherlands) at a concentration of 10 nM siRNA and 1.5 µL transfection reagent for a 24-wells plate. To examine the impact of *Pcsk3* knockdown on LPL cleavage, mature 3T3-L1 adipocytes were washed with PBS, trypsinized and collected in DMEM. Following centrifugation at 1250 rpm for 5 minutes, pelleted cells were re-suspended and filtered through a 70 µM cell strainer. Adipocytes were plated at 70% confluency and two hours later siRNAs complexed to Lipofectamine were added. Cleavage of LPL was examined after 48 hours of incubation.

Mouse studies

Tissue samples from *Angptl4* knockout mice *(Angptl4*$^{-/-}$) and wild-type mice were taken to assess LPL cleavage. *Angptl4*$^{-/-}$ mice and wild-type mice have been on a C56BL/6J background for > 10 generations. To assess regulation of *Pcsk3* during different physiological conditions, WAT from previously published studies was used. WAT from wild-type mice exposed to cold or thermoneutral temperature for 10 days were from a study by Dijk et al. (8). WAT from fed and fasted mice were from a study by Kersten et al. (23). WAT from mice injected with 1 mg/kg CL316,243 were from a study by Dijk et al. (submitted for publication). For the tissue panel, wild-type mice (n=4) were euthanized and multiple tissues were harvested, snap-frozen in liquid nitrogen and stored at -80˚ C until further analyses.

Western blots

Fat pads, WAT explants and differentiated primary adipocytes were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 1% SDS; Thermo Fisher-Scientific, Landsmeer, The Netherlands) supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Almere, The Netherlands). Following homogenization, lysates were placed on ice for 30 minutes and centrifuged 2–3 times at 13,000 rpm for 10 min at 4˚C to remove fat and cell debris. Concentration of protein lysates was determined by using a bicinchoninic acid (BCA) assay (Thermo Fisher-Scientific). Protein lysates (10–30 µg protein per lane) or medium aliquots (10–15 µL) were loaded onto 8-16% or 10% Criterion gels (Bio-Rad, Veenendaal, The Netherlands). Next, proteins were transferred onto a PVDF membrane using the Transblot Turbo System (Bio-Rad). Membranes were probed with a goat anti-mouse LPL antibody (24); a rabbit anti-mouse HSP90 antibody (Cell Signaling Technology, #4874); a rat anti-mouse ANGPTL4 antibody (Kairos 142-2, Adipogen); a mouse anti-mouse β-TUBULIN (sc-5274, Santa Cruz); or a rabbit anti-mouse PCSK3 (Abcam, ab183495) at 1:5000 (LPL), 1:2000 (HSP90) or 1:1000 (PCSK3) dilutions. Blocking, primary antibody incubations and secondary antibody incubations were all done in Tris-buffered saline, pH 7.5, 0.1% Tween-20 (TBS-T), and 5% w/v

skimmed milk. In between, membranes were washed in TBS-T. Quantification was performed with the ChemiDoc MP system (Bio-Rad) and Clarity ECL substrate (Bio-Rad). Equal loading of medium samples was verified by Coomassie blue staining.

RNA isolation and qPCR

To isolate RNA, tissues or cells were homogenized using TRIzol (Thermo Fisher-Scientific) in a Qiagen Tissue lyser II (Qiagen, Venlo, The Netherlands) or by pipetting up and down. RNA was reverse transcribed using the First Strand cDNA synthesis kit (Thermo Fisher-Scientific). qPCR analyses were done on a CFX384 Real-Time PCR platform (Bio-Rad) with the SensiMix PCR mix from Bioline (GC biotech, Alphen aan de Rijn, The Netherlands).

RESULTS

LPL is cleaved in adipose tissue by PCSKs

Examination of western blots of LPL in different adipocyte and adipose tissue models consistently revealed a band of ~33 kDa below the band of full-length LPL of ~55 kDa and corresponding to the amino-terminal domain of LPL (**Figure 1A**) (10, 25) (A. Beigneux, personal communications). Previously, members of the proprotein convertase subtilisin/kexin (PCSK) family were suggested to cleave LPL at residues 321-324 to yield nearly complete amino-terminal and carboxyl-terminal domains (21). To examine whether the observed amino-terminal LPL was the result of PCSK-mediated cleavage, mature 3T3-L1 and 3T3-F442a adipocytes, as well as primary adipocytes and WAT explants were treated with the inhibitor Dec-RVKR-CMK (dec-CMK) to block PCSK activity (26). In all adipose tissue models, incubation with dec-CMK consistently resulted in the almost complete disappearance of amino-terminal LPL in cell culture medium and cell lysates, indicating that LPL is cleaved by PCSKs in adipocytes (**Figure 1A**).

PCSK3 is expressed in adipose tissue and cleaves adipocyte LPL

To assess whether adipocyte LPL is cleaved by PCSKs intracellularly or upon secretion, we treated 3T3-L1 adipocytes with heparin to release LPL bound to heparin sulphate proteoglycans (HSPGs) from the cell surface. As expected, heparin treatment resulted in a pronounced increase of LPL in the cell culture medium, along with a concomitant reduction of LPL in the cell lysates (**Figure 2A & Figure 2B**). However, in line with the reported importance of carboxyl-terminal LPL for HSPG binding, the amount of amino-terminal LPL observed in cell culture medium and cell lysates was unaltered (**Figure 2A & Figure 2B**) (25, 27). These data indicate that no or little amino-terminal LPL is bound to HSPGs on the cell surface and that amino-terminal LPL in cell lysates must originate from intracellular LPL cleavage.

Given that LPL is cleaved by PCSKs intracellularly, a promising candidate for LPL cleavage in adipocytes is PCSK3, as it is activated and active intracellularly in the *trans*-Golgi (16). Expression profiling showed that PCSK3 is most highly expressed in liver and kidney, with comparatively low but clearly detectable PCSK3 expression in adipose tissue and in adipocytes (**Figure 3A & 3B**). In the adipose tissue, PCSK3 appears to be present in its mature ~98 kDa form and its proteolytically cleaved, shed ~90 kDa form (**Figure 3A**) (28). To assess whether PCSK3 might be involved in LPL cleavage in adipocytes, we knocked down *Pcsk3* in mature 3T3-L1 adipocytes by means of siRNA. SiRNA-mediated knockdown resulted in a 90% reduction in *Pcsk3* expression levels and a near complete loss of the mature PCSK3 protein, as assessed by western blot (**Figure 3C & Figure 3D**). Surprisingly, however, knockdown of PCSK3 did not substantially impact protein levels of shed PCSK3 (**Figure 3D**). In line with a role for PCSK3 in LPL cleavage, the knockdown of *Pcsk3* significantly reduced the amount of LPL cleavage in cell culture medium and cell lysates (**Figure 3D**).

Amino-terminal LPL is cleared by the lysosomes

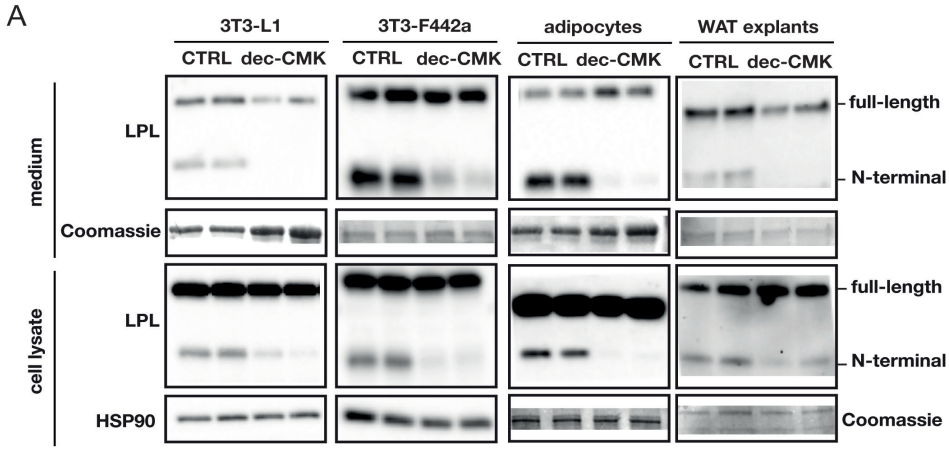


Figure 1. LPL is cleaved by PCSKs in adipocytes.

(A) Western blots of cell culture medium (*upper panels*) and cell lysates (*lower panels*) of mature 3T3-L1 adipocytes, mature 3T3-F442a adipocytes, primary adipocytes differentiated from the stromal vascular fraction of WAT and WAT explants, that were treated with 50 μM Dec-RVKR-CMK (dec-CMK) for 9h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). Coomassie blue staining was performed as a loading control for cell culture medium, primary adipocytes and WAT explants.

Previously, PCSK-mediated cleavage was shown to promote the inactivation of endothelial lipase, a family member of LPL (21). Since the PCSK-cleavage site in LPL is situated in the middle of the protein, it may be suggested that PCSK-mediated cleavage might also serve to inactivate LPL (21). Since full-length LPL is known to be degraded in the lysosomes in adipocytes, we incubated mature 3T3-L1 adipocytes and primary adipocytes with bafilomycin A1 (BafA1) and ammonium chloride (NH_4Cl) to inhibit lysosomal degradation and to assess the fate of amino-terminal LPL (29). In agreement with previous studies, treatment of adipocytes with the lysosomal inhibitors BafA1 and NH_4Cl significantly increased proteins levels of full-length LPL in cell lysates and to a lesser extent in the cell culture medium (**Figure 4A & Figure 4B**). More surprisingly, both compounds also consistently increased the amount of amino-terminal LPL in the cell lysates, suggesting that at least part of the amino-terminal LPL is cleared by the lysosomes (**Figure 4A & Figure 4B**). In contrast, BafA1 and NH_4Cl differentially impacted the accumulation of amino-terminal LPL in the cell culture medium (**Figure 4A & Figure 4B**).

ANGPTL4 promotes PCSK-mediated cleavage of adipocyte LPL

The observation that amino-terminal LPL is cleared by the lysosomes suggests that PCSK-mediated cleavage might promote LPL degradation and thereby affect LPL activity levels. To explore whether regulation of PCSK3 might contribute to the phys-

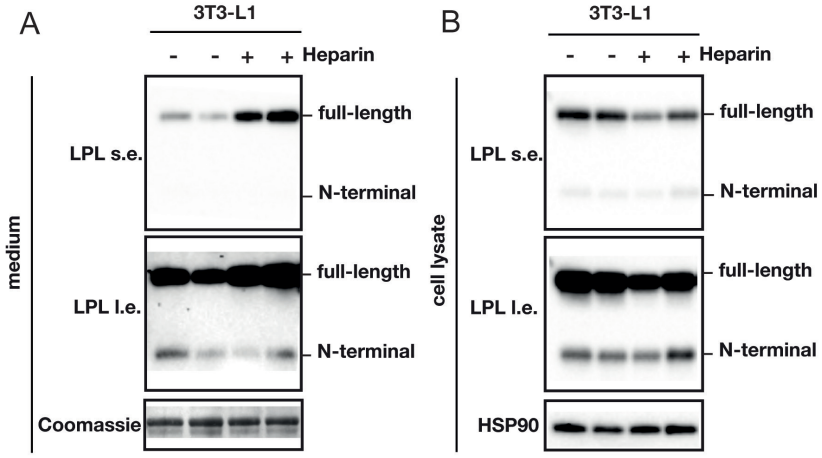


Figure 2. Amino-terminal LPL cannot be released from the cell surface by heparin.

(A) Western blots of cell culture medium of mature 3T3-L1 adipocytes that were treated with 10 IU/ mL heparin for 20 minutes. Western blot was probed with an antibody against LPL and a Coomassie blue staining was performed as a loading control. (B) Western blots of cell lysates of mature 3T3-L1 adipocytes that were treated with 10 IU/ mL heparin for 20 minutes. Western blots were probed with antibodies against LPL and HSP90 (as a loading control).

iological changes in LPL activity observed in the adipose tissue, we assessed *Pcsk3* expression levels in adipose tissue of mice that were either fasted for 24h, treated with the β3-adrenergic agonist CL316,243 or cold-exposed for 10 days, conditions that are known to influence LPL activity (8, 9). Fasting and treatment with the β3-adrenergic agonist CL316,243 resulted in a significant reduction in the expression of *Pcsk3*, while cold exposure did not affect *Pcsk3* expression (**Figure 5A**). However, the decrease in *Pcsk3* expression upon fasting is in apparent conflict with the reduction of adipose tissue LPL activity during fasting (9). Similarly, treatment of mature 3T3-L1 adipocytes with a variety of compounds did not result in any significant changes in the expression of PCSK3 (**Figure 5B**). Together, these data suggest that the regulation of PCSK3 is most likely not responsible for the variation in LPL activity levels during various physiological conditions.

From these data it cannot be inferred that cleavage of LPL by PCSK3 is relevant for the regulation of LPL activity. It may hypothesized that LPL needs to be destabilized to unmask the PCSK3 cleavage site and to enable cleavage. Recently, we identified ANGPTL4 as a protein that promotes the intracellular degradation of LPL (10). To investigate whether ANGPTL4 could promote PCSK3-mediated cleavage, we assessed the accumulation of amino-terminal LPL in adipose tissue and isolated adipocytes from *Angptl4*$^{-/-}$ and wild-type mice. Confirming previous studies, we observed higher levels of full-length LPL in adipocytes and adipose tissue *Angptl4*$^{-/-}$ mice (**Figure 5D & Figure 5E**) (10). More surprisingly, we detected significantly lower levels of amino-terminal LPL in adipocytes and adipose tissue of *Angptl4*$^{-/-}$ mice (**Figure 5D & Figure 5E**). Treatment with the PCSK inhibitor dec-CMK fully abrogated these

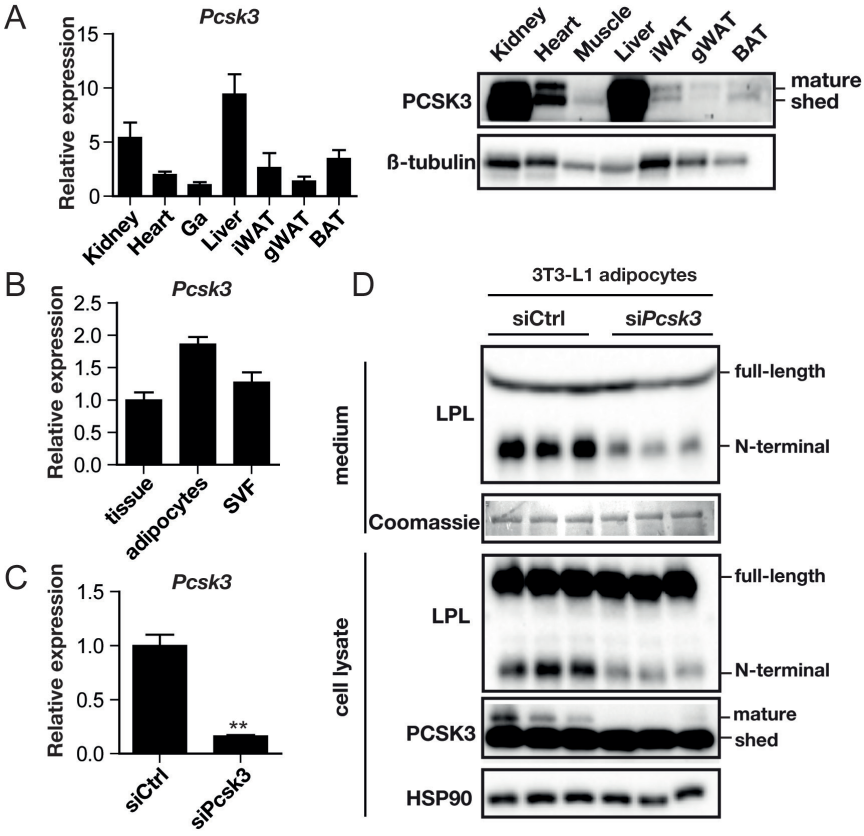


Figure 3. PCSK3 is well expressed in adipose tissue and cleaves adipocyte LPL.

(A) *Pcsk3* mRNA levels (*left*) and PCSK3 protein levels (*right*) in different tissues from C57BL/6 mice (n=4 for mRNA levels, n=1 for protein levels). (B) *Pcsk3* mRNA levels in WAT, isolated primary adipocytes and isolated stromal vascular fractions from WAT of C57BL/6 mice (n=4). (C) *Pcsk3* mRNA levels in fully differentiated 3T3-L1 adipocytes that were trypsinized, replated at 70% confluency, and treated with si*Pcsk3* or si*Ctrl* for 48h. (D) Western blots of cell culture medium (upper panels) and cell lysates (lower panels) of fully differentiated 3T3-L1 adipocytes that were trypsinized, replated at 70% confluency, and treated with si*Pcsk3* or si*Ctrl* for 48h. Western blots were probed with antibodies against LPL, PCSK3 and HSP90 (as a loading control). Coomassie blue staining was performed as a loading control for cell culture medium. Data are mean ± SEM. Asterisks indicate significant differences according to Student's t-test; ** $p<0.01$, * $p<0.05$.

differences, indicating that PCSK-mediated LPL cleavage mediates the differences in amino-terminal LPL between *Angptl4*$^{-/-}$ and wild-type mice (**Figure 5F**). Of interest, PCSKs might have a double function in the regulation of LPL activity, as ANGPTL4 is also cleaved in the adipose tissue to yield an amino-terminal domain that is known to potently inhibit LPL activity (**Figure 5G**) (18, 19, 30).

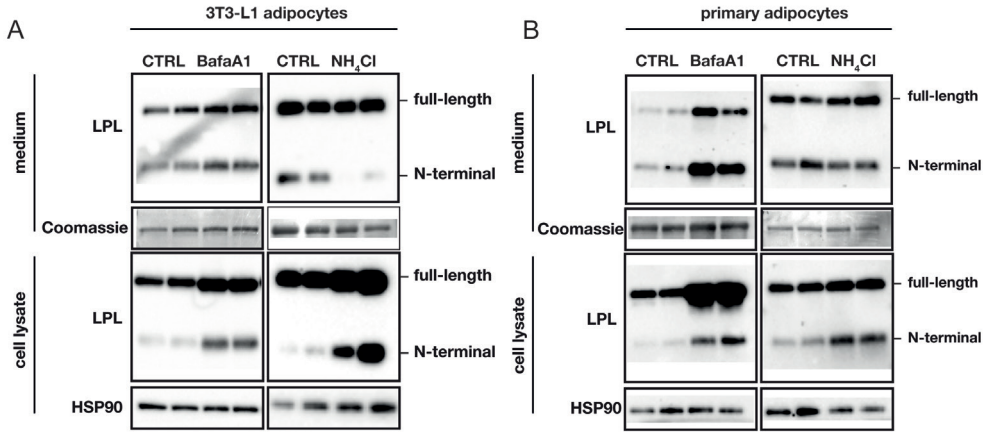


Figure 4. Amino-terminal LPL is cleared by the lysosomes.

(A) Western blots of cell culture medium (*upper panels*) and cell lysates (*lower panels*) of mature 3T3-L1 adipocytes, that were treated with 100 nM Bafilomycin A1 (BafA1), a vaculaor-type H (+)-ATPase inhibitor, and 10 mM NH_4Cl for 10h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). Coomassie blue staining was performed as a loading control for cell culture medium. (B) Western blots of cell culture medium (upper panels) and cell lysates (lower panels) of primary adipocytes differentiated from the stromal vascular fraction of WAT that were treated with 100 nM Bafilomycin A1 (BafA1), a vaculaor-type H (+)-ATPase inhibitor, and 10 mM NH_4Cl for 12h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). Coomassie blue staining was performed as a loading control for cell culture medium.

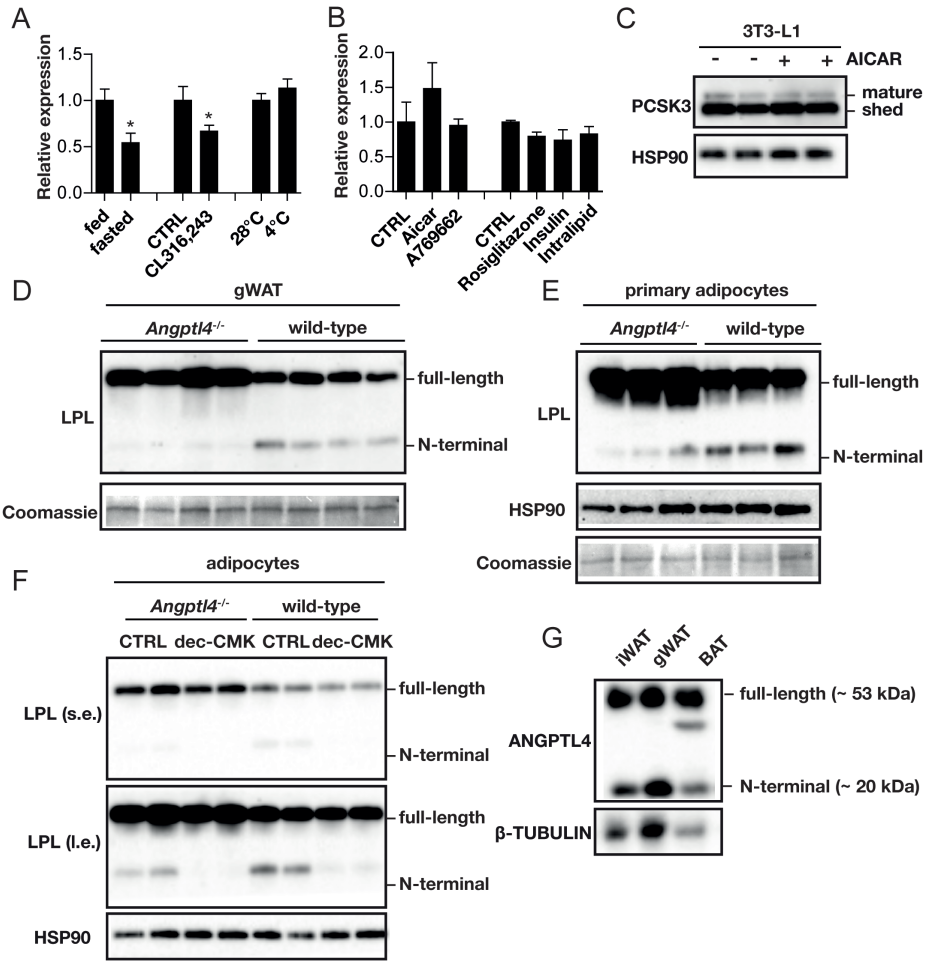


Figure 5. ANGPTL4 promotes PCSK-mediated cleavage of adipocyte LPL.

(A) *Pcsk3* mRNA levels in gonadal WAT of three different mice experiments: 1) a 24h fast; 2) injection of 1 mg/kg CL316,243; 3) cold exposure to 4˚C for 10 days (B) *Pcsk3* mRNA levels of mature 3T3-L1 adipocytes that were treated with 0.5 mM AICAR, 100 μM A769662, 10 μM rosiglitazone, 500 nM insulin, and 2 mM intralipid for 6h. (C) Western blots of cell lysates of mature 3T3-L1 adipocytes that were treated with 0.5 mM AICAR. Western blots were probed with antibodies against PCSK3 and HSP90 (as a loading control). (D) Western blots of cell lysates of gonadal WAT (gWAT) from *Angptl4*$^{-/-}$ and wild-type mice. Western blot was probed with an antibody against LPL and a Coomassie blue staining was performed as a loading control. (E) Western blots of primary adipocytes differentiated from the stromal vascular fraction of WAT from *Angptl4*$^{-/-}$ and wild-type mice. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (F) Western blots of primary adipocytes differentiated from the stromal vascular fraction of WAT from *Angptl4*$^{-/-}$ and wild-type mice that were treated with 50 μM dec-CMK for 9h. Western blots were probed with antibodies against LPL and HSP90 (as a loading control). (G) Western blots of inguinal WAT (iWAT), gonadal WAT (gWAT) and brown adipose tissue (BAT) of a C57BL/6 mouse. Western blots were probed with antibodies against ANGPTL4 and β-tubulin (as a loading control). Data are mean ± SEM. Asterisks indicate significant differences according to Student's t-test; ** $p<0.01$, * $p<0.05$

DISCUSSION

In this paper, we demonstrate that PCSK-mediated cleavage of LPL is a physiological process in adipocytes that may be promoted by the LPL inhibitor ANGPTL4. We show that treatment with the PCSK inhibitor dec-CMK prevents the appearance of amino-terminal LPL in various adipocyte models. At least part of the LPL cleavage occurs intracellularly and is mediated by PCSK3. We did not find evidence to suggest that regulation of *Pcsk3* expression explains the physiological regulation of LPL activity. However, loss of *Angptl4* resulted in a significant reduction in the amount of amino-terminal LPL, suggesting a role for ANGPTL4 in the PCSK3-mediated cleavage of LPL in adipocytes.

Given that LPL cleavage occurs intracellularly and PCSK3 localizes to the *trans*-Golgi and the endosomes, we focused on PCSK3 as a potential mediator of LPL cleavage. Other PCSKs, such as PCSK5 (PC5/6) and PCSK6 (PACE4), are mainly activated and present on the cell surface, anchored to HSPGs (31). However, PCSK3, PCSK5 and PCSK6 are widely expressed, recognize similar target sequences and, hence, share multiple target proteins (16). Indeed, *in vitro* studies have indicated that LPL may also be cleaved by PCSK5 and PCSK6 (20, 21). It is likely, though, that the spatial and temporal separation of PCSKs, as well as other cell-specific factors make that differences exist between *in vitro* and *in vivo* studies (16). Nonetheless, we did observe LPL cleavage products in the cell culture medium of adipocytes and we cannot formally exclude that PCSK5 and PCSK6 cleave LPL extracellularly or on the cell surface.

An intriguing observation is that PCSK-mediated cleavage of LPL is promoted by ANGPTL4. ANGPTL4 is a well-established inhibitor of LPL and changes in ANGPTL4 expression are responsible for the physiological regulation of LPL activity that is observed during exercise, fasting and cold exposure (7–9, 32). Interestingly, the liver-expressed family member ANGPTL3 has been demonstrated to enhance the cleavage of LPL, resulting in the inactivation of LPL and release of LPL from the endothelial cell surface (20). Based on these data, it was suggested that ANGPTL3 functions as a co-factor for PCSKs and thereby inhibits LPL activity (20). In contrast, it has been proposed that ANGPTL4 inhibits LPL by binding to LPL and converting the catalytically active, dimeric form of LPL to catalytically inactive monomers (13). To what extent the ANGPTL4-mediated conversion of the LPL dimer to monomers can be merged with our observations that ANGPTL4 promotes LPL cleavage by PCSKs remains to be determined. Hypothetically, ANGPTL4 and possibly ANGPTL3 bind to LPL and convert LPL to inactive monomers, thereby unmasking the PCSK cleavage site at residues 321-324 and rendering LPL more susceptible for cleavage and subsequent degradation (13, 21). Another interesting point is that both ANGPTL4 and ANGPTL3 are cleaved by PCSKs, liberating their amino-terminal domains (17–19, 33). Since the amino-terminal domain of ANGPTL4 inhibits LPL more potently than the full-length protein, the PCSKs might promote the loss of LPL activity by two complementary mechanisms (19, 30). To further establish the link between ANGPTL4 and PCSK-mediated cleavage of LPL, future studies should assess the impact of ANGPTL4 on LPL cleavage and LPL activity through extensive *in vitro* studies, as were performed for ANGPTL3 (20).

An interesting question concerns the rationale behind PCSK3-mediated cleavage of LPL. PCSK3 catalyzes the maturation of a variety of proproteins, including growth factors and pathogen recognizing proteins (31). In fact, PCSK3 is essential for proper embryonal development with *Pcsk3* deletion resulting in defective ventral closure and axial rotation (34). However, in contrast to the several proproteins, PCSK3-mediated cleavage of LPL likely does not serve to activate but rather to inactivate LPL, as was previously observed for endothelial lipase (20, 21). Indeed, cleaved LPL products that were found in human pre-heparin plasma had little LPL activity (35). Furthermore, mutations G409R or E410V in the human *LPL* gene that are characterized by enhanced cleavage of LPL *in vitro* cause a severe chylomicronaemia (36). Our data support this notion and indicate that amino-terminal LPL is efficiently cleared by the lysosomes. An alternative explanation is that some degree of LPL cleavage may be required for the proper secretion of LPL, as inhibition of LPL cleavage resulted in the reduced secretion of total LPL (full-length and cleaved LPL) in an *in vitro* model (21). Similarly, in the setting of the human G409R or E410V mutations, pharmacologic inhibition of cleavage increased the degradation of full-length LPL (36). Although these observations are counterintuitive, it may be speculated that cleaved amino-terminal LPL forms transient heterodimers with full-length LPL monomers, thereby stabilizing and preventing the inactivation of LPL monomers (13). Indeed, a rapid exchange of subunits exists between LPL dimers, with the intermediate monomer being metastable and more easily acted upon by factors that impair monomer re-association (37). Furthermore, the rapid association/dissociation of the LPL dimer might be an important aspect of the intracellular transport of LPL, as covalently linked LPL homodimers were found to be less efficiently secreted compared to unlinked homodimers (38). Evidently, more in-depth studies will need to reveal why some degree of LPL cleavage would be required for the effective secretion of LPL secretion.

Another explanation for the PCSK-mediated cleavage of LPL would be that the separate amino- and carboxyl-termini of LPL have specific physiological functions that complement or go beyond LPL's capacity to hydrolyze triglycerides from triglyceride-rich lipoproteins. For example, the carboxyl-terminal domain of LPL was previously found to mediate the margination of triglyceride-rich lipoproteins to the endothelium (39). However, it is questionable whether the carboxyl-terminal domain alone is capable of translocating onto the endothelium *via* GPIHBP1 (40). Likewise, the separate termini are unlikely to bind to HSPGs on the cell surface as both monomeric LPL and carboxyl-terminal LPL have a much lower affinity for HSPGs compared to full-length dimeric LPL (41, 42). As briefly mentioned above, however, cleaved LPL might hypothetically form a heterodimer with a full-length LPL monomer. It has been postulated repeatedly that LPL may function as a functional heterodimer in that the two LPL monomers serve distinct roles when dimerized, although this hypothesis remains to be confirmed experimentally (43, 44). As such, amino-terminal or carboxyl-terminal LPL might be transferred onto the endothelium to influence, for example, the margination of circulating triglyceride-rich lipoproteins. Alternatively, secreted amino-terminal or carboxyl-terminal domains may bind to circulating triglyceride-rich lipoproteins to enhance the hepatic uptake of remnant particles (45). Possibly, future studies on the specific physiological functions of amino-terminal of

carboxyl-terminal LPL could employ adipose tissue-specific *Pcsk3*$^{-/-}$ knockout mice to investigate the functional consequences of loss of LPL cleavage.

In conclusion, we demonstrate that LPL is cleaved in adipocytes by PCSK3, a process that is promoted by the LPL inhibitor ANGPTL4. Our data suggest a novel pathway by which ANGPTL4 regulates LPL in the adipose tissue. Given the importance of LPL for the regulation of plasma triglyceride levels and the causal role of plasma triglyceride levels in the development of cardiovascular disease, future studies need to address the functional consequences of PCSK-mediated cleavage of LPL and should aim to further examine the link between ANGPTL4 and the PCSK-mediated cleavage of LPL.

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7

ANGPTL4 STIMULATES BILE ACID UPTAKE WHICH IS ASSOCIATED WITH CHANGES IN GUT MICROBIAL COMPOSITION

Wieneke Dijk*
Aafke W.F. Janssen*
Jos Boekhorst
Albert K. Groen
Sabina Lukovac
Guido Hooiveld
Sander Kersten

* Authors contributed equally

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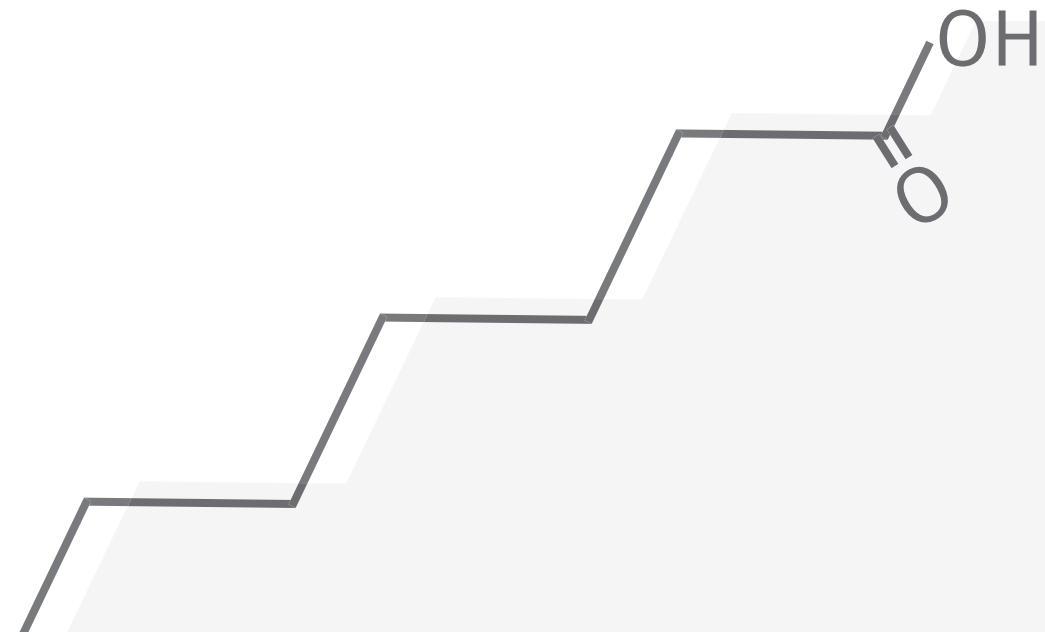

ABSTRACT

An intricate relationship exists between the gut microbiota, the host and host bile acid (BA) metabolism. Angiopoietin-like 4 (ANGPTL4) is abundantly expressed in the intestine, is regulated by BAs and bacterial metabolites in intestinal cell lines, and potently inhibits the activity of pancreatic lipase. Here, our objective was to examine the impact of intestinal ANGPTL4 on gut microbial composition and host BA metabolism. To that end, we fed wild-type and *Angptl4*$^{-/-}$ mice either a control diet or a diet enriched in taurocholic acid (TCA) for seven days. We found that total plasma BA concentrations were significantly lower while the faecal excretion of specifically primary BAs was significantly higher in the *Angptl4*$^{-/-}$ mice fed TCA as compared to the wild-type mice fed TCA, suggesting that the loss of ANGPTL4 reduces BA re-absorption. The lower absorption of BAs in the *Angptl4*$^{-/-}$ mice significantly impacted BA signaling *via* FXR, as demonstrated by the reduced expression of multiple FXR-target genes in the ileum of the *Angptl4*$^{-/-}$ mice fed TCA. Inasmuch as the gut microbiota convert primary bile acids into secondary bile acids, the higher excretion of specifically primary BAs in the faeces of *Angptl4*$^{-/-}$ mice indicated that loss of ANGPTL4 may impact gut microbial composition. Indeed, analysis of the colonic microbial composition by 16s rRNA gene sequencing revealed substantial differences between the *Angptl4*$^{-/-}$ mice and the wild-type mice on the control and the TCA-enriched diet. In conclusion, we demonstrate that loss of *Angptl4* significantly reduces plasma BA levels and increases BA excretion in TCA-fed mice, which is associated with differences in gut microbial composition.

INTRODUCTION

The gastro-intestinal tract harbours a large community of micro-organisms of which the composition is influenced by numerous factors, including diet, environment, stress, geography and age (1, 2). The gut microbial community has a mutualistic relationship with the host. For example, intestinal bacteria play an essential role in the energy metabolism and the immune system of the host (3). As a result, disturbances in gut microbiota have been associated with diseases such as obesity-associated metabolic disease, liver disease, inflammatory bowel disease and cancer (1, 2).

An intricate relationship exists between the gut microbiota, the host and host bile acid (BA) metabolism. While the gut microbiota deconjugate BAs and transform primary BAs into secondary BAs, alterations in BA composition in turn affect the gut microbial composition (4–7). By shifting BA composition, the gut microbiota may significantly impact host metabolism, as BAs affect the emulsification and absorption of dietary lipids and fat-soluble vitamins, act as signalling molecules and impact host cholesterol and triglyceride metabolism (7–12). For example, dietary BA supplementation significantly reduces plasma triglyceride levels (8, 9, 12, 13).

Angiopoietin-like 4 (ANGPTL4) is a ubiquitously expressed protein that potently inhibits the activity of lipoprotein lipase (LPL), the enzyme responsible for hydrolysis of fatty acids from circulating triglycerides (14). Accordingly, mice lacking *Angptl4* have reduced plasma triglyceride levels, whereas mice overexpressing *Angptl4* have elevated plasma triglyceride levels as compared with wild-type mice (15–18). In addition, physiological regulation of ANGPTL4 is responsible for the changes in LPL activity observed in white adipose tissue during fasting, in brown adipose tissue during cold, and in muscle during exercise (16, 19, 20). Given the prominent role of ANGPTL4 in the regulation of plasma triglyceride levels and our previous observation that BAs potently lower ANGPTL4 secretion from the human duodenal cell line Hutu-80, we hypothesized that the triglyceride-lowering effect of BAs might perhaps be mediated *via* regulation of ANGPTL4 (21).

Several studies have also linked the expression of intestinal ANGPTL4 to the gut microbiota and host metabolism. For example, specific bacterial strains as well as the bacterial fermentation end products short-chain fatty acids potently stimulate *Angptl4* expression in colonic cell lines (21–24). Conversely, conventionalization of germ-free mice significantly suppressed the expression of *Angptl4* in the intestine (25–27). More recently, intestinal ANGPTL4 was demonstrated to inhibit the activity of pancreatic lipase, a family member of LPL responsible for the hydrolysis of dietary triglycerides in the gastro-intestinal tract (28). The effects of various microbiota strains and bacterial metabolites on intestinal *Angptl4* expression, as well as the inhibitory effect of ANGPTL4 on pancreatic lipase activity suggest that intestinal ANGPTL4 might be part of the intricate cross-talk between gut microbiota and host. To test this hypothesis and to address whether the triglyceride-lowering effect of BAs might be mediated *via* ANGPTL4, we challenged *Angptl4*$^{-/-}$ and wild-type mice with a diet enriched in taurocholic acid (TCA) and assessed the differences in gut microbial composition and BA metabolism between *Angptl4*$^{-/-}$ and wild-type mice.

MATERIALS AND METHODS

Animals and diet

Animal studies were performed using pure-bred wild-type and *Angptl4*$^{-/-}$ mice on a C57BL/6 background. *Angptl4*$^{-/-}$ mice have been obtained *via* homologous recombination of embryonic stem cells and lack part of the *Angptl4* gene, resulting in a non-functional ANGPTL4 protein (17, 29). Mice were individually housed in temperature- and humidity-controlled conditions. 4 month-old male mice received either chow (control) or chow supplemented with 0.5% (wt/wt) taurocholic acid (TCA) (Calbiochem) for 7 days. The mice had *ad libitum* access to food and water. Body weight and food intake were assessed daily. At the end of the study, mice were anesthetized using isoflurane and blood was collected by orbital puncture. Mice were sacrificed *via* cervical dislocation after which tissues were excised and weighed. The small intestine was divided in three equal parts along the distal-to-proximal axis and epithelial cells were obtained by scraping the mucosal lining. All animal experiments were approved by the local animal ethics committee of Wageningen University.

Plasma parameters

Blood was collected in EDTA-coated tubes and centrifuged for 15 minutes at 3000 rpm to obtain plasma. Plasma free fatty acid and triglyceride concentrations were determined according to manufacturer's instructions using commercially available kits (Wako chemicals, Neuss, Germany and HUMAN Diagnostics, Wiesbaden, Germany). Plasma cholesterol was quantified using a kit from Elitech (Sees, France). Plasma concentrations of total bile acids were determined using a colorimetric assay kit (Diazyme Laboratories, Poway, USA). Free and conjugated bile acid subspecies were quantified by liquid chromatography tandem MS (LC-MS/MS) using a SHIMADZU liquid chromatography system (SHIMADZU, Kyoto, Japan) and tandem AB SCIEX API-3200 triple quadrupole mass spectrometry (AB SCIEX, Framingham, USA) as previously described (30).

RNA isolation and qPCR

Total RNA was extracted from scrapings of the distal small intestine and liver using TRIzol reagent (Life technologies, Bleiswijk, Netherlands). Subsequently, 500 ng RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Veenendaal, the Netherlands). Changes in gene expression were determined by real-time PCR on a CFX384 Real-Time PCR detection system (Bio-Rad) by using SensiMix (Bioline, GC biotech, Alphen aan den Rijn, the Netherlands). The housekeeping genes *36b4* and *β-actin* were used for normalization. Sequences of the used primers are listed in **Table 1**.

Faecal measurements

Prior to sacrifice, faeces were collected over a period of 48 hours. Total lipids and free fatty acids were measured in the faecal samples as described by Govers *et al* (31).

Briefly, 100 mg of faecal samples were weighed, dried and acidified using HCl. Fatty acids were then extracted using petroleum and diethyl ether. The ether fraction was collected, evaporated and total lipids were weighed. Lipids were re-solubilised in ethanol and, next, the free fatty acid concentration was determined using a commercially available kit (Wako chemicals, Neuss, Germany). Faecal bile salt composition and faecal neutral sterols were determined by capillary gas chromatography (Agilent 6890, Amstelveen, the Netherlands) as described previously (32, 33).

Bacterial DNA extraction and Library preparation for 16S rRNA pyrosequencing

DNA was extracted from the freeze-dried luminal content of the colon as described previously (34). Universal primers were used for initial amplification of part of the 16S rRNA gene: forward primer, '5-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG-3' (broadly conserved bacterial primer 357F) and reverse primer, '5-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTACNVGGGTATCTAAKCC' (broadly conserved bacterial primer 802R). To the purified PCR product, Illumina sequencing adapters were added and the 16S rRNA gene was subsequently sequenced on the Illumina MiSeq (PE300 cycles run) platform.

16s rRNA gene sequencing

Sequences were analyzed using a workflow based on QIIME 1.8 (35). OTU clustering, taxonomic assignment and reference alignment were done with the pick_open_reference_otus.py workflow script of QIIME, using uclust as clustering method (97% identity) and GreenGenes v13.8 as reference database for taxonomic assignment. Reference-based chimera removal was done with uchime. Redundancy analysis (RDA) was performed in Canoco version 5.

Statistical analysis

Data are presented as mean ± SEM. Statistical significant differences were determined with two-way analysis of variance with Bonferroni post hoc tests or by Student's t-test where appropriate using GraphPad Prism (GraphPad Software, Inc., La Jolla, USA). $P<0.05$ was considered as statistically significant. For microbiota analysis, false discovery rate (FDR)-corrected P-values were estimated for each taxon to correct for multiple testing. Bacterial taxa with a FDR-corrected P-value < 0.20 were considered significant.

RESULTS

ANGPTL4 is not implicated in the triglyceride-lowering effects of BAs

To investigate the role of ANGPTL4 in the lowering of plasma triglycerides by BAs, we fed wild-type and *Angptl4*$^{-/-}$ mice either a control diet or a diet enriched in taurocholic acid (TCA) for seven days (**Figure 1A**). The TCA-enriched diet slightly reduced food intake and body weight. However, no differences were observed between the genotypes (**Supp. Figure 1**). In contrast to our hypothesis that BAs may down-regulate *Angptl4* expression, mRNA levels of *Angptl4* were either unchanged (liver; **Figure 1B**) or increased (ileum; **Figure 1C**) by TCA feeding. Furthermore, although the TCA treatment effectively lowered plasma TG levels, the reduction was similar in the wild-type and *Angptl4*$^{-/-}$ mice (**Figure 1D**). Other plasma parameters, including plasma free fatty acids (**Figure 1E**) and plasma cholesterol (**Figure 1F**) were also not different between the genotypes. These data indicate that ANGPTL4 is not responsible for the plasma triglyceride-lowering effect of BAs.

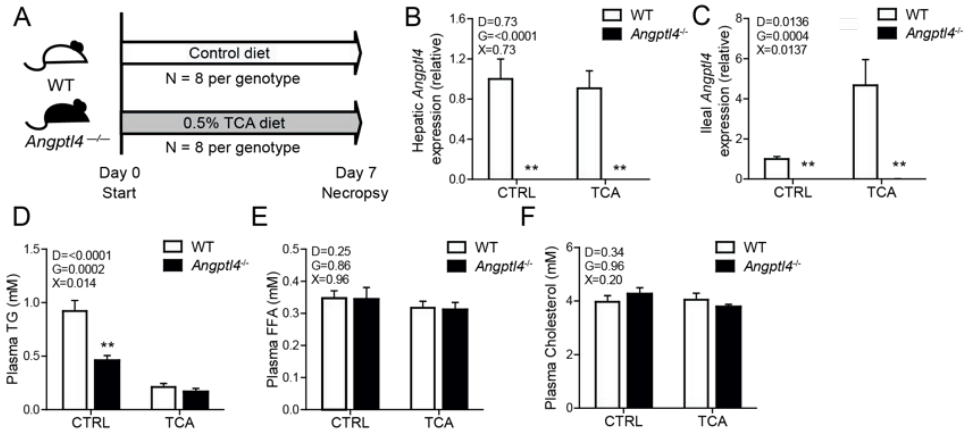

Figure 1: ANGPTL4 is not part of the triglyceride-lowering effects of BAs.

(A) Schematic representation of dietary intervention with 0.5% w/w taurocholic acid (TCA) in wild-type and *Angptl4*$^{-/-}$ mice. Relative gene expression of (B) hepatic *Angptl4*, (C) ileal *Angptl4* in the liver and ileum of wild-type and *Angptl4*$^{-/-}$ mice fed (control) CTRL or TCA diet for 7 days. Gene expression levels in wild-type mice fed the control diet were set at 1. (D) Plasma triglycerides, (E) plasma free fatty acids (FFA), and (F) plasma cholesterol concentration of wild-type and *Angptl4*$^{-/-}$ mice fed a control (CTRL) or TCA diet for 7 days. Data are presented as mean ± SEM. Statistically significant differences were assessed using two-way ANOVA for diet (D), genotype (G) or interaction between both parameters (X), as indicated at the top of each figure. Asterisks represent significant differences compared with WT mice. *p<0.05, **p<0.001.

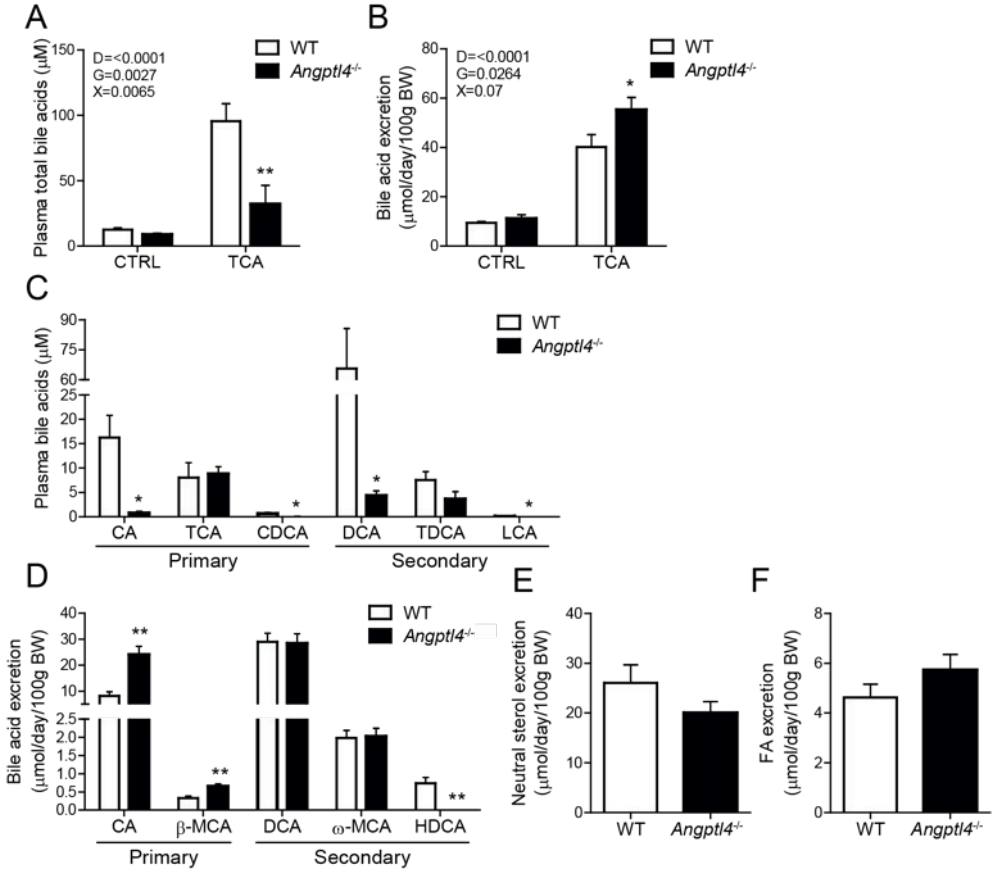

Figure 2: ANGPTL4 promotes an increase in BA re-absorption upon TCA feeding.

(A) Total plasma bile acid concentration of WT and *Angptl4*$^{-/-}$ mice fed control (CTRL) or TCA diet for 7 days. Statistically significant differences were observed using two-way ANOVA for diet (D), genotype (G) or interaction between both parameters (X), as indicated at the top of the figure. (B) Total bile acid excretion of wild-type and *Angptl4*$^{-/-}$ mice fed control (CTRL) or TCA diet for 7 days. Statistically significant differences were observed using two-way ANOVA for diet (D), genotype (G) or interaction between both parameters (X), as indicated at the top of the figure. (C) Concentration of free and conjugated bile acid subspecies in the plasma of wild-type and *Angptl4*$^{-/-}$ mice fed a TCA diet for 7 days. (D-F) Mean excretion of (D) free and conjugated bile acid subspecies, (E) neutral sterols and (F) fatty acid excretion of WT and *Angptl4*$^{-/-}$ mice fed TCA diet for 7 days. Data are presented as mean ± SEM. Asterisks represent significant differences compared with WT mice. *p<0.05, **p<0.001.

ANGPTL4 promotes an increase in BA re-absorption upon TCA feeding

As expected, the TCA-enriched diet significantly increased total BA levels in plasma and total BA excretion in the faeces (**Figure 2A & 2B**). Interestingly, total plasma BA concentrations were significantly lower while the faecal BA excretion was significantly higher in the *Angptl4*$^{-/-}$ mice fed TCA as compared to the wild-type mice fed TCA (**Figure 2A & 2B**). These observations suggest that the loss of ANGPTL4 reduces the absorption of BAs. To study this potential effect of *Angptl4* deletion on BA absorption in more detail, we measured the levels of individual BAs in plasma and faeces. The analysis revealed that the lower levels of total plasma BAs in the *Angptl4*$^{-/-}$ mice as compared to the wild-type mice were primarily accounted for by lower plasma levels of cholic acid and deoxycholic acid, and to a lesser extent chenodeoxycholic acid and lithocholic acids (**Figure 2C**). In the faeces, the excretion of the primary BAs cholic acid and β-muricholic acid was significantly higher in the *Angptl4*$^{-/-}$ mice as compared to wild-type mice fed the TCA-containing diet, whereas faecal excretion of the secondary BAs deoxycholic acid and α-muricholic acid, as well as excretion of neutral sterols and fatty acids was not different between the genotypes (**Figure 2D – 2F**). Overall, these data indicate that the loss of *Angptl4* in mice fed TCA reduces BA absorption, leading to lower levels of BAs in the plasma and higher excretion of BAs in the faeces.

The lower absorption of BAs in the *Angptl4*$^{-/-}$ mice is expected to significantly impact BA signaling *via* FXR. Consistent with this notion, the expression in the ileum of several target genes of FXR, including *Fgf15*, *Slc51a* (*Ost*α) and *Slc51b* (*Ost*β), as well as expression of *Fxr* itself, was significantly lower in the *Angptl4*$^{-/-}$ mice than the wild-

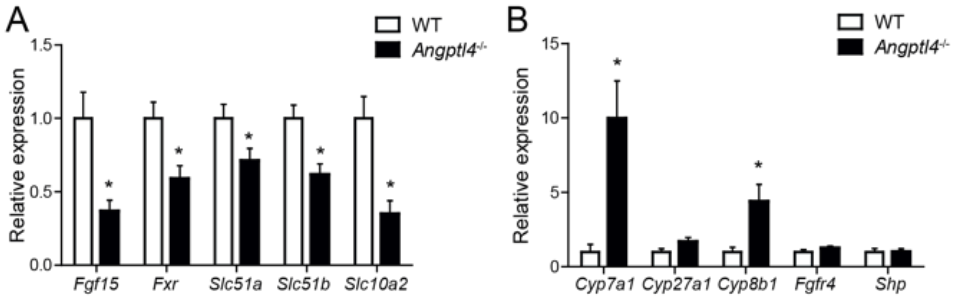

Figure 3: Differences in BA re-absorption between wild-type and *Angptl4*$^{-/-}$ mice translate into differences in ileal and hepatic gene expression.

(A) Relative expression of ileal BA transporters and BA-responsive genes, *Fgf15*, *Fxr*, *Slc51a*, *Slc51b* and *Slc10a2*, in wild-type and *Angptl4*$^{-/-}$ mice fed TCA diet for 7 days. Gene expression levels in WT mice fed TCA diet were set at 1. (B) Relative hepatic expression of genes involved in BA synthesis in wild-type and *Angptl4*$^{-/-}$ mice fed TCA diet for 7 days. Gene expression levels in WT mice fed TCA diet were set at 1. Data are presented as mean ± SEM. Asterisks represent significant differences compared with WT mice. *p<0.05, **p≤0.001.

type mice (**Figure 3A**, **Supp. Figure 2**). The lower expression of *Fgf15* in the ileum of *Angptl4*$^{-/-}$ mice was associated with markedly higher hepatic expression of genes involved in bile acid synthesis, including *Cyp7a1*, *Cyp27a1* and *Cyp8b1*, whereas hepatic expression of other genes involved in BA metabolism was not or only slightly different between the *Angptl4*$^{-/-}$ and wild-type mice (**Figure 3B**, **Supp. Figure 2**). Interestingly, expression of the BA transporter *Slc10a2* (*Asbt*) in the ileum was markedly lower in the *Angptl4*$^{-/-}$ than in the wild-type mice, potentially contributing to the lower BA absorption (**Figure 3A**).

ANGPTL4 affects changes in gut microbial composition upon bile acid feeding

As pointed out above, the excretion of the primary BAs cholic acid and β-muricholic acid was significantly higher in *Angptl4*$^{-/-}$ mice, whereas the excretion of secondary BAs and neutral sterols was not different between the genotypes. Inasmuch as the conversion of primary BAs into secondary BAs is mediated by the gut microbiota, the higher excretion of specifically primary BAs in the faeces of *Angptl4*$^{-/-}$ mice indicates that loss of ANGPTL4 may impact gut microbial composition. Analysis of the colonic microbial composition by 16s rRNA gene sequencing revealed substantial differences between the *Angptl4*$^{-/-}$ mice and the wild-type mice on the control diet and on the

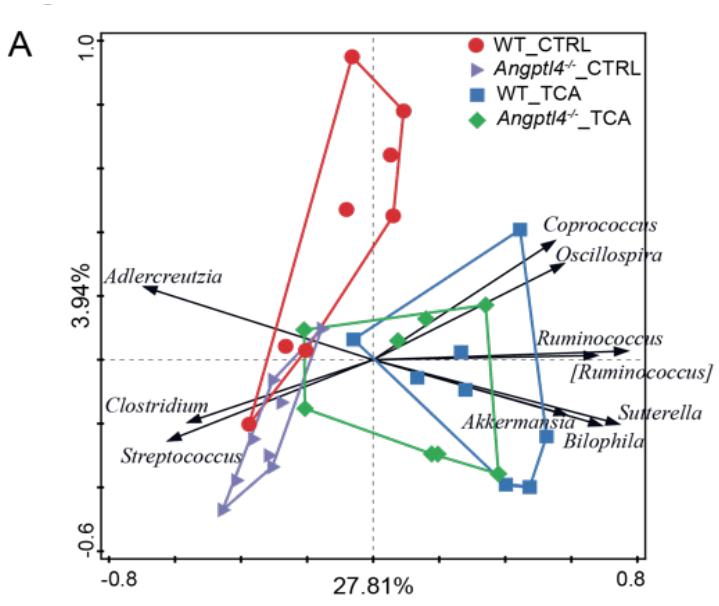

Figure 4: TCA feeding and *Angptl4* genotype affect gut microbial composition.

(A) Redundancy analysis (RDA) correlation biplot describing the relationship between colonic luminal microbiota composition and the differences induced by genotype and diet. The explanatory variables diet and genotype explained 34.9% of the total variation in the microbiota composition.

TCA-enriched diet. Redundancy analysis (RDA) indicated that the explanatory variables diet and genotype explained 34.9% of the total variation in the colonic microbiota composition (**Figure 4A**). To determine which variable was the most important determinant of colonic microbial composition, a partial RDA was performed. While genotype explained 10.9% of the total variation in microbial composition, diet was more important and explained 25.1% of the microbial variation (**Figure 5A & 5B**).

Although *Angptl4* genotype also affected gut microbial composition on the control diet (**Supp. Figure 3**), differences in BA metabolism between *Angptl4*$^{-/-}$ and wild-type mice were only observed upon TCA feeding, suggesting that microbial changes occurring upon a TCA diet might be important for the observed differences. To that end, the composition of the colonic microbiota of wild-type and *Angptl4*$^{-/-}$ mice fed the TCA diet was compared in more detail. At the phylum level, the relative abundance of Actinobacteria was higher in the *Angptl4*$^{-/-}$ mice fed TCA, whereas the relative abundance of Bacteroidetes and Cyanobacteria was lower as compared with the wild-type mice fed TCA (**Figure 6**). An increase within the genus Adlercreutzia largely explained the increased abundance of the phylum Actinobacteria, whereas a decrease in the genus Prevotella mainly accounted for the decrease within the phylum Bacteroidetes (**Figure 6**). Interestingly, while the relative abundance of the phylum Proteobacteria was comparable between the genotypes, the family of Enterobacteriaceae was, with a relative proportion of 3.6%, 158-fold more abundant in the colon of the *Angptl4*$^{-/-}$ mice as compared with wild-type mice fed the TCA diet (**Figure 6**). Conversely, the abundance of Bilophila was significantly higher in the colon of wild-type mice as compared with the *Angptl4*$^{-/-}$ mice fed the TCA diet. Finally, the species Lactobacillus reuteri was present in the colon of wild-type mice, whereas it was almost absent in the *Angptl4*$^{-/-}$ mice fed (**Figure 6**).

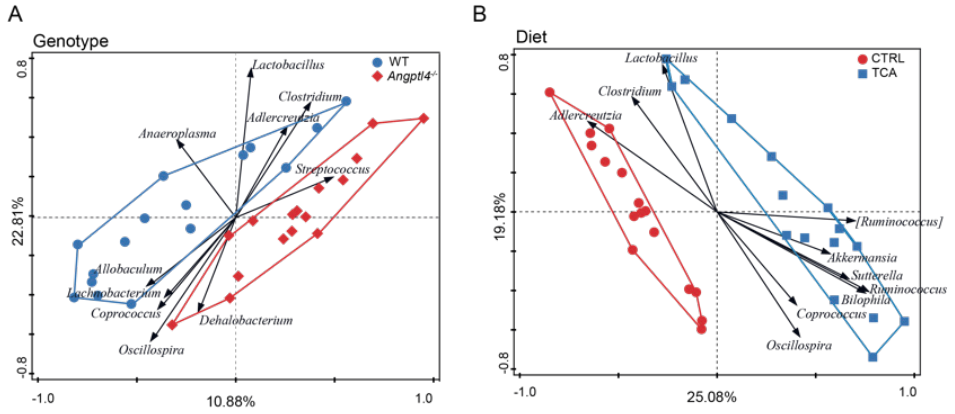

Figure 5: Bile acid feeding has more impact on gut microbial composition than *Angptl4* genotype

(A-B) Partial RDA correlation biplot describing the relationship between colonic luminal microbiota composition and explanatory variables (A) genotype and (B) diet. Genotype explained 10.9% and diet 25.1% of total variation in microbiota composition.

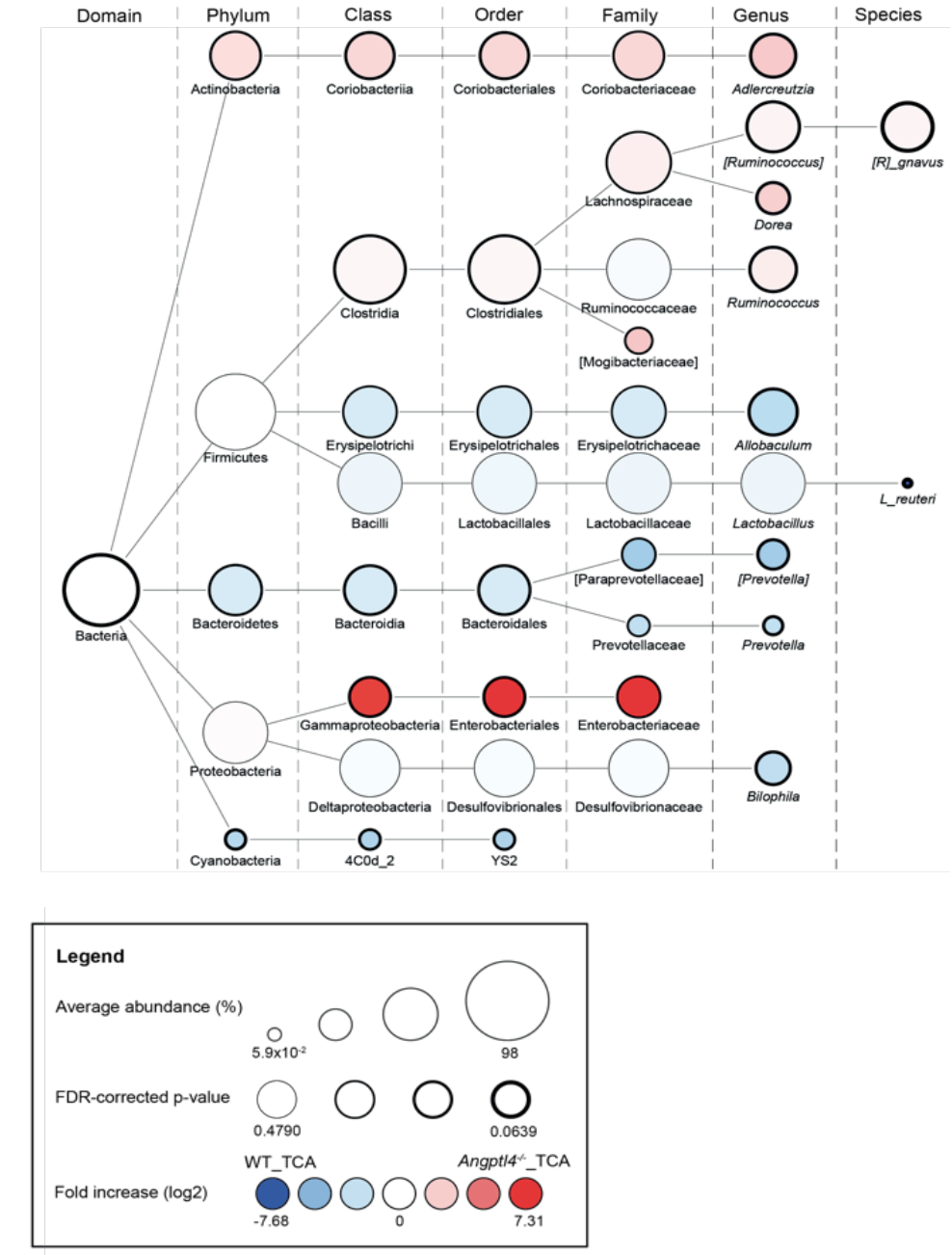

Figure 6: Differences in microbial composition between wild-type and *Angptl4*$^{-/-}$ mice upon TCA feeding.

Nodes represent taxa and the edges link the different taxonomic levels. The fold increase is calculated as the 2log of the ratio of the relative abundance in *Angptl4*$^{-/-}$ and wild-type mice upon TCA feeding. Significance is expressed as the FDR-corrected p-valu

DISCUSSION

In the present paper, we examined the impact of ANGPTL4 on gut microbial composition and host BA metabolism. We demonstrate that TCA feeding dramatically increased plasma BA levels in the wild-type but not the *Angptl4*$^{-/-}$ mice, which may be explained by lower ileal BA re-absorption in *Angptl4*$^{-/-}$ mice (see **Figure 7** for an overview). The higher plasma BA levels found in the wild-type mice are likely responsible for the reduction in genes involved in BA synthesis *via* the well-known feedback mechanism mediated by FXR. Although causality remains to be proven, we believe that the differences in gut microbial composition between the *Angptl4*$^{-/-}$ mice and the wild-type mice fed TCA provide a reasonable explanation for the lower ileal BA re-absorption.

We initially hypothesized that the well-established triglyceride-lowering effect of BAs might be mediated by ANGPTL4 (14, 21). Although TCA feeding effectively lowered plasma triglyceride levels, the reduction in plasma triglyceride levels was similar between the wild-type and the *Angptl4*$^{-/-}$ mice, indicating that ANGPTL4 does not mediate the triglyceride-lowering effect of BAs. Instead, it is probable that another circulating factor that impacts LPL activity, such as ANGPTL3 or APOCII, mediates the triglyceride-lowering effect of BAs (12, 36). Our data indicate that it is unlikely that circulating BAs directly impact LPL activity in peripheral tissues, as the pronounced differences in plasma BA levels between wild-type and *Angptl4*$^{-/-}$ mice fed TCA did not translate into differences in plasma triglyceride levels.

A potential explanation for the higher plasma BA levels as observed in the wild-type mice fed TCA may lie in the increased expression of *Slc10a2*, the main active transporter for BA re-absorption, in the ileum of wild-type mice fed TCA compared to the *Angptl4*$^{-/-}$ mice fed TCA (37). Expression of *Slc10a2* in the ileum is tightly controlled by FXR *via* a negative feedback mechanism that involves activation of the small heterodimer partner (SHP) and subsequent repression of liver related homolog-1 (LRH-1) (37–39). Various BAs activate intestinal FXR, but their potency to activate FXR varies greatly (38, 40). Indeed, a recent study demonstrated that the expression levels of *Fxr* and FXR-target genes such as *Fgf15* and *Slc10a2* were differentially regulated in ileum and liver upon feeding of different BA species (41). Furthermore, more hydrophilic BAs such as tauro-β-muricholic acid may in fact act as antagonists of FXR (42). Since *Angptl4*$^{-/-}$ mice had a significantly higher excretion of specifically primary BAs, our studies suggest that the differences in intestinal BA composition between wild-type and *Angptl4*$^{-/-}$ mice might explain the differences in ileal *Slc10a2* expression and BA re-absorption.

Gut microbial composition potently influences intestinal BA composition and BA metabolism. Indeed, the gut microbiota convert primary BAs to secondary BAs by 7α-dehydroxylation, thereby increasing the hydrophobicity of BAs, and deconjugate BAs, thereby preventing the active re-absorption of BAs by SLC10A2 (4, 7, 43). As a result, alterations in gut microbial composition, for example by treatment with antibiotics, probiotics or a high-fat diet, significantly impact the abundance of secondary BAs and BA homeostasis (6, 41, 42, 44, 45). The gut microbiota may also directly impact BA metabolism, as recent studies demonstrate that bacterial metabolites modulate FXR activity and that conventionalization of germ-free mice increases

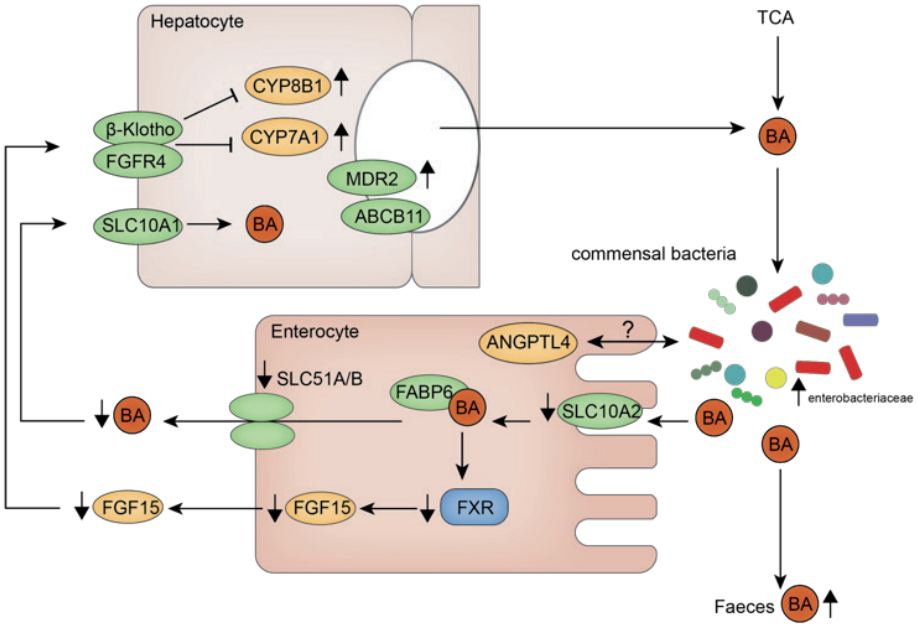

Figure 7: Schematic overview of differences in gene expression between wild-type and *Angptl4*$^{-/-}$ mice in liver and intestine.

Compared to wild-type mice, TCA feeding in *Angptl4*$^{-/-}$ mice significantly increased faecal primary BA levels and changed gut microbial composition. A reduction in the ileal expression of *Slc10a2* may have resulted in the reduced reuptake of BAs into the ileum and a decrease in plasma BA levels in the *Angptl4*$^{-/-}$ mice fed the TCA-enriched diet as compared to wild-type mice. The lower plasma BA levels, as well as the lower expression of *Fgf15* as observed in the *Angptl4*$^{-/-}$ mice fed the TCA-enriched diet might have stimulated the expression of *Cyp7a1* and *Cyp8b1* and might have increased hepatic BA synthesis in the *Angptl4*$^{-/-}$ mice as compared to the wild-type mice.

the expression of FXR-target genes (42, 46). More specifically, suppression of the gut microbiota by antibiotics treatment reduced activation of GATA4, a transcription factor that negatively regulates the expression of the *Slc10a2* transporter, resulting in the increased expression of *Slc10a2* and increased BA re-absorption, while conventionalization of germ-free mice lowered *Slc10a2* expression (47, 48). Given the intricate relationship between the gut microbiota and BA metabolism, it may be hypothesized that the microbial changes that occur specifically upon a TCA diet significantly contribute to the differences in BA metabolism observed between the *Angptl4*$^{-/-}$ fed TCA and the wild-type mice fed TCA. For example, we detected a higher abundance of the Enterobacteriaceae, a family that includes several pathogenic strains such as *Salmonella* and *E.Coli*, and a lower abundance of bile-loving, sulphite-reducing *Bilophila* in the colon of *Angptl4*$^{-/-}$ mice fed TCA compared with the wild-type mice fed TCA (49). In human subjects, Enterobacteriaceae have been associated with the presence of primary BAs in the faeces and have been linked to pathologies such as hepatic encephalopathy and Crohn's disease (50–52). It remains to be investigated, however, if and what microbial changes that occur upon TCA feeding are responsible for the

differences in intestinal BA composition and BA re-absorption between the wild-type and the *Angptl4*$^{-/-}$ mice fed TCA.

The origin of the differences in gut microbial composition between *Angptl4*$^{-/-}$ mice and wild-type mice is currently unclear, which is partially due to the uncertainty about the function(s) of intestinal ANGPTL4. Levels of intestinal ANGPTL4 are comparatively high and numerous studies have demonstrated that BAs, bacteria and bacterial metabolites potently influence intestinal ANGPTL4 expression (21–24, 28). In contrast, what intestinal cells produce and secrete ANGPTL4 and whether intestinal ANGPTL4 is secreted into the blood stream and/or into the intestinal tract is unknown (21, 28). For example, conventionalization of germ-free mice was associated in some, yet not all studies with enhanced peripheral adiposity in wild-type mice but not *Angptl4*$^{-/-}$ mice *via* the purported loss of endocrine inhibition on adipose tissue LPL activity (25, 27, 53). In contrast, in line with observations that suggest a local instead of endocrine function for ANGPTL4, a recent study demonstrated that intestinal ANGPTL4 has the capacity to inhibit pancreatic lipase, the main enzyme responsible for the hydrolysis of dietary triglycerides in the gastro-intestinal tract (14, 28, 54). In agreement with this study, we observed a tendency towards increased fat excretion in *Angptl4*$^{-/-}$ mice. Multiple studies indicate that elevated levels of lipids in the ileum and colon significantly impact the gut microbial composition, indicating that the differences in dietary fat metabolism might possibly explain the differences in gut microbial composition between wild-type and *Angptl4*$^{-/-}$ mice (55, 56). In fact, the origin of the differences in gut microbial composition between wild-type and *Angptl4*$^{-/-}$ mice might lie during suckling, when pups receive a high amount of lipid-containing milk. Future studies employing an intestine-specific knock-out of ANGPTL4 should confirm that inhibition of pancreatic lipase is the primary function of intestinal ANGPTL4 and should clarify what cells are responsible for secretion of ANGPTL4 into the intestinal tract.

In conclusion, we demonstrate that loss of *Angptl4* significantly reduces plasma BA levels and increases BA excretion in TCA-fed mice. Although causality remains to be proven, these differences are associated with significant differences in gut microbial composition between the wild-type mice and the *Angptl4*$^{-/-}$ mice. Our observations further underline the importance of the gut microbiota for BA homeostasis.

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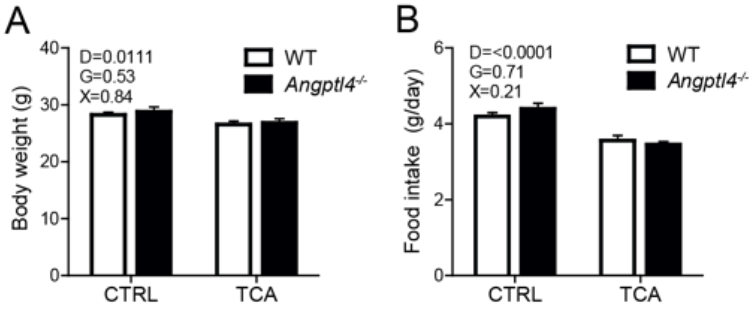
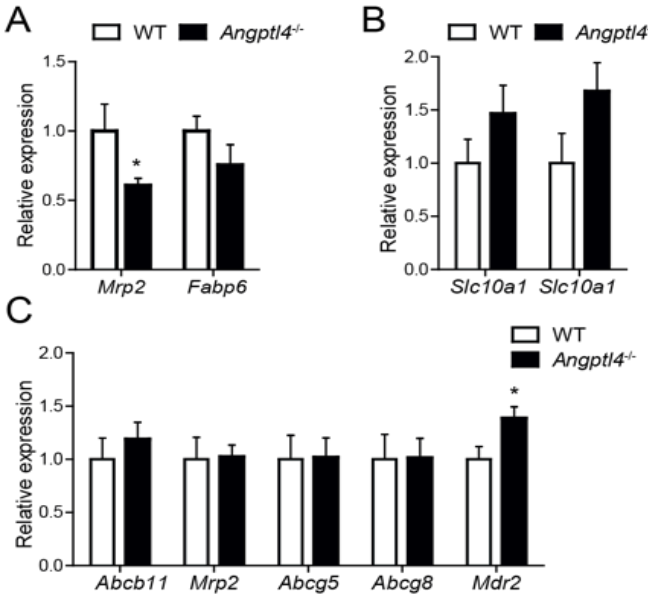
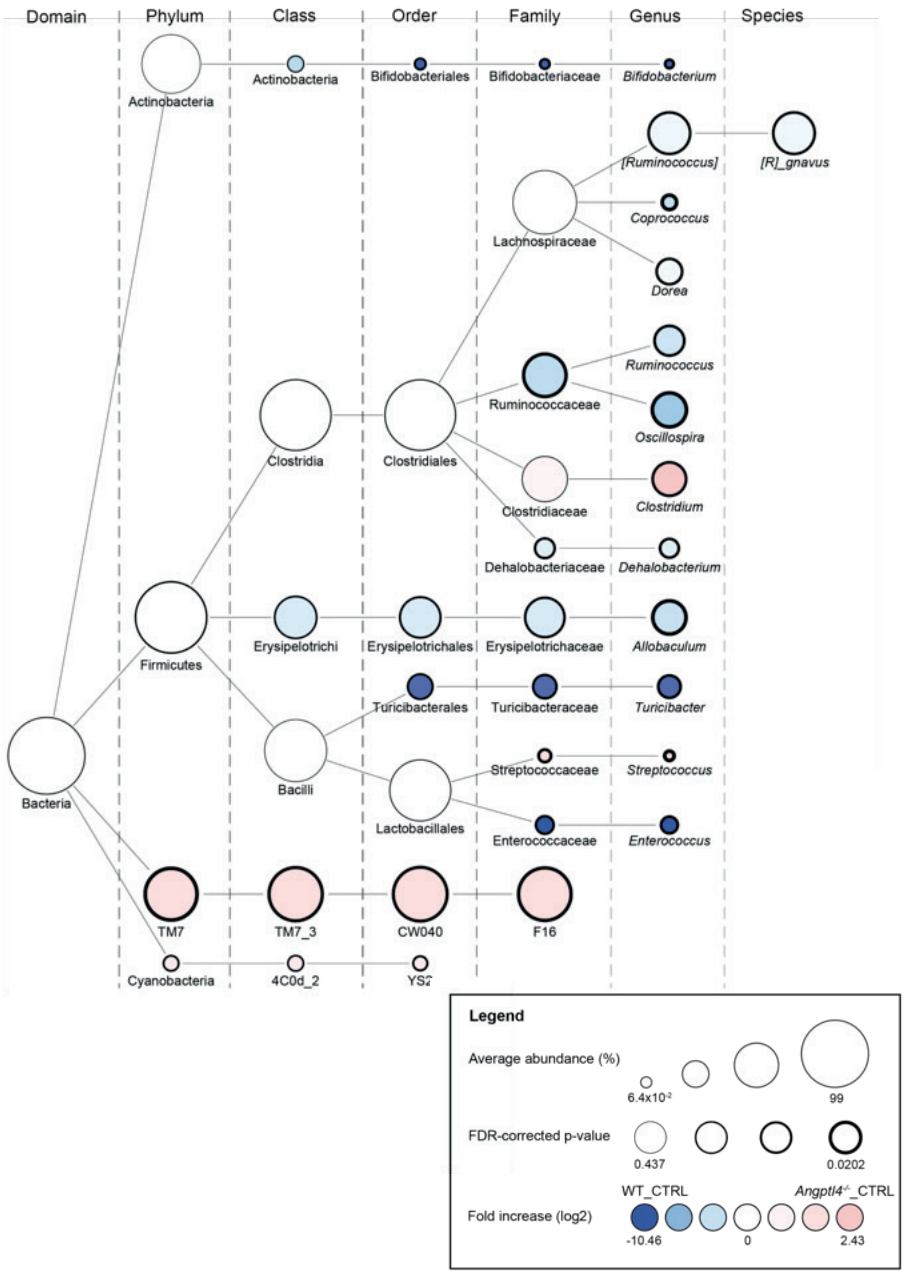
SUPPLEMENTAL FIGURES



Supplemental Figure 1: TCA feeding affect body weight and food intake.
(A) Final body weight of wild-type and *Angptl4*$^{-/-}$ mice fed control (CTRL) or TCA diet for 7 days. (B) Mean food intake per day during dietary intervention. Data are presented as mean ± SEM.



Supplemental Figure 2: Ileal and hepatic gene expression in wild-type and *Angptl4*$^{-/-}$ mice fed the TCA diet.
(A-C) Relative gene expression of (A) ileal BA transporters, (B) portal BA transporters, (C) genes involved in canalicular bile acid, cholesterol and phosphatidylcholine export in the ileum (A) or liver (B &C) of wild-type and *Angptl4*$^{-/-}$ mice fed TCA diet for 7 days. Gene expression levels in WT mice fed TCA diet were set at 1. Data are presented as mean ± SEM. Asterisks represent significant differences compared with WT mice. *p<0.05, **p≤0.001.



Supplemental Figure 3: Differences in microbial composition between wild-type and *Angptl4*$^{-/-}$ mice fed the control diet.

Nodes represent taxa and the edges link the different taxonomic levels. The fold increase is calculated as the 2log of the ratio of the relative abundance in *Angptl4*$^{-/-}$ and wild-type mice fed the control (CTRL) diet. Significance is expressed as the FDR-corrected p-value

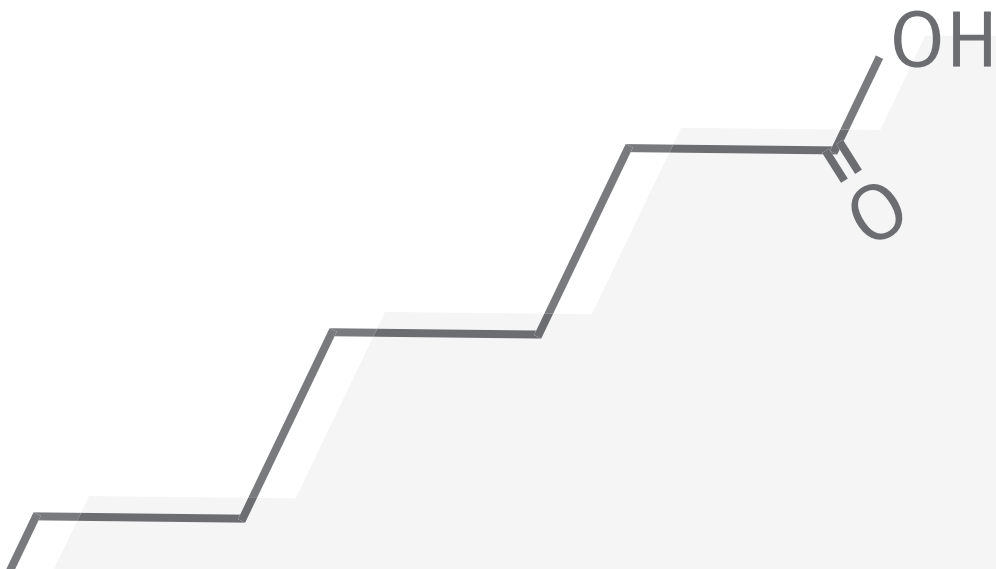
8

ROLE AND REGULATION OF HILPDA IN ADIPOSE TISSUE

Wieneke Dijk*
Frits Mattijssen*
Montserrat de la Rosa Rodriguez
Angel Loza Valdes
Anne Loft
Susanne Mandrup
Eric Kalkhoven
Ling Qi
Jan Willem Borst
Sander Kersten

* Authors contributed equally

Submitted for publication



ABSTRACT

Triglycerides are stored in specialized organelles called lipid droplets. Numerous proteins have been shown to be physically associated with lipid droplets and to govern their function. Previously, hypoxia-inducible lipid droplet-associated (HILPDA) was localized to lipid droplets in HeLa cells and hepatocytes. Studies in hepatocytes suggested that HILPDA may inhibit triglyceride lipolysis. Here, our aim was to explore the regulation and possible function of HILPDA in adipose tissue. We found that HILPDA is abundant in adipose tissue and is associated with lipid droplets. Expression of HILPDA in adipocytes was controlled by the peroxisome proliferator-activated receptor γ and by β-adrenergic stimulation. Expression of HILPDA markedly increased during 3T3-L1 adipocyte differentiation. Nevertheless, knockdown of HILPDA using siRNA or overexpression of HILPDA using adenovirus did not influence 3T3-L1 adipogenesis. Knockdown of HILPDA in adipocytes did not significantly alter the release of NEFAs following β-adrenergic stimulation. By contrast, adenoviral-mediated overexpression of HILPDA modestly attenuated the release of NEFAs from adipocytes following β-adrenergic stimulation. In mice, however, adipocyte-specific deletion of HILPDA had no effect on plasma levels of NEFAs and glycerol after fasting, cold exposure or pharmacological β-adrenergic stimulation. In addition, other relevant metabolic parameters were unchanged by adipocyte-specific deletion of HILPDA. Taken together, we find that HILPDA is highly abundant in adipose tissue, where its expression is regulated by PPARγ and β-adrenergic stimulation. In contrast to the purported inhibition of lipolysis by HILPDA in hepatocytes, our data do not support a major role of HILPDA in the regulation of lipolysis in adipose tissue.

INTRODUCTION

In humans and many other animal species, excess energy is efficiently stored in white adipose tissue as triglycerides, providing a buffer against periods of low energy intake. Triglycerides are stored in the adipose tissue in lipid droplets, which are dynamic intracellular organelles that are regulated by the organism's nutritional status. Lipid droplets consist of a core of triglycerides and cholesteryl esters surrounded by a monolayer of phospholipids and proteins (1). Many lipid droplet-associated proteins are involved in the release of non-esterified fatty acids (NEFAs) from lipid droplets or the incorporation of triglycerides into lipid droplets (1–4). Other lipid droplet-associated proteins include regulatory proteins such as members of the perilipin (PLIN) family, and proteins that affect lipid droplet size such as members of the cell death–inducing DFF45-like effector (CIDE) protein family (5, 6). For example, CIDEC was suggested to promote the enlargement of lipid droplets by facilitating lipid transfer from small to larger lipid droplets and/or by acting as a barrier for adipose triglyceride lipase, the rate-limiting enzyme of lipolysis (5, 7–9). A better understanding of the function of lipid droplet-associated proteins is important to gain more insight into the regulation of lipid storage and mobilization.

Peroxisome Proliferator Activated Receptors (PPARs) represent an important subgroup within the nuclear receptor superfamily, consisting of PPARα, PPARδ and PPARγ. An intricate relationship exists between lipid storage and PPARs. On the one hand, changes in intra- and extracellular lipid metabolism impact the activation of PPARs by influencing the availability of ligands in the form of fatty acids and fatty acid derivatives (10–13). On the other hand, PPARs influence lipid metabolism by activating the transcription of numerous target genes, including many genes encoding lipid droplet-associated proteins such as adipose triglyceride lipase (ATGL/PNPLA2), G0/G1 switch gene 2 (G0S2), PLIN2 and CIDEC (11, 14–16). Functional studies of (novel) PPAR-target genes may help to further uncover the intricate regulation of lipid storage and mobilization in adipose tissue.

Recently, hypoxia-inducible lipid droplet-associated (HILPDA) was identified as a novel lipid droplet-associated protein in HeLa cells and hepatocytes (17, 18). We and others have identified *Hilpda* as a novel target gene of PPARα in liver involved in hepatic triglyceride metabolism (17, 19). HILPDA overexpression resulted in a fatty liver due to a reduction in very-low density lipoprotein (VLDL) triglyceride secretion, whereas *Hilpda* deletion lowered hepatic lipid accumulation (17, 19). More specifically, loss of HILPDA was found to lead to enhanced triglyceride lipolysis and triglyceride turnover in isolated primary hepatocytes (17). Considering the reported localization of HILPDA to lipid droplets and the importance of lipolysis in adipocytes, we examined the role and regulation of HILPDA in adipose tissue (18, 19). In particular, we investigated the potential role for HILPDA in adipocyte lipolysis using *in vitro* and *in vivo* tools of HILPDA overexpression and inactivation. We find that *HILPDA* is a sensitive PPARγ target gene in adipocytes and is highly responsive to β-adrenergic stimulation. In contrast to the purported inhibition of lipolysis by HILPDA in hepatocytes, our data do not support a major role of HILPDA in the regulation of lipolysis in adipose tissue.

MATERIALS AND METHODS

All animal experiments were performed in accordance with Directive 2010/63/EU from the European Union. All animal studies were reviewed and approved by the Animal Ethics Committee of Wageningen University.

Animal experiments

Mice were housed in temperature- and humidity-controlled specific pathogen-free conditions. Mice had *ad libitum* access to food (chow) and water, except where specified otherwise. To generate mice with adipocyte-specific deletion of *Hilpda*, heterozygous floxed *Hilpda* animals on a mixed C57BL/6 and Sv129 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). LoxP sites were introduced to flank the second exon of *Hilpda* followed by the open reading frame for membrane-tethered human placental alkaline phosphatase (*ALPP*) after the second loxP site. Following Cre-recombination, *ALPP* is expressed under control of the *Hilpda* promoter. Floxed *Hilpda* mice were crossed with Adiponectin-Cre mice (The Jackson Laboratory) on a C57BL/6 background. After initial crosses, *Hilpda*$^{flox/flox}$ mice were crossed with *Hilpda*$^{flox/flox}$ mice heterozygous for Adiponectin-Cre, yielding 50% wild-type and 50% adipocyte-specific *Hilpda* null animals (*Hilpda*$^{\Delta AT}$), equally distributed among males and females. For experiment 1, in which the adipocyte-specific deletion of HILPDA was verified by separation of the adipose tissue into adipocytes and stromal vascular fractions, four 20-week old female *Hilpda*$^{flox/flox}$ and four 20-week old female *Hilpda*$^{\Delta AT}$ mice were sacrificed by cervical dislocation and epididymal adipose tissue depots were excised and placed in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) supplemented with 1% fatty acid-free bovine serum albumin (BSA) until further isolation as described below. For the CL316,243 experiment (2), 15- to 18-week old male *Hilpda*$^{flox/flox}$ (n=16) and *Hilpda*$^{\Delta AT}$ (n=16) littermates were fasted for 3h and subcutaneously injected with 1 mg/kg CL316,243 (n=8 per genotype) or saline solution (n=8 per genotype). During the experiment, all mice were housed individually. At 10 minutes, 30 minutes, and 60 minutes after injection, blood was drawn *via* the tail vein. 3h after injection, the animals were anesthesized with isoflurane, blood was collected and following cervical dislocation several tissues were excised and snap-frozen or placed in DMEM + 1% BSA for separation into adipocytes and stromal vascular fractions. The animals remained fasted until sacrifice. For the fasting experiment (3), 9- to 13-week old male *Hilpda*$^{flox/flox}$ (n=6) and *Hilpda*$^{\Delta AT}$ (n=8) littermates were fasted for 24 hours and killed by cervical dislocation, after which several tissues were excised and snap-frozen. During the fasting period, all mice were housed individually. For the *in vitro* lipolysis experiment with explants (4), adipose tissue of male *Hilpda*$^{flox/flox}$ (n=3) and *Hilpda*$^{\Delta AT}$ (n=3) mice was excised and minced with scissors. Adipose tissue explants were plated, serum-starved for 30 minutes in DMEM (Lonza, Verviers, Belgium) with 1% fatty acid-free BSA, and subsequently treated with 10 µM isoproterenol for 3h. Medium samples were assayed for NEFAs as described below and corrected for total protein. For the *in vitro* lipolysis experiment with primary adipocytes (5), inguinal adipose tissue of male *Hilpda*$^{flox/flox}$ (n=3) and *Hilpda*$^{\Delta AT}$ (n=3) mice was excised and minced with scissors. Stromal vas-

cular fractions were isolated and differentiated to mature adipocytes as described previously (20). Mature adipocytes were serum-starved for 2h in DMEM with 1% fatty acid-free BSA, and subsequently treated with 10 µM isoproterenol or 10 µM CL316,243 for 3h. Medium samples were assayed for NEFAs as described below and corrected for total protein.

Recombinant adenoviruses

Adenoviruses (AV) were generated by cloning GFP or *Hilpda* cDNA in human adenovirus type 5 (dE1/E3). Expression was under the control of the CMV promotor. Viruses were produced and titrated by Vector Biolabs (Philadelphia, PA, USA).

Chemicals

Isoproterenol, norepinephrine, 3-isobutyl-1-methylxanthine (IBMX), insulin, dexamethasone, fatty acid-free BSA and rosiglitazone were purchased from Sigma-Aldrich (Schnelldorf, Germany). Forskolin, procaterol and CL316,243 were from Tocris (Tocris Bioscience, Bristol, United Kingdom).

Separation SVF & Adipocytes

Dissected adipose tissue depots from the different experiments were kept on ice in DMEM supplemented with 1% fatty acid-free BSA (Sigma-Aldrich). Samples were minced into small 1-2 mm^3 pieces and incubated with collagenase solution (DMEM, 3.2 mM $CaCl_2$, 15 mM HEPES, 0.5% BSA, 10% fetal calf serum (FCS), 1.5 mg/mL collagenase type II (Sigma-Aldrich #C6885)) at 37˚C for 45 minutes. Mixtures were strained through a 100 µm cell strainer and centrifuged at 300*g for 10 minutes at room temperature. Floating adipocytes were collected and snap-frozen for RNA isolation. The pelleted stromal vascular fractions (SVF) were re-suspended in Trizol (Thermo Fisher-Scientific, Landsmeer, the Netherlands) and snap-frozen for RNA isolation.

Cell Culture

3T3-L1 fibroblasts were maintained in DMEM supplemented with 10% newborn calf serum (NCS) (Lonza) and 1% penicillin/streptomycin (P/S) (Lonza) and differentiated as described previously (21). The day at which differentiation was started is indicated as day 0. Dharmacon ON-TARGETplus SMARTpool siRNAs were purchased from Thermo Fisher-Scientific. siRNAs were diluted in Dharmacon 1x siRNA buffer (final concentration 20 mM KCl, 6 mM HEPES pH 7.5, 0.2 mM $MgCL_2$). Transfections were performed with Lipofectamine RNAiMAX transfection reagent (Life Technologies, Bleiswijk, The Netherlands). All siRNA transfections were carried out at a concentration of 40 nM siRNA and 2 µL of transfection reagent for a 12-wells plate. To study the effect of HILPDA knockdown on 3T3-L1 differentiation, siRNAs were added two days before initiation of differentiation, at the start of differentiation

and subsequently every 3rd day. To knock down HILPDA, G0S2 or PPARγ in mature 3T3-L1 adipocytes, cells were washed with PBS, trypsinized and collected in PBS or DMEM. After centrifugation at 400*g for 5 minutes cells were strained over a 70 µM cell strainer and plated to 70% confluency. siRNAs were added two hours later, and experiments were carried out after an additional 48-72 hours of incubation. siCtrl-, siHILPDA- and siG0S2-treated adipocytes were incubated in DMEM with 1% fatty acid-free BSA for 2 hours and subsequently exposed as described in figure legends. Medium samples were assayed for NEFAs as described below.

For overexpression experiments in differentiating 3T3-L1 adipocytes, recombinant viruses were diluted in DMEM (low glucose, 1 g/L) supplemented with 0.5 µg/mL poly-L-lysine and incubated at room temperature for 100 minutes. Mixtures were added to cells and allowed to incubate for 90 minutes followed by the addition of normal culture medium. Differentiation was initiated two days later. Adenoviruses were used at a multiplicity of infection (MOI) of 500. To study isoproterenol-induced NEFA release, mature 3T3-L1 adipocytes were re-plated at 70% confluency as described above and subsequently starved in DMEM (low glucose) + 1% FCS for 12 hours followed by DMEM (low glucose) + 0.1% FCS for an additional 12 hours. Adenoviruses diluted in 0.5 µg/mL poly-L-lysine were added to serum starved cells at an MOI of 750. Regular culture medium containing 10% FCS was added after 90 minutes. Three days later cells were incubated in DMEM + 1% BSA for 2 hours and subsequently exposed as described in figure legends. Medium samples were assayed for NEFA as described below.

Stromal vascular fractions derived from eWAT or iWAT of wild-type male C57BL/6 mice (n=3/type of WAT) were differentiated as described previously and treated as described in figure legends (20). SGBS cells and hMADs cells were cultured and differentiated according to published methods (22, 23). For microarray analysis, cells were exposed to 0.5 µM rosiglitazone or DMSO control for 6 hours as described previously (24).

Serum analyses

Triglycerides were determined using a triglyceride liquicolor mono kit (Human, Wiesbaden, Germany). NEFA was measured with the NEFA-HR(2) kit (Wako, Neuss, Germany). Cholesterol and glucose were measured with commercially available kits from Diasys (Holzheim, Germany). Glycerol was measured with a kit from Sigma-Aldrich. Plasma adiponectin was determined using the Mouse Ultrasensitive Insulin ELISA (ALPCO Diagnostics, Salem, NH, USA). Plasma insulin levels were quantified using the Quantikine ELISA (R&D Systems, Abingdon, United Kingdom).

Oil-red-O staining

3T3-L1 adipocytes were fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. Cells were subsequently washed with PBS and incubated with filtered Oil-red-O solution (30 mg/mL in 60% isopropanol) for 10 minutes. Cells were washed 2-3 times with ddH_2O twice before taking pictures.

ChIP-seq data

ChIP-seq data sets from 3T3-L1 cells (GSE13511) (25), hMADS cells (GSE59703) (26) and mouse eWAT and BAT, either whole tissue (GSE43763) (27) or primary *in vitro* differentiated adipocytes (GSE41481) (28) were obtained from the Gene Expression Omnibus database. PPARγ peaks in mouse and human adipocytes were identified with default settings and scanned for the presence of known motifs using HOMER (29). The location of PPARγ super-enhancers was identified using annotatePeaks.pl–style super in HOMER. Conservation of PPARγ binding sites was determined using the University of California at Santa Cruz (UCSC) LiftOver tool from the mm9 to hg19 genome assemblies. The UCSC genome browser (30) was used for visualization.

Western Blot

Proteins were extracted from adipose tissue using homogenization buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% v/v/ NP-40, 0.5% v/v sodium deoxycholate, 0.1% SDS) supplemented with Complete EDTA-free protease inhibitor cocktail and phosSTOP tablets (Roche Diagnostics, Almere, The Netherlands), and the Qiagen Tissuelyser II (Qiagen, Venlo, The Netherlands). Lysates were rotated for 15 minutes at 4 ˚C and subsequently centrifuged at 11.000*g for 15 minutes at 4 ˚C. For extraction of protein from cells, homogenization buffer was added to PBS-washed cells and the cells were subsequently scraped. Equal amounts of protein were diluted with 2x or 5x Laemmli sample buffer, boiled, and separated on 8-16% or 4-20% Criterion gradient gels (Bio-Rad, Veenendaal, The Netherlands). Proteins were transferred to PVDF membrane using a Transblot turbo system (Bio-Rad). Antibodies to detect HILPDA (1:1000, Santa Cruz Biotechnology, #sc-137518), β-TUBULIN (1:1000, Santa Cruz Biotechnology, #sc-23949), ACTIN (1:2000, Sigma-Aldrich #A2066), HSP90 (1:2000-4000, Cell Signaling Technology #4874), HSL (1:5000, Cell Signaling Technology #4107), ATGL (1:1000, Cell Signaling Technology #2138), phospho-HSL ser660 (1:2000, Cell Signaling Technology #4126) and corresponding secondary antibodies were diluted in PBS-T or TBS-T containing 5% w/v skimmed milk powder. Quantification of western blots was performed with the ChemiDoc MP system (Bio-Rad) and either Pierce ECL plus (Thermo Fisher-Scientific) or Clarity ECL substrate (Bio-Rad).

RNA isolation and qPCR

RNA was isolated from tissues and cells using TRIzol (Thermo Fisher-Scientific). Homogenization of tissues was performed using a Qiagen Tissue lyser II, whereas cells were lysed by pipetting up and down several times. Isolated RNA and RNA from the FirstChoice Human Total RNA Survey Panel (Ambion, via Thermo Fisher-Scientific) was reverse transcribed using the First Strand cDNA synthesis kit (Thermo Fisher-Scientific) or iScript cDNA Synthesis Kit (Bio-Rad). Gene expression analysis was performed on a CFX384 Real-Time PCR platform (Bio-Rad). SensiMix PCR mix was purchased from Bioline (GC biotech, Alphen aan de Rijn, The Netherlands). Gene expression values were normalized with expression values for 36B4 (housekeeping gene).

Microarray analysis

RNA from SGBS and hMADS (24) cells was purified with an RNAeasy Minikit (Qiagen). RNA quality was verified with the RNA 6000 Nano assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands). Hybridization, washing and scanning of the Affymetrix Human Gene 1.1 ST array plate was performed according to standard protocols on an Affymetrix GeneTitan platform. Bioconductor packages were used to analyse the scans of the arrays (31). Robust multiarray normalization was applied to obtain raw signal intensities. Probe sets were defined using remapped chip definition file (CDF) based on the Entrez gene database. Fold changes were calculated by dividing expression values of rosiglitazone-treated adipocytes by expression values of DMSO-treated adipocytes.

Confocal Microscopy

A HILPDA-Turquoise2 plasmid and a HILPDA-mCherry plasmid were constructed by fusing full-length HILPDA DNA with the 3′end of eGFP in pEGFP-N2 (CLONTECH). A CIDEB-mCherry plasmid was constructed by fusing full-length CIDEB cDNA with 3′end of eGFP in pEGFP-N2 (CLONTECH). Next, the eGFP region was substituted with cDNA encoding the fluorescent protein mTurquoise2 or the fluorescent protein mCherry (32). 3T3-L1 fibroblasts were transfected with HILPDA-mTurquoise2 plasmid DNA and CIDEB-mCherry plasmid DNA or HILPDA-mCherry plasmid DNA with Fugene (Promega). 24 hours post-transfection, 3T3-L1 cells were lipid-loaded with 400 µM oleic acid. Lipid droplets were stained with BODIPY® 493/503(4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene) (Molecular Probes, Leiden, The Netherlands). BODIPY was diluted in PBS at a concentration of 1 mg/mL and incubated for 45 mins. Confocal laser scanning microscopy imaging was performed using a confocal laser scanning microscope (LSM510 Carl Zeiss, Jena, Germany). Cells were maintained in medium for cellular imaging (20 mM HEPES pH=7.4, 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM D-Glucose). For mTurquoise2, an excitation light of 458 nm was used and a band-pass filter of 470-500 nm was used for detection. mCherry was excited using a He/Ne diode laser (543nm) and and a band-pass filter 560-615 nm was used for detection. A PlanNeofluar 63x oil immersion objective with a numeric aperture of 1.25 was used. The pinhole setting was set at 1 to 1.3 Airy units. Images were collected as 2048×2048 pixel scans at 12-bit intensity resolution. Pixel saturation was avoided using the range indicator view during acquisition.

Statistical analyses

Student's t-tests or two-way ANOVAs with Tukey's post-hoc test were performed in GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The significance level was set at $p < 0.05$.

RESULTS

HILPDA is highly expressed in adipose tissue and localizes to lipid droplets

First, we determined the distribution of hypoxia-inducible lipid droplet-associated (HILPDA*)* mRNA and protein expression across various mouse and human tissues and found that HILPDA protein is highly abundant in adipose tissue (**Figure 1A & Figure 1B**). In agreement with previous reports, fluorescently labelled HILPDA localized primarily, though not exclusively, to the lipid droplets in lipid-loaded 3T3-L1 pre-adipocytes, as demonstrated by the co-localization with BODIPY and CIDEB, an established lipid droplet protein (**Figure 1C & Figure 1D**) (17, 18, 33).

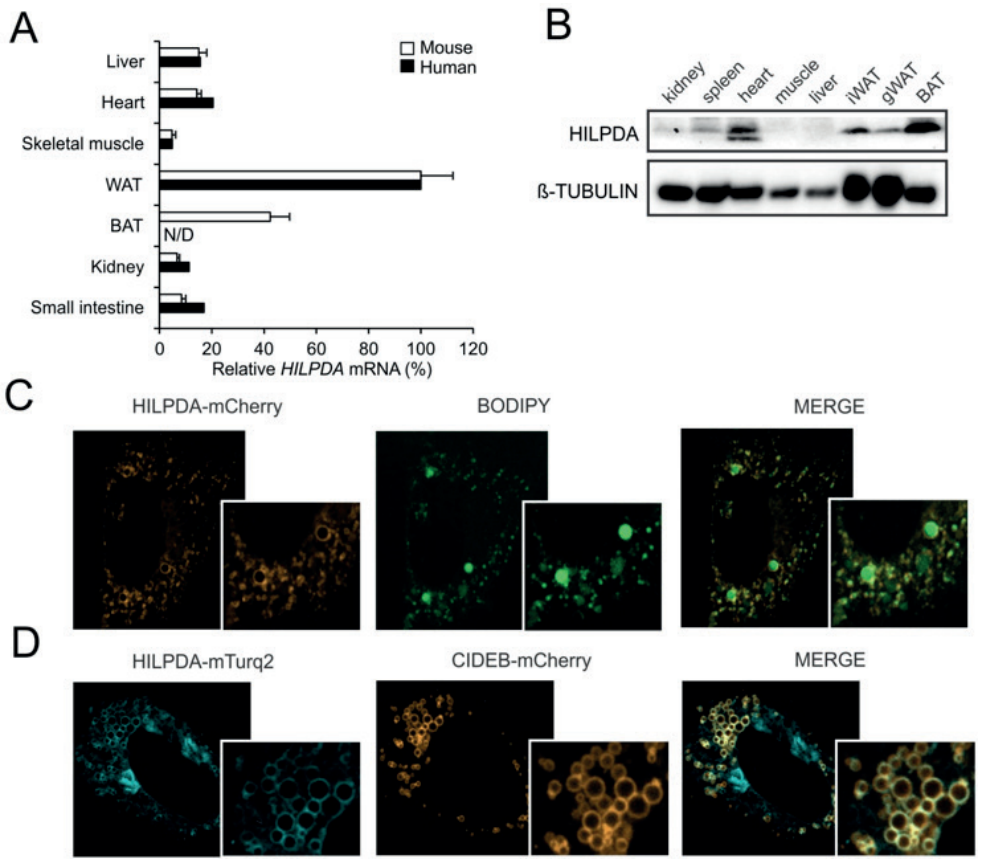


Figure 1. HILPDA is highly expressed in adipose tissue and localizes to lipid droplets.
(A) *HILPDA* mRNA levels in human (n=1) and mouse (n=4) tissues. (B) Immunoblot for HILPDA in various mouse tissues (n=1). (C) 3T3-L1 pre-adipocytes were transfected with HILPDA-mCherry plasmid, loaded with 400 µM of oleic acid, and 48h post-transfection stained with BODIPY and analysed by confocal microscopy. (D) 3T3-L1 pre-adipocytes were transfected with HILPDA-Turquoise2 plasmid and CIDEB-mCherry plasmid, loaded with 400 µM of oleic acid, and analysed by confocal microscopy 48h post-transfection. Data are mean ± SEM.

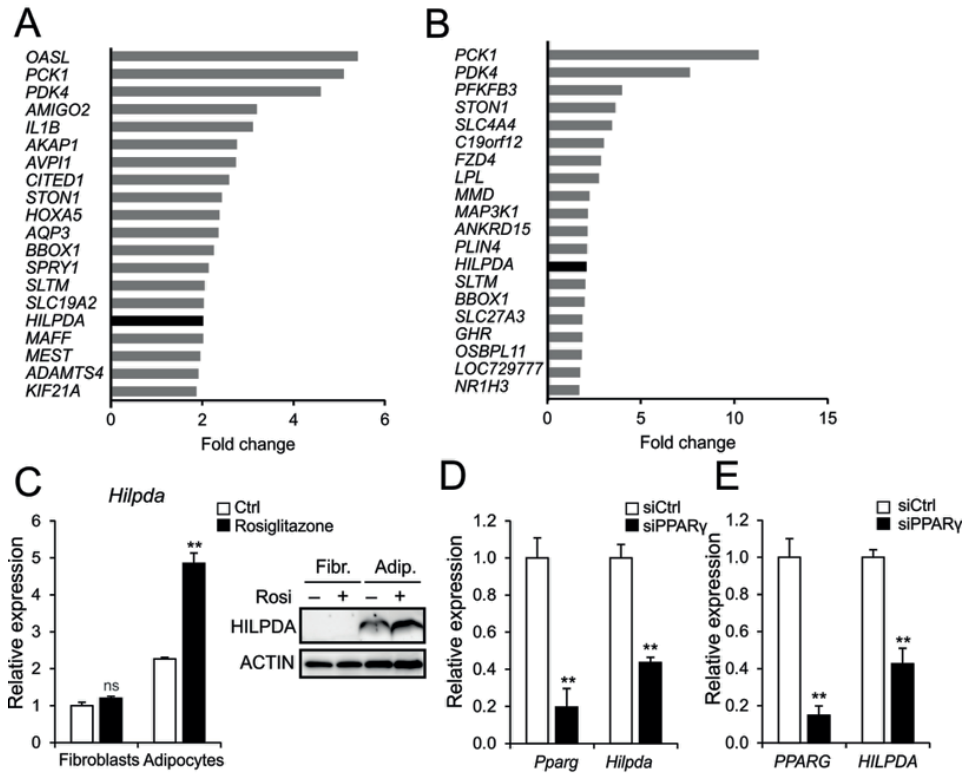


Figure 2. HILPDA is a PPARγ target gene in adipocytes.

(**A & B**) Top 20 of most highly induced genes following gene expression profiling of SGBS (**A**) and hMADS adipocytes (**B**) treated with 0.5 µM rosiglitazone or DMSO control for 6h. Fold change was calculated by dividing gene expression values of rosiglitazone-treated adipocytes by values of control-treated adipocytes. (**C**) Undifferentiated (*fibroblasts*) and fully differentiated 3T3-L1 adipocytes (*adipocytes*) were exposed to 10 µM rosiglitazone or DMSO control for 24h and subsequently analysed for HILPDA mRNA (*left*) and protein (*right*). Gene expression levels of control-treated fibroblasts were set at 1. ACTIN was used as a loading control. (**D**) Fully differentiated 3T3-L1 adipocytes were trypsinized, re-plated at 70% confluency, and incubated with control siRNA (siCtrl) or siRNA against PPARγ (siPPARγ) and analysed for expression of PPARγ and HILPDA after 72h. Gene expression levels of siCtrl-treated adipocytes were set at 1 (**E**) Fully differentiated SGBS adipocytes were trypsinized, re-plated at 70% confluency, and incubated with control siRNA (siCtrl) or siRNA against PPARγ (siPPARγ) and analysed for expression of PPARγ and HILPDA after 72h. Gene expression levels of siCtrl-treated adipocytes were set at 1. Data are mean ± SEM. Asterisks indicate significant differences according to Student's t-test relative to control-treated fibroblasts or adipocytes (Figure 2C) or relative to siCtrl-treated adipocytes (Figure 2D & Figure 2E); ** $p<0.01$, * $p<0.05$.

(**B**) Screenshot of the human *HILPDA* locus showing RNA-seq and PPARγ ChIP-seq profiles in hMADS preadipocytes and adipocytes. PPARγ binding sites that are conserved between mouse and human and the position of PPREs within the PPARγ binding sites are highlighted. The sequences indicate conserved PPREs in the respective binding sites. The red bar indicates the position of adipocyte-specific PPARγ super-enhancers in 3T3-L1 and hMADS adipocytes. The PPARγ binding site thathas been reported to bind PPARα in liver and that harbors a highly conserved PPRE is marked by an asterisk.

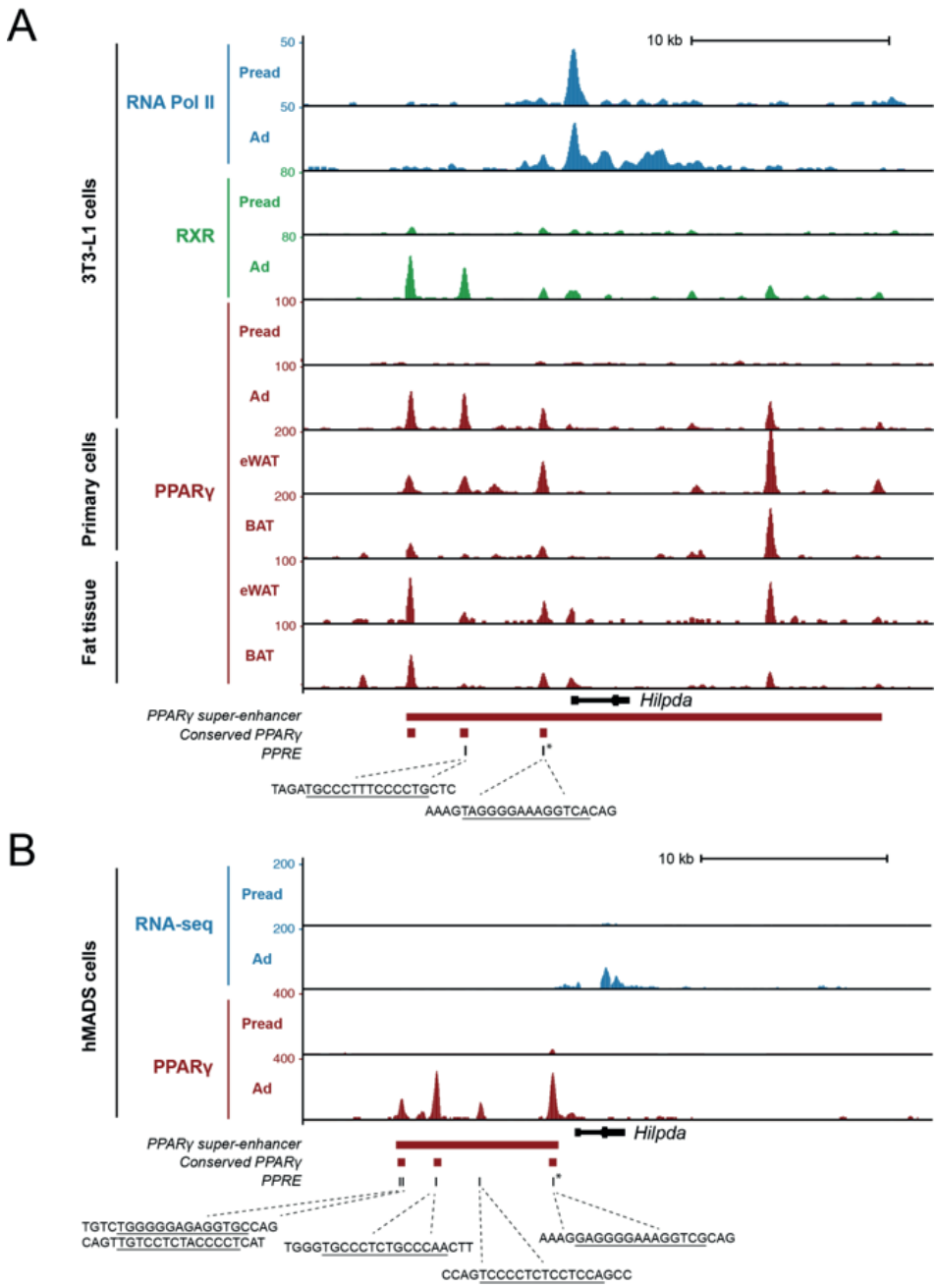


Figure 3. PPARγ is associated with the HILPDA locus in mouse and human adipocytes.

(**A**) Screenshot of the mouse *Hilpda* locus showing ChIP-seq profiles of RXR, PPARγ, and RNA polymerase II in 3T3-L1 preadipocytes and adipocytes, and PPARγ ChIP-seq profiles from eWAT and BAT (primary adipocytes and whole tissue). *See previous page for continuation.*

HILPDA is a PPARγ target gene in adipocytes

Hilpda was recently identified as a target gene of peroxisome proliferator-activated receptor (PPAR) α in liver (19). To explore whether PPARγ impacts HILPDA expression in adipocytes, we treated different types of adipocytes with the PPARγ agonist rosiglitazone. Interestingly, microarray analyses indicated that HILPDA was among the 20 most significantly upregulated genes by rosiglitazone in differentiated SGBS adipocytes and differentiated human multipotent adipose-derived stem cells (hMADS), together with the well-known PPARγ targets PCK1, PDK4 and LPL (**Figure 2A & Figure 2B**). Rosiglitazone also significantly induced HILPDA mRNA and protein in mature 3T3-L1 adipocytes, but not 3T3-L1 pre-adipocytes (**Figure 2C**). Consistent with PPARγ-dependent regulation, HILPDA expression in 3T3-L1 and human SGBS adipocytes was markedly decreased upon knockdown of PPARγ (**Figure 2D & Figure 2E**).

Figure 4. SiRNA-mediated knockdown or adenoviral-mediated overexpression of HILPDA does not affect adipogenesis.

(A) HILPDA mRNA (top) and protein (bottom) levels during 3T3-L1 adipogenesis. Cells were harvested at indicated days, with day 0 as the day at which differentiation was started. Gene expression levels of Day -3 were set at 1. ACTIN was used as a loading control. (B) HILPDA mRNA (top) and protein (bottom) levels during 3T3-L1 adipogenesis. Control (siCtrl) and HILPDA siRNAs (siHILPDA) were added two days before initiation of adipogenesis, at the start of differentiation and on day 3 and day 6 of differentiation. Cells were harvested at indicated days, with day 0 as the day at which differentiation was started. Gene expression levels of siCtrl-treated adipocytes at day 3 of differentiation were set at 1. ACTIN was used as a loading control. (C) Oil-Red-O staining of of 3T3-L1 adipocytes treated with control (siCtrl) and HILPDA siRNA (siHILPDA). siCtrl and siHILPDA were added two days before initiation of adipogenesis, at the start of differentiation and on day 3 and day 6 of differentiation. Cells were harvested at indicated days, with day 0 as the day at which differentiation was started. (D) FABP4, G0S2, and PPARγ mRNA levels during adipogenesis of 3T3-L1 adipocytes treated with control (Ctrl) and HILPDA siRNA. siCtrl and siHILPDA were added two days before initiation of adipogenesis, at the start of differentiation and on day 3 and day 6 of differentiation. Cells were harvested at indicated days, with day 0 as the day at which differentiation was started. Gene expression levels of siCtrl-treated adipocytes at day 3 of differentiation were set at 1. (E) HILPDA mRNA (top) and protein (bottom) during 3T3-L1 adipogenesis of AV-*gfp*- or AV-*Hilpda*-treated cells. Recombinant adenoviruses expressing *gfp* or *Hilpda* were added at an MOI of 500 two days before initiation of differentiation. Cells were harvested at indicated days, with day 0 as the day at which differentiation was started. Gene expression levels of AV-*gfp*-treated adipocytes at day 3 of differentiation were set at 1. ACTIN was used as a loading control. (F) Oil-Red-O staining of AV-*gfp* or AV-*Hilpda* treated cells at indicated days during the process of differentiation. Recombinant adenoviruses expressing gfp or Hilpda were added at an MOI of 500 two days before initiation of differentiation. Cells were harvested at indicated days, with day 0 as the day at which differentiation was started. (G) FABP4, G0S2, and PPARγ mRNA levels during adipogenesis of 3T3-L1 adipocytes treated with AV-*gfp* or AV-*Hilpda*. Recombinant adenoviruses expressing *gfp* or *Hilpda* were added at an MOI of 500 two days before initiation of differentiation. Cells were harvested at indicated days, with day 0 as the day at which differentiation was started. Gene expression levels of AV-*gfp*-treated adipocytes at day 3 of differentiation were set at 1. Data are mean ± SEM. Asterisks indicate significant differences according to Student's t-test relative to Day -3 (Figure 4A), relative to siCtrl-treated adipocytes (Figure 4B, 4D), or relative to AV-gfp-treated adipocytes (Figure 4E, 4G); ** $p<0.01$, * $p<0.05$.

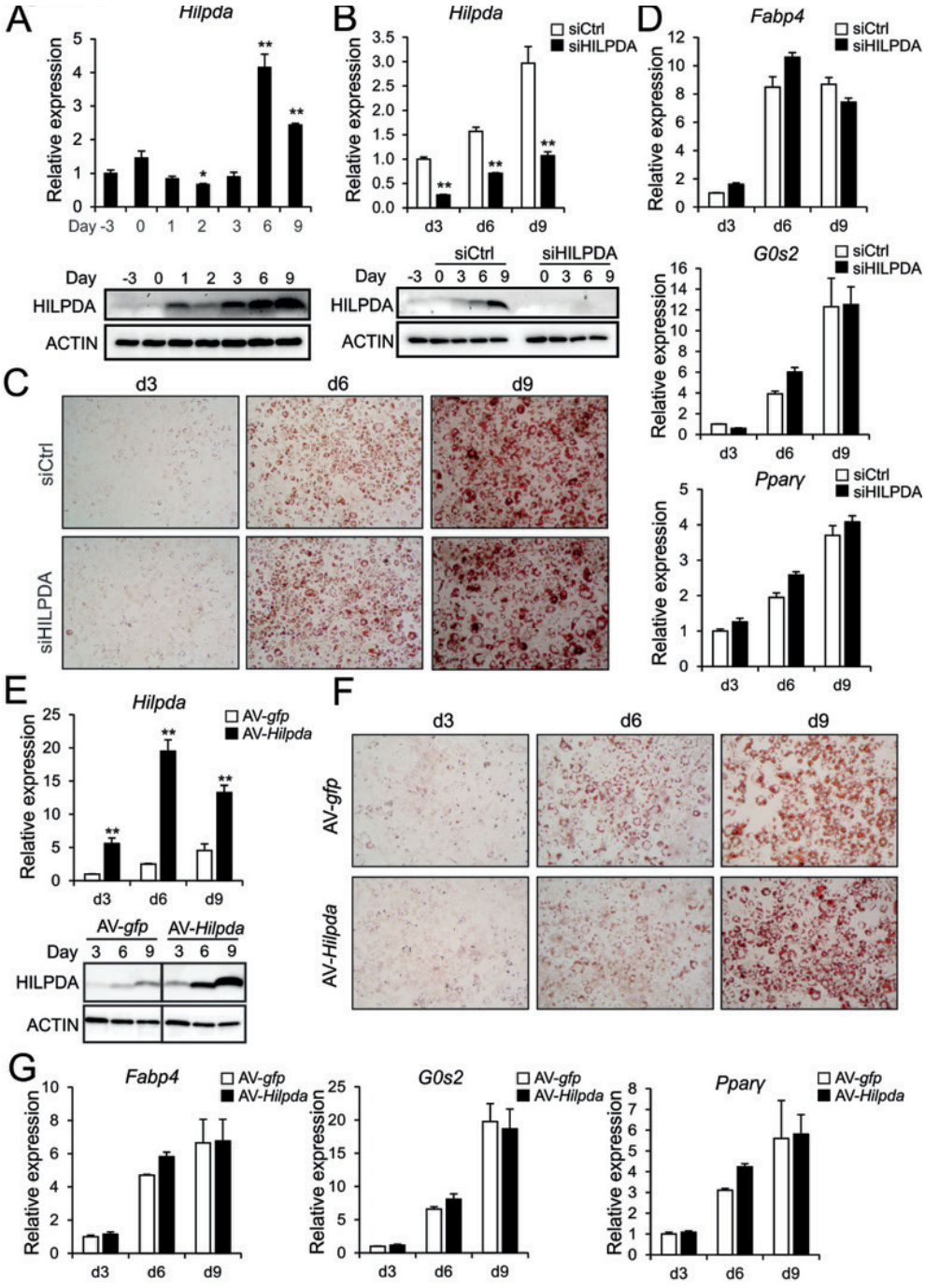


Figure 4.

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Previously, a functional PPAR response element (PPRE) was identified around 1200 base pairs upstream of the transcriptional start site (TSS) of the *Hilpda* gene (19). Investigation of PPARγ ChIP-seq data from 3T3-L1, primary epididymal WAT- and brown adipose tissue (BAT)-derived adipocytes, and hMADS adipocytes demonstrated that PPARγ binds to three conserved sites upstream of the TSS (**Figu-**

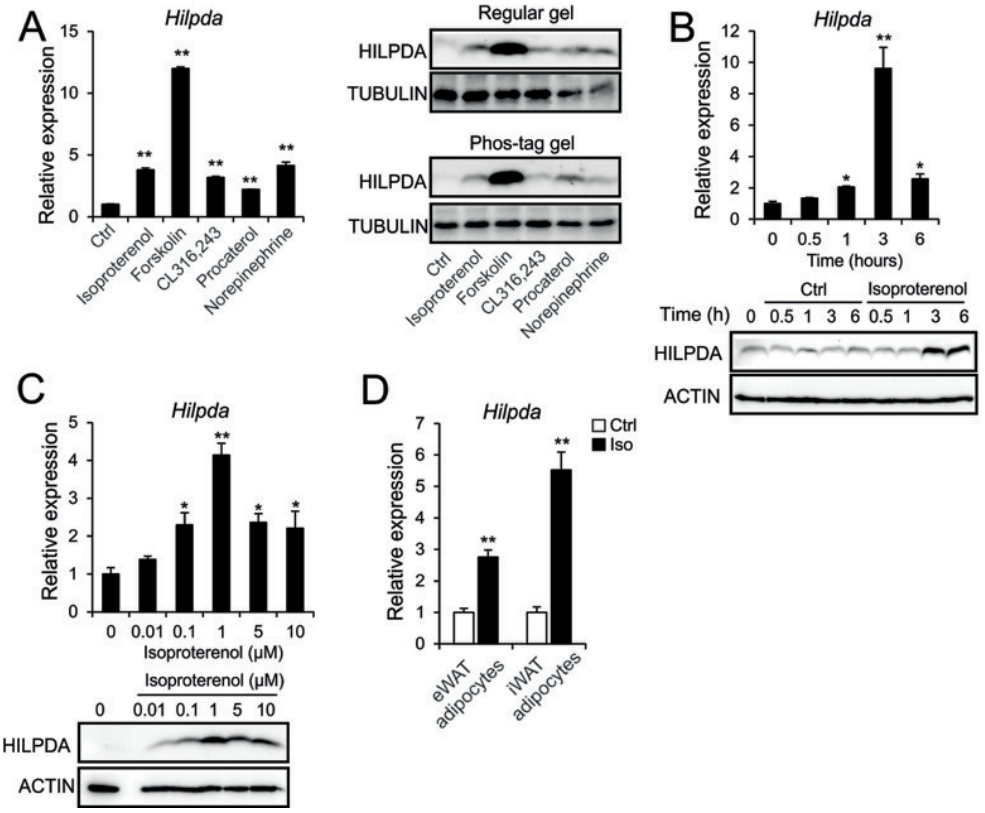

Figure 5. HILPDA is highly induced upon activation of β-adrenergic signaling.

(A) HILPDA mRNA (left) and protein (right) levels in fully differentiated 3T3-L1 cells exposed to control medium, 10 μM isoproterenol, 5 μM forskolin, 10 μM CL316,243, 10 μM procaterol, 10 μM norepinephrine for 3h. Protein levels of HILPDA were assessed on a regular gel and on a Phos-tag gel to assess HILPDA phosphorylation. Gene expression levels of control-treated adipocytes were set at 1. TUBULIN was used as a loading control. (B) HILPDA mRNA (top) and protein (bottom) levels in fully differentiated 3T3-L1 cells exposed to 10 μM isoproterenol for indicated time points. Gene expression levels at time point 0h were set at 1. ACTIN was used as a loading control. (C) HILPDA mRNA (top) and protein (bottom) levels in fully differentiated 3T3-L1 cells incubated with control medium or indicated concentrations of isoproterenol for 6h. Gene expression levels of control-treated adipocytes were set at 1. ACTIN was used as a loading control. (D) HILPDA mRNA levels in primary adipocytes differentiated from the stromal vascular fractions of epididymal or inguinal white adipose tissue and exposed to 10 μM isoproterenol for 3h. Gene expression levels of control-treated epididymal adipocytes and inguinal adipocytes were set at 1. Data are mean ± SEM. Asterisks indicate significant differences according to Student's t-test relative to control-treated adipocytes (all figures); ** $p<0.01$, * $p<0.05$.

re 3A & Figure 3B). The PPARγ binding site closest to the TSS overlaps with the location of a highly conserved PPRE that was previously shown to mediate PPARγ-dependent transcriptional activation *in vitro* (**Figure 3A & Figure 3B**) (19). Since previous studies have shown that clusters of nearby enhancers (i.e. super-enhancers) (34) control important cell type-defining genes, we next asked whether the *Hilpda* locus is associated with a PPARγ super-enhancer. Interestingly, in both mouse and human adipocytes a prominent PPARγ super-enhancer is located near *Hilpda* (**Figure 3A & Figure 3B**), suggesting that *Hilpda* is an important PPARγ target gene that might control key adipocyte functions.

HILPDA does not affect 3T3-L1 adipogenesis

With *Hilpda* being a PPARγ target gene, we explored a potential role for HILPDA in adipocyte differentiation. Expression of HILPDA mRNA significantly increased during mouse 3T3-L1 adipocyte differentiation, which was confirmed at the protein level (**Figure 4A**). To assess the role of HILPDA in 3T3-L1 adipocyte differentiation, we employed siRNA-mediated knockdown of HILPDA. When compared with control siRNA, siRNA targeting HILPDA effectively reduced HILPDA mRNA and protein levels (**Figure 4B**). However, knockdown of HILPDA did not substantially influence 3T3-L1 adipogenesis, as shown by Oil-Red-O staining and expression of adipogenic marker genes (**Figure 4C & Figure 4D**). Similarly, adenoviral-mediated overexpression of HILPDA did not influence 3T3-L1 adipogenesis, as revealed by Oil-Red-O staining, and the expression of adipogenic marker genes (**Figure 4E-G**). Together, these data suggest that HILPDA does not regulate 3T3-L1 adipogenesis.

HILPDA is highly induced upon activation of β-adrenergic signaling

Several lipid droplet proteins are responsive to β-adrenergic signaling, the key signaling pathway that activates adipocyte lipolysis (35, 36). To assess whether β-adrenergic signaling regulates HILPDA expression, 3T3-L1 adipocytes were treated with various β-adrenergic receptor agonists. HILPDA mRNA and protein expression were significantly induced upon treatment with the non-selective β-receptor agonist isoproterenol, the β3-receptor agonist CL316,243, the β2-receptor agonist procaterol, the non-selective adrenergic receptor agonist norepinephrine and the adenylate cyclase activator forskolin, suggesting that HILPDA is responsive to β-adrenergic stimulation (**Figure 5A**). The remainder of the experiments was performed with isoproterenol. HILPDA mRNA and protein levels were induced by isoproterenol in a time- and concentration-dependent manner, with HILPDA levels peaking after 3 hours of stimulation and an isoproterenol concentration of 1 μM (**Figure 5B & Figure 5C**). Induction of HILPDA mRNA by isoproterenol was confirmed in primary mouse gonadal and inguinal adipocytes, as well as in brown adipocytes (**Figure 5D, Supplemental Figure 1**).

SiRNA-mediated knockdown of HILPDA in adipocytes does not affect lipolysis

Loss of HILPDA was previously shown to increase triglyceride hydrolysis in primary hepatocytes (17). Also, the protein that shares the highest sequence homology with HILPDA is G0S2, an inhibitor of intracellular lipolysis (17) (unpublished observations). To explore a possible role of HILPDA in adipose tissue lipolysis, we studied the effect of siRNA-mediated HILPDA knockdown on intracellular lipolysis in differentiated 3T3-L1 adipocytes. Although treatment with siRNA targeting HILPDA effectively reduced HILPDA mRNA and protein levels in the adipocytes, it did not influence the release of NEFA upon stimulation with isoproterenol (**Figure 6A & Figure 6B**). To determine whether loss of HILPDA may be functionally compensated by G0S2, we assessed the impact of siRNA-mediated knockdown of HILPDA and/or G0S2 on intracellular lipolysis in 3T3-L1 adipocytes. Again, while treatment with siRNA targeting HILPDA and/or G0S2 effectively reduced RNA levels of HILPDA

Figure 6. Overexpression of HILPDA in adipocytes inhibits lipolysis.

(A) HILPDA mRNA (left) and protein levels (right) in fully differentiated 3T3-L1 adipocytes that were trypsinized, replated at 70% confluency, and treated with siHILPDA or siCtrl for 48h. Gene expression levels of siCtrl-treated adipocytes were set at 1. ACTIN was used as a loading control. (B) NEFA release in medium of fully differentiated 3T3-L1 adipocytes that were trypsinized, replated at 70% confluency, and treated with siHILPDA or siCtrl for 48h. After 48h, adipocytes were serum starved for 2h and incubated with 5 μM isoproterenol or control medium for 2h. NEFA levels were corrected for protein levels in cell lysates. (C) HILPDA and G0S2 mRNA levels in fully differentiated 3T3-L1 adipocytes that were trypsinized, replated at 70% confluency, and treated with siCtrl, siHILPDA or siG0S2 for 48h. Gene expression levels of siCtrl-treated adipocytes were set at 1. (D) NEFA release in medium of fully differentiated 3T3-L1 adipocytes that were trypsinized, replated at 70% confluency, and treated with siCtrl, siHILPDA or siG0S2 for 48h. After 48h, adipocytes were serum starved for 2h and incubated with 5 μM isoproterenol or control medium for 2h. NEFA levels were corrected for protein levels in cell lysates. (E) HILPDA mRNA (left) and protein levels (right) in fully differentiated 3T3-L1 adipocytes transduced with AV-gfp or AV-Hilpda. Fully differentiated 3T3-L1 adipocytes were trypsinized, re-plated at 70% confluency, serum starved for 24h and transduced with recombinant adenoviruses expressing gfp or Hilpda at an MOI of 750 for 72h. Gene expression levels of AV-gfp- treated adipocytes were set at 1. HSP90 was used as a loading control. s.e., short exposure; l.e., long exposure. (F) NEFA release in fully differentiated 3T3-L1 adipocytes that were trypsinized, re-plated at 70% confluency, serum starved for 24h and transduced with recombinant adenoviruses expressing gfp or Hilpda at an MOI of 750 for 72h. Transduced differentiated 3T3-L1 adipocytes were serum starved for 2h, and incubated with 5 μM isoproterenol for 3h. (G) NEFA release in fully differentiated 3T3-L1 adipocytes that were trypsinized, re-plated at 70% confluency, serum starved for 24h and transduced with recombinant adenoviruses expressing gfp or Hilpda at an MOI of 750 for 72h. Transduced differentiated 3T3-L1 adipocytes were serum starved for 2h, and incubated either with 10 μM isoproterenol, 5 mM 3-methyladenine or 10 μM CL316,243 for 3h. (H) Representative immunoblots for HSL (LIPE), phospho-HSL, ATGL (PNPLA2), PLIN1 and HILPDA in fully differentiated 3T3-L1 adipocytes that were trypsinized, re-plated at 70% confluency, serum starved for 24h and transduced with recombinant adenoviruses expressing gfp or Hilpda at an MOI of 750 for 72h. Transduced 3T3-L1 adipocytes were serum starved for 2h, and incubated with 10 μM isoproterenol for 3h. HSP90 was used as a loading control. s.e., short exposure; l.e., long exposure. Data are mean ± SEM. Asterisks indicate significant differences according to student's t-test relative to siCtrl-treated or AV-gfp-treated adipocytes (Figure 6A, 6C, 6E). Asteriks or hashtags indicate significant differences according to two-way ANOVA (#) followed by Tukey's HD post-hoc test (*) relative to control-treated adipocytes (#) or relative to siCtrl- or AV-gfp-treated adipocytes (*); ** or ## $p<0.01$, * or # $p<0.05$.

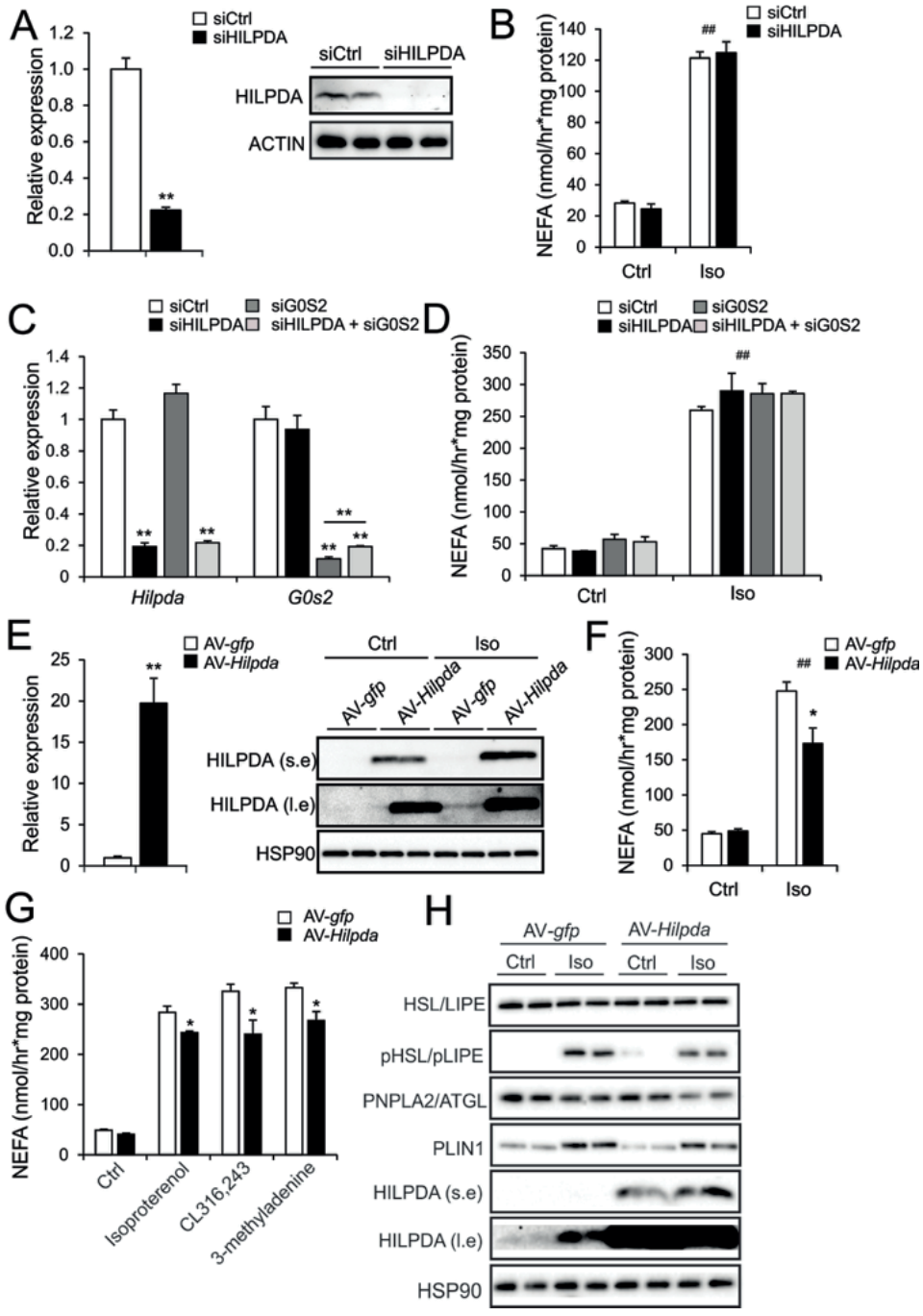

Figure 6.

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and G0S2 (**Figure 6C)**, it did not affect NEFA release upon stimulation with isoproterenol (**Figure 6D**). Together, these results indicate that knockdown of HILPDA in adipocytes—alone or in combination with G0S2—does not have a major impact on the *in vitro* lipolytic response to pharmacologic β3-adrenergic stimulation.

Overexpression of HILPDA in adipocytes *in vitro* reduces lipolysis

We reasoned that a possible effect of HILPDA on lipolysis might be more evident when HILPDA is overexpressed as opposed to knocked down. Accordingly, we studied the effect of adenoviral-mediated HILPDA overexpression on lipolysis in differentiated 3T3-L1 adipocytes (**Figure 6E**). Remarkably, HILPDA overexpression modestly but significantly reduced NEFA release induced by isoproterenol (**Figure 6F**). The inhibitory effect of HILPDA overexpression on NEFA release was reproduced with the β3-adrenergic agonist CL316,243 and the PI3K inhibitor 3-methyladenine (**Figure 6G**). To explore the potential mechanism underlying the observed anti-lipolytic effect of HILPDA, we studied the effect of AV-mediated HILPDA overexpression on the relative abundance and activation status of key lipolytic enzymes, including adipose triglyceride lipase (ATGL/PNPLA2) and hormone sensitive lipase (HSL/LIPE). Interestingly, HILPDA overexpression modestly decreased the abundance of phosphorylated HSL following isoproterenol induction and, to a lesser extent, ATGL (**Figure 6H**). These data suggest that overexpression of HILPDA has a mild attenuating effect on isoproterenol-induced lipolysis in 3T3-L1 adipocytes.

Figure 7. Adipocyte-specific knockdown of HILPDA has no effect on indicators of lipolysis in mice injected with CL316,243.

(A) HILPDA mRNA levels in whole adipose tissue, and in freshly separated adipocytes and stromal vascular fractions (SVF) of *Hilpda*$^{flox/flox}$ (n=4) and *Hilpda*$^{\Delta AT}$ mice (n=4). Gene expression levels of adipose tissue from *Hilpda*$^{flox/flox}$ mice were set at 1. (B) NEFA release in medium of primary adipocytes differentiated from the stromal vascular fraction of inguinal white adipose tissue from *Hilpda*$^{\Delta AT}$ mice (n=3) and *Hilpda*$^{flox/flox}$ (n=3) mice, serum starved for 2h, and incubated with 10 μM isoproterenol or 10 μM CL316,243 for 3h. (C) NEFA release in medium of WAT explants of *Hilpda*$^{\Delta AT}$ mice (n=3) and *Hilpda*$^{flox/flox}$ (n=3) mice, serum starved for 30 min, and incubated with 10 μM isoproterenol for 3h. (D) HILPDA mRNA levels in epididymal WAT of *Hilpda*$^{flox/flox}$ (n=8/treatment) and *Hilpda*$^{\Delta AT}$ (n=8/treatment) mice excised and snap-frozen 3h after subcutaneous injection with 1 mg/kg CL316,243 or saline control. Gene expression levels of saline-treated Hilpdaflox/flox mice were set at 1. (E) HILPDA mRNA levels in freshly isolated adipocytes or stromal vascular fraction isolated from epididymal WAT of *Hilpda*$^{flox/flox}$ (n=8/treatment) and *Hilpda*$^{\Delta AT}$ mice (n=8/treatment) excised and snap-frozen 3h after subcutaneous injection with 1 mg/kg CL316,243 or saline control. Gene expression levels of the stromal vascular fractions of saline-treated *Hilpda*$^{flox/flox}$ mice were set at 1. (F) Plasma levels of NEFAs, glycerol, triglycerides and glucose of *Hilpda*$^{flox/flox}$ (n=8/treatment) and *Hilpda*$^{\Delta AT}$ (n=8/treatment) mice 3h after subcutaneous injection with 1 mg/kg CL316,243 or saline control. (G) Tissue weights of inguinal WAT and epididymal WAT of *Hilpda*$^{flox/flox}$ (n=8/treatment) and *Hilpda*$^{\Delta AT}$ mice (n=8/treatment) 3h after subcutaneous injection with 1 mg/kg CL316,243 or saline control. Data are mean ± SEM. Asterisks indicate significant differences according to student's t-test (*) relative to adipose tissue or SVF of saline-treated *Hilpda*$^{flox/flox}$ mice (Figure 7A, 7E). Asteriks or hashtags indicate significant differences according to two-way ANOVA (#) followed by Tukey's HD post-hoc test (*) relative to control-treated adipocytes (Figure 7B, 7C) or saline-treated mice (Figure 7D, 7F, 7G) (#) and relative to *Hilpda*$^{flox/flox}$ mice (Figure 7D, 7F, 7G) (*); ** or ## $p<0.01$, * or # $p<0.05$.

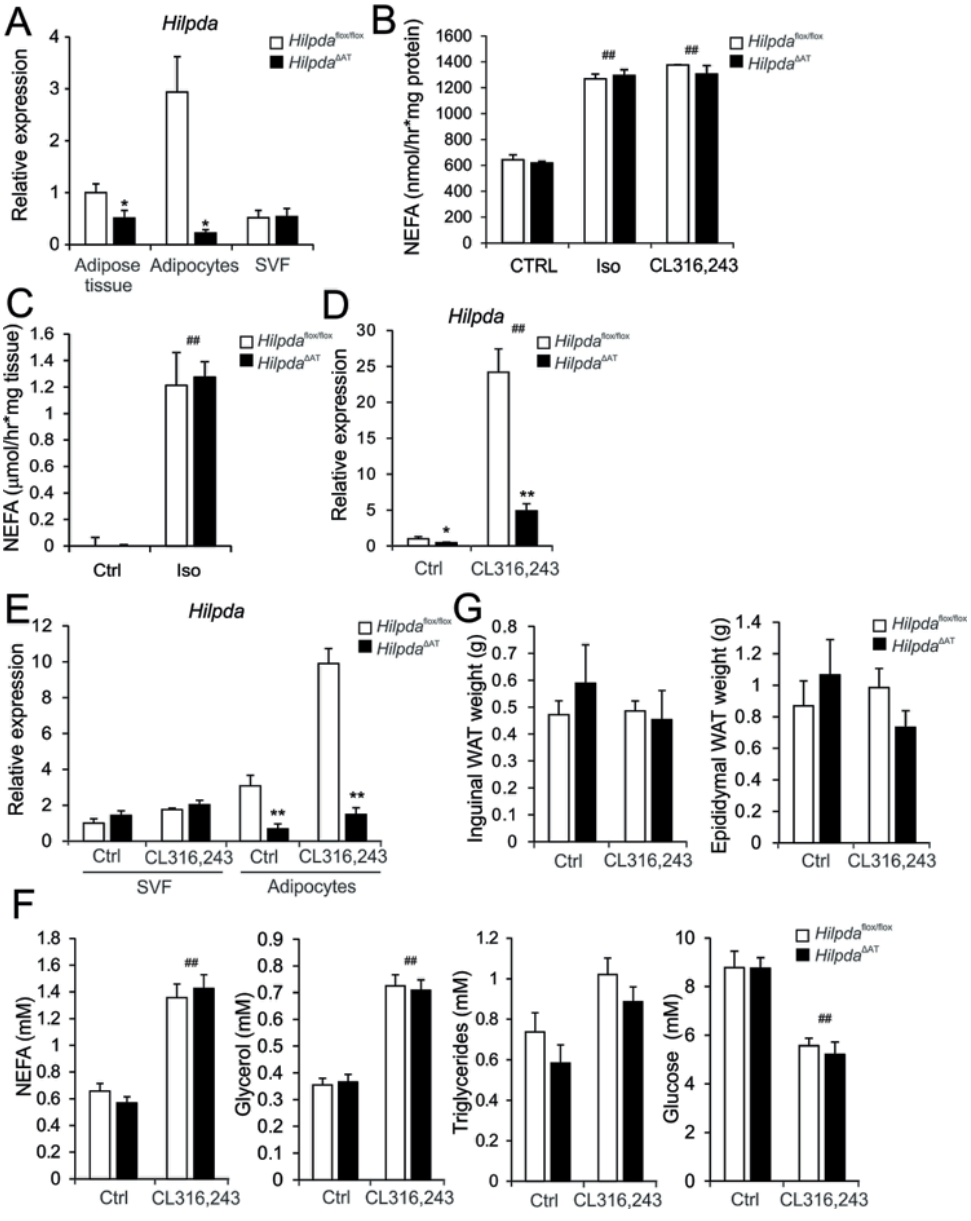

Figure 7.

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Adipocyte-specific knockdown of HILPDA has minimal effects on metabolic parameters following pharmacological β-adrenergic stimulation and fasting

To examine a potential role for HILPDA in adipocyte lipolysis *in vivo*, we generated adipocyte-specific *Hilpda* mutant mice (*Hilpda*$^{\Delta AT}$) by crossing floxed *Hilpda* mice (*Hilpda*$^{flox/flox}$) with Adiponectin-Cre mice. HILPDA expression was about 50% lower in adipose tissue of *Hilpda*$^{\Delta AT}$ mice as compared to *Hilpda*$^{flox/flox}$ mice, which was accounted for by an almost complete loss of *Hilpda* expression in adipocytes (**Figure 7A**). Upon examination of the *ex vivo* NEFA release from primary adipocytes or adipose tissue explants stimulated with isoproterenol, we found that NEFA levels were comparable between adipocytes and adipose tissue explants of *Hilpda*$^{\Delta AT}$ mice and *Hilpda*$^{flox/flox}$ mice, in line with our data of *in vitro* knockdown of HILPDA (**Figure 7B & Figure 7C**). To examine a potential role of HILPDA in the *in vivo* lipid metabolic response to β-adrenergic stimulation, we injected *Hilpda*$^{\Delta AT}$ and *Hilpda*$^{flox/flox}$ with the β3-adrenergic agonist CL316,243 to induce lipolysis. In agreement with our *in vitro* data, injection with CL316,243 potently induced HILPDA expression in white adipose tissue of *Hilpda*$^{flox/flox}$ mice (**Figure 7D**). Induction of HILPDA expression upon CL316,243 injection was specifically observed in the adipocytes and not in the stromal vascular fraction (**Figure 7E**). While injection with CL316,243 significantly increased plasma NEFA and glycerol levels—suggesting that CL316,243 effectively induced lipolysis—no differences were observed between *Hilpda*$^{\Delta AT}$ and *Hilpda*$^{flox/flox}$ mice (**Figure 7F**). Weight of inguinal and epididymal WAT (**Figure 7G**) and plasma levels of other metabolites (**Figure 7F**) were also not different between *Hilpda*$^{\Delta AT}$ and *Hilpda*$^{flox/flox}$ mice.

More physiological conditions that are associated with the activation of β-adrenergic signaling and adipose tissue lipolysis are fasting and cold exposure. Indeed, fasting caused a marked increase in HILPDA mRNA and protein in WAT of wild-type mice (**Figure 8A**). After a 24h fast, however, WAT weight was not different between *Hilpda*$^{\Delta AT}$ and *Hilpda*$^{flox/flox}$ mice (**Figure 8B**). No major differences in plasma NEFA and glycerol, as well as other metabolites were observed between the two genotypes (**Figure 8C**). Also, plasma insulin and adiponectin levels and adipose tissue morphology were not different between *Hilpda*$^{\Delta AT}$ and *Hilpda*$^{flox/flox}$ mice (**Figure 8D, Supplemental Figure 2**). Similarly, cold exposure of *Hilpda*$^{\Delta AT}$ and *Hilpda*$^{flox/flox}$ mice did not result in significant differences in plasma NEFA levels or other metabolic parameters (**Supplemental Figure 3**). We did, however, observe a small but consistent increase in the expression of several genes involved in lipolysis and lipid droplet morphology in WAT of *Hilpda*$^{\Delta AT}$ compared to *Hilpda*$^{flox/flox}$ mice (**Figure 8E**).

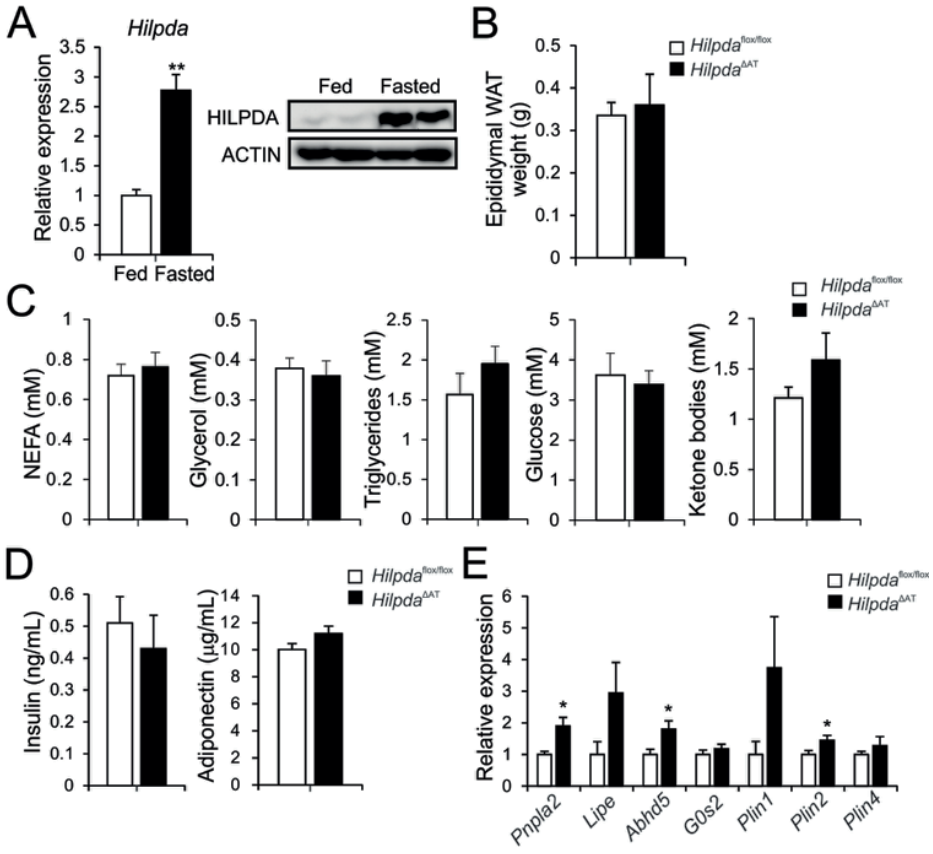

Figure 8. Adipocyte-specific knockdown of HILPDA has no effect on tissue weights or plasma parameters following a 24h fast.

(A) HILPDA mRNA (*left*) and protein levels (*right*) in epididymal WAT from C57BL/6 mice fed or fasted for 18h (n=6/group). Gene expression levels of fed mice were set at 1. ACTIN was used as a loading control. (B) Weight of epididymal WAT of male *Hilpda*$^{flox/flox}$ (n=6) and *Hilpda*$^{\Delta AT}$ (n=8) fasted for 24h (in grams). (C) Plasma levels of non-esterified fatty acids (NEFAs), glycerol, triglycerides, glucose and ketone bodies of male *Hilpda*$^{flox/flox}$ (n=6) and *Hilpda*$^{\Delta AT}$ (n=8) mice fasted for 24h. (D) Plasma levels of insulin and adiponectin of *Hilpda*$^{flox/flox}$ (n=6) and *Hilpda*$^{\Delta AT}$ (n=8) mice fasted for 24h. (E) mRNA levels of genes involved in lipolysis or lipid droplet morphology (PNPLA2, LIPE, ABHD5, G0S2, PLIN1, PLIN2, PLIN4) in epididymal WAT of *Hilpda*$^{flox/flox}$ (n=6) and *Hilpda*$^{\Delta AT}$ (n=8) mice fasted for 24h. Gene expression levels for *Hilpda*$^{flox/flox}$ mice were set at 1 for each gene. Data are mean ± SEM. Asterisks indicate significant differences according to Student's t-test relative to fed mice (Figure 8A) or *Hilpda*$^{flox/flox}$ mice; ** $p<0.01$, * $p<0.05$.

DISCUSSION

Our paper shows that HILPDA is abundant in adipocytes, where its expression is stimulated by PPARγ and β-adrenergic signaling. Whereas adenoviral overexpression of HILPDA modestly lowered NEFA release from adipocytes, the absence of an effect of *Hilpda* deletion on NEFA release *in vitro* and plasma NEFA and glycerol levels *in vivo* suggest that HILPDA is not a physiological regulator of lipolysis in adipocytes, at least under conditions of β-adrenergic stimulation, cold exposure or fasting. It should be mentioned, though, that based on our data, we cannot exclude that HILPDA may influence adipose tissue lipolysis under different conditions, such as obesity or physical exercise.

The reason for the apparent discrepancy between the effect on lipolysis of *in vitro* HILPDA overexpression versus *in vivo/in vitro Hilpda* deletion or knockdown remains unclear. It can be hypothesized that the lack of effect of HILPDA deletion or knockdown on any of the parameters studied may be because the loss of HILPDA is functionally compensated by other genes. One protein that might be suspected to be able to functionally compensate for HILPDA is G0S2, a small ~11 kDa protein that is known to inhibit ATGL activity and influence ATGL localization (35, 37). Remarkable similarities exist between HILPDA and G0S2: HILPDA and G0S2 are both highly sensitive PPAR targets, share striking sequence homology in their N-terminal domains, and increase hepatic lipid accumulation when overexpressed in liver (14, 17, 19, 35, 38). In our studies, however, knockdown of *G0s2* did not significantly alter isoproterenol-induced NEFA release in 3T3-L1 adipocytes, whether alone or in combination with knockdown of HILPDA, suggesting that G0S2 does not functionally compensate for HILPDA upon HILPDA knockdown.

Since HILPDA does not seem to be a direct inhibitor of adipocyte lipolysis—which is supported by our previous studies indicating that HILPDA does not directly inhibit HSL or ATGL—the modest inhibitory effect of HILPDA overexpression on NEFA release by adipocytes might reflect an indirect mechanism [19]. It may be speculated that HILPDA, similar to other lipid droplet-associated PPAR targets such as CIDEC and PLIN2, plays a role in lipid droplet coating, lipid droplet growth or in the regulation of interactions of lipid droplets with other organelles, such as the ER (5, 6, 39, 40). An important future research strategy should be to further examine the exact localization of HILPDA in adipocytes and hepatocytes and to study the potential influence of relevant stimuli on HILPDA localization. Also, efforts should be undertaken to identify binding partners of HILPDA. One possible binding partner for HILPDA is PLIN2, as PLIN2 and HILPDA are both sensitive PPAR targets, have a similar tissue expression profile, and co-localize on the surface of some, but not all lipid droplets (16, 18, 19).

Our data on the role of HILPDA in adipocytes add to the current literature on HILDA function. The observation that HILPDA is physically associated with lipid droplets in 3T3-L1 cells is in line with previous findings in lipid-loaded HeLa cells and primary hepatocytes (17, 18). In those studies, overexpression of *Hilpda* in HeLa cells promoted cellular lipid accumulation, whereas loss of *Hilpda* in hepatocytes reduced lipid accumulation and resulted in more yet smaller lipid droplets (17, 18). The reduced lipid accumulation was attributed to increased triglyceride lipolysis and tri-

glyceride turnover (17). Similarly, hepatic overexpression of HILPDA *in vivo* increased hepatic lipid accumulation, whereas liver-specific deletion of *Hilpda* lowered hepatic lipid accumulation, which was observed specifically in mice fed a chow diet but not in mice fed a high fat diet (17, 19). By contrast, we did not observe any effect of adipocyte-specific deletion of *Hilpda* in mice on adipocyte lipolysis, weight of adipose tissue depots or other relevant parameters. While the collective data thus point toward a role for HILPDA in lipid droplet morphology and lipid storage/mobilization, the exact molecular function of HILPDA remains to be elucidated.

An intriguing observation is that HILPDA is rapidly and very potently upregulated upon β-adrenergic stimulation. β-adrenergic signaling generally leads to activation of proteins that promote lipolysis (36). As discussed above, our data do not support such a function for HILPDA. The exact biological rationale and mechanisms underlying the upregulation of HILPDA mRNA by β-adrenergic stimulation require further investigation. Besides β-adrenergic stimulation, our data show that HILPDA expression is potently stimulated by treatment with PPARγ agonists. Previously, we demonstrated that HILPDA expression in liver is under transcriptional control of PPARα (19). In the current paper we show the presence of several conserved PPARγ binding sites in the vicinity of *HILPDA*, indicating that *HILPDA* is a bona-fide PPARγ target gene in mouse and human adipocytes. The preservation of three conserved PPARγ sites, one of which is also conserved at the sequence level, is remarkable given that only approximately 17% of all mouse adipocyte PPARγ binding sites (and 28% of PPARγ sites in the vicinity of adipocyte selective genes) are preserved in human adipocytes (41). Furthermore, we demonstrate the existence of a conserved PPARγ super-enhancer in association with the *HILPDA* locus in both mouse and human adipocytes.

Only a few genes and pathways are induced by both β-adrenergic signaling and PPARγ. Interestingly, a shared downstream effect of activation of PPARγ and β-adrenergic signaling is stimulation of adipocyte lipolysis (36, 42). Whereas β-adrenergic signaling stimulates lipolysis mainly by promoting the phosphorylation of key lipolytic proteins, PPARγ induces lipolysis at the level of gene transcription (36, 42–44). Whether β-adrenergic signaling and PPARγ interact in the regulation of HILPDA mRNA remains unclear. Using Phos-tag gels, we did not find any evidence that HILPDA becomes phosphorylated upon β-adrenergic stimulation. Overall, the combined regulation of HILPDA by β-adrenergic signaling and PPARγ in adipocytes hints at an important yet not fully understood role of HILPDA in adipocyte biology.

In conclusion, we demonstrate that HILPDA is well-expressed in adipocytes, where it is potently induced by PPARγ and β-adrenergic signaling. While overexpression of HILPDA modestly inhibited lipolysis in adipocytes, adipocyte-specific knockdown of HILPDA had minimal effects on intracellular lipolysis and other relevant metabolic parameters upon pharmacological β-adrenergic stimulation, cold exposure and fasting. Taken together, our data indicate that HILPDA does not appear to be a major physiological regulator of adipocyte lipolysis. Future studies should reveal the exact molecular function of HILPDA in adipocytes.

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SUPPLEMENTAL MATERIALS & METHODS

Animal experiments

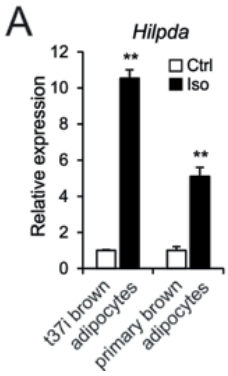
For the cold exposure experiment (**Supp. Figure 3**), 15- to 18-week old male *Hilpda*$^{flox/flox}$ and *Hilpda*$^{\Delta AT}$ littermates were exposed to a cold environmental temperature (4 ˚C) (n=10 per genotype) or a thermoneutral temperature (28 ˚C) (n=8 per genotype) for 10 days. After 10 days animals were anesthesized with isoflurane, blood was collected and following cervical dislocation several tissues were excised and snap-frozen. Food intake, body weight and body temperature were monitored daily. Body temperature of cold-exposed mice was monitored via read-out of transponders (IPTT-300) that were injected subcutaneously prior to the experiment (Bio Medic Data Systems, Seaford, USA).

Cell culture

T37i cells (P31-36; kind gift of Marc Lombès) were cultured in DMEM/F-12 (Gibco, Life Technologies, Blijswijk, the Netherlands), supplemented with 10% FBS and 1% P/S. Two days post-confluency, cell culture medium was supplemented with 112 ng/mL insulin and 2 nM T3 (Sigma-Aldrich) to induce differentiation. After 7 days of differentiation, cells were switched back to regular medium and used for experiments 2/3 days after (1).

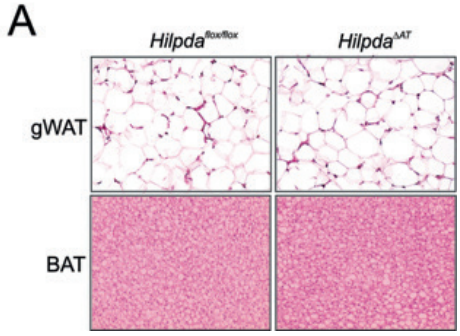
For isolation of primary brown adipocytes, BAT from 1-month-old pups was used. Tissues of 5-10 pups were pooled, minced with scissors and digested for 30 min in collagenase-containing medium at 37˚C (DMEM w/o serum, 2 mg/mL Collagenase type II, 2% BSA, 25 mM HEPES). After digestion, cells were passed through a 70 or 100 µM filter, mature adipocytes were discarded and cells were centrifuged at 800g for 5 min. Cells were re-suspended in differentiation medium (DMEM, 10% FBS, 20 nM insulin, 1 nM T3) and plated. Upon confluence, cells were treated with induction medium (differentiation medium supplemented with 0.5 mM IBMX, 0.5 µM dexamethasone, 0.125 mM indomethacin) for two days. After washing, cells were incubated in differentiation medium for another 5 to 7 days.

SUPPLEMENTAL FIGURES



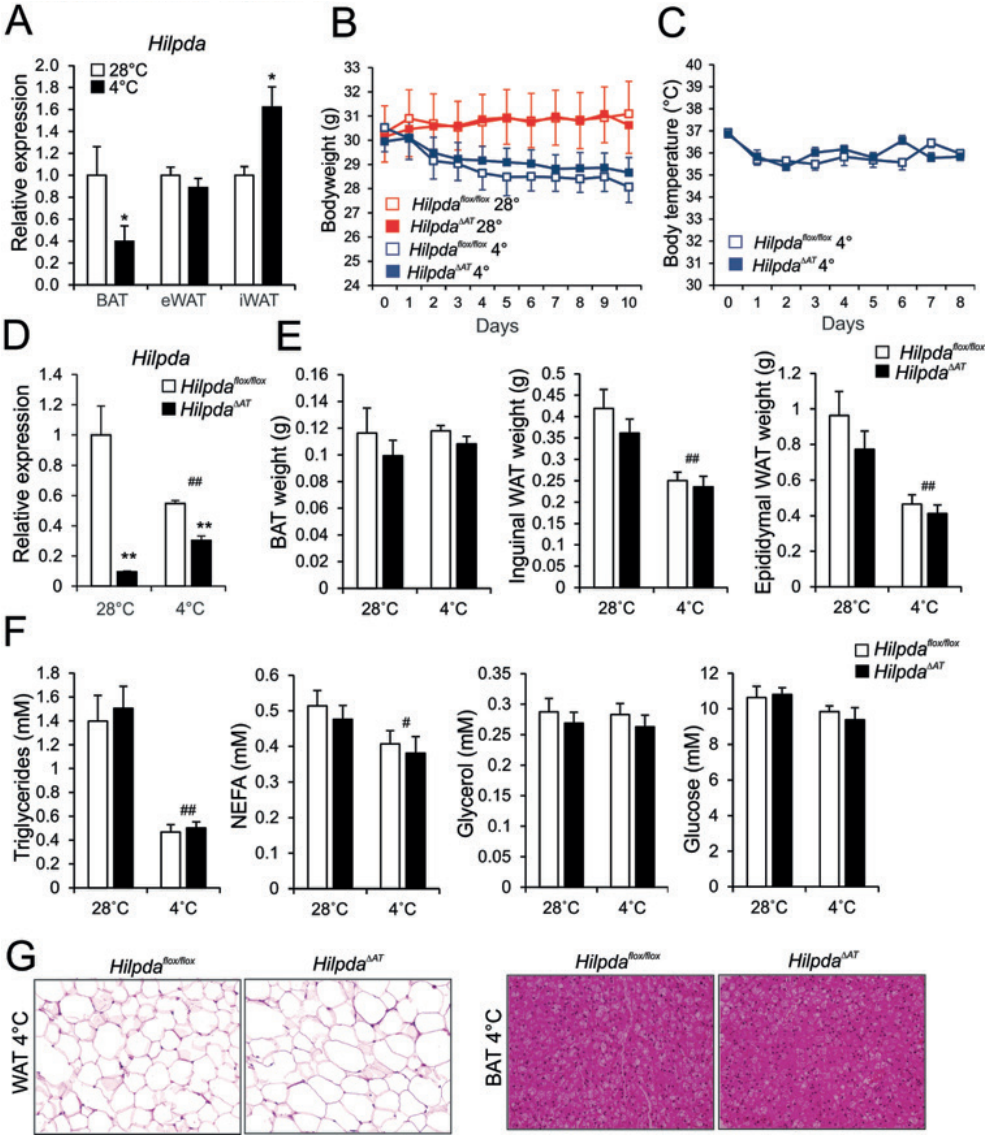
Supplemental Figure 1.

(A) HILPDA mRNA levels in fully differentiated T37i adipocytes (*left*) and primary brown adipocytes (*right*) exposed to control medium or 10 μM isoproterenol. Gene expression levels of control-treated adipocytes were set at 1. Data are mean ± SEM. Asterisks indicate significant differences according to Student's t-test relative to control-treated adipocytes (all figures); ** $p<0.01$, * $p<0.05$.



Supplemental Figure 2.

(A) Representative H&E stainings of epididymal WAT and BAT (7 μM) of male *Hilpda*$^{flox/flox}$ (n=6) and *Hilpda*$^{\Delta AT}$ (n=8) fasted for 24h.



Supplemental Figure 3.

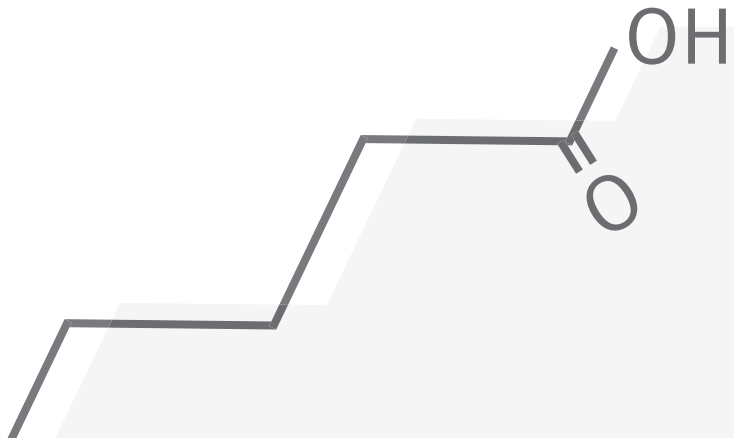
(A) HILPDA mRNA levels in BAT, epididymal WAT and inguinal WAT of C57BL/6 mice exposed to 4 °C or 28 °C for 10 days (n=8-10 animals per group). Gene expression levels of mice exposed to 28 °C were set at 1. (B) Body weight of *Hilpda*$^{flox/flox}$ and *Hilpda*$^{\Delta AT}$ mice exposed to 4 °C or 28 °C for 10 days (n=8-10 animals per group). (C) Body temperature of *Hilpda*$^{flox/flox}$ and *Hilpda*$^{\Delta AT}$ mice exposed to 4 °C or 28 °C for 10 days (n=10 animals per group). (D) HILPDA mRNA levels in BAT of *Hilpda*$^{flox/flox}$ and *Hilpda*$^{\Delta AT}$ mice exposed to 4 °C or 28 °C for 10 days (n=8-10 animals per group). Gene expression levels of *Hilpda*$^{flox/flox}$ mice exposed to 28 °C were set at 1. (E) Tissue weights of BAT, epididymal WAT and inguinal WAT of *Hilpda*$^{flox/flox}$ and *Hilpda*$^{\Delta AT}$ mice exposed to 4 °C or 28 °C for 10 days (n=8-10 animals per group) (in grams).

See next page for continuation.

(F) Triglycerides, non-esterified fatty acids (NEFA), glucose and glycerol levels in plasma of *Hilpda*$^{flox/flox}$ and *Hilpda*$^{\Delta AT}$ mice exposed to 4 °C or 28 °C for 10 days (n=8-10 animals per group). (G) Representative H&E stainings of eWAT and BAT of *Hilpda*$^{flox/flox}$ and *Hilpda*$^{\Delta AT}$ mice exposed to 4°C or 28°C for 10 days. Data are mean ± SEM. Asterisks indicate significant differences according to student's t-test (*) relative to mice exposed to thermoneutrality (Supp. Figure 3A). Asteriks or hash-tags indicate significant differences according to two-way ANOVA (#) followed by Tukey's HD post-hoc test (*) relative to mice exposed to thermoneutrality (Supp. Figure 3D, 3E, 3F) (#) and relative to *Hilpda*$^{flox/flox}$ mice (Supp. Figure 3D, 3E, 3F) (*); ** or ## $p<0.01$, * or # $p<0.05$.

9

GENERAL DISCUSSION



Over a century ago, Whitehead's observation that triglycerides are unable to cross cellular membranes in their unhydrolyzed form highlighted the crucial role of lipolysis for general metabolism (1). Since then, considerable progress has been made in identifying the key players of gastro-intestinal lipolysis, intravascular lipolysis and intracellular lipolysis (2–5). Notwithstanding the significant advances made, this thesis highlights that many of the molecular mechanisms underlying the physiological regulation of lipolysis had and have not been fully characterized. In the previous chapters, we have unveiled and clarified the functions of the peroxisomal-proliferator activated receptor (PPAR)-target genes angiopoietin-like 4 (*ANGPTL4*) and hypoxia-inducible lipid droplet-associated (*HILPDA*) in the regulation of extracellular and intracellular lipolysis, respectively. We uncover ANGPTL4 as the main regulator of lipoprotein lipase (LPL) activity in the brown adipose tissue (BAT) during sustained cold and establish a novel mechanism by which ANGPTL4 inhibits LPL activity in adipocytes. In contrast, our data do not support a major role for HILPDA in the regulation of intracellular lipolysis in adipocytes, in apparent contrast to a study that identified a role for HILPDA in intracellular lipolysis in the liver (6).

EXTRACELLULAR LIPOLYSIS

ANGPTL4 regulates lipid partitioning during sustained cold

Upon cold exposure, the sympathetic nervous system activates BAT to promote the combustion of large amounts of fatty acids to generate heat (7). To replenish intracellular lipid stores and to provide fatty acids for combustion, lipid fuel is specifically directed towards BAT and away from other metabolic organs following cold exposure. Indeed, cold exposure promotes the release of fatty acids from the white adipose tissue (WAT) and enhances the production of very low-density lipoproteins (VLDLs) by the liver (8, 9). At the same time, the activity of LPL in BAT is dramatically increased upon cold exposure, enabling the uptake of fatty acids derived from triglyceride-rich lipoproteins (TRLs) such as VLDLs into BAT (10–14). In **Chapter 4**, we show that the increase in LPL activity and associated TRL-derived fatty acid uptake is primarily mediated by the marked downregulation of the LPL inhibitor ANGPTL4 in BAT upon sustained cold. By contrast, the expression of ANGPTL4 in the white adipose tissue (WAT) was increased rather than decreased following cold exposure, thereby limiting WAT LPL activity and the uptake of TRL-derived fatty acids into WAT (**Figure 1**). Our data thus identify ANGPTL4 as an integral component of the physiological response that directs lipid fuel towards BAT during sustained cold, as ANGPTL4 promotes the uptake of TRL-derived fatty acids into BAT and prevents their uptake into WAT. However, several aspects merit further investigation.

First of all, the mechanisms that underlie the opposite regulation of ANGPTL4 in BAT and WAT in response to sustained cold are of great interest. Many of the metabolic changes in response to cold exposure are directly orchestrated by the sympathetic nervous system *via* the activation of adrenergic receptors by noradrenaline (15, 16). Indeed, repeated injections with the β3-adrenergic agonist CL316,243 potently activate BAT and promote TRL-derived fatty acid uptake into BAT (15). Our *in vitro* studies, however, surprisingly indicated that treatment with β-adrenergic agonists in-

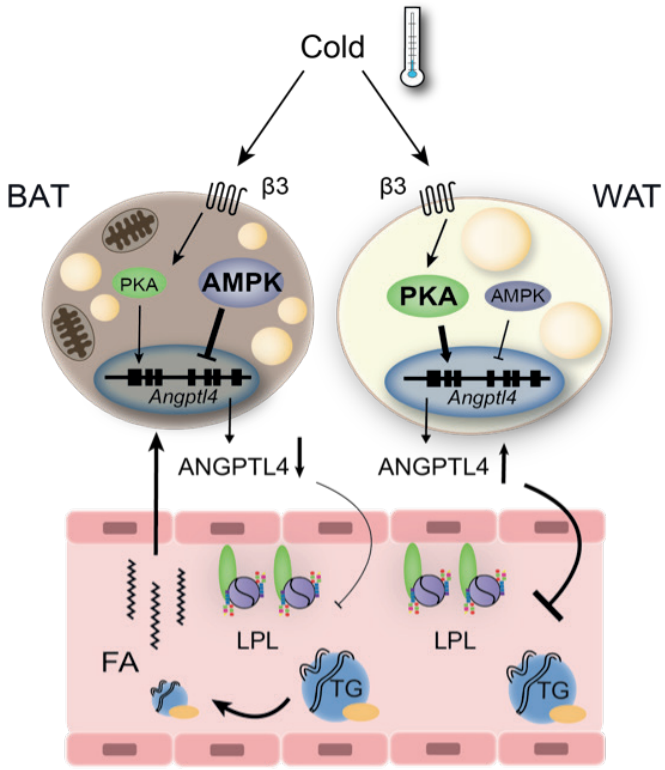


Figure 1. Schematic model of the role of ANGPTL4 in the shuttling of lipids towards BAT during sustained cold. Regulation of ANGPTL4 in BAT and WAT is an integral part of the physiological response that re-directs fatty acids from circulating TRLs, such as VLDL and chylomicrons, preferentially to the BAT during cold exposure. Down-regulation of ANGPTL4 in BAT upon cold exposure facilitates the increase in BAT LPL activity, which results in an increased uptake of TRL-derived fatty acids. Down-regulation of ANGPTL4 in BAT is likely due to specific activation of AMPK in BAT, but not WAT. On the other hand, the up-regulation of ANGPTL4 in WAT upon cold exposure limits LPL activity in WAT and the concomitant uptake of TRL-derived fatty acids into WAT. The increase in ANGPTL4 expression in WAT is likely mediated *via* activation of the β-adrenergic signalling pathway

creased the expression of ANGPTL4 in both white and brown adipocytes (**Chapter 4**). Instead, we propose that the opposite regulation of ANGPTL4 in BAT and WAT upon sustained cold exposure is mediated by the differential activation of AMP-activated protein kinase (AMPK) in BAT and WAT (**Chapter 4**). AMPK is a well-conserved energy sensing kinase that is composed of a catalytic α subunit and regulatory β and γ subunits that together form a heterotrimeric complex (17). AMPK is activated upon a reduction in cellular energy status and phosphorylates downstream targets that inhibit energy-consuming processes and/or stimulate energy-generating pathways such as fatty acid oxidation and the uptake of TRL-derived fatty acids from the circulation (17–19). Indeed, AMPK activation has been linked to a local increase in

LPL activity *via* the repression of ANGPTL4 expression (20, 21) (**Chapter 4**). Given AMPK's function as an energy sensor, it is perhaps not surprising that we and others have found that AMPK is progressively activated in the energy-combusting BAT and only to a lesser extent in WAT during sustained cold (18, 22, 23) (**Chapter 4**). The underlying mechanisms for the specific activation of AMPK in BAT upon cold exposure remain, however, unclear; AMPK in BAT may be either directly activated by β-adrenergic signaling or indirectly activated due to a decrease in cellular energy status (24). Possibly, the elevated expression of the catalytic AMPKα2 isoform in BAT but not WAT may explain part of the tissue-specific activation of AMPK in BAT upon cold exposure (**Chapter 4**). In any case, our findings highlight that the cold-induced activation of AMPK in BAT is indispensable for the effective shuttling of lipid fuel towards BAT during cold exposure *via* the negative regulation of ANGPTL4 in BAT. Future studies should further clarify the role of AMPK in the uptake of lipid fuel into BAT and investigate the relative contributions of the catalytic AMPKα1 and AMPKα2 isoforms (22). Also, the mechanisms by which AMPK represses ANGPTL4 expression require further study.

A second point of importance is the moderate increase in LPL activity levels that we observed in BAT of *Angptl4*$^{-/-}$ mice in response to sustained cold. This observation implies that besides ANGPTL4 additional mechanisms contribute to the cold-induced increase in BAT LPL activity levels. Possibly, changes in BAT LPL activity upon cold exposure are regulated by ANGPTL3 and ANGPTL8, two proteins that share a common structure with ANGPTL4 and are known to potently inhibit LPL activity (25–29). However, literature indicates that the physiological regulation of ANGPTL3 is limited, which likely precludes a functional role for ANGPTL3 in the cold-induced increase of BAT LPL activity levels in *Angptl4*$^{-/-}$ mice (27, 30). Similarly, the reported induction of ANGPTL8 expression upon cold exposure in BAT is in seeming conflict with the increased activity of LPL in BAT (31). A more reasonable explanation is that BAT LPL activity is regulated at the transcriptional level during sustained cold, as we observed a significant increase in *Lpl* mRNA levels in BAT in all cold-exposed mice (**Chapter 4**). Indeed, several studies suggest that BAT LPL mRNA levels are directly induced by the cold-induced, noradrenaline-mediated activation of β_3-adrenergic receptors in BAT (32–34). In support, short-term cold exposure significantly increases BAT LPL activity levels *via* an increase in LPL mRNA expression and independent of changes in ANGPTL4 expression (**Chapter 4**) (12, 13, 34–36). Currently, the relative contributions of transcriptional regulation and ANGPTL4-mediated post-translational regulation to the dynamic changes in BAT LPL activity during specific periods of cold exposure are not clear. To gain further insight, ideally, an elaborate cold exposure time-course experiment with wild-type and *Angptl4*$^{-/-}$ mice should be performed.

Besides TRL-derived fatty acids, circulating glucose and fatty acids represent an important energy source for BAT during cold exposure (7). In fact, we propose in **Chapter 4** that the lower uptake of TRL-derived fatty acids into BAT of *Angptl4*-transgenic mice may be compensated by the increased uptake of free fatty acids and glucose. This suggestion implies that glucose and fatty acids should also be preferentially shuttled to and/or taken up by BAT during cold exposure, similar to TRL-derived fatty acids. However, as opposed to TRLs, glucose and fatty acids can

directly cross cellular membranes and their uptake is therefore largely determined by the localization and abundance of transporters that shuttle between intracellular compartments and the cell membrane (37–39). Yet, there is some data to suggest that mechanisms promoting the specific uptake of glucose and fatty acids into BAT following cold exposure exist. For example, the expression of the glucose transporters GLUT1 and GLUT4 significantly increases in BAT upon cold exposure, which together with the purported cold-induced insulin resistance in peripheral tissues might promote the uptake of glucose specifically into BAT (13, 40). Furthermore, the glucose transporter GLUT1 (but not GLUT4) translocates to the brown adipocyte cell surface upon β-adrenergic stimulation, although no inhibitory or stimulatory protein akin to ANGPTL4 has currently been identified to regulate this translocation (38, 41). The uptake of the circulating free fatty acids released from WAT and of the TRL-derived fatty acids is primarily mediated by the fatty acid transporter cluster of differentiation 36 (CD36) in BAT (13, 42). Similar to GLUT1 and GLUT4, the expression of CD36 in BAT rapidly increases upon cold exposure (13). The impact of cold exposure on CD36 translocation, as well as on the expression levels of CD36 in other tissues remains, however, unclear. Given the crucial role of CD36 in the uptake of (TRL-derived) fatty acids, it would be of great interest to identify the regulatory mechanisms driving the expression of CD36 in endothelial cells and brown adipocytes, as well as the mechanisms underlying a possible translocation of CD36 to the cell surface upon cold exposure (43). Similarly, the potential changes in the expression and translocation of glucose and fatty acid transporters in other metabolically active tissues during cold exposure would merit further investigation.

ANGPTL4 as the master regulator of intravascular lipolysis

The identification of ANGPTL4 as the main regulator of LPL activity in BAT during sustained cold further establishes ANGPTL4 as the master regulator of tissue LPL activity during different physiological conditions. Indeed, recent studies convincingly demonstrated that the decrease in LPL activity in WAT observed during fasting is mediated by the increased expression of ANGPTL4 (originally called fasting-induced adipose factor) in WAT (44–46). Similarly, changes in the expression of muscle ANGPTL4 coordinate LPL-dependent uptake of fatty acids into exercising and nonexercising skeletal muscle during acute exercise (21). Together, these data point to a scenario wherein local ANGPTL4 coordinates local LPL-mediated hydrolysis of plasma TRLs and subsequent fatty acid uptake in response to changes in cellular fuel demand and lipid availability (21, 44, 47) (**Chapter 4**). By safeguarding the proper distribution of plasma triglycerides over various tissues during different physiological states, ANGPTL4 assures that tissues take up adequate amounts of fatty acids for rapid oxidation or storage, while preventing excess lipid uptake and cellular lipotoxicity. Several key questions regarding the physiology of ANGPTL4 remain, however, to be answered.

A local versus circulatory function for ANGPTL4

An intriguing question is how the local, tissue-specific regulation of LPL activity by ANGPTL4 can be reconciled with the non-specific regulation of endothelial LPL activity by circulating ANGPTL4. Indeed, on the one hand, multiple *in vitro* experiments that investigated the effects of secreted ANGPTL4, recombinant ANGPTL4 and anti-ANGPTL4 antibodies on extracellular LPL activity suggest that freshly secreted ANGPTL4 may act on LPL within the subendothelial spaces (48–50). Moreover, we propose in **Chapter 5** that ANGPTL4 inhibits LPL intracellularly in the adipose tissue. On the other hand, ANGPTL4 is abundantly present in the circulation where it can potently inhibit endothelial-bound LPL, as illustrated by the impact of either anti-ANGPTL4 antibodies or recombinant ANGPTL4 on plasma triglycerides and plasma LPL activity levels in experimental animals (26, 51–53). Most conceivably, the endocrine and local functions of ANGPTL4 are complementary and together assure that LPL activity is effectively regulated in a variety of tissues that differ in their overall physiology and energy requirements. Such complementary mechanisms are not uncommon in lipid metabolism, as illustrated by the hepatic protein proprotein convertase subtilisin/kexin 9 (PCSK9). PCSK9 promotes the degradation of the low-density lipoprotein receptor (LDLR) in the liver by acting upon the LDLR within the hepatocyte and on the cell surface, but also promotes the degradation of the related VLDL receptor in distal tissues upon secretion into the circulation (54). For ANGPTL4, its function (local or endocrine) may be determined by the relative expression levels of ANGPTL4 and LPL in a particular tissue (**Figure 2**). The low expression of LPL in the liver makes it conceivable that liver-expressed ANGPTL4 is primarily secreted into the circulation to act upon LPL that is either captured on the liver endothelium or bound to the endothelium in distal tissues (46, 55, 56). It should be noted, however, that a recent paper suggested that liver-expressed LPL might significantly impact triglyceride metabolism (57) (**Chapter 4**). By contrast, in the adipose tissue the expression levels of LPL and ANGPTL4 are high and ample data from our lab as well as others indicate that the physiological regulation of adipose tissue ANGPTL4 controls adipose tissue LPL activity during cold exposure and fasting (44, 58) (**Chapter 4, Chapter 5**). To what extent ANGPTL4 is secreted from the adipose tissue is, however, not clear. In the heart, the expression of ANGPTL4 is low compared to the expression of LPL (**Chapter 4**). Given that the energy requirements of the heart are primarily met by the oxidation of (TRL-derived) fatty acids, the physiological changes in the activity of heart LPL are tightly regulated by the binding affinity of LPL to the heart endothelium as well as a transcription-regulated factor (58–61). This transcription-regulated factor is, however, unlikely to be ANGPTL4, as during fasting the increase in cardiac LPL activity is accompagnied by an increase in cardiac ANGPTL4 expression (46, 58–61). By contrast, ANGPTL4 is capable of regulating LPL activity in the heart, as highlighted by the reduced LPL activity in the heart of mice with cardiac-specific over-expression of *Angptl4* (62). Similarly, following a post-prandial lipid challenge, cardiac ANGPTL4 expression was significantly increased and protected the heart from fatty acid-induced oxidative stress (47). Collectively, these data suggest that depending on the tissue, a) ANGPTL4 is secreted into the circulation, where it inhibits endothelial LPL (liver), b) ANGPTL4 regulates

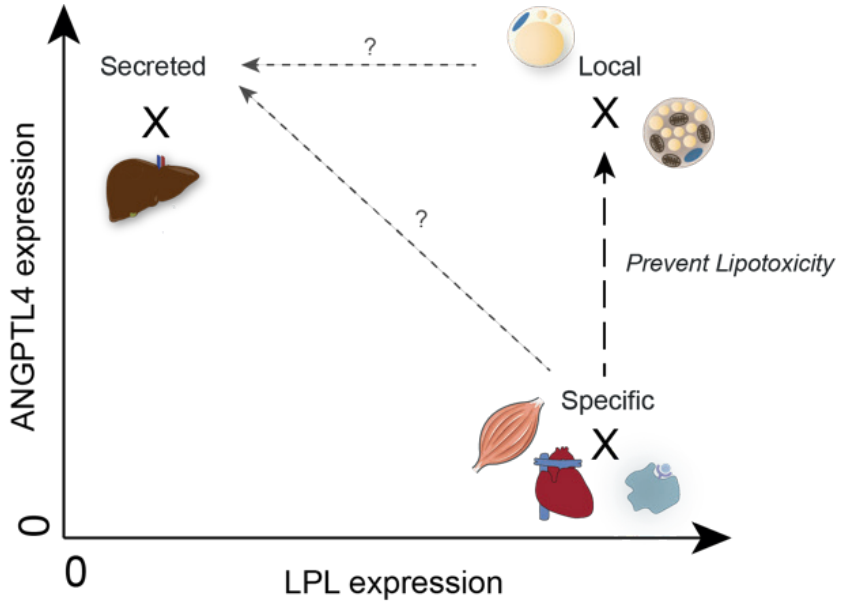


Figure 2. Model of the local versus circulatory role of ANGPTL4.

Dependent on the relative expression levels of ANGPTL4 and LPL in the different tissues, the function of ANGPTL4 may differ. In liver, which has a comparatively low expression of LPL, it conceivable that liver-expressed ANGPTL4 is primarily secreted into the circulation to act upon LPL that is either captured on the liver endothelium or bound to the endothelium in distal tissues (46, 55, 56). By contrast, in the adipose tissue (brown and white) the expression levels of LPL and ANGPTL4 are comparatively high, and multiple lines of evidence suggest that ANGPTL4 regulates the physiological changes in LPL activity in BAT and WAT following cold exposure or fasting, respectively. In heart, but also muscle and macrophages, the expression of ANGPTL4 is low compared to the expression of LPL. In these tissues, it is unlikely that ANGPTL4 is responsible for the physiological regulation of LPL. However, the expression of ANGPTL4 may be rapidly induced in these tissues upon fatty acid overload or another physiological stressor to prevent, for example, lipotoxicity (47, 203).

the physiological changes in LPL activity (BAT, WAT), c) a significant induction of ANGPTL4 expression prevents lipotoxicity during specific conditions (heart, muscle, macrophages) (see **Figure 2** for further details). The overall picture is, however, far from complete and many aspects warrant further study. For example, it is currently unclear what the exact physiological function of circulating ANGPTL4 is and what tissues contribute to circulating ANGPTL4. Also, the extent to which ANGPTL4's function depends on inherent differences in tissue LPL biology remains to be determined. Turnover of LPL protein in the heart is, for example, slower than in adipocytes and LPL mRNA species differ between heart and adipocytes (63, 64). Similarly, a study that compared LPL secretion from muscle and adipose tissue indicated that muscle mostly released active LPL into plasma, while adipose tissue mainly released inactive LPL (65). Future studies would greatly benefit from more in-depth knowledge on tissue-specific differences in LPL biology, as well as the generation of cell type-specific *Angptl4* knockout mice. Cell type-specific *Angptl4* knockout mice could

also resolve other burning questions related to ANGPTL4 physiology, including what cells are responsible for the severe ascites observed in full-body *Angptl4* knockout mice fed a saturated fat diet (66).

The respective functions of ANGPTL3, ANGPTL4 and ANGPTL8

Another important question is how the physiological function of ANGPTL4 relates to the functions of its family members ANGPTL3 and ANGPTL8. ANGPTL3, ANGPTL4 and ANGPTL8 share a common architecture and influence plasma triglyceride levels by impacting LPL activity, yet their tissue distribution, regulation and mode of action differ substantially (25–27). ANGPTL3 is mainly expressed in liver and is abundantly secreted into the circulation, whereas ANGPTL4 and ANGPTL8 are more ubiquitously expressed (25, 27, 46) (**Chapter 4**). Similarly, while physiological regulation of ANGPTL3 is limited, the expression levels of ANGPTL4 and ANGPTL8 are potently, although oppositely, regulated during physiological conditions such as fasting/refeeding and cold exposure (30, 31, 67) (**Chapter 4**). These data suggest that ANGPTL3, ANGPTL4 and ANGPTL8 regulate LPL activity during different physiological situations and/or in different tissues. Indeed, the impact of ANGPTL3 and ANGPTL8 (*feeding-induced*) on plasma triglyceride levels is most pronounced upon refeeding, while the impact of ANGPTL4 (*fasting-induced*) on plasma triglyceride levels is more pronounced during fasting (30, 52, 67). Given the limited physiological regulation of ANGPTL3, it has been proposed that ANGPTL3 collaborates with ANGPTL8 during refeeding to inhibit LPL activity and TRL-derived fatty acid uptake specifically in oxidative tissues and to promote the replenishment of WAT (25, 30, 67, 68) (**Chapter 2**). Such a role for ANGPTL3 and ANGPTL8 would fit with the model wherein ANGPTL4 is responsible for the physiological regulation of LPL activity in the adipose tissue depots, but not in oxidative tissues (**Figure 2**). Intriguingly, it had already been suggested in 1983 that the physiological regulation of adipose tissue and heart LPL activity is under independent genetic control (69). Together, these data suggest that ANGPTL4 and ANGPTL3/ANGPTL8 have complementary functions that ensure the adequate distrubtion of circulating triglycerides during different physiological conditions. However, the circulating nature ANGPTL3 and ANGPTL8 questions how these proteins are able to specifically inhibit LPL in oxidative tissues during refeeding. Similarly, the model is challenged by the observation that the expression of ANGPTL8 and the activity of LPL in oxidative tissues simultaneously increase upon cold exposure (9, 70, 71) (unpublished observations). Evidently, further studies are needed to clarify the respective functions ANGPTL3, ANGPTL4 and ANGPTL8 in the regulation of LPL activity. Such investigations would be helped by the generation of mice knockout for multiple ANGPTL proteins and/or tissue-specific knockout mice.

Mechanism of ANGPTL4-mediated inhibition of LPL

In line with a more local regulatory role for ANGPTL4 in the adipose tissue, we propose in **Chapter 5** that ANGPTL4 inhibits the activity of LPL intracellularly in adipocytes. Intracellular regulation of LPL by ANGPTL4 complements the established regulation of LPL by ANGPTL4 on the endothelium and in the subendothelial spaces

and may account for a substantial part of the physiological regulation of LPL activity upon fasting and cold exposure in WAT and BAT (44, 51, 52, 72) (**Chapter 5**). ANGPTL4 inhibits LPL activity intracellularly by promoting the degradation of LPL, possibly *via* the lysosomes (**Chapter 5**). Indeed, lysosomal degradation may account for the rapid degradation of up to 80% of the newly synthesized LPL in adipocytes (73, 74). The degradation of LPL is most likely initiated in the *trans*-Golgi or beyond, as ANGPTL4 solely impacts mature-glycosylated LPL (**Chapter 5**). From the (*trans*-) Golgi, inactivated LPL may be send towards the endosomes to eventually end up in the lysosomes, possibly as part of the so-called Golgi protein quality control system. This quality control system is initiated upon protein aggregation, oligomerization and maturation, depends on nutrient availability and surveys secreted proteins, such as LPL, whose mature conformation is acquired following post-translational modifications or assembly in the Golgi (75, 76). Indeed, a mutant form of human LPL that failed to be secreted was efficiently targeted directly from the Golgi to the lysosomes (77). Moreover, the endosomal sorting receptor Sortilin has been demonstrated to target LPL for degradation (78). Alternative pathways by which LPL may be targeted to the lysosomes include the endocytosis of cell surface-bound LPL or (chaperone-mediated) autophagy of inactivated LPL (73, 79–81). Lastly, we cannot formally exclude the involvement of proteasomal degradation, although we and others did not observe any impact of proteasomal inhibitors on LPL degradation (unpublished observations) (82). Future studies into the fate of the LPL molecules targeted by ANGPTL4 could employ advanced microscopy techniques, such as fluorescence resonance energy transfer (FRET), fluorescence time-lapse imaging and cellular co-localization of LPL with markers of the Golgi (GM130), early endosomes (RAB5), late endosomes (RAB7) and/or autophagosomes (LC3) (83). Alternatively, one could knock down key components of the different degradatory pathways in adipocytes, alone or in combination with ANGPTL4 (*e.g.* ATG5 and ATG7 (*autophagy*), RAB5 and RAB7 (*endosomes*), or the sorting receptors SORLA and Sortilin) (78, 79, 81). Another question that should be addressed is whether the intracellular degradation of LPL by ANGPTL4 is limited to the adipose tissue or occurs also in other tissues. Possibly, a high expression level of ANGPTL4, such as in the adipose tissue, is required for intracellular degradation of LPL to occur, a level that may only be reached in other tissues upon a significant increase in ANGPTL4 expression (**Figure 2**). In support, we did not observe intracellular regulation of LPL in the hearts of fed and fasted wild-type and *Angptl4*$^{-/-}$ mice (**Chapter 5**).

Most likely, the ANGPTL4-mediated intracellular degradation of LPL is secondary to the main action of ANGPTL4. It is surprising to note that, although ANGPTL4 has been known to inhibit LPL for nearly 15 years, the exact biochemical mechanism by which ANGPTL4 acts upon LPL remains hotly debated (26). A relatively short amino acid sequence close to the amino-terminal of ANGPTL4 inhibits LPL and, as a consequence, only amino-terminal ANGPTL4 and full-length ANGPTL4 impact LPL activity (28, 84–87). In contrast, carboxyl-terminal ANGPTL4 does not appear to impact lipid metabolism (88). In 2006, Sukonina and colleagues proposed the now widely accepted dimer-dissociation model in which amino-terminal ANGPTL4 converts the catalytically active, dimeric form of LPL to catalytically inactive monomers in an irreversible manner (89). This model was recently challenged

by the observation that LPL could regain activity upon detachment of ANGPTL4, suggesting that ANGPTL4 is a reversible, non-competitive inhibitor of LPL (90). These purported mechanisms are, however, based on data from biochemical *in vitro* techniques and the exact mechanisms by which ANGPTL4 destabilizes/inhibits LPL *in vivo* remain to be elucidated. One possible consequence of the destabilization of LPL by ANGPTL4 *in vivo* may be that LPL's binding affinity towards LPL-stabilizing proteins becomes altered. It is well-established that LPL rapidly loses its catalytic activity unless stabilized by lipase maturation factor 1 (LMF1) in the endoplasmic reticulum (ER), by heparan sulphate proteoglycans (HSPGs) on the cell surface, and by glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) on the endothelium (91, 92). Indeed, ANGPTL4 has been proposed to lower the affinity of LPL to GPIHBP1 (93). Of note, no (chaperoning) protein has so far been identified for stabilization of LPL during its intracellular transport towards the cell surface. Another potential consequence of the destabilization of LPL by ANGPTL4 *in vivo* is proposed in **Chapter 6**, where ANGPTL4 is shown to promote the cleavage of LPL by members of the PCSK family, serine endopeptidases that either activate or inactivate proteins (94, 95). Most conceivably, destabilization of LPL by ANGPTL4 renders LPL more susceptible for PCSK-mediated cleavage, which likely ensues the inactivation and subsequent degradation of LPL, as was previously observed for the related endothelial lipase (96, 97). In support, mutations in the human *LPL* gene that are characterized by enhanced PCSK-mediated cleavage of LPL result in severe chylomicronaemia (98). However, to better understand the impact of ANGPTL4 on either LPL's interaction with stabilizing proteins or on PCSK-mediated cleavage of LPL, the exact biochemical mechanism by which ANGPTL4 acts on LPL first needs to be clarified. Possibly, techniques similar to those used to study the impact of GPIHBP1 on LPL stability may be employed (99).

An intriguing analogy between the multi-level regulation of LPL by ANGPTL4 and the mechanisms of regulation of other key proteins in lipid metabolism can be made. To regulate plasma cholesterol levels, the amount of LDLR on the hepatocyte surface is tightly controlled by the protein PCSK9. PCSK9 prevents the recycling of internalized LDLR from the endosomes to the cell surface by binding to cell surface LDLR, thereby targeting LDLR for lysosomal degradation. However, PCSK9 acts also on the LDLR in the *trans*-Golgi from where the LDLR can be directly targeted for degradation in the lysosomes (100–102). Similarly, apolipoprotein B (APOB), the major non-exchangeable apolipoprotein on VLDL, is subjected to extensive post-translational regulation that includes a late-stage degradatory process in a post-ER compartment (103–105). Conceivably, the induction of intracellular degradation *via* a post-ER, post-translational mechanism is a common mechanism by which levels of proteins crucial for lipid metabolism are controlled.

Clinical perspectives of ANGPTL4

Risk for cardiovascular disease, the current leading cause of death worldwide, is determined by a plethora of factors, including plasma lipoprotein levels (106). The view on the role of TRLs in the development of cardiovascular disease has fluctuated over the past decades, but recent epidemiological and genetic studies unequivocally indi-

cate that elevated plasma TRLs strongly predict the risk for cardiovascular disease and all-cause mortality (106–109). In order to promote cardiovascular disease, TRLs must enter the arterial intima, the location of atherosclerotic plaque development. Influx of lipoproteins into the arterial wall is largely determined by lipoprotein size and, as a result, large TRL particles such as chylomicrons are incapable of entering the intima (explaining why *LPL*-deficient individuals with severe chylomicronaemia are largely protected against cardiovascular disease) (110–112). In contrast, medium-sized TRLs that are generated upon LPL-mediated hydrolysis (*e.g.* chylomicron remnants) are capable of entering the arterial wall, albeit at lower speed than smaller lipoproteins such as low-density lipoproteins (LDLs) (110). Upon entrance and possible trapping of TRLs in the arterial wall, LPL at the endothelial surface or within the arterial wall may hydrolyse the incorporated triglycerides to yield fatty acids that can promote local and possibly systemic inflammation (107, 113). Alternatively, TRL particles may be directly taken up by macrophages in the arterial wall *via* the VLDL receptor in concert with LPL, resulting in foam cell formation– a hallmark of the development of atherosclerosis (114–116). Given the postulated roles of LPL in TRL-mediated atherosclerotic plaque development, it is perhaps not suprising that mendelian randomization studies revealed that deleterious or loss-of-function variants in either the *LPL* gene itself or in genes that encode regulators of LPL, such as *APOA5*, *APOC3, ANGPTL3* and *ANGPTL4*, impact risk for cardiovascular disease (106, 117–120). More specifically, these genetic data suggest that a reduced clearance rate of TRLs promotes the risk for cardiovascular disease (*e.g.* due to deleterious variants in *APOA5* and *LPL*), whereas the increased clearance of TRLs protects against cardiovascular disease (*e.g.* due to inactivating variants in *ANGPTL3*, *ANGPTL4* and *APOC3*) (106, 117–120).

The re-appreciation of TRLs as contributors to cardiovascular disease risk has renewed the interest of pharmaceutical companies in regulators of LPL, such as ANGPTL4, as potential therapeutic targets to reduce cardiovascular disease risk. Currently, ample data support a role for ANGPTL4 in the inhibition of LPL activity in humans: 1) the E40K inactivating variant of *ANGPTL4* lowers circulating triglyceride levels (68, 118, 121–124), 2) *in vitro* human ANGPTL4 inhibits human LPL activity (50, 86, 125), 3) treatment of mice and non-primate monkeys with an antibody against human ANGPTL4 potently lowers plasma triglyceride levels (53). In contrast, no positive association has been found between plasma ANGPTL4 and plasma triglycerides in human subjects, possibly because the employed ELISAs detect carboxyl-terminal ANGPTL4 and not the LPL-inhibiting amino-terminal domain (50, 126–128). More detailed physiological studies on the role of ANGPTL4 in humans are, however, scarce. Indeed, it is unclear whether the high expression of ANGPTL4 in the liver of humans as compared to mouse liver signifies a more prominent circulatory function for human ANGPTL4 (46, 55). Similarly, no studies have investigated the association of the expression of ANGPTL4 in human adipose tissue and adipose tissue LPL activity upon fasting and refeeding (44, 129). From a clinical perspective, however, the most important question to resolve is whether the loss or inhibition of ANGPTL4 in humans will result in the lymphadenopathy and severe ascites that was observed in *Angptl4*$^{-/-}$ mice fed a diet high in saturated fat (51, 53, 66). So far, there are no indications that human carriers of the inactivating E40K variant in the *ANGPTL4*

gene present with increased rates of ascites, peritonitis or abdominal discomfort compared to the general population, but more in-depth investigations of these subjects are required (53). Further studies on the physiological role of ANGPTL4 in humans will need to indicate whether ANGPTL4 might indeed be a safe therapeutic target to lower plasma triglycerides and the risk for cardiovascular disease. If so, ANGPTL4 may be the second member of the ANGPTL family that is targeted to lower cardiovascular disease risk, with an anti-sense oligonucleotide and a monoclonal antibody against ANGPTL3 currently being in Phase 2 clinical trials (130, 131).

Gastro-intestinal lipolysis and the function of intestinal ANGPTL4

The expression of ANGPTL4 in the human intestine is relatively high (55). However, despite the observation that enterocytes take up circulating lipids at their basolateral membrane, there are currently no indications that LPL is involved in intravascular lipolysis in the intestine, suggesting that the primary function of intestinal ANGPTL4 is likely not the local inhibition of LPL (132). It has been hypothesized that intestinal ANGPTL4 serves an endocrine function by inhibiting LPL in distal tissues, but so far a direct link remains to be established (133, 134). A clue about the function of intestinal ANGPTL4 came from a recent paper that identified ANGPTL4 as a potent inhibitor of pancreatic lipase and, thus, implicated ANGPTL4 in gastro-intestinal lipolysis (135). In **Chapter 7**, we extend these observations and demonstrate that ANGPTL4 impacts intestinal bile acid uptake and gut microbial composition. More specifically, deletion of *Angptl4* in mice significantly attentuated the increase in plasma bile acid levels induced by dietary taurocholic acid supplementation, as compared to wild-type mice, *via* a reduction in bile acid uptake in the ileum (**Chapter 7**). At the same time, gut microbial composition was significantly different between wild-type and *Angptl4*$^{-/-}$ mice (**Chapter 7**).

An intricate crosstalk exists between bile acids and the gut microbial composition. Bile acids impact the composition of the gut microbiota, while the gut microbiota deconjugate bile acids and convert primary bile acids to secondary bile acids by 7α-dehydroxylation (136–139). As a result, it is challenging to identify the origin of the differences in gut microbial composition and bile acid uptake between the wild-type and the *Angptl4*$^{-/-}$ mice. Given the established impact of ANGPTL4 on gastro-intestinal lipolysis, it may be hypothesized that the impact of *Angptl4* genotype on bile acid uptake and gut microbial composition originates from an altered intestinal dietary fat metabolism. Indeed, multiple studies indicate that elevated levels of lipids in the ileum and colon significantly impact the gut microbial composition (140, 141). Furthermore, the treatment of human subjects with the lipase inhibitor orlistat altered gut microbial composition and significantly reduced the secretion of bile acids in the faeces (142, 143). One may speculate that the origin of differences in gut microbial composition between wild-type and *Angptl4*$^{-/-}$ mice may commence during the suckling period when pups receive a high amount of lipid-containing milk. Future studies should further establish the influence of intestinal ANGPTL4 on the bile acids – gut microbes – dietary fat crosstalk by, for example, modulating the gut microbiota of wild-type and *Angptl4*$^{-/-}$ mice with antibiotics.

An intriguing question relates to the origin of intestinal ANGPTL4. Recent-

ly, we demonstrated that carboxyl-terminal ANGPTL4 is specifically present in the entero-endocrine cells of the human intestinal tract, suggesting secretion of carboxyl-terminal ANGPTL4 into the bloodstream (144). In analogy with LPL, however, it is most reasonable to expect that the amino-terminal portion of ANGPTL4 inhibits pancreatic lipase activity (87). However, what cells in the intestinal tract produce and secrete amino-terminal ANGPTL4 into the intestinal lumen remains unclear and should be studied once an antibody that detects amino-terminal ANGPTL4 becomes available. Most likely, amino-terminal ANGPTL4 is secreted by the enterocytes to function as a negative feedback signal to regulate the uptake of dietary fat into enterocytes. Indeed, ANGPTL4 is potently regulated by the fatty acid-sensing PPARα and PPARγ in the small intestine and colon, respectively (145, 146). Furthermore, both the localization and regulation of intestinal ANGPTL4 following a meal should be addressed in future studies, possibly by employing enterocyte-specific *Angptl4* knockout mice.

INTRACELLULAR LIPOLYSIS

Recently, *HILPDA* was identified as a novel PPAR-regulated gene that promotes hepatic lipid accumulation by lowering triglyceride hydrolysis and triglyceride turnover in hepatocytes (6, 147). In **Chapter 8**, we show that HILPDA is abundantly expressed in the adipose tissue and is associated with lipid droplets. However, in contrast to the reported inhibitory effect of HILPDA on triglyceride hydrolysis in the liver, we did not find a major impact of adipocyte-specific deletion of *Hilpda* on adipose tissue lipolysis following fasting, cold exposure or pharmacological β-adrenergic activation in mice (**Chapter 8**). Nonetheless, we did observe potent regulation of HILPDA by β-adrenergic signaling and PPARγ in adipocytes, hinting at an important yet not fully understood role of HILPDA in adipocyte biology.

Given HILPDA's small size (~8 kDa) and expression in both liver and adipose tissue, it may be hypothesized that HILPDA collaborates with or regulates a protein common to hepatocyte and adipocyte lipid droplet metabolism (148, 149). PLIN2 is a PPAR-regulated lipid droplet-associated protein that has a similar tissue expression profile as HILPDA (147, 150, 151). Important work by Gimm *et al*. suggests that HILPDA colocalizes with PLIN2 at the surface of some, but not all lipid droplets (152). Similar to HILPDA, liver-specific knockdown of PLIN2 significantly reduced hepatic lipid accumulation, whereas PLIN2 overexpression resulted in the development of a fatty liver (6, 150, 153, 154). Furthermore, again similar to HILPDA, knockout of PLIN2 did not alter adipose tissue lipolysis or adipocyte differentiation, an observation that may be linked to the observation that PLIN2 primarily localizes to the surface of smaller, possibly less mature lipid droplets (155, 156). Indeed, replacement of PLIN1 by PLIN2 on the lipid droplets of *Plin1*-null adipocytes significantly reduced adipocyte lipolysis *via* the exclusion of adipose triglyceride lipase (ATGL/PNPLA2) from the lipid droplet (153, 157–159).

Another protein that HILPDA might collaborate with or regulate is CIDEC, also an established PPAR target gene (148, 149). Adenoviral-mediated silencing of CIDEC prevented the development of fasting-induced steatosis in liver, while silencing in human adipocytes significantly increased both basal and stimulated lipolysis

(148, 160). CIDEC thus promotes lipid accumulation in the adipose tissue and liver, conceivably by facilitating lipid transfer and the formation of unilocular lipid droplets and/or by acting as a barrier for ATGL (160–163). In support of a possible connection between HILPDA and CIDEC, loss of either HILPDA or CIDEC in hepatocytes resulted in more, yet smaller lipid droplets (6, 161). Hypothetically, HILPDA may participate in or regulate the lipid droplet fusion complex that comprises Rab8A – AS160 – CIDEC (164).

A common denominator between the PPAR-targets PLIN2 and CIDEC is that they promote lipid accumulation by preventing access of ATGL (PNPLA2) to the lipid droplets. Although HILPDA does not directly impact ATGL activity, it may be hypothesized that HILPDA prevents access of ATGL to the lipid droplet, resulting in the mild reduction in adipocyte lipolysis that we observed upon HILPDA overexpression (147) (**Chapter 8**). Alternatively, HILPDA might regulate or prevent access of the ATGL-related lipase patatin-like phospholipase domain-containing protein 3 (PNPLA3) to the lipid droplet, a protein that is upregulated following intracellular lipid accumulation and that affects lipid droplet morphology (165). Indeed, HILPDA's protein structure is remarkably similar to G0S2, a small ~11 kDa protein that is known to inhibit ATGL activity and to influence ATGL localization (6, 166, 167) (*unpublished observations*). Whether HILPDA cooperates with and/or physically interacts with PLIN2, CIDEC, ATGL or PNPLA3 requires further investigation, possibly by employing state-of-the-art techniques such as FRET-FLIM. Alternatively, it may be that HILPDA simply competes for lipid droplet access with other lipid droplet proteins, such as ATGL, *via* protein crowding (168). Protein crowding is a major determinant of lipid droplet protein composition and describes the phenomenon that proteins are gradually expelled from the shrinking surfaces of lipid droplets during lipolysis dependent on their binding strength to the lipid droplet surface (168).

An important step to further elucidate the functional significance of HILPDA is to clarify HILPDA's intracellular localization and mobility. HILPDA has been shown to localize to the lipid droplet surface numerous times (6, 152) (**Chapter 8**). However, as opposed to other lipid droplet-associated proteins, HILPDA is not solely localized to the lipid droplet surface and has so far not been detected in proteomic screens of lipid droplet-associated proteins (169–171) (*unpublished observations*). At this moment, no information is available about the mobility of HILPDA upon, for example, β-adrenergic stimulation and the localization of HILPDA to other organelles. In our microscopy studies, we frequently observe intense staining of HILPDA in what we believe to be the ER, an observation that is perhaps not surprising given that lipid droplets originate from and interact with the ER (172, 173). In fact, several lipid droplet proteins accumulate in the ER upon the induction of lipid droplet formation before migrating onto lipid droplets (173). Alternatively, ER-localized HILPDA might regulate ER proteins involved in either the formation of triglycerides (*e.g.* glycerol-3-phosphate acyltransferases (GPATs), Lipin, or acyl-coA:diacylglycerol acyltransferases (DGAT)) or the formation of lipid droplets (*e.g.* Seipin, fat storage–inducing transmembrane protein (FITM/FIT) or members of the PLIN family) (174–177). Future microscopy and cellular fractionation studies should confirm whether (endogenous) HILPDA indeed localizes to the ER and whether HILPDA is involved in the ER-lipid droplet interface.

Despite the minor phenotype observed in mice with an adipocyte-specific deletion of *Hilpda*, we cannot exclude a functionally significant role for HILPDA in adipocytes. Possibly, we did not employ the appropriate stimulus or perhaps overexpression may more effectively reveal HILPDA's function in adipocytes (**Chapter 8**). Alternatively, it may be that HILPDA's function is more important in other lipid droplet-containing cells such as hepatocytes, myocytes, adrenocortical cells, enterocytes and macrophages (178). Indeed, although the underlying mechanisms in lipid droplet formation and triglyceride mobilization are similar, the levels of regulatory proteins that cover the surface of lipid droplets vary between cell types (159, 179, 180). It would thus be of great interest to assess the function of HILPDA and HILPDA's impact on intracellular lipid accumulation in these different cell types. For example, we initially identified HILPDA as a gene that is highly induced upon lipid loading of macrophages, which is observed in obese adipose tissue and in atherosclerotic plagues *in vivo* (*unpublished observations*) (181, 182). To investigate the role of HILPDA in macrophages, mice with a macrophage-specific deletion of *Hilpda* might be fed a high-fat diet to assess adipose tissue inflammation or crossed with APOE3-Leiden mice to assess atherosclerotic plaque development. Another area of exploration is the function of HILPDA in humans. We know that the *HILPDA* gene is highly conserved and that human HILPDA is potently regulated by PPARs (147) (**Chapter 8**). Besides that, HILPDA expression has been associated with the activation of hypoxia-inducible factor 1-α and the progression of different types of cancers in humans (152, 183–185). To examine a potential function for HILPDA in human lipid metabolism, it would be of interest to assess the assocation of HILPDA expression in adipose tissue with plasma free fatty acid and glycerol levels, as well as other relevant metabolic parameters such as insulin resistance and plasma cholesterol and triglyceride levels.

PPAR-MEDIATED REGULATION OF LIPOLYSIS

A common denominator in the regulation of ANGPTL4 and HILPDA is their potent regulation by the PPAR transcription factors (46, 147, 186) (**Chapter 4, Chapter 8**). Indeed, the expression of ANGPTL4 in WAT, BAT, heart, liver, intestine, muscle and macrophages is potently regulated by different isoforms of the PPAR family (see **Chapter 3** for an overview). Similarly, HILPDA is strongly regulated by PPARα in the liver and PPARγ in the adipose tissue (147) (**Chapter 8**). PPARα, PPARδ and PPARγ are key regulators of lipid partitioning and lipid utilization and their activation by fatty acids and fatty acid derivatives directly links changes in lipid metabolism to changes in gene expression (187, 188). Although their differential tissue distributions and ligand specificities make that PPARα, PPARδ and PPARγ have distinct physiological roles, the three transcription factors all regulate genes that participate in metabolic processes that involve the handling of fatty acids (189). For example, PPARs control the effective storage of incoming fatty acids by regulating the expression of genes involved in triglyceride synthesis and lipid droplet formation, but also promote the expression of genes involved in fatty acid oxidation and fatty acid uptake (148, 190–192) (**Chapter 8**). Perhaps not suprising given their pivotal role in fatty acid metabolism (and as highlighted by this thesis), PPARs also tightly control the rates of intracellular and extracellular lipolysis, *via* both positive and negative feed-

back loops. Indeed, PPARs potently regulate the transcription of genes that promote lipolysis, such as LPL and ATGL, of which the resultant fatty acids positively feed back to activate PPARs (193–195). At the same time, PPAR activation also promotes the expression of genes that inhibit lipolysis and that promote lipid storage, such as ANGPTL4, G0S2 and possibly HILPDA (46, 147, 186) (**Chapter 4**, **Chapter 8**). Also, treatment of mice and humans with the PPARα agonist fenofibrate potently lowers plasma triglyceride levels by promoting the clearance of TRLs by LPL, but at the same time paradoxically increases levels of ANGPTL4 (55, 196–198). Currently, the rationale for the simultaneous, opposite regulation of lipolysis by PPARs is unclear, but one may speculate that this type of regulation attenuates or tempers the effective uptake and/or the release of fatty acids following PPAR activation to prevent cellular lipotoxicity and/or lipid depletion. An intriguing question is how PPARs balance the expression levels of genes that positively and negatively regulate lipolysis. Unfortunately, the complexity of PPAR-mediated gene regulation renders it difficult, if not impossible to provide a unified answer, with gene regulation being context dependent and determined by factors such as ligand availability, the abundance of specific isoforms, the sensitivity of target genes to specific PPAR isoforms and the degree of PPAR activation (199). In fact, even the source of the fatty acid ligands might play part, as it has been proposed that, during fasting, liver PPARα is mainly activated by lipoprotein-derived fatty acids, whereas circulating free fatty acids primarily activate liver PPARδ (200). Another layer of complexity is added by the co-activators and co-repressors of PPARs, such as CBP/P300 and proteins of the p160 family, that may finetune the expression of genes involved in the positive and negative feedback loops during specific physiological conditions (201). Indeed, we show in **Chapter 4** that the inactivation of P300 upon sustained cold exposure is involved in the reduction in ANGPTL4 expression in BAT. Similarly, the PPARγ co-activators SRC-1 and TIF2 have been reported to impact intracellular lipolysis (202). Overall, it is evident that many details of the PPAR-mediated regulation of lipolysis require further investigation. Especially, studies into the activity of co-regulators might provide better insight into how PPARs delicately balance the different fatty acid-driven processes, including intracellular and intravascular lipolysis, within a cell. Alternatively, the identification of novel PPAR-target genes may uncover regulators of extracellular or intracellular lipolysis.

CONCLUSION

In this thesis, we investigated the molecular mechanisms that underlie extracellular and intracellular lipolysis. In **Figure 3**, an overview of our findings related to intravascular and intracellular lipolysis in adipocytes is provided. We demonstrate that the regulation of ANGPTL4 expression is an integral component of the shuttling of lipid fuel towards BAT during sustained cold exposure (**Figure 1**). Furthermore, our studies show that ANGPTL4 regulates intravascular lipolysis in the adipose tissue *via* a mechanism that comprises the intracellular degradation of LPL (**Figure 3**). We also

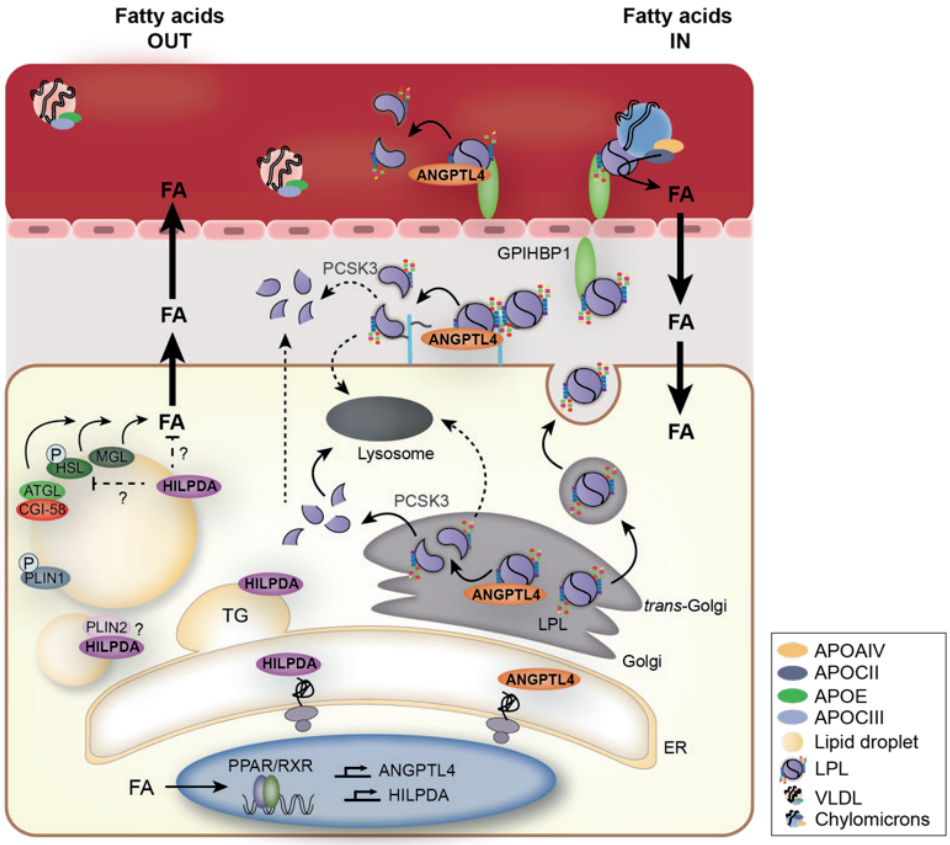


Figure 3: The roles of HILPDA and ANGPTL4 in intracellular and extracellular lipolysis in the adipose tissue.
On the left side, the potential roles of HILPDA in adipocytes are highlighted. HILPDA is potently regulated by PPARγ and β-adrenergic signaling in adipose tissue. Following translation, HILPDA is found in the ER and on lipid droplets. Overexpression of HILPDA lowers the release of fatty acids from adipocytes. upon induction of intracellular lipolysis. However, adipocyte-specific deletion of *Hilpda* did not reveal a major impact of plasma non-esterified fatty acid levels, which questions whether HILPDA is a physiological regulator of intracellular lipolysis in the adipose tissue. A possible interacting partner of HILPDA is PLIN2, but also a role for HILPDA in lipid droplet formation or morphology cannot be excluded. On the right side, the role of ANGPTL4 in extracellular lipolysis is depicted. Similar to HILPDA, ANGPTL4 is regulated by PPARs. Upon translation, ANGPTL4 inhibits LPL at multiple levels: in the *trans*-Golgi, on the cell surface, and at the endothelium. Although the exact mechanism by which ANGPTL4 inhibits LPL remains to be clarified, the inhibition may include the de-dimerization of LPL and subsequent cleavage by PCSKs. By acting upon LPL, ANGPTL4 limits the amount of fatty acids that enter adipocytes.

extend our knowledge on the function of ANGPTL4 in the intestine by showing that intestinal ANGPTL4 impacts bile acid metabolism and gut microbial composition. Lastly, we identify the lipid droplet-associated protein HILPDA as a novel target of PPARγ and β-adrenergic signalling in the adipose tissue, although we did not find a major role for HILPDA in the regulation of intracellular lipolysis in adipocytes (**Figure 3**). Taken together, our studies clarified several of the regulatory pathways underlying lipolysis, but also highlight that knowledge gaps remain to be filled. Further insights into the physiological regulation of extracellular and intracellular lipolysis may improve our understanding of metabolic disorders such as cardiovascular disease and may uncover possible treatment strategies. Hopefully, future mechanistic studies and translational studies will side-by-side clarify the function of HILPDA in the adipose tissue, as well as the physiological role of ANGPTL4.

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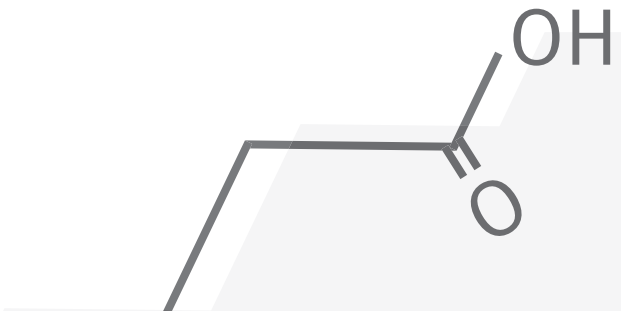
ENGLISH ABSTRACT AND SUMMARY



ABSTRACT

The body efficiently stores energy in the form of triglyceride (fat) molecules. However, triglycerides cannot directly enter or exit our cells, but first need to be degraded to so-called fatty acids before moving in or out. This degradation process, called lipolysis, is crucial for human physiology and is tightly regulated to prevent the accumulation of fats either within organs or within the bloodstream - hallmarks of diseases such as obesity and cardiovascular disease.

To allow for uptake by underlying organs, triglycerides in the circulation are efficiently broken down to fatty acids by an enzyme called lipoprotein lipase that sits in the bloodstream of multiple organs (also called *extracellular lipolysis*). In this thesis, we characterized a protein named angiopoietin-like 4 (ANGPTL4) that potently inhibits lipoprotein lipase (LPL) and, thereby, inhibits the breakdown of triglycerides in the bloodstream. Our data show that by adjusting the tissue expression levels of ANGPTL4, different organs collaborate to ensure that triglycerides are distributed to organs in need of energy. Moreover, we uncovered that, in the fat tissue, ANGPTL4 starts to inhibit LPL before LPL arrives in the bloodstream. By preventing the arrival of LPL in the bloodstream, ANGPTL4 is capable of rapidly adjusting the rates of triglyceride degradation and the concomitant uptake of fatty acids to the energy requirements of the underlying organ.

To exit our cells, stored triglycerides that are for example present in our fat tissue need to be broken down (also called *intracellular lipolysis*). Subsequently, the released fatty acids can fuel other organs in need of energy. To further clarify the mechanisms underlying this process of intracellular lipolysis, we investigated the role of a promising new protein called HILPDA. Our data show, however, that a loss of HILPDA did not impact the release of fatty acids from the fat tissue, while a high abundance of HILPDA only had a mild attenuating effect on the release of fatty acids. This suggests that HILPDA is not a major physiological regulator of intracellular lipolysis in fat cells.

In conclusion, in this thesis, we have clarified the regulation of intracellular and extracellular lipolysis by studying the respective roles of the proteins ANGPTL4 and HILPDA. Such efforts are clinically relevant, as regulators of lipolysis are potential therapeutic targets to lower cardiovascular disease risk.

SUMMARY

Fatty acids are highly efficient energy substrates that are mostly found incorporated into triglycerides to facilitate their transport and storage. The inability of triglycerides to move across cellular membranes necessitates that they are hydrolysed to fatty acids before moving in or out a cell. This degradation process, called lipolysis, is crucial for human physiology: lipolysis in the intestine (*gastro-intestinal lipolysis*) makes that dietary triglycerides can be taken up by our body, lipolysis in the bloodstream (*intravascular lipolysis*) assures that circulating triglycerides can move into tissues that require energy, and lipolysis within cells (*intracellular lipolysis*) mobilizes stored triglycerides that are used for energy or distributed to provide fuel to other organs. Perhaps not surprisingly, dysregulation of gastro-intestinal, intravascular or intracellular lipolysis may have severe consequences for health, including ectopic fat storage and dyslipidemia– hallmarks of metabolic disorders such as obesity and cardiovascular disease. Despite the importance of a tight regulation of lipolysis for metabolic health, however, the underlying molecular mechanisms are far from being completely understood.

In the first part of this thesis, we focused on the regulation of lipoprotein lipase (LPL), the enzyme responsible for intravascular lipolysis. LPL sits on the endothelium of many metabolically active tissues, such as the heart and the adipose tissue. At the endothelium, LPL releases fatty acids from circulating triglycerides to assure that these tissues obtain adequate amounts of lipid fuel. Since the fuel demands of tissues continuously change, the activity of endothelial LPL needs to be dynamically regulated in a tissue-specific manner. For example, following a prolonged fast, the heart and not our adipose tissue should preferentially take up fatty acids derived from circulating triglycerides. Accordingly, the activity of LPL in the heart increases upon fasting, while the activity of LPL in the adipose tissue decreases. This fasting-induced reduction in LPL activity in the adipose tissue is mediated by the protein angiopoietin-like 4 (ANGPTL4), a protein that potently inhibits LPL (**Chapter 2**, **Chapter 3**). Another physiological situation that significantly impacts the metabolic demand of tissues is cold exposure. During cold exposure, the activity of the brown adipose tissue increases dramatically, as the brown adipose tissue combusts large amounts of fatty acids to generate heat for the maintenance of core body temperature. To replenish intracellular lipid stores and to supply fatty acids for combustion, the activity of LPL in the brown adipose tissue increases significantly upon cold exposure. However, the underlying regulatory mechanisms had remained unclear. In **Chapter 4**, we hypothesized that the changes in LPL activity in the brown adipose tissue upon cold exposure might be controlled by changes in the abundance of the LPL inhibitor ANGPTL4. To test this hypothesis, we exposed mice without ANGPTL4 (so-called *Angptl4* knockout mice), control mice (so-called wild-type mice), and mice with more ANGPTL4 (so-called *Angptl4*-transgenic mice) to a cold environmental temperature (4 °C) or a thermoneutral temperature (28 °C) for 10 days. We found that the amount of ANGPTL4 was significantly decreased in the brown adipose tissue of wild-type mice upon cold exposure, concurrent with the increase in LPL activity. In contrast, the abundance of ANGPTL4 in the brown adipose tissue of *Angptl4*-transgenic mice did not change following cold exposure and, confirming our initial hypothesis, the in-

crease in LPL activity in *Angptl4*-transgenic mice was significantly blunted compared to the wild-type mice. Surprisingly, upon examination of the white adipose tissue of these mice, we found that the amount of ANGPTL4 in the white adipose tissue was increased rather than decreased, resulting in the repression of LPL activity in the white adipose tissue. Together, these data suggest that ANGPTL4 promotes the shuttling of lipid fuel towards the brown adipose tissue upon sustained cold exposure by enhancing the uptake of triglyceride-derived fatty acids into the brown adipose tissue and by inhibiting their uptake into the white adipose tissue. To confirm the role of ANGPTL4 in regulating LPL activity and concomitant fatty acid uptake in the brown and white adipose tissue upon cold exposure, we intravenously injected radiolabeled and fluorescently labeled triglycerides into cold-exposed *Angptl4* knockout mice, wild-type mice, and *Angptl4*-transgenic mice and tracked the fate of the injected triglycerides. Consistent with our initial experiments, the uptake of fatty acids released by LPL from the radiolabeled and fluorescently labeled triglycerides was inversely associated with the amount of ANGPTL4 in the brown and white adipose tissue. Taken together, our data show that the opposite regulation of ANGPTL4 in the brown and white adipose tissue assures an adequate provision of fatty acids derived from circulating triglycerides to the brown fat during cold exposure.

Although we convincingly showed that ANGPTL4 potently regulates the activity of LPL in the brown adipose tissue, our data did not shed light on the mechanism by which ANGPTL4 inhibits LPL activity in the adipose tissue. Accordingly, our objective in **Chapter 5** was to investigate the cellular location and mechanism by which ANGPTL4 inhibits LPL in the adipose tissue by employing a combination of cell culture studies and studies with adipocytes and adipose tissue from wild-type and *Angptl4* knockout mice. We found that co-transfection of ANGPTL4 and LPL markedly reduced the amount of LPL within cells, whereas co-plating of independently transfected cells did not. These observations suggested that ANGPTL4 lowers the amount of LPL protein within cells, but only when expressed in the same cell as LPL. Conversely, in adipocytes, the absence of ANGPTL4 resulted in a pronounced accumulation of LPL on the adipocyte cell surface. Together, our data show, for the first time, that ANGPTL4 has the capacity to inhibit LPL intracellularly in adipocytes, possibly by promoting the intracellular degradation of LPL. Our identification of the intracellular regulation of LPL by ANGPTL4 complements the established regulation of LPL by ANGPTL4 in the subendothelial spaces and at the endothelium. In **Chapter 6**, our objective was to further clarify the mechanism by which ANGPTL4 promotes the intracellular degradation of LPL. Here, we showed that adipose tissue LPL is potently cleaved intracellularly and extracellularly by proteins of the so-called proprotein convertase subtilisin/kexin (PCSK) family, resulting in the inactivation of LPL. Furthermore, we provide evidence that the cleavage of LPL by PCSKs in adipocytes can be promoted by ANGPTL4, suggesting that PCSK-mediated cleavage may at least partially explain how ANGPTL4 promotes the intracellular degradation of LPL in the adipose tissue.

Besides LPL, ANGPTL4 is also known to inhibit the activity of pancreatic lipase, the enzyme responsible for the gastro-intestinal lipolysis of dietary triglycerides. In the intestine, the abundance of ANGPTL4 is modulated by the gut microbiota and by bile acids. Bile acids emulsify dietary triglycerides in the intestinal tract and facilitate triglyceride digestion. In **Chapter 7**, we examined the impact of intestinal ANGPTL4 on gut microbial composition and host bile acid metabolism. To that end, we challenged *Angptl4* knockout mice and wild-type mice with a diet enriched in taurocholic acid, a conjugated bile acid, and assessed the differences in gut microbial composition and bile acid metabolism between *Angptl4* knockout and wild-type mice. We found that taurocholic acid feeding dramatically increased the levels of bile acids in the blood of wild-type mice but not of *Angptl4* knockout mice, which could be attributed to a lower re-absorption of bile acids in the intestine of *Angptl4* knockout mice. Furthermore, we found significant differences in gut microbial composition between the wild-type and the *Angptl4* knockout mice, which we believe may provide a reasonable explanation for the observed differences in bile acid metabolism between *Angptl4* knockout and wild-type mice. Conceivably, the differences in bile acid uptake and gut microbial composition between *Angptl4* knockout and wild-type mice might originate from differences in gastro-intestinal lipolysis, as elevated levels of fat in the intestine significantly impact the gut microbial composition. This hypothesis remains, however, to be tested.

Next, we investigated the function of a newly-identified protein called HILPDA in the regulation of intracellular lipolysis in adipocytes. HILPDA is abundantly present in the adipose tissue where it localizes to the lipid droplets. Given that HILPDA was previously shown to prevent intracellular lipolysis in hepatocytes, we hypothesized in **Chapter 8** that HILPDA might regulate intracellular lipolysis in the adipose tissue. Our initial experiments indicated that the abundance of HILPDA in adipocytes is regulated by the fatty acid-inducible transcription factor peroxisome proliferator-activated receptor γ (PPARγ) and by β-adrenergic signaling, the key signaling pathway initiating intracellular lipolysis. However, loss of HILPDA did not significantly impact intracellular lipolysis and the concomitant release of fatty acids from adipocytes, whereas overexpression of HILPDA modestly attenuated the release of fatty acids from adipocytes. To examine a potential role for HILPDA in adipocyte lipolysis *in vivo*, we subsequently generated mice that are deficient for *Hilpda* specifically in the adipocytes. We challenged these mice either by the injection of a β-adrenergic agonist, by cold exposure or by prolonged fasting, in order to promote adipocyte lipolysis and the concomitant release of fatty acids from the adipose tissue. In contrast to our expectations, however, we did not observe an effect of *Hilpda* deletion on the amount of fatty acids in the plasma nor on any other relevant metabolic parameters. Taken together, our data suggest that HILPDA is not a major physiological regulator of intracellular lipolysis in adipocytes.

In conclusion, the studies presented in this thesis clarify several of the regulatory pathways underlying lipolysis. We demonstrate that the regulation of ANGPTL4 expression in brown and white adipose tissue is an integral component of the shuttling of lipid fuel towards BAT during cold exposure *via* a mechanism that comprises the

intracellular degradation of LPL, the key enzyme for intravascular lipolysis. We also extend our knowledge on the function of ANGPTL4 in the intestine by showing that intestinal ANGPTL4, possibly *via* its impact on gastro-intestinal lipolysis, impacts bile acid metabolism and gut microbial composition. Lastly, we identify the lipid droplet-associated protein HILPDA as a novel target of PPARγ and of β-adrenergic signalling in the adipose tissue. However, in contrast to the proposed inhibition of lipolysis by HILPDA in the liver, our data did not reveal a major role of HILPDA in the regulation of intracellular lipolysis in the adipose tissue. A better understanding of the molecular mechanisms underlying lipolysis is clinically relevant. For example, physiological regulators of LPL activity, such as ANGPTL4, have recently regained interest from pharmaceutical companies as potential therapeutic targets to lower cardiovascular disease risk. Hopefully, future mechanistic studies and translational studies will side-by-side clarify the underlying regulatory processes, as well as their relevance for human physiology.

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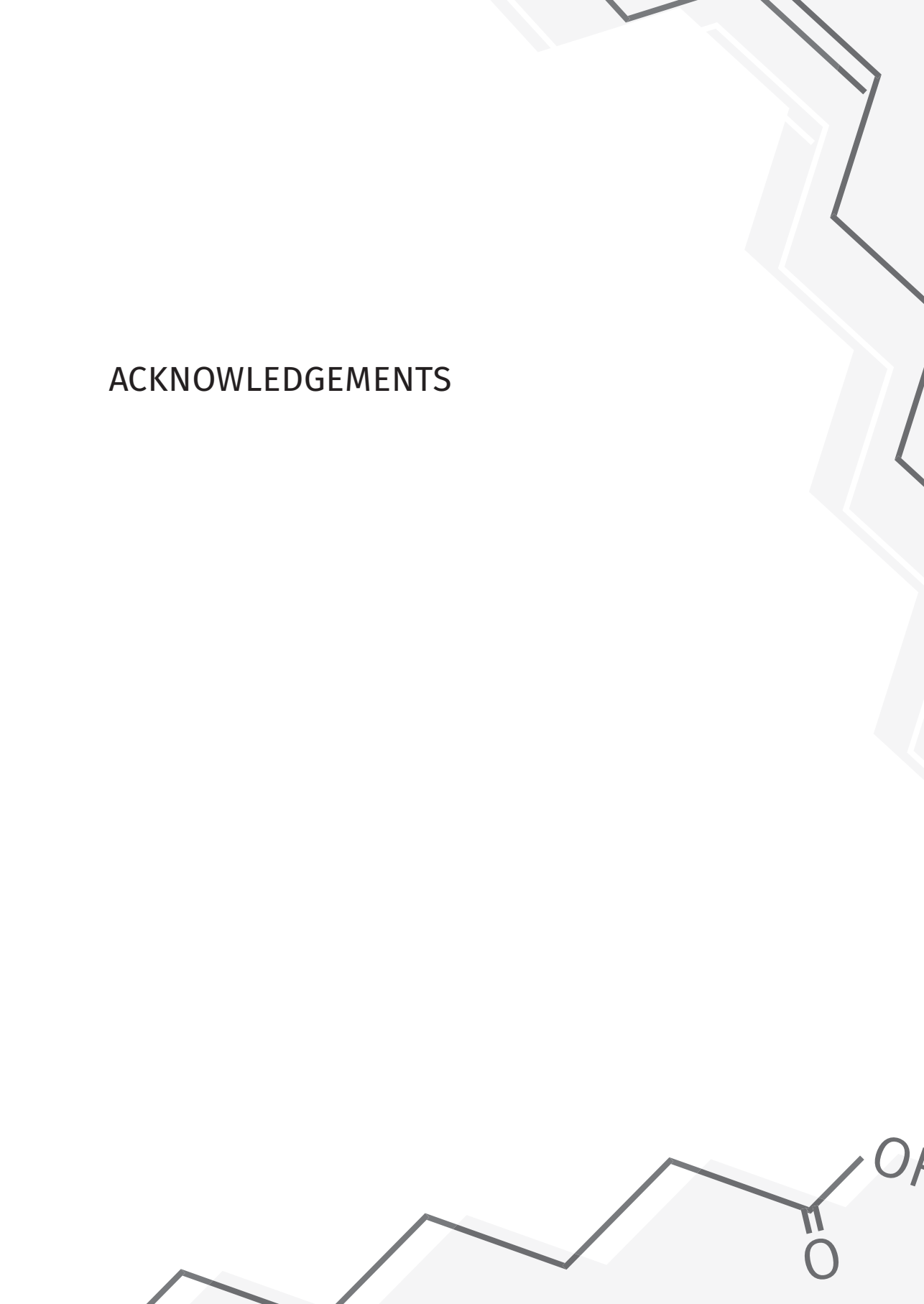

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ABOUT THE AUTHOR

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Wieneke Dijk was born on July 4th 1989 in Beuningen, the Netherlands. In 2007, she completed secondary school (Athenaeum, *cum laude*) at O.S.G. Sevenwolden in Heerenveen. In that same year, she started her Bachelor at University College Utrecht, where she specialized in Biochemistry and Molecular Cell Biology. Upon completion of her Bachelor's degree (*cum laude*) in 2010, she continued with a Master in Molecular Life Sciences at Wageningen University that she finished in 2012 (*cum laude*). Meanwhile, she developed a keen interest in Molecular Nutrition and, as a result, she completed her Master's thesis and research internship in that field of study.

During her Master's thesis, Wieneke investigated the role of the protein Mannose Binding Lectin in the development of obesity, under supervision of Dr. Rinke Stienstra at the Nutrition, Metabolism & Genomics group at Wageningen University. Subsequently, she completed her research internship in the lab of Philippe Costet at INSERM UMR 1087 in Nantes, where she worked on the molecular mechanisms underlying Trans-Intestinal Cholesterol Excretion (TICE).

In December 2012, Wieneke started her PhD project entitled "Intracellular & Extracellular Lipolysis. Regulation by the PPAR targets ANGPTL4 & HILPDA" under the supervision of Prof. Sander Kersten at the Nutrition, Metabolism & Genomics group of the Division of Human Nutrition at Wageningen University. Her PhD project was part of a transatlantic network of excellence on triglyceride metabolism, funded by the Leducq Foundation. In October 2016, she received a grant from the Fondation de la Recherche Médicale to work on the role of the protein PCSK9 in triglyceride metabolism. She will conduct this research in the lab of Bertrand Cariou at INSERM UMR 1087 in Nantes, where she will start as a post-doc in February 2017.

LIST OF PUBLICATIONS

This thesis

Dijk W*, Janssen AWF*, Boekhorst J, Groen AK, Lukovac S, Hooiveld GJEJ, Kersten S. ANGPTL4 stimulates bile acid uptake which is associated with changes in gut microbial composition. *In preparation.*

Dijk W, Oost LJ, Kersten S. ANGPTL4 promotes the intracellular cleavage of lipoprotein lipase by PCSK3 in adipocytes. *In preparation.*

Dijk W*, Mattijssen F*, De La Rosa Rodriguez M, Loza Valdes A, Loft A, Mandrup S, Kalkhoven E, Qi L, Borst JW, Kersten S. Role and regulation of HILPDA in adipose tissue. *Submitted.*

Dijk W, Kersten S. (2016). Regulation of lipid metabolism by angiopoietin-like proteins. *Curr Opin Lipidol.* 27 (3): 249-256.

Dijk W, Beigneux AP, Larsson M, Bensadoun A, Young SG, Kersten S. (2016). Angiopoietin-like 4 (ANGPTL4) promotes intracellular degradation of lipoprotein lipase in adipocytes. *J Lipid Res.* 57 (9): 1670-1683.

Dijk W, Heine M, Vergnes L, Boon MR, Schaart G, Hesselink MK, Reue K, van Marken Lichtenbelt WD, Olivecrona G, Rensen PC, Heeren J, Kersten S. (2015) ANGPTL4 mediates shuttling of lipid fuel to brown adipose tissue during sustained cold exposure. *elife.* 4; e08428.

Dijk W, Kersten S. (2014) Regulation of lipoprotein lipase by Angptl4. *Trends Endocrinol Metab.* 25 (3): 146-155.

Others

Khedoe PP, Hoeke G, Kooijman S, **Dijk W**, Buijs JT, Kersten S, Havekes LM, Hiemstra PS, Berbée JF, Boon MR, Rensen PC. (2015) Brown adipose tissue takes up plasma triglycerides mostly after lipolysis. J Lipid Res. 56(1):51-59.

Stienstra R, **Dijk W**, van Beek L, Jansen H, Heemskerk M, Houtkooper RH, Denis S, van Harmelen V, Willems van Dijk K, Tack CJ, Kersten S. (2014) Mannose-binding lectin is required for the effective clearance of apoptotic cells by adipose tissue macrophages during obesity. *Diabetes.* 63(12):4143-4153.

Alex S, Lichtenstein L, **Dijk W**, Mensink RP, Tan NS, Kersten S. (2014) ANGPTL4 is produced by entero-endocrine cells in the human intestinal tract. *Histochem Cell Biol.* 141(4): 383-391.

Huber M, Bakker MH, **Dijk W**, Prins HA, Wiegant FA. (2012) The challenge of evaluating health effects of organic food; operationalisation of a dynamic concept of health. *J Sci Food Agric.* 92(14): 2766-2773.

TRAINING ACTIVITIES

Discipline specific activities

Conferences

Deuel Conference	ASBMB	San Diego, USA	2014	oral
Deuel Conference	ASBMB	Monterey, USA	2015	poster
Deuel Conference	ASBMB	Napa Valley, USA	2016	poster
European Lipoprotein Club	EAS	Tutzing, Germany	2014	oral
NVDO meeting	NVDO	Oosterbeek	2013	oral
NVDO meeting	NVDO	Oosterbeek	2014	oral
NVDO meeting	NVDO	Oosterbeek	2015	oral

Training

Training period at University of Hamburg-Eppendorf, Hamburg, Germany.

General courses

VLAG PhD week	VLAG	Baarlo	2013
Laboratory Animal Science	WUR	Wageningen	2013
Techniques for Writing & Presenting a Scientific Paper	WGS	Wageningen	2015
Writing Grant Proposals	WGS	Wageningen	2016

Others

Writing research proposal	WUR	Wageningen	2012-2013
Six-monthly project meetings	Leducq	various locations	2013-2016
NMG/Pharma lab meetings	WUR	Wageningen	2012-2016

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