The assembly of cytosolic lipid droplets and its effect on insulin sensitivity

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Abstract

Accumulation of neutral lipids, in particular triglycerides, in non-adipocytes is highly related to the development of insulin resistance and its consequences, type-2 diabetes and cardiovascular diseases. The accumulation of triglycerides occurs in so-called lipid droplets in the cytosol. The lipid droplet is a highly dynamic organelle consisting of a core of neutral lipids surrounded by a monolayer of amphipathic lipids and proteins. The mechanism of assembly of these droplets is poorly understood and the main aim of this thesis was to investigate this mechanism at the molecular level. Another aim was to determine the relationship between lipid storage and insulin sensitivity of the cell.

In paper I, gain- and loss-of-function experiments showed that phospholipase D1 promotes the formation of lipid droplets. In addition, a cytosolic protein required for assembly of the droplets was isolated and identified as the extracellular regulated kinase 2 (ERK2). The importance of ERK2 in the formation of lipid droplets was confirmed in intact cells using gain- and loss-of-function experiments. Both PLD1 and ERK2 were shown to be necessary for the effect of insulin on lipid droplet biosynthesis. Finally, ERK2 was shown to exert its effects through phosphorylation of the motor protein dynein.

Lipid droplets are formed as primordial structures with a diameter of 0.1–0.4 μm. In paper II, it was found that these primordial droplets grow in size by a fusion process that is independent of triglyceride biosynthesis. This conclusion was based on investigations in a cell-free system, on pulse-chase experiments in intact cells, and by 3D reconstructions of time-lapse studies of fluorescent droplets in intact cells. Intact microtubules and dynein were found to be essential for fusion between the droplets. The mechanism behind the fusion process was investigated further in paper III. The SNARE proteins SNAP23, VAMP4, and syntaxin5 were shown to be present on lipid droplets and to mediate their fusion. Previously described co-factors for SNARE-mediated fusion events (NSF and α-SNAP) were also found to be present on droplets.

It is well known that SNAP23 also mediates the insulin-stimulated fusion between transport vesicles containing the glucose transporter 4 (GLUT4) and the plasma membrane—a process that is essential for insulin-stimulated glucose uptake. Treatment of cells with oleic acid caused massive accumulation of lipid droplets, and also translocation of SNAP23 from the plasma membrane to sites within the cell, including lipid droplets. This was paralleled by an ablation of insulin-stimulated glucose uptake—an effect that was totally reversed by overexpression of SNAP23. Thus, SNAP23 may be a molecular link between insulin resistance and neutral lipid storage.

List of publications

This thesis is based on the following publications, referred to in the text by their Roman numerals

I

PLD1 and ERK2 regulate cytosolic lipid droplet formation
Andersson L*, ***Bostrom P****, Ericson J, Rutberg M, Magnusson B, Marchesan D, Ruiz M, Asp , Huang P, Frohman MA, Boren J, Olofsson SO.
Journal of Cell Science. 2006 Jun 1;119(Pt 11):2246-57.
**** Contributed equally***

II

Cytosolic lipid droplets increase in size by microtubule-dependent complex formation
Bostrom P, Rutberg M, Ericsson J, Holmdahl P, Andersson L, Frohman MA, Boren J, Olofsson SO.
Arteriosclerosis Thrombosis Vascular Biology. 2005 Sep;25(9):1945-51. 2005.

III

SNAP23 is important for the fusion between lipid droplets: a novel role for the SNARE system with implications for the insulin sensitivity of muscle cells
Boström P, Andersson L, Rutberg M, Perman J, Lidberg U, Johansson BR, Fernandez-Rodriguez J, Ericsson J, Nilsson T, Borén J and Olofsson S-O.
Nature Cell Biology, 2007 Nov;9(11):1286-93.

List of abbreviations

Acyl CoA	Acyl Co-Enzyme A
ADFP	Adipocyte Differentiation Related Protein
ADP	Adenosine Di-Phosphate
ADRP	Adipocyte Differentiation Related Protein
ATP	Adenosine Tri Phosphate
BMI	Body Mass Index
DGAT	Diacylglycerol acyltransferase
EM	Electron Microscopy
ER	Endoplasmatic Reticulum
ERK	Extracellular Regulated Kinase
GFP	Green Flourescent Protein
GLUT	Glucose Transporter
GPAT	Glycerol phosphate acyl transferase
GTP	Guanosine Tri-Phosphate
HC	Heavy chain
HSL	Hormone Sensitive Lipase
IDF	International Diabetes Foundation
JNK	c-Jun N-terminal Kinase
LDL	Low Denisty Lipoprotein
LSDP5	Lipid Storage Droplet Protein 5
MALDI	Matrix-Assisted Laser Desorption/Ionisation
MAPK	Mitogen-Activated Protein Kinase
MLDP	Myocardial Lipid Droplet Protein
MPAT	Monoacylglycerol acyltransferase
MPR	Mannose Phosphate receptors
NSF	N-ethylmaleimide sensitive factor
PAT	Perilipin ADFP and TIP47
PAT	Perilipin Amino Terminal
PI4,5P	Phosphatidyl Inositol 4,5-bisphosphate
PIP3	Phosphatidyl Inositol 3-Phosphate
PKA	Protein Kinase A
PLD	Phospholipase D
PPAR	Peroxisomal Proliferator-Activated Receptor
PPRE	PPAR response element
RNA	Ribonucleic Acid
RNAi	Rna interference
RXR	Retinoid X receptor

siRNA	small interference Ribonucleic acid
SNARE	SNAP receptor
SNP	Single Nucleotide Polymorphism
T2D	Type 2 Diabetes
TIP47	Tail Interacting Protein 47
TOF	Time of Flight
TS1	Time Step 1
TS2	Time Step 2
VAMP	Vesicle-Associated Membrane Protein
WAT	White Adipose Tissue
VLDL	Very Low Denisity Lipoprotein
α-SNAP	Soluble NSF adaptor protein

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Introduction

General introduction

Some diseases have one very simple underlying mechanism that directly causes the development and progression of symptoms. Examples of such diseases are those in which one single gene is affected, causing a specific protein defect that in turn causes development of the disease.

Many diseases have more complex causality, however, where multiple factors contribute to their development and progression. Among these complex diseases, the development of type-2 diabetes (T2D) might be the ultimate challenge. The complexity of T2D has prevented rapid scientific progress, as the condition is characterized by an intriguing relationship between the adipose tissue, the liver, the pancreas, and skeletal muscle. Even more challenging is the long period between the initial disturbance and onset of symptoms, with the involvement of both environmental and genetic factors.

Advances in new techniques during the past decade, has provided us with new insights into the mechanisms underlying T2D. The importance of turnover and storage of lipids in this condition has been highlighted recently.

In this thesis, my aim has been to elucidate the mechanism of cellular lipid storage, and also to study how that process may be connected to the development of insulin resistance, which is the first sign of development of diabetes.

Insulin Resistance and Type-2 Diabetes

The world faces a pandemic of type-2 diabetes mellitus (T2D), something that has attracted the attention not only of scientists, but also of the popular media. In the United States, diabetes is already the leading cause of blindness in adults of working age[2], end-stage renal disease, and non-traumatic loss of limbs[3]. It is also the fifth leading cause of death[4]. The most accurate data regarding prevalence in Europe come from Italy, where the cost of T2D was recently estimated to be 5 billion euros, which amounts to more than 6% of the total private and public healthcare expenditure[5]. The International Diabetes Federation (IDF) anticipates that the worldwide incidence of diabetes among those aged 20–79 years will increase by around 70% in the next 20 years, from 184 million in 2003 to 333 million in 2025. This increase will affect all global regions, with projected increases ranging from 21% in Europe to 111% in Africa[6].

T2D is characterized by an inability to secrete sufficient amounts of insulin into the bloodstream. This causes defective glucose uptake and results in increased circulating concentrations of glucose, which in turn leads to major organ dysfunction and death. The development of T2D occurs over more than a decade, and is initiated by a defect in the response to insulin — insulin resistance. As the cellular insulin signal is reduced, glucose cannot be cleared from the blood, which causes the pancreatic beta cells to produce more insulin. This overproduction compensates for the reduced insulin sensitivity, and can disguise the pathological condition for years. Eventually, however, the pancreas fails to overcome the ablated insulin response, and glucose concentrations rise in the bloodstream. At this point,

T2D has developed and can be diagnosed. Thus, the initial event in the development of type-2 diabetes is insulin resistance (reviewed in[7-9]).

There are no clear explanations as to what causes this initial disturbance, which drives the development of T2D. Many associations have been established between insulin resistance and other factors, but no causative relationship has been proven in man. One important factor is the increased concentration of plasma-free fatty acids derived from adipose tissue triglycerides. Such fatty acids adversely affect the insulin signal. Another important factor is inflammation of the adipose tissue and the release of inflammatory cytokines—factors also known to disrupt insulin signaling (reviewed in[8,9]). In recent years, a new, important factor has been identified: lipid accumulation within muscle cells (intramyocellular lipids).

A possible relationship between skeletal muscle triglycerides (the dominant neutral lipid) and insulin resistance independently of obesity was first proposed in the mid-1980s, when increased triglyceride content in skeletal muscle was found in normoglycemic, hyperinsulinemic dogs with low plasma triglycerides[10]. Further and more direct support for this concept was provided in 1991, when a correlation between insulin resistance and the amount of triglycerides was observed in muscles of mice[11]. In 1988, the first evidence of a possible role of increased skeletal muscle triglycerides in the development of insulin resistance in humans was published. Hyperinsulinemic subjects with T2D had several-fold increased triglyceride concentrations in muscle extracts[12].

More recently, non-invasive methods such as proton magnetic resonance spectroscopy for detection and quantification of triglycerides (and other lipids) in man have been developed. This allows discrimination between triglycerides within muscle cells and neutral lipids in extramyocellular adipose cells[13]. A number of studies have thereafter shown a strong association between intramyocellular lipids and insulin resistance, independently of obesity, fasting plasma glucose, and age[14,15]. Thus, accumulation of neutral lipids within muscle cells is a major marker of insulin resistance. This marker precedes any other measurable analytical marker in the early development of insulin resistance leading to T2D.

It is, however, unclear whether intramyocellular neutral lipids cause the insulin resistance, or whether these are merely a marker of disease. It is clear that metabolites derived from increased triglyceride turnover in muscle could cause ablation of the insulin signal. Diacylglycerol, long-chain fatty acids, and ceramides have been especially implicated in affecting the kinase cascade following insulin stimulation[16]. It is also clear that the levels of such metabolites are elevated in individuals with insulin resistance[17], but whether or not there is a *causative* association between intramyocellular accumulations and insulin resistance remains controversial. Recently, this question has been addressed in animal studies by specific overexpression of triglyceride synthesizing enzymes within the liver. The results have been contradictory; one study involving overexpression of glyceraldehyde-3-phosphate acyl transferase (GPAT) in the liver resulted in major accumulation of triglycerides and in insulin

resistance[18]. Another study, however, showed that overexpression of diacylglycerol acyltransferase 1 (DGAT1), which is the enzyme that catalyses the final step in triglyceride synthesis, did not have any effect on insulin resistance[19]. To date, no studies involving increased quantities of neutral lipids within skeletal muscle, with concomitant insulin signaling, have been published. Thus, there is no clear evidence at present to show whether neutral lipids in muscle cause insulin resistance or whether they simply act as a marker of disease.

Triglyceride biosynthesis

For a proper understanding of cellular storage of neutral lipids and its consequences, an overview of neutral lipid biosynthesis is necessary. The most abundant neutral lipids in all cells are triglycerides. These are the major constituents of lipid droplets (see below), forming the neutral core. This organization allows efficient storage of highly hydrophobic molecules, which would be difficult to handle in any other way. Also, the formation of triglycerides neutralizes free fatty acids—which are extremely hazardous to the cell in the free form, as they can act as detergents at low concentrations.

Triglyceride biosynthesis begins with glycerol 3-phosphate, which is acylated by acyl coenzyme A (acyl CoA)—a reaction catalyzed by glycerol phosphate acyltransferase (GPAT). The next step is one additional acylation by acyl CoA, catalyzed by monoacylglycerol acyltransferase (MPAT). The di-acitylated product from these reactions is called phosphatidic acid. Phosphatidic acid can either be used for phospolipid biosynthesis or hydrolyzed by a phosphatidase to generate diacylglycerol

(DG). This molecule can then be acylated with acyl CoA to triacylglycerol, a reaction catalyzed by diacylglycerol acyl transferases 1 and 2 (DGAT).

Storage of cellular neutral lipids: lipid droplets

Virtually all cells have the ability to store neutral lipids as lipid droplets in the cytoplasm. These droplets have a core of neutral lipids (cholesterol esters and triglycerides), surrounded by a monolayer of phospholipids[20]. The surface of the droplets is also coated with various proteins. To date, using proteomic approaches, more than 100 proteins have been reported to localize to the surface of the droplet, but only a small proportion of these have been verified using other techniques[21]. The most well-known proteins associated with lipid droplets are the PAT domain proteins, which include perilipin, adipocyte differentiation-related protein (ADFP), tail interacting protein 47 (TIP47), lipid storage droplet protein 5 (LSDP5, also known as MLDP or OXPAT) and S3-12[22]. These proteins will be discussed in detail later. Beyond these, Rab 18[23,24], caveolin[25], vimentin[26], and enzymes involved in the synthesis of inflammatory cyotkines have been found on the droplet surface[27]. Enzymes involved in the hydrolyzation of triglycerides, together with co-factors, have also been reported to be present—at least transiently—on droplets[28-31].

In vitro studies on purified microsomal membranes have shown that lipid droplets can be formed from this compartment in response to cytosolic activators, independently of triglyceride synthesis. Thus, lipid droplets originate from the endoplasmatic reticulum or the Golgi apparatus[32]. This corresponds well with

the intracellular distribution of the diacylglycerol acyl transferase (DGAT) protein[33], which catalyzes the last step in triglyceride biosynthesis—the formation of triglycerides from diacylglycerol and acyl-CoA. Studies using freeze-fracture electron microscopy also support the idea of droplets originating from the microsomal membrane, as continuous sheets of membrane between droplets and the membrane of the endoplasmatic reticulum can be found, suggesting a possible site of droplet formation[34]. There have also been studies reporting lipid droplet formation from the plasma membrane of isolated adipocytes[35].

The exact mechanism of formation of lipid droplets is still not fully understood, but the most cited model involves synthesis of triglycerides into the microsomal membrane, oiling out between the luminal and cytoplasmatic membranes, and then budding of the cytoplasmatic leaflet (Figure 1), forming a cytosolic lipid droplet[20,36].

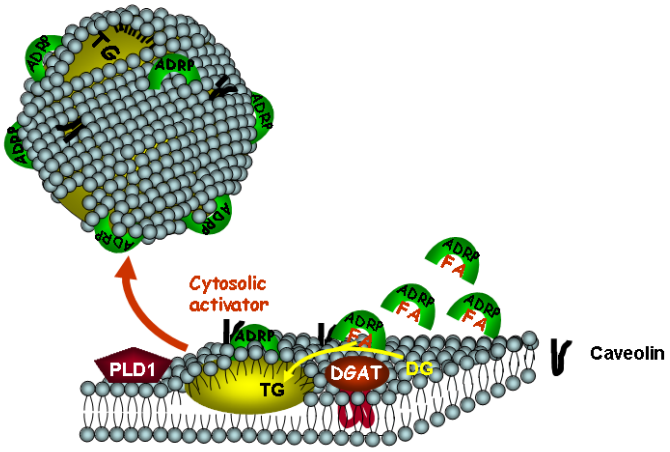

Figure 1. Model of lipid droplet biosynthesis, based on previous studies using an in vitro system for lipid droplet biosynthesis from microsomal membranes. Diacylglycerol acyltransferase (DGAT) catalyses the formation of triglycerides, that resides between the leaflets of the microsomal membrane. Thereafter,a lipid droplet is formed in response to the activity of Phospholipase D (PLD) and a cytosolic activator.

The lipid droplets are formed as small (0.2-µm diameter) spherical structures[32], and when observed in the cell they usually have a very heterogeneous size distribution in the 0.2- to 10-µm diameter range. In adipocytes, however, the lipid droplet can become more than 25 µm in diameter. The cause of these size differences has not been investigated yet; nor has the underlying mechanism. The small, newly formed droplets could either grow by synthesis of core and surface lipids or expand by fusing with each other.

Once formed, the lipid droplets are involved in the turnover of triglycerides in the cell[37]. Lipases can bind reversibly to the droplets and catalyse the hydrolysis of triglycerides into diglycerides and fatty acids[22]. This process is dependent on hormonal activation and protein-protein interactions between lipases and specific proteins on the surface of the lipid droplet[22].

Lipid droplets in different types of cell are not identical. For example, as mentioned above, there are very large droples in adipocytes (around 25 μm in diameter) and there are very small droplets in skeletal muscle (less than 1 μm in diameter).

The PAT-domain protein family

The biology of lipid droplets is determined by the proteins they interact with. Five structural proteins (PAT proteins) have been reported to be associated with the droplet surface, and therefore deserve special attention.

The PAT-domain protein family is named after the initial letters of the first three members identified; perilipin, ADFP (adipocyte differentiation-related protein) and TIP47 (tail interacting protein 47). Alternatively, the name may originate from the close similarity between the N-terminal regions of these proteins: perilipin amino-terminal (PAT). The members of the family are characterized by 1) a localization to lipid droplets, and 2) a similarity to ADFP or perilipin at either the amino terminus or the carboxy terminus[22]. Apart from perilipin, ADFP and TIP47, S3-12 and lipid storage droplet protein 5 (LSDP5) have also been added to the group. LSDP5 is also known as OXPAT, MLDP, or PAT-1[38,39].

Apart from binding to lipid droplets, PAT-domain proteins also share several common features regarding structure, genome distribution, and regulation (described below).

Perlipin, ADFP, and TIP47 have high sequence identity within the amino-terminal PAT-1 domain, but weaker homology in the central region and carboxy-terminal PAT-2 domain[40,41]. The amino-terminal segment of S3-12 shares limited identity with the PAT-1 domain, but the remainder of the protein shows significant homology with ADFP and TIP47 at the carboxy terminus. LSDP5 is similar to ADFP and TIP47 (55% and 51% identity/homology [choose one] over the entire protein), with highest homology at the PAT-1 domain[42,43]. In mice, the genes encoding perilipin and ADFP are located on chromosomes 7[41] and 4[44], respectively, and the genes for TIP47, S3-12, and LSDP5 are on chromosome 17[38]. Genetic analysis suggests that the S3-12 and LSDP5 genes might have evolved from gene duplication of the TIP47-gene[38].

There is also similarity at the exon-intron level, where ADFP, TIP47, and LSDP5 have identical exon-intron boundaries[38,41,44]. Perilipin and S3-12 also have similar exon-intron bounderies at the amino terminus, but they differ at the carboxy-terminal end of the protein[38].

Perilipin was the first PAT-domain protein to be identified, and impressive functional studies have helped to reveal a specific role of the protein in the regulation of lipolysis (see below).

However, the roles of ADFP, TIP47, S3-12, and LSDP5 have not been elucidated in such great detail.

Perilipin

Perilipin was initially identified as a protein phosphorylated by protein kinase A (PKA) in lipolytically stimulated adipocytes[45]. The protein was found to be located in lipid droplets, and to be expressed only in adipocytes and steriogenic cells[46]. Perilipin has a single-copy gene that gives rise to three isoforms (A, B, and C) as a result of alternative splicing[41]. Perilipin A is by far the most abundant, and shares its N-terminal sequence with perilipin B. Perilipin C has not been studied yet in any detail[47].

Functional studies on perilipin A in the past two decades have elucidated a specific role for perilipin in the turnover of adipocyte triglycerides. Perilipin A replaces ADFP as the major protein coating lipid droplets in adipocytes during late differentiation[48]. Perilipin A can then be phosphorylated in response to lipolytic stimulation (beta-adrenergic ligands). This phosphorylation, together with phosphorylation of the hormone-sensitive lipase (HSL), causes HSL to translocate to the lipid droplet and catalyze the hydrolyzation of triglycerides into diacylglycerides and fatty acids (reviewed in[49]). Ectopic overexpression of perilipin A in cell lines lacking perilipin expression causes a dramatic reduction in lipolytic activity, suggesting that unphosphorylated perilipin A may protect lipid droplets from lipolysis[50].

The vital role of perilipin in adipocyte triglyceride turnover was confirmed in perilipin knockout mice, which showed a

substantial increase in basal lipolysis. These mice had a 75% reduction in adipose mass, and were resistant to diet-induced obesity. Furthermore, the mice did not respond to lipolytic stimulation by beta-adrenergic stimulation, and cells derived from these mice failed to show translocation of HSL to the lipid droplet[51,52].

As the role of perilipin in lipolysis became more clear, the gene became interesting in human studies on obesity and metabolic diseases. The gene for human perilipin is located on chromosome 15q26, at a locus linked to diabetes, hypertriglyceridemia, and obesity. In a study designed to examine the regulation of lipolysis in obese and non-obese women, it was found that perilipin expression was reduced in obese women (with two- to fourfold increased basal and stimulated lipolysis). In fact, there was an inverse relationship between adipocyte perilipin and plasma glycerol levels. Apart from this, several single-nucleotide polymorphisms (SNPs) in the perilipin gene have been strongly linked to gender-specific obesity in a variety of population studies (reviewed in[53]).

ADFP

The second PAT protein to be discovered was ADFP—a fatty acid binding protein implicated in adipocyte differentiation, which was identified due to its striking homology with perilipin at the amino-terminal end [54]. The gene was shown to be ubiquitously expressed[48], and there was also a high correlation between levels of expression of the protein and the amount of neutral lipid in the cell[55].

ADFP is located on lipid droplets, and mutational studies have ruled out the PAT-1 and PAT-2 domains as being important for the binding of ADFP to lipid droplets. Instead, the middle domain—including putative alpha-helical regions between amino acids 189 and 205—appears to function in directing ADFP to droplets[56,57].

ADFP is expressed in increasing amounts early in adipocyte differentiation, but is then replaced by perilipin at later stages[48]. The phenotype of the ADFP knockout mice does, however, suggest that ADFP does not have a vital role in adipocyte differentiation (below). ADFP is regulated at the transcriptional level by PPARα[58-60], but also through post-translational degradation by the proteosomal system[61,62].

ADFP knockout mice (ADFP -/-) does not have any dramatic phenotype. The major effect was seen in the liver, with reduced liver triglyceride content[63]. Furthermore, ADFP -/- mice were found to be resistant to diet-induced hepatosteatosis. There were no effects on adipocyte differentiation or lipolysis[63]. Further studies on cells from ADFP -/- mice showed that TIP47 was directed to the droplets, replacing ADFP[64]. When TIP47 function was ablated using RNAi in ADFP -/- cells, the ability to store neutral lipids as lipid droplets was abolished, and exogenously added fatty acids were directed to phospholipid biosynthesis[64].

Overexpression and RNA interference of ADFP in hepatocyte cell lines and primary hepatocytes showed that the formation of ADFP-coated cytoplasmatic lipid droplet sequestered

triglycerides from the biosynthesis and assembly of very low density lipoproteins (VLDL)[65]. This was supported by studies using antisense RNA directed against expression of liver ADFP, which had the effect of reducing liver triglyceride content, dyslipidemia, and insulin resistance[66].

ADFP is highly expressed in macrophages challenged with different lipoprotein species[67,68], and in a recent publication ADFP was shown to be expressed in macrophage foam cells from human atherosclerotic lesions[69]. The expression of ADFP in atherosclerotic plaque also seemed to correlate with the degree of symptoms. Recent publications from our group have also demonstrated that hypoxia, which can be found in the central regions of atherosclerotic plaques, increases the amount of lipid droplets together with ADRP protein expression[67].

The precise role of ADFP on lipid droplets is not clear, even though several reports have provided important clues. There is a discrepancy in the literature concerning the importance of ADRP in the formation of lipid droplets. Thus, while some authors have claimed that elevated levels of ADRP increase the assembly of such droplets[65], conflicting results have been obtained by others[49]. Moreover, in most cells and animal models, ADFP protein expression is increased with treatments that induce lipid droplet formation[61,62,70]. Thus, it is unclear whether ADFP increases the amount of lipid droplets or whether lipid droplet biosynthesis stabilizes ADFP and thereby increases protein expression. But in summary – ADFP is accepted as a marker for lipid droplets in non-adipocytes.

TIP47

TIP47 was the third PAT protein to be identified; it was initially described as a ubiquitously expressed cytosolic and endosomal 47-kDa protein involved in the intracellular transport of mannose 6-phosphate receptors (MPRs) between the trans-Golgi and endosomes[71,72]. TIP47 is believed to act as an effector for the Rab9 protein in this process[73].

In 2001, however, TIP47 was reported to be present on lipid droplets in HeLa cells[74]. This association was greatly increased with fatty acid-stimulated biosynthesis of lipid droplets. TIP47 appeared to move from the cytosol and onto lipid droplets as a response to lipid loading[74]. This has not been observed for ADFP, which is always associated with droplets, and is degraded in the absence of neutral lipid. The localization of TIP47 on droplets was challenged by other groups[75], and even though the most recent publications favor the idea of TIP47 being a lipid droplet protein, the controversy still remains[76-78].

As TIP47 is a relatively soluble protein compared to ADFP and perilipin, the carboxy-terminal end has been crystallized and its structure determined. Surprisingly, the structure was very similar to that of apolipoproteins, with an alpha/beta domain of novel topology and four helix bundles resembling the LDL receptor binding domain of apolipoprotein E (ApoE)[79]. The structure suggests an analogy between PAT proteins and apolipoproteins, with helical repeats interacting with lipid and the ordered C-terminal region being involved in protein-protein interactions. This structural resemblance to ApoE further strengthens the role of TIP47 as a lipid droplet protein.

To date, there are no known pathways for regulation of TIP47, and further studies will be required for us to gain an insight into its function in lipid droplets.

LSDP5/OXPAT/MLDP

LSDP5 was identified and described by three independent groups within a year, and therefore received three different names: lipid droplet storage protein 5 (LSDP5), OXPAT, and myocardial lipid droplet protein (MLDP)[38,39,80]. This protein is mainly expressed in tissues with high rates of beta oxidation—muscle, heart, liver, and brown adipose tissue (BAT)[39].

The protein shares high homology with the other PAT-domain proteins (especially ADFP, as described above), and is transcriptionally regulated by PPARα in striated muscle and liver, and by PPARγ in white adipose tissue[38,39,80].

The accumulation of triglycerides in response to fatty acid treatment of cultured cells is greatly increased with forced overexpression of LSDP5[39]. This might possibly be explained by a decrease in both basal and stimulated lipolysis[38]. Thus, the protein seems to protect the triglyceride core of lipid droplets from degradation in a manner similar to that known for perilipin. This has also been suggested by the fact that there was found to be a negative correlation between LSDP5 mRNA expression in subcutaneous white adipose tissue (WAT) and BMI in nondiabetic human subjects. A positive correlation between LSDP5 mRNA expression and insulin sensitivity was also found[39].

S3-12

Only a small number of reports have investigated S3-12 as a lipid droplet protein, even though it was initially described in this context[81]. S3-12 is mainly expressed in white adipose tissue, and there is a rather weak sequence homology between this protein and the other proteins in the family[81].

S3-12 expression is transcriptionally regulated by PPARγ[42], and appears to function in a manner similar to TIP47—translocating to newly formed lipid droplets in response to fatty acid stimulation. Apart from this, functional studies are required to determine the role of S3-12 in lipid droplets.

The peroxisome proliferator-activated receptors and regulation of PAT-domain proteins

The tissue expression of the PAT-domain proteins varies (see above for details), but their regulation is somewhat similar. Several of the PAT-domain proteins are transcriptionally regulated by members of the peroxisomal proliferator-activated receptors (PPARs). The PPAR family consists of three isoforms: PPARα, PPAR ß/δ, and PPARγ which regulate transcription by heterodimerization with retinoid X receptors (RXRs) and binding to specific PPAR response elements (PPREs) in the promoter region of target genes. PPARγ is highly expressed in white adipose tissue and macrophages, PPARα is expressed in the liver, muscle, and kidney, and PPAR ß/δ is more ubiquitously expressed (reviewed in[82]). LSDP5[39], S3-12[42], and perilipin[83] are regulated by PPARγ; ADFP[59,60] and LSDP5[80] are regulated by PPARα, whereas TIP47 does not appear to be regulated by PPARs.

Besides transcriptional regulation, the amounts of perilipin protein[84] and ADFP protein[61,62] are regulated post-transcriptionally by the proteosomal system—a process that is dependent on the amount of neutral lipid in the cell. To date, post-transcriptional regulation of protein expression has not been described for the other PAT proteins.

Microtubules and motor protein transport

As most organelles—including lipid droplets—move in a coordinated manner within the cell, a description of intracellular transport is necessary in order to give the reader a full understanding of neutral lipid storage.

One of the major systems for intracellular movement is microtubule transport. Microtubules are tube-like structures composed of heterodimers of the protein tubulin. They are the largest type of cytoskeletal filament with an outer diameter of about 25 nm and a wall thickness of 5 nm (reviewed in Brinkley -87).

Microtubules are polar structures, a characteristic arising from the asymmetry of the tubulin dimer, which is made up of α and β subunits[85]—proteins that are expressed ubiquitously. This polarization usually puts the "minus ends" of microtubules at the microtubule-organizing center, close to the nucleus, while the "plus ends" spread outwards from the organizing center, leading to a radial organization. The detail of this radial organization differs between cell types. Microtubules are highly dynamic, polymerizing and depolymerizing rapidly in response to changes

in ion concentration, GTP levels, and different protein interactions (reviewed in Brinkley -87).

Cellular organelles are transported on these intracellular microtubule "transport lanes" by the motor proteins dynein and kinesin. Transport occurs when the appropriate motor binds to a cargo at one end, and simultaneously binds to the microtubule-rail through the other end. The motor then moves along the "rail" by using repeated cycles of coordinated binding and unbinding of its two heads, powered by energy derived from hydrolysis of ATP.

Motor proteins are able to recognize the microtubule polarity; so the organization of the rails combined with the specific motor employed determines the direction of transport. Most kinesin-family motors that have been studied move toward the plus end of the microtubules, and thus kinesin-mediated transport is usually used to bring cargo toward the cell periphery. In contrast, dynein moves in the other direction—toward the minus end of the microtubule—and is typically used to move cargo toward the center of the cell (and the nucleus) (for review, see[86]).

Motors also often appear to work together locally; intracellular transport often employs multiple motors of different classes on the same organelle. For example, multiple dyneins and kinesins attach to, and move, single lipid droplets along microtubules in bidirectional fashion (back and forth) inside the syncytial *Drosophila* embryo[87].

Dynein

Dyneins are motor proteins that are active on the microtubule network. They are found in many different kinds of cells, and can be classified into two forms: axonemal and cytoplasmic dyneins. Axonemal dyneins are restricted to cileal and flagellar movement,while cytoplasmatic dyneins (also called "processive", as they actually generate movement) are active in intracellular transport [88].

Only two cytoplasmic dyneins are known: cytoplasmic dynein 1b, which mainly drives slow transport within the flagellum and cytoplasmic dynein 1, which shows an immense range of functions during mitosis, in neuronal transport, and in maintenance of the Golgi and transport of a wide variety of intracellular cargoes such as mRNA, endosomes, viruses, etc. *In vitro* studies have shown that cytoplasmic dynein 1 is a processive motor[88].

Dynein 1 is a 1.2-MDa protein formed from several subunits. Three heavy chains (HCs) of > 500 kDa correspond to the number of morphologically identifiable heads, which contain the motor domains of the molecule. The dynein HC forms two main structures: (1) a ~160-kDa N-terminal domain that forms the base of the molecule, to which most of the accessory subunits bind, and (2) the ~380-kDa motor domain. A diversity of accessory subunits, referred to as intermediate, light intermediate, and light chains are bound to this dynein head. Most, but not all, of these subunits are associated with the cargo-binding base of the dynein molecule[89].

It has been difficult to elucidate the different regulatory mechanisms behind dynein motor activity, due to the complexity of the protein. It is, however, clear that different protein-protein interactions and covalent modifications (e.g. phosphorylation) can inhibit or increase dynein activity.

In *Drosophila*, dynein has been shown to mediate minus-end motion of lipid droplets, suggesting that lipid droplets are motile on microtubules. No studies on lipid droplet transport in mammalian cells have been published[87].

Phospholipase D

Phospholipase D (PLD) exists in two different isoforms (1 and 2) that are ubiquitously expressed; this enzyme catalyzes the hydrolysis of phosphatidyl choline into phosphatidic acid and choline. Data from different labs have suggested different subcellular localizations of PLD1, but the most common observation is that it is located at the endoplasmatic reticulum (ER)[90]. The protein has also been reported to translocate between the plasma membrane and the ER in response to activation of epidermal growth factor stimulation [91]. PLD2 has been reported to be located only on the plasma membrane[92].

PLD2 has a high basal enzymatic activity, while PLD1 requires activation through modifications and/or interactions with other proteins[93].

Molecules known to activate PLD1 include the phosphoinositides PI4, 5P2, and PIP3, which all binds to and activates PLD1 both *in vivo* and *in vitro*[94]. Besides these,

different protein kinase C (PKC) isoforms activate PLD1 both dependent and independent on their phosphorylation activity. The ADP ribosylation factor 1 and other small GTP-dependent proteins such as RhoA, Cdc42Hs, and Rac1 activate PLD1 in a GTP-dependent manner in many cell types and tissues (activation reviewed in [90]).

Extracellular signal-regulated kinase

Extracellular signal-regulated kinases 1 and 2 (ERK1 and 2) belong to a family of protein kinases named mitogen-activated protein kinases (MAPKs). The family also includes c-Jun N-terminal kinase (JNK(1-3)) and p38 (α, β, γ, and δ). ERK1 and 2 are 43 and 41 kDa in size, have 83% identity, and are ubiquitously expressed. They are part of large signaling transduction cascades for a diversity of different cellular functions: cell motility, proliferation, differentiation, and survival. ERK1 and 2 are located throughout the cell—but as much as half of the total amount of protein is situated at the microtubules, where it affects polymerization dynamics (reviewd in[95]).

ERK1 and 2 are phosphorylated and thereby activated by MAPK kinases (MAP2Ks) named MEK1 and 2, in response to activation of different cell receptors: G-protein-coupled receptors, cytokines, transforming growth factors, osmotic stress and microtubule depolymerization[95].

Once activated, ERK1 and 2 exert a variety of effects through phosphorylation of different targets. The signal transduction secondary to growth factor stimulation is one of the most well-

studied pathways of ERK1/2 activation, where ERK1 and 2 are translocated to the nuclei and phosphorylate different transcription factors that affect expression of target genes[95].

Fusion between transport vesicles and membranes

One of the central observations in this thesis work has been that lipid droplets increase in size by a fusion process. The most well-characterized fusion process in the cell is the one between transport vesicles and target membranes.

Through transport vesicles, in which an aqueous interior is surrounded by a membrane, cargo can be shipped from a source organelle to specific target membranes[96]. The vesicles are formed from a source membrane using specific protein machinery, causing budding into the cytosol. The vesicle is then transported to its target membrane and fused with that membrane, delivering the cargo to that organelle or to the outside of the cell (if the target membrane is the plasma membrane)[96].

This fusion process has been studied extensively and characterized in detail. The first discovery was that a protein named N-ethylmaleimide-sensitive factor (NSF), a cytosolic ATPase temporarily bound to membranes, was shown to be vital for vesicle-to-membrane fusion[97]. Thereafter, an important co-factor for NSF was described—the "soluble NSF adaptor protein" (α-SNAP)—which was shown to bind NSF to membranes[98]. Even though these factors were found to be vital for the fusion process, their precise function was not clear until a family of "SNAP-receptors" (SNAREs) was discovered[99]. The SNAREs act as functional mediators of the fusion event, forcing

membranes together (see below for details). The discovery of NSF, α-SNAP, and SNAREs made it possible to outline the detailed process in which vesicles fuse to membranes.

First, the vesicle binds loosely to the target membrane through weak protein-protein interactions with so-called tethering proteins[100]. This is then followed by more close interactions between SNAREs, which are situated both on the vesicle (v-SNAREs) and on the target membrane (t-SNAREs)[101]. The SNAREs then undergo a conformational change (cis to trans), which exert a force on the two membranes—moving them close, and forcing them to fuse[102]. This trans-SNARE complex is characterized by four alpha helix bundles twined together (the so-called "four-helix bundle)[103] with a central affinity point called "the zero-plane". The four alpha helices belong to three different SNARE proteins, and their different contributions of amino-acid residues to the zero-plane constitutes a basis for grouping of the SNAREs: some SNAREs contribute with an glutamine (Q-SNAREs), and others contribute with an arginine (R-SNAREs)[103,104]. In order to form a functional SNARE complex that is able to fuse membranes, the three SNAREs must consist of one Qa-SNARE (contributing with one glutamine at the a-position in the zero-plane), one Qbc-SNARE (contributing with two glutamines at the b-and c-positions in the zero-plane), and one R-SNARE (contributing with one arginine in the zero-plane)[104,105].

Finally, to be ready for the subsequent rounds of transport, the SNARE complex must be disassembled. This process is catalyzed by the combined action of α-SNAP and NSF.

Interaction of NSF and three α-SNAPs with the SNARE complex through ATP hydrolysis leads to the disassembly, through a transient 20-S complex. The freed v-SNAREs can then be recycled to the donor compartment by retrograde transport, while the t-SNARE subunits can be reorganized into functional t-SNAREs for the next round of fusion events[106-108].

SNAREs

There are a large number of SNAREs in mammalian cells, and they are grouped based on whether they are found on vesicles (v-SNAREs) or target membranes (t-SNAREs). An alternative scheme uses the terminology R- or Q-SNAREs, reflecting the presence of an arginine or a glutamine, respectively, at a characteristic position within the SNARE motif [109]. SNAREs also differ in the way they bind to membranes; some bind through an integral, transmembrane region while others are synthesized on free ribosomes, followed by insertion of the C-terminal region into the membrane by chaperones[110]. One small group of SNAREs is soluble and binds to membranes using a palmoityl anchor, covalently attached to central cysteine residues. The VAMPs are examples of tail-inserted SNAREs, and SNAP23 of palmoitylated[109].

The insulin-regulated glucose transporter

GLUT4 is a membrane protein containing 12 transmembrane domains. One of the ways in which insulin can influence the uptake of glucose in a muscle or fat cell is by increasing the amount of the glucose transporter GLUT4 in the plasma membrane. Of importance for this increase is the translocation of GLUT4 from a cellular storage from to the plasma membrane, a

process that involves a SNARE-mediated fusion process(reviewed in[111]).

Although the GLUT4 activity can be regulated at the transcriptional level in some cases (obesity[112] and exercise[113]), the major control of GLUT4-mediated glucose uptake is through translocation of intracellular GLUT4 to the plasma membrane. Under basal conditions, most of the cellular GLUT 4 resides in low-density fractions of intracellular membranes. In response to stimuli, however, vesicles containing GLUT4 are transported from this compartment toward the plasma membrane[114]. These vesicles become tethered to the membrane, and fuse using a SNARE complex-containing VAMP4, syntaxin 2 and SNAP23[115]. The stimulus that causes GLUT4 to move to the cell surface also inhibits the recycling pathway, where GLUT4 vesicles are internalized using an endocytotic mechanism. The increased translocation to the plasma membrane together with reduced internalization causes a net increase in GLUT 4 on the cell surface, thus facilitating glucose uptake[111].

Aim

The aim of this thesis has been to describe the biosynthesis of lipid droplets, and to identify a molecular link between lipid droplets and insulin resistance. Specifically, the following issues have been addressed:

1. Identification of insulin-dependent mediators of lipid droplet formation.
2. Clarification of the mechanism by which lipid droplets grow in size.
3. The molecular background behind such growth.
4. The molecular link between lipid droplet biosynthesis and insulin sensitivity.

Summary of results

Paper I

PLD1 but not PLD2, is active in lipid droplet biosynthesis

In previous studies, lipid droplets were shown to be synthesised from purified microsomes in an *in vitro* system. Phospholipase D activity and a partially purified cytosolic activator were needed to form small lipid droplets[32]. In this paper, the cytosolic activator was identified and its role was studied in living cells. The role of PLD was also investigated in living cells – especially regarding what isoform was active in lipid droplet biogenesis. As insulin induces lipid droplet formation, the question of whether PLD and/or the cytosolic activator have any role in the cellular response to insulin stimuli was then investigated.

In order to study the intracellular localization of PLD1 and 2, immunocyotchemistry was used, which showed that PLD1 was located in intracellular compartments while PLD2 was located at the plasma membrane. Overexpression of ectopic PLD1 in NIH 3T3 cells caused a 5.1 ± 2.6-fold increase (mean ± s.d.; $n = 7$; $P < 0.001$, Mann-Whitney rank sum test) in the total area of oil red o-stained lipid droplets, while overexpression of PLD2 had no effect on the pool of lipid droplets. Overexpression of PLD1 also resulted in increased amounts of triglycerides and ADFP, effects that were not seen with PLD2 overexpression. These results indicate that PLD1, but not PLD2, regulates the intracellular formation of lipid droplets.

The importance of PLD1 was confirmed by the use if loss–of-function experiments. The siRNA knockdown of PLD1 reduced both the amount of oil red o-stained lipid droplets in the cell compared to control siRNA ($P < 0.001$, Mann-Whitney rank sum test; 6 independent experiments) and the accumulation of triglycerides and ADFP. Taken together, these results indicate that PLD1 has an important, rate-limiting role in lipid droplet assembly in intact cells.

Identification of a cytosolic activator

A cytosolic component had been previously reported to activate lipid droplet formation in vitro[32]. This activator was partially purified using a chromatographic procedure[32]. For each chromatography step, all fractions were analyzed for ability to activate the in vitro system for lipid droplet formation. Most of the activity was recovered between 62 and 0 mM $(NH_4)_2SO_4$ in the final hydrophobic interaction chromatography. This fraction was analyzed by SDS polyacrylamide gel electrophoresis where all bands were cut out, trypsinized and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). Three of the proteins identified (ERK2, citrate lyase, and adiponectin) were added to the in vitro system as recombinant proteins. Citrate lyase and adiponectin did not promote lipid droplet formation, whereas ERK2 showed a dramatic effect: 12 ± 2-fold higher activity than the control fraction. This result suggested that ERK2 might be the unidentified cytosolic factor working in conjunction with PLD1 to promote lipid droplet formation.

ERK2 is active on droplet formation in intact cells

Transfection of NIH 3T3 cells with the ERK2 gene increased the formation of cytosolic lipid droplets 3.0 ± 1.9-fold (mean ± s.d; n = 5; P = 0.008, Mann-Whitney rank sum test) when compared to a vector encoding GFP as control. As seen for PLD1, ERK2 also increased the amount of triglycerides and ADFP protein expression.

The role of ERK2 was also examined in loss-of-function experiments using siRNA followed by quantification of lipid droplets. Compared to control siRNA, ERK2 siRNA significantly reduced the formation of Oil Red O-stained lipid droplets by 56 ± 14% (mean ± s.d.; n = 6; P = 0.002, Mann-Whitney rank sum test). Also, levels of ADFP and triglycerides were reduced.

Microinjection of active ERK2 protein into NIH 3T3 cells resulted in an increase in the amount of lipid droplets in comparison to injection of control GFP protein (P<0.001).

Treatment of NIH 3T3 cells with Ste-Mek1$_{13}$[116], which inhibits the activation of ERK2, reduced the amount of lipid droplets by 59 ± 14% (n = 5). Together, these results indicate that ERK2 is vital for lipid droplet formation in living cells.

The ERK2 siRNAs were isoform-specific, indicating that ERK1 does not promote lipid droplet formation in the absence of ERK2. To further examine whether ERK1 might act in this process, ERK1 was overexpressed - which resulted in a severalfold increase in the level of expression of ERK1 without having any

effect on lipid droplet formation. SiRNAs against ERK1 significantly reduced the amount of ERK1 but had no effect on the amount of lipid droplets in the cell.

Finally, the effects of JNK1, JNK2, p38, and p38ß on lipid droplet formation were investigated using siRNA and/or inhibitors. SiRNA to p38 or p38ß failed to affect the formation of lipid droplets, as was the case for an inhibitor of p38. SiRNAs to JNK1 and JNK2 also had no effect, as was the case for JNK inhibitor.

Taken together, these results suggest that the role of ERK2 in promoting lipid droplet formation is specific to this MAP kinase family member

Insulin promotes lipid droplet formation through PLD1 and ERK2

Insulin induced lipid droplet formation in serum-starved cells in a dose-dependent manner. Knockdown of PLD1 using siRNA inhibited the insulin-induced lipid droplet formation by 138 ± 43% (mean ± s.d.; $n = 3$; $P = 0.005$). The ERK2 inhibitor Ste-Mek1$_{13}$ also reduced the insulin-induced increase in the formation of lipid droplets by 73 ± 40% ($n = 5$, $P = 0.004$). SiRNA to ERK2 gave a similar result, suggesting that inhibition of ERK2 inhibits the insulin-stimulated rate of lipid droplet formation. Together, these results indicate that PLD1 and ERK2 are essential for insulin-stimulated lipid droplet biosynthesis.

ERK2 phosphorylates the motor protein dynein, which directs it to droplets

Next, the question of whether ERK2 could phosphorylate lipid droplet proteins was investigated. ADFP, caveolin, vimentin, PLD1, and dynein were analyzed for ERK2 phosphorylation using recombinant ERK2 in NIH 3T3 homogenates, followed by the PhosphoProtein purification kit and immunoblotting of retained fractions. ERK2 phosphorylated dynein only. Furthermore, it was shown that dynein is directed to lipid droplets through ERK2 phosphorylation. Thus, ERK2 possibly exerts its action by phosphorylation of the motor protein dynein.

In order to assess whether dynein might promote the assembly of lipid droplets, 3T3 NIH cells were microinjected either with an antibody directed against dynein or a control immunoglobulin. The results showed that the antibody to dynein caused a significant decrease in the amount of lipid droplets in the cell - by 63 ± 16% (mean ± s.d.; $P = 0.002$, *t*-test).

Finally, the role of dynein in the fusion between lipid droplets was investigated. Cells were injected either with antibodies to dynein or with the control immunoglobulin. The droplets were then stained with BODIPY and followed using confocal microscopy in time-lapse studies, as described in paper II. The cells injected with anti-dynein antibodies significantly reduced the frequency of fusions ($P < 0.001$; $n = 16$ for anti-dynein, $n = 13$ for control immunoglobulin). Together, these results indicate that dynein is an important player in lipid droplet formation, and a potential mediator of the effect of ERK2 effect on the assembly of lipid droplets.

Paper II

Lipid droplets are synthesized as small structures, 200 nm in diameter[32]. When observed in the cell, however, they are much larger and heterogenous in size. Thus, in this paper, the mechanism of the enlargement of lipid droplets was investigated.

The possibility that lipid droplets grow in size after being formed was tested by pulsing NIH 3T3 cells with oleic acid for 2 hours followed by a chase for 2 hours in the presence of 20 μmol/L triacsin C, a potent inhibitor of triglyceride biosynthesis. After the pulse, most lipid droplets were small (< 3 μm). After the chase in the presence of triacsin C, however, there were fewer small droplets - with a compensatory increase in larger particles. The total area of oil red o–stained lipid droplets per cell did not change during the chase. These results indicate that small droplets can increase in size independently of triglyceride biosynthesis.

A likely explanation is that the increase occurs via a fusion between droplets. This was tested in confocal analysis of live cells by labeling droplets with Nile red dye, or overexpressed fluorescent ADFP. Both approaches showed clear fusion events (see example in figure 2). Close-up zooms after 3D reconstructions ruled out the possibility that the droplets moved on top of each other.

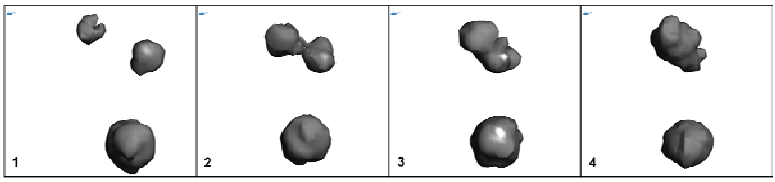

Figure 2. Fusion between two lipid droplets after 3D reconstruction. Complete z-stacks were obtained at 30 seccons interval (1-4) followed by 3D-reconstruction. The top two droplets are seen fusion with each other.

An *in vitro* system was also established to study the process of fusion of lipid droplet. NIH 3T3 cells were either transfected with ADFP-GFP or incubated with [^{3}H]-palmitic acid to label triglycerides. Homogenates from both dishes were then mixed and incubated together, followed by immunoprecipitation with antibodies to GFP, and isolation of triglycerides. Thirty per cent of the [^{3}H]-labeled triglycerides were recovered using such precipitations, compared to 6% in control precipitations. This indicates that droplets from ADFP-GFP-labeled cells had fused with droplets from [^{3}H]-labeled cells.

Calcium, a known activator of vesicle fusion, increased the recovery of labeled triglycerides after ADFP-GFP precipitation (63% of the radioactivity could be precipitated with the anti-GFP antibody). Thus, the fusion process appeared to be calcium-dependent.

Nocodazole, which disrupts the microtubule network *in vivo* reduced the rate of fusions between droplets - as judged from pulse chase experiments with Triacsin C and also the *in vitro*

system for droplet fusion. Thus, a functional microtubule network is vital for fusion of lipid droplets.

Dynein was shown to be vital for droplet formation in the time-lapse system in paper I, and this was investigated further by the use of vanadate—an inhibitor of the ATPase activity of dynein. Vanadate also ablated lipid droplet fusions, both *in vivo* and *in vitro*. Thus, the microtubule network and the motor protein dynein are vital factors for the fusion between lipid droplets.

Paper III

The aim of this paper was to clarify the molecular mechanism behind lipid droplet fusion, and the connection between lipid droplet fusions and insulin signaling.

The SNARE machinery is present on lipid droplets

The SNARE proteins SNAP23, syntaxin5 and VAMP4 were observed on isolated lipid droplets together with NSF and α-SNAP. This was shown by immunoblot of purified lipid droplets, co-precipitations against ADFP, and immuno-electron microscopy.

SNAP23, VAMP4, and syntaxin-5 are present in a functional complex on lipid droplets

Using immunoprecipitation of cell lysate, VAMP4 and SNAP23 co-precipitated with syntaxin-5, demonstrating that the three SNARE proteins form stable complexes with each other. No reactions were seen when control immunoglobulins were used for precipitation (data not shown). SNARE complexes are

stabilized with α-SNAP when the ATPase activity of NSF is inhibited. Isolated droplets were therefore solubilized and incubated with His-tagged recombinant α-SNAP in the presence of ATP-γ-S and EDTA. The complexes were then purified using immunoaffinity chromatography against the His-tag. The retained fractions were immunoblotted against several SNAREs. Only SNAP23, syntaxin-5, and VAMP4 were detected with this method, indicating that these three SNARE proteins form four-helix bundles that interact with α-SNAP on lipid droplets. Furthermore, it was observed that syntaxin-5 and SNAP23 co-precipitated with VAMP4 from solubilized lipid droplets, supporting a stable interaction between the three SNARE proteins. No reaction was seen when control immunoglobulins were used for precipitation.

Thus, the SNARE proteins identified on lipid droplets are in a functional complex.

SNAP23, VAMP4, and syntaxin-5 mediate fusion between lipid droplets

To study whether the SNARE proteins were active on lipid droplet fusion, siRNA knockdown of each protein was performed, followed by time-lapse analysis of the rate of lipid droplet fusion. Such ablation of lipid droplet SNAREs greatly reduced the rate of fusions. (A truncated, dominant negative SNAP23 was overexpressed, which also caused reduced lipid droplet fusion). In addition, microinjection of a dominant-negative mutant of α-SNAP reduced the rate of lipid droplet fusion. As a control, siRNA against syntaxin-2 was also used,

which did not affect lipid droplet fusion. Thus, the SNARE proteins found on lipid droplets are vital for the fusion activity.

We also investigated whether the SNAREs found on the droplets affected their sizes. SiRNA-mediated depletion of SNAP23, syntaxin-5, or VAMP4 resulted in smaller lipid droplets—as assessed in three dimensions after confocal microscopy. No effect on triglyceride content or biosynthesis was observed, though. Microinjection of a dominant-negative mutant of α-SNAP into cells reduced the size of the lipid droplets, but did not influence the total pool of lipid droplets. In summary, these results indicate that SNAP23, syntaxin-5, VAMP4, and α-SNAP are not only present on the droplets but are also active in the fusion between these droplets.

Fatty acid treatment of HL-1 cardiomyocytes divert SNAP23 from the plasma membrane to intracellular compartments, including lipid droplets

SNAP23 is important both for the fusion between GLUT4-carrying vesicles and the plasma membrane, and for the fusion step in the assembly of lipid droplets. The question of whether SNAP23 participates in fatty-acid-induced insulin resistance was therefore investigated.

As expected, oleic acid treatment of the cells caused a large increase in the pool of lipid droplets. This was paralleled by reduced insulin-dependent glucose uptake and translocation of GLUT4 to the plasma membrane. Furthermore, the plasma membrane pool of SNAP23 was reduced upon oleic acid

treatment, as assessed by both immunocytochemistry and subcellular fractionation. There was, however, no effect on the total pool of SNAP23. SNAP23 was also increased on lipid droplets with fatty acid treatment. Thus, it seems that oleic acid-treatment results in dissociation of SNAP23 from the plasma membrane—to structures in the interior of the cell, including lipid droplets. The majority of SNAP23 molecules appear to be translocated to the cytosol or to the intracellular membranes, and the amount that appears on lipid droplets seems to be dependent on the oleic acid-induced increase in the pool size of these droplets.

Fatty acid treatment of muscle cells causes insulin resistance, which is reversed by overexpression of SNAP23

As shown by other authors, ablation of SNAP23 function by siRNA results in a deficiency in insulin-stimulated glucose uptake[117]. This implies that reduction in SNAP23 at the plasma membrane during fatty acid treatment might possibly cause insulin resistance. In order to test this conclusively, SNAP23 was transiently overexpressed during oleic acid treatment while observing insulin-dependent glucose uptake and plasma membrane translocation of GLUT4. The results showed that the fatty acid-induced insulin resistance could be completely restored by ectopic SNAP23 expression.

In conclusion, it was shown that SNAP23 has a key role in the fatty acid-mediated regulation of the insulin sensitivity of the cell.

Discussion

The effect of PLD1 on lipid droplets

In paper I, PLD1 was shown to be important for the formation of lipid droplets. This conclusion was based on siRNA knockdown in NIH 3T3 fibroblasts, which reduced the amount of lipid droplets, and also on overexpression of the enzyme, which increased the amount of lipid droplets compared to an inactive mutant. The exact mechanism by which PLD1 increases the pool of lipid droplets has not been fully elucidated, however. One possibility is that PLD1 acts by facilitating the fusion between lipid droplets. This is supported by the fact that PLD1 has been identified on lipid droplets in NIH 3T3 cells[118], and that phosphatidic acid has been reported to mediate fusion events between transport vesicles and target membranes[119]. As newly formed lipid droplets are undetectable under light microscopy, due to their size (2000 Å)[32], it may be that PLD1 promotes the fusion between such small structures into larger ones (see discussion below) - visible by light microscopy. Arguing against PLD1 acting only on lipid droplet fusion is the finding that siRNA knockdown of PLD1 reduced the amount of cellular triglycerides, while overexpression of the enzyme increased the amount of triglycerides. This effect cannot be explained solely by lipid droplet fusion.

Another possibility is that PLD1 acts by promoting the influx of glucose into the cell, which is supported by the fact that PLD1 has been proven to be active on the plasma membrane in the fusion of GLUT4 transport vesicles[120], thus increasing glucose uptake. Increased glucose uptake may promote triglyceride

synthesis. However, data from an *in vitro* system suggesting that PLD1 acts directly on the microsomal membrane for lipid droplet formation[32] conflicts with such a hypothesis. Also, PLD1 was shown to be mainly located in intracellular compartments such as the endoplasmatic reticulum (paper I, supplemental information), which argues against a specific role of this protein at the plasma membrane in this system.

A third possibility is that PLD1 enzyme activity is vital for the formation of lipid droplets from the microsomal membrane to the cytosol. This suggestion is supported by the intracellular localization of PLD1 at the site where lipid droplets form (the endoplasmatic reticulum and the Golgi apparatus; Supplemental information – paper I). Some further support for this idea is that PLD1 and the product of its enzyme activity, phosphatidic acid, are vital for the formation of lipid droplets in an *in vitro* system[32]. The effect of PA may be related to its ability to influence the structure of membranes and facilitate budding reactions[119], or it may recruit or activate other proteins involved in the process[121].

In summary, PLD1 has a central role in the formation of cytosolic lipid droplets, most likely by a specific effect on lipid droplet formation at the endoplasmic reticulum. An additional role in promoting fusion between lipid droplets cannot be excluded, however.

PLD1, but not PLD2, is the active isoform in lipid droplet biogenisis

Using specific overexpression, and dominant negative constructs of both PLD1 and PLD2, PLD1 was identified as the isoform active in lipid droplet biosynthesis.

This finding is in agreement with published data regarding the localization of PLD1 at the endoplasmatic reticulum and the Golgi apparatus[90].

PLD2 has been reported to be located on the plasma membrane exclusively[92]. Such a location does not fit in with ER synthesis of lipid droplets. Some investigators have reported that lipid droplet formation is also carried out in the plasma membrane[35]. However, the results presented in paper I are not compatible with a role of PLD2 in the assembly of lipid droplets.

Thus, PLD1 is linked to the assembly of lipid droplets and it is located at the site of droplet formation. Based on this, one can conclude that PLD1 is the most likely isoform to be active in lipid droplet biosynthesis.

ERK2 but not ERK1 is the active isoform for lipid droplet biosynthesis

The previously identified cytosolic protein required for lipid droplet assembly[32] was isolated and identified as ERK2. Using specific siRNAs against ERK1 and ERK2, combined with enzyme inhibitors, overexpression and microinjection of purified

enzyme, ERK2 was shown to be the active isoform in lipid droplet biosynthesis. We were also able to demonstrate that ERK2 phosphorylates the motor protein dynein, thereby translocating it to droplets. This was shown *in vitro* by incubating cell homogenates with active ERK2, followed by isolation of lipid droplets and phosphoprotein purification.

As to the finding that ERK2 (and not ERK1) is the active isoform in lipid droplet formation, one possibility might be that the siRNAs used were not specific for ERK2. Thus, the specificities of the respective isoforms were tested by immunoblotting against both ERK1 and ERK2 after siRNA knockdown. The data presented in paper I show that siRNA against ERK2 does not affect ERK1, or control proteins. Thus, the effect seen with siRNA against ERK2 could not be explained by ablation of ERK1 protein expression. Also, specific siRNA-mediated knockdown of ERK1 (without affecting ERK2 protein levels) failed to affect the amount of lipid droplets. Thus, ERK2 is most likely to be the isoform specific for lipid droplet formation.

ERK2 could act either by promoting budding of small lipid droplets from the microsomal membrane, or by increasing the amount of fusion events, providing more droplets that would be visible by light microscopy. Such a hypothesis is supported by the fact that ERK2 was shown to phosphorylate the motor protein dynein, directing it to droplets. Dynein was also shown to be vital for the fusion between lipid droplets (papers I and II). However, it is possible that ERK2 might influence other mechanisms involved in the assembly process. Indeed, the

observation that overexpression of ERK2 increased the triglyceride levels while siRNA against ERK2 decreased them, argue against a role for ERK2 only in fusion between lipid droplets. Unless there is a difference in triglyceride turnover between small and large droplets, such an effect cannot be explained by an effect only on lipid droplet enlargement.

This would indicate that ERK2 acts directly on the formation of the droplet from the microsomal membrane. This possibility is supported by the fact that purified ERK2 alone can activate the *in vitro* system for lipid droplet formation (paper I).

Thus, based on the results and discussion above, ERK2 most likely promotes fusion between lipid droplets by phosphorylation of dynein. The possibility that ERK2 might also act earlier in the process of lipid droplet biosynthesis cannot be excluded, however. Other methods will be required to address this question.

ERK2 and PLD1 as effectors of Insulin stimulated lipid droplet biogenesis

In paper I, data are presented which suggest that insulin acts through ERK2 and PLD1 on lipid droplet biosynthesis. This was based on the observation that siRNA knockdown of PLD1—or inhibitors of ERK2—were able to reduce the effect of insulin on lipid droplet formation.

One possibility is that ERK2 and PLD1 really are activated by the signaling cascade downstream of the insulin receptor. As discussed previously, ERK2 and PLD1 could then act either on

GLUT4 translocation, lipid droplet budding, or lipid droplet fusion. This is supported by the fact that both ERK kinases and PLD1 have already been reported to be activated by insulin signaling kinases[120,122].

The data presented in paper I do not provide evidence for direct activation of PLD1 or ERK2, only ablation of the effect of insulin on droplets when the enzymes are inhibited. It cannot, therefore, be excluded that PLD1 and/or ERK2 act in parallel with the insulin signal—being vital for lipid droplet biogenesis without direct activation of the insulin signaling cascade. In support of this is the fact that lipid droplet formation increases when ERK2 or PLD1 are overexpressed without additional activation by insulin.

The movement of lipid droplet on microtubules

In paper II, we demonstrated a rapid plus-end motion of small droplets using time-lapse microscopy with 3D reconstructions. This is in agreement with reports from *Drosophila*[87], where motor protein-mediated microtubule transport has been demonstrated.

Interestingly, we could show that the transport on microtubules and the motor protein dynein (see above) were vital for the fusion between lipid droplets. Thus, the observed fusion activity between droplets was ablated when the microtubule network was disrupted using nocodazole, or when dynein was inhibited with microinjected antibodies.

Lipid droplet fusion

In paper II, it was demonstrated that lipid droplets become enlarged by fusing with each other. This was demonstrated by three different techniques: confocal microscopy of live cells followed by 3D reconstruction, analysis of lipid droplet sizes with inhibition of triglyceride biosynthesis, and *in vitro* fusion between purified lipid droplets.

It is well known that lipid droplets increase in size secondary to fatty acid treatment, for example. One explanation is that the droplets contain the complete machinery for synthesis of both core and surface material, and enlarge individually; another explanation is that they grow by fusion.

Arguing against the possibility that droplets enlarge by themselves are data from paper II, which show that lipid droplets enlarge independently of triglyceride biosynthesis. Also, no complete machinery for lipid biosynthesis has been identified on droplets in any of the proteomic studies performed on purified droplets[31,76,123-125]. It would also be difficult to fit the membrane-spanning triglyceride biosynthesis enzymes with large hydrophilic regions at both sides of the membranes, onto a lipid droplet with a hydrophobic core surrounded by a monolayer. Together, these circumstances would argue against lipid droplet enlargement by lipid biosynthesis on the droplet surface.

Instead, the most plausible mechanism for droplet enlargement is fusion—i.e. small droplets becoming larger by fusion. This is supported by the data presented in paper II. Using time-lapse, individual lipid droplets could be followed in living cells, and the

fusion between droplets could be observed in detail. These results—especially the movies provided—clearly demonstrate fusions between lipid droplets.

In summary, the data available strongly indicate that droplets do indeed grow in size by a fusion process, and not by individual enlargement.

SNARE proteins are present in a complex on lipid droplets

In paper III, data are presented that demonstrate that the SNARE proteins SNAP23, syntaxin-5, and VAMP4 are present on lipid droplets. Co-factors for the SNARE fusion machinery—α-SNAP and NSF—were also found on droplets. Beyond just being present on droplets, the SNAREs were found to be in a functional complex on the droplets.

As syntaxin-5 and VAMP4 are integral membrane proteins, their attachment to the lipid droplet surface would be problematic if they were inserted during translation. Syntaxin-5 and VAMP4 are so-called tail-anchor proteins. They are formed as cytosolic proteins and their C-terminal hydrophobic domains are anchored to membranes by interaction with chaperone-like proteins as well as membrane proteins[110]. Such a mechanism may also anchor SNAREs in the amphipathic monolayer that surrounds lipid droplets. The C-terminal domain of syntaxin-5 is highly hydrophobic, suggesting that it could fit into a monolayer with a hydrophobic phase below. In contrast to syntaxin-5, VAMP4 contains two charged amino acids at its luminally-oriented carboxy terminus, which would be difficult to incorporate into

the core of a lipid droplet. However, it is unclear whether lipid droplets contain a perfect hydrophobic core. Freeze-fracture experiments indicate the presence of internal membrane systems. Even a small area containing a lipid-bilayer structure at the surface of lipid droplets would be sufficient for VAMP4 anchoring, and might even provide a target for localization of VAMP4. Alternatively, the transmembrane region of VAMP4 might dip into the lipid-droplet monolayer, leaving the hydrophilic tail exposed to the cytosol.

Isolation of lipid droplets with their large amphipathic surface covering the extremely hydrophobic core poses problems. Thus, such isolations always result in the presence of some contaminants. This could possibly pose a problem regarding interpretation of the finding of SNAREs on purified droplets. This question was assessed by analyzing the presence of other (highly expressed) SNAREs, and control proteins. None of the other SNAREs (or the control proteins) were present on droplets.

The SNARE proteins identified on droplets in paper III were not found in the published articles of proteomic analysis of purified lipid droplets[31,76,123-125]. This could be explained by the immuno-electron microscopy findings, which demonstrate that only a few molecules of each SNARE protein are present on each droplet. Thus, the total amount of SNAREs on lipid droplets is low, even though most droplets were found to have a positive signal.

It can therefore be concluded that the SNARE proteins SNAP23, syntaxin-5, and VAMP4—together with α-SNAP and NSF—are

present on the surface on lipid droplets, where they form a functional SNARE complex.

Lipid droplets fuse using SNARE proteins

SNAREs are present on lipid droplets and form the expected complex. Moreover, it was demonstrated that they are also active in the fusion between lipid droplets. This conclusion was based on the analysis of the effect of siRNA-mediated ablation of the respective protein followed by both lipid droplet size analysis and live time-lapse analysis of lipid droplet fusion. Apart from siRNA ablations, dominant negative overexpression and microinjections were used to reduce protein amounts and to assess loss-of-function effects. All reductions in protein levels resulted in a reduced frequency of lipid droplet fusion and smaller droplets in NIH3T3 cells.

One alternative possibility would be that the fusion between droplets is not protein-dependent, and thereby uncoordinated. This hypothesis is supported by the fact that pure triglycerides in an aqueous solution *in vitro* readily fuse to form one large fat droplet. This is also seen with lipid droplets lacking PAT domain protein associations[64]. Within the cell, however, such an organization would be chaotic—with hydrophobic droplets fusing in an uncontrolled manner, disrupting cellular architecture and clogging all the hydrophobic molecules. A more likely situation is the one seen in live cells with lipid droplets coated with PAT-domain proteins, which provides an effective way to control and organize the droplets. Such a protein coat would inhibit spontaneous fusion events between lipid droplets,

providing a means of controlling the fusion process using protein machinery.

This study is the first report of a functional v-/t-SNARE complex containing syntaxin-5, SNAP-23, and VAMP4, which would be consistent with the discovery of a previously unknown fusion reaction. Because the putative t-SNARE complex, consisting of syntaxin-5 and SNAP-23, is new, it is likely to be involved in a novel fusion reaction.

Transport vesicles are surrounded by a double phospholipid membrane, whereas the lipid droplets are surrounded by a single membrane. It is therefore likely that the mechanism of fusion between lipid droplets differs from that between transport vesicles and target membranes. The most strongly prevailing hypothesis of vesicle fusion is the so-called “stalk hypothesis”. According to the hypothesis, fusion is the result of an ordered sequence of transition states (reviewed in[126]).

Based on this, a model is therefore proposed in which fusion between lipid droplets requires only the first two steps in the process proposed for fusion between vesicles and target membranes, and is completed at a stage equivalent to the creation of the “fusion stalk”, i.e. when the hydrophobic parts of two membranes are connected but no aqueous connection has been formed (shown in Figure 3).

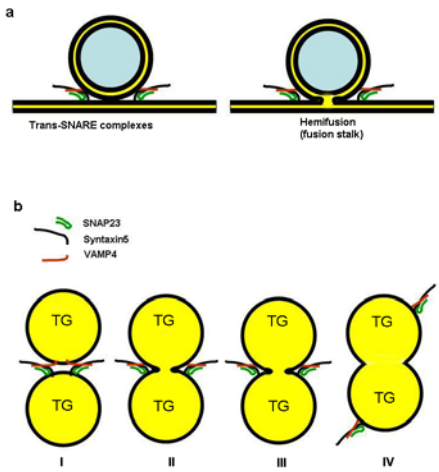

Figure 3. A model of fusion. (a) The first step in the stalk hypothesis for the fusion of two bi-layers (adapted from[126]). The outer leaflets fuse and connect the two hydrophobic regions of the bi-layer (yellow). The hydrophilic portions of each monolayer of the membranes are shown in black. (b) A possible model for fusion between two droplets. The formation of the four-helix bundle between syntaxin-5 (Qa), SNAP23 (Qbc), and VAMP4 (R) forces the two monolayers to fuse with each other, resulting in a connection between the hydrophobic phases (yellow). This is equivalent to the creation of a fusion stalk in (a). TG: triglycerides.

SNAP23 as a modulator of fatty acid-induced insulin resistance

The results from paper III reveal that fatty acid treatment reduces the insulin-dependent uptake of glucose and the translocation of GLUT4 to the plasma membrane by diverting SNAP23 from the plasma membrane to the interior of the cell and lipid droplets.

Even though SNAP23 is clearly reduced on the plasma membrane upon fatty acid treatment and increased on the lipid droplets, the disappearance at the plasma membrane, however, represents only 30% of the total pool. Also, the increase in SNAP23 on droplets is only a small proportion of the total intracellular amount of SNAP23. Thus, the quantitative changes in SNAP23 levels would not completely explain the huge effect seen on insulin-stimulated glucose uptake and GLUT4 translocation. There are, however, other mechanisms that might additionally explain the dramatic effect of SNAP23. The t-SNARE complex between SNAP23 and syntaxin-4 that mediates the fusion of GLUT4 vesicles to the plasma membrane can be disrupted by a protein named Munc18c[127]. Munc18c binds to syntaxin-4 and thereby prevents SNAP23 from binding, thus causing insulin resistance[127-129]. Munc18 is also known to be upregulated by fatty acid treatment[130]. Thus, apart from the translocation of SNAP23 seen in paper III, treatment with fatty acids might inhibit the t-SNARE complex by increasing the protein amount of Munc18c. These two mechanisms together could explain the great effect of fatty acids on insulin resistance, and also the vital role for SNAP23 in the process.

It has been shown that fatty acid-induced insulin resistance is mediated by inactivation of vital signalling kinases downstream of the insulin receptor. An example of such a kinase is Protein Kinase C δ (PKCδ)[131]. This could possibly argue against SNAP23 as being the only mechanistic mediator of fatty acid-mediated insulin resistance. On the other hand, it is not unlikely that the disruption of the t-SNARE complex for GLUT4-translocation by fatty acid stimulation would be mediated by

signaling kinases. In fact, SNAP23 has been shown to be phosphorylated by PKC[132]. Also, a protein named Synip has been shown to undergo protein-protein interactions with SNAP23, and its phosphorlyation is necessary for insulin-dependent glucose uptake. Thus, the previously described kinases that are active in fatty acid-induced insulin resistance could possibly act by disrupting the t-SNARE complex of SNAP23 and syntaxin-4, thereby being compatible with the data in paper III.

Taken together, the results presented suggest that translocation of SNAP23 is a major mediator of fatty acid-induced insulin resistance.

Proposed model

The data and discussion from this thesis can be summarized in a model in which lipid droplets are formed in association with microsomes. The assembly is promoted by insulin and required enzymes involved in triglyceride biosynthesis as well as phospholipase D1 and ERK2. The two latter proteins are necessary for the insulin effect. ERK2 acts by phosphorylating dynein, which then translocates to the lipid droplets and participates in enlargement of the newly formed droplets. This process is illustrated in Figure 4.

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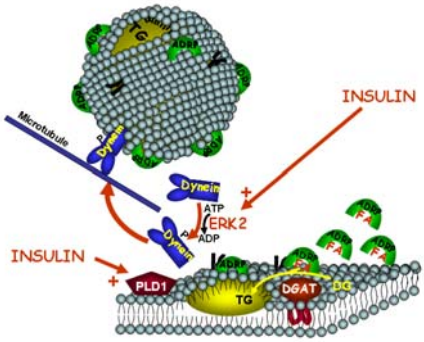

Figure 4. A model for initial lipid droplet formation, where DGAT produces triglycerides between the leaflets of the microsomal membrane. A lipid droplet is then formed in response to insulin signaling and the activities of PLD1 and ERK2. This droplet contains dynein, which mediates the enlargement of newly synthesized lipid droplets.

After the initial formation, the droplets increase in size by fusing with each other. This fusion is performed on microtubules, and is mediated by SNARE proteins—SNAP23, syntaxin-5, and VAMP4. Apart from the SNAREs, co-factors such as α-SNAP are necessary for the continuation of fusion events. This process is illustrated in Figure 5.

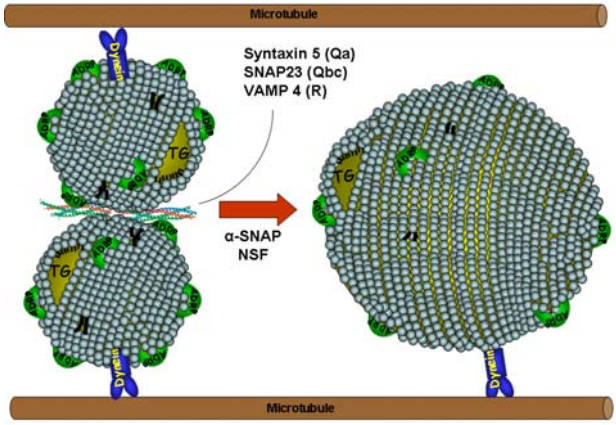

Figure 5. Model of fusion between small lipid droplets, mediated by the SNARE proteins SNAP23, syntaxin-5, and VAMP4.

The final data in this thesis indicate that SNAP23 also participates in insulin-mediated translocation of GLUT4 to the plasma membrane, thus causing glucose uptake. When lipid biosynthesis is activated, SNAP23 is redistributed from the plasma membrane to lipid droplets—causing a defect in the insulin signalling and thereby insulin resistance.

References

1. King, H., Aubert, R. E. & Herman, W. H. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* **21**, 1414-31 (1998).
2. Williamson, D. F., Vinicor, F. & Bowman, B. A. Primary prevention of type 2 diabetes mellitus by lifestyle intervention: implications for health policy. *Ann Intern Med* **140**, 951-7 (2004).
3. Ulbrecht, J. S., Cavanagh, P. R. & Caputo, G. M. Foot problems in diabetes: an overview. *Clin Infect Dis* **39 Suppl 2**, S73-82 (2004).
4. Hogan, P., Dall, T. & Nikolov, P. Economic costs of diabetes in the US in 2002. *Diabetes Care* **26**, 917-32 (2003).
5. Barcelo, A., Aedo, C., Rajpathak, S. & Robles, S. The cost of diabetes in Latin America and the Caribbean. *Bull World Health Organ* **81**, 19-27 (2003).
6. Federation, I. D. in *Diabetes atlas* (2005).
7. Petersen, K. F. & Shulman, G. I. Etiology of insulin resistance. *Am J Med* **119**, S10-6 (2006).
8. Eckel, R. H., Grundy, S. M. & Zimmet, P. Z. The metabolic syndrome. *Lancet* **365**, 1415-28 (2005).
9. Stumvoll, M., Goldstein, B. J. & van Haeften, T. W. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* **365**, 1333-46 (2005).
10. Falholt, K., Cutfield, R., Alejandro, R., Heding, L. & Mintz, D. The effects of hyperinsulinemia on arterial wall and peripheral muscle metabolism in dogs. *Metabolism* **34**, 1146-9 (1985).
11. Storlien, L. H. et al. Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and omega-3 fatty acids in muscle phospholipid. *Diabetes* **40**, 280-9 (1991).

12. Falholt, K. et al. Carbohydrate and lipid metabolism of skeletal muscle in type 2 diabetic patients. *Diabet Med* **5**, 27-31 (1988).
13. Machann, J., Haring, H., Schick, F. & Stumvoll, M. Intramyocellular lipids and insulin resistance. *Diabetes Obes Metab* **6**, 239-48 (2004).
14. Krssak, M. et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia* **42**, 113-6 (1999).
15. Jacob, S. et al. Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. *Diabetes* **48**, 1113-9 (1999).
16. Yu, C. et al. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* **277**, 50230-6 (2002).
17. Morino, K. et al. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest* **115**, 3587-93 (2005).
18. Nagle, C. A. et al. Hepatic overexpression of glycerol-sn-3-phosphate acyltransferase 1 in rats causes insulin resistance. *J Biol Chem* **282**, 14807-15 (2007).
19. Monetti, M. et al. Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. *Cell Metab* **6**, 69-78 (2007).
20. Martin, S. & Parton, R. G. Lipid droplets: a unified view of a dynamic organelle. *Nat Rev Mol Cell Biol* **7**, 373-8 (2006).
21. Welte, M. A. Proteins under new management: lipid droplets deliver. *Trends Cell Biol* **17**, 363-9 (2007).
22. Brasaemle, D. L. The perilipin family of structural lipid droplet proteins: Stabilization of lipid droplets and control of lipolysis. *J Lipid Res* (2007).

23. Ozeki, S. et al. Rab18 localizes to lipid droplets and induces their close apposition to the endoplasmic reticulum-derived membrane. *J Cell Sci* **118**, 2601-11 (2005).
24. Martin, S., Driessen, K., Nixon, S. J., Zerial, M. & Parton, R. G. Regulated localization of Rab18 to lipid droplets: effects of lipolytic stimulation and inhibition of lipid droplet catabolism. *J Biol Chem* **280**, 42325-35 (2005).
25. Ostermeyer, A. G. et al. Accumulation of caveolin in the endoplasmic reticulum redirects the protein to lipid storage droplets. *J Cell Biol* **152**, 1071-8 (2001).
26. Almahbobi, G., Williams, L. J. & Hall, P. F. Attachment of steroidogenic lipid droplets to intermediate filaments in adrenal cells. *J Cell Sci* **101 (Pt 2)**, 383-93 (1992).
27. Weller, P. F. & Dvorak, A. M. Lipid bodies: intracellular sites for eicosanoid formation. *J Allergy Clin Immunol* **94**, 1151-6 (1994).
28. Dolinsky, V. W., Gilham, D., Alam, M., Vance, D. E. & Lehner, R. Triacylglycerol hydrolase: role in intracellular lipid metabolism. *Cell Mol Life Sci* **61**, 1633-51 (2004).
29. Carey, G. B. Mechanisms regulating adipocyte lipolysis. *Adv Exp Med Biol* **441**, 157-70 (1998).
30. Smirnova, E. et al. ATGL has a key role in lipid droplet/adiposome degradation in mammalian cells. *EMBO Rep* **7**, 106-13 (2006).
31. Liu, P. et al. Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. *J Biol Chem* **279**, 3787-92 (2004).
32. Marchesan, D. et al. A phospholipase D-dependent process forms lipid droplets containing caveolin, adipocyte differentiation-related protein, and vimentin in a cell-free system. *J Biol Chem* **278**, 27293-300 (2003).

33. Farese, R. V., Jr., Cases, S. & Smith, S. J. Triglyceride synthesis: insights from the cloning of diacylglycerol acyltransferase. *Curr Opin Lipidol* **11**, 229-34 (2000).
34. Blanchette-Mackie, E. J. et al. Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J Lipid Res* **36**, 1211-26 (1995).
35. Ost, A., Ortegren, U., Gustavsson, J., Nystrom, F. H. & Stralfors, P. Triacylglycerol is synthesized in a specific subclass of caveolae in primary adipocytes. *J Biol Chem* **280**, 5-8 (2005).
36. Murphy, D. J. & Vance, J. Mechanisms of lipid-body formation. *Trends Biochem Sci* **24**, 109-15 (1999).
37. Brasaemle, D. L., Levin, D. M., Adler-Wailes, D. C. & Londos, C. The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. *Biochim Biophys Acta* **1483**, 251-62 (2000).
38. Dalen, K. T. et al. LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues. *Biochim Biophys Acta* **1771**, 210-27 (2007).
39. Wolins, N. E. et al. OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* **55**, 3418-28 (2006).
40. Miura, S. et al. Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, Drosophila, and Dictyostelium. *J Biol Chem* **277**, 32253-7 (2002).
41. Lu, X. et al. The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. *Mamm Genome* **12**, 741-9 (2001).
42. Dalen, K. T. et al. Adipose tissue expression of the lipid droplet-associating proteins S3-12 and

perilipin is controlled by peroxisome proliferator-activated receptor-gamma. *Diabetes* **53**, 1243-52 (2004).
43. Scherer, P. E., Bickel, P. E., Kotler, M. & Lodish, H. F. Cloning of cell-specific secreted and surface proteins by subtractive antibody screening. *Nat Biotechnol* **16**, 581-6 (1998).
44. Eisinger, D. P. & Serrero, G. Structure of the gene encoding mouse adipose differentiation-related protein (ADRP). *Genomics* **16**, 638-44 (1993).
45. Greenberg, A. S. et al. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J Biol Chem* **266**, 11341-6 (1991).
46. Londos, C. et al. Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells. *Biochem Soc Trans* **23**, 611-5 (1995).
47. Londos, C., Brasaemle, D. L., Schultz, C. J., Segrest, J. P. & Kimmel, A. R. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin Cell Dev Biol* **10**, 51-8 (1999).
48. Brasaemle, D. L. et al. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J Lipid Res* **38**, 2249-63 (1997).
49. Londos, C., Sztalryd, C., Tansey, J. T. & Kimmel, A. R. Role of PAT proteins in lipid metabolism. *Biochimie* **87**, 45-9 (2005).
50. Brasaemle, D. L. et al. Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J Biol Chem* **275**, 38486-93 (2000).
51. Martinez-Botas, J. et al. Absence of perilipin results in leanness and reverses obesity in Lepr(db/db) mice. *Nat Genet* **26**, 474-9 (2000).

52. Tansey, J. T. et al. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc Natl Acad Sci U S A* **98**, 6494-9 (2001).
53. Tai, E. S. & Ordovas, J. M. The role of perilipin in human obesity and insulin resistance. *Curr Opin Lipidol* **18**, 152-6 (2007).
54. Jiang, H. P. & Serrero, G. Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. *Proc Natl Acad Sci U S A* **89**, 7856-60 (1992).
55. Heid, H. W., Moll, R., Schwetlick, I., Rackwitz, H. R. & Keenan, T. W. Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. *Cell Tissue Res* **294**, 309-21 (1998).
56. Nakamura, N. & Fujimoto, T. Adipose differentiation-related protein has two independent domains for targeting to lipid droplets. *Biochem Biophys Res Commun* **306**, 333-8 (2003).
57. Nakamura, N. et al. ADRP is dissociated from lipid droplets by ARF1-dependent mechanism. *Biochem Biophys Res Commun* **322**, 957-65 (2004).
58. Dalen, K. T., Ulven, S. M., Arntsen, B. M., Solaas, K. & Nebb, H. I. PPARalpha activators and fasting induce the expression of adipose differentiation-related protein in liver. *J Lipid Res* **47**, 931-43 (2006).
59. Edvardsson, U. et al. PPARalpha activation increases triglyceride mass and adipose differentiation-related protein in hepatocytes. *J Lipid Res* **47**, 329-40 (2006).
60. Targett-Adams, P. et al. A PPAR response element regulates transcription of the gene for human adipose differentiation-related protein. *Biochim Biophys Acta* **1728**, 95-104 (2005).
61. Xu, G. et al. Post-translational regulation of adipose differentiation-related protein by the

ubiquitin/proteasome pathway. *J Biol Chem* **280**, 42841-7 (2005).
62. Masuda, Y. et al. ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells. *J Lipid Res* **47**, 87-98 (2006).
63. Chang, B. H. et al. Protection against fatty liver but normal adipogenesis in mice lacking adipose differentiation-related protein. *Mol Cell Biol* **26**, 1063-76 (2006).
64. Sztalryd, C. et al. Functional compensation for adipose differentiation-related protein (ADFP) by Tip47 in an ADFP null embryonic cell line. *J Biol Chem* **281**, 34341-8 (2006).
65. Magnusson, B. et al. Adipocyte differentiation-related protein promotes fatty acid storage in cytosolic triglycerides and inhibits secretion of very low-density lipoproteins. *Arterioscler Thromb Vasc Biol* **26**, 1566-71 (2006).
66. Imai, Y. et al. Reduction of hepatosteatosis and lipid levels by an adipose differentiation-related protein antisense oligonucleotide. *Gastroenterology* **132**, 1947-54 (2007).
67. Bostrom, P. et al. Hypoxia converts human macrophages into triglyceride-loaded foam cells. *Arterioscler Thromb Vasc Biol* **26**, 1871-6 (2006).
68. Wang, X. et al. Induced expression of adipophilin mRNA in human macrophages stimulated with oxidized low-density lipoprotein and in atherosclerotic lesions. *FEBS Lett* **462**, 145-50 (1999).
69. Nuotio, K. et al. Adipophilin expression is increased in symptomatic carotid atherosclerosis: correlation with red blood cells and cholesterol crystals. *Stroke* **38**, 1791-8 (2007).
70. Andersson, L. et al. PLD1 and ERK2 regulate cytosolic lipid droplet formation. *J Cell Sci* **119**, 2246-57 (2006).

71. Diaz, E. & Pfeffer, S. R. TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. *Cell* **93**, 433-43 (1998).
72. Krise, J. P., Sincock, P. M., Orsel, J. G. & Pfeffer, S. R. Quantitative analysis of TIP47-receptor cytoplasmic domain interactions: implications for endosome-to-trans Golgi network trafficking. *J Biol Chem* **275**, 25188-93 (2000).
73. Carroll, K. S. et al. Role of Rab9 GTPase in facilitating receptor recruitment by TIP47. *Science* **292**, 1373-6 (2001).
74. Wolins, N. E., Rubin, B. & Brasaemle, D. L. TIP47 associates with lipid droplets. *J Biol Chem* **276**, 5101-8 (2001).
75. Barbero, P., Buell, E., Zulley, S. & Pfeffer, S. R. TIP47 is not a component of lipid droplets. *J Biol Chem* **276**, 24348-51 (2001).
76. Brasaemle, D. L., Dolios, G., Shapiro, L. & Wang, R. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J Biol Chem* **279**, 46835-42 (2004).
77. Wolins, N. E. et al. S3-12, Adipophilin, and TIP47 package lipid in adipocytes. *J Biol Chem* **280**, 19146-55 (2005).
78. Robenek, H., Lorkowski, S., Schnoor, M. & Troyer, D. Spatial integration of TIP47 and adipophilin in macrophage lipid bodies. *J Biol Chem* **280**, 5789-94 (2005).
79. Hickenbottom, S. J., Kimmel, A. R., Londos, C. & Hurley, J. H. Structure of a lipid droplet protein; the PAT family member TIP47. *Structure* **12**, 1199-207 (2004).
80. Yamaguchi, T., Matsushita, S., Motojima, K., Hirose, F. & Osumi, T. MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-

activated receptor alpha. *J Biol Chem* **281**, 14232-40 (2006).
81. Wolins, N. E. et al. Adipocyte protein S3-12 coats nascent lipid droplets. *J Biol Chem* **278**, 37713-21 (2003).
82. Ahmed, W. et al. PPARs and their metabolic modulation: new mechanisms for transcriptional regulation? *J Intern Med* **262**, 184-98 (2007).
83. Arimura, N., Horiba, T., Imagawa, M., Shimizu, M. & Sato, R. The peroxisome proliferator-activated receptor gamma regulates expression of the perilipin gene in adipocytes. *J Biol Chem* **279**, 10070-6 (2004).
84. Xu, G., Sztalryd, C. & Londos, C. Degradation of perilipin is mediated through ubiquitination-proteasome pathway. *Biochim Biophys Acta* **1761**, 83-90 (2006).
85. Luduena, R. F., Shooter, E. M. & Wilson, L. Structure of the tubulin dimer. *J Biol Chem* **252**, 7006-14 (1977).
86. Soldati, T. & Schliwa, M. Powering membrane traffic in endocytosis and recycling. *Nat Rev Mol Cell Biol* **7**, 897-908 (2006).
87. Gross, S. P., Welte, M. A., Block, S. M. & Wieschaus, E. F. Coordination of opposite-polarity microtubule motors. *J Cell Biol* **156**, 715-24 (2002).
88. Mallik, R. & Gross, S. P. Molecular motors: strategies to get along. *Curr Biol* **14**, R971-82 (2004).
89. Hook, P. & Vallee, R. B. The dynein family at a glance. *J Cell Sci* **119**, 4369-71 (2006).
90. Jenkins, G. M. & Frohman, M. A. Phospholipase D: a lipid centric review. *Cell Mol Life Sci* **62**, 2305-16 (2005).
91. Han, J. M. et al. Localization of phospholipase D1 to caveolin-enriched membrane via palmitoylation: implications for epidermal growth factor signaling. *Mol Biol Cell* **13**, 3976-88 (2002).

92. Sarri, E., Pardo, R., Fensome-Green, A. & Cockcroft, S. Endogenous phospholipase D2 localizes to the plasma membrane of RBL-2H3 mast cells and can be distinguished from ADP ribosylation factor-stimulated phospholipase D1 activity by its specific sensitivity to oleic acid. *Biochem J* **369**, 319-29 (2003).
93. Colley, W. C. et al. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr Biol* **7**, 191-201 (1997).
94. Hammond, S. M. et al. Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. *J Biol Chem* **270**, 29640-3 (1995).
95. Raman, M., Chen, W. & Cobb, M. H. Differential regulation and properties of MAPKs. *Oncogene* **26**, 3100-12 (2007).
96. Bonifacino, J. S. & Glick, B. S. The mechanisms of vesicle budding and fusion. *Cell* **116**, 153-66 (2004).
97. Glick, B. S. & Rothman, J. E. Possible role for fatty acyl-coenzyme A in intracellular protein transport. *Nature* **326**, 309-12 (1987).
98. Clary, D. O., Griff, I. C. & Rothman, J. E. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* **61**, 709-21 (1990).
99. Sollner, T. et al. SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318-24 (1993).
100. Whyte, J. R. & Munro, S. Vesicle tethering complexes in membrane traffic. *J Cell Sci* **115**, 2627-37 (2002).
101. Chen, Y. A. & Scheller, R. H. SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol* **2**, 98-106 (2001).

102. Jahn, R., Lang, T. & Sudhof, T. C. Membrane fusion. *Cell* **112**, 519-33 (2003).
103. Sutton, R. B., Fasshauer, D., Jahn, R. & Brunger, A. T. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* **395**, 347-53 (1998).
104. Fasshauer, D., Sutton, R. B., Brunger, A. T. & Jahn, R. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci U S A* **95**, 15781-6 (1998).
105. Bock, J. B., Matern, H. T., Peden, A. A. & Scheller, R. H. A genomic perspective on membrane compartment organization. *Nature* **409**, 839-41 (2001).
106. Hohl, T. M. et al. Arrangement of subunits in 20 S particles consisting of NSF, SNAPs, and SNARE complexes. *Mol Cell* **2**, 539-48 (1998).
107. Wimmer, C. et al. Molecular mass, stoichiometry, and assembly of 20 S particles. *J Biol Chem* **276**, 29091-7 (2001).
108. Brunger, A. T. & DeLaBarre, B. NSF and p97/VCP: similar at first, different at last. *FEBS Lett* **555**, 126-33 (2003).
109. Hong, W. SNAREs and traffic. *Biochim Biophys Acta* **1744**, 120-44 (2005).
110. High, S. & Abell, B. M. Tail-anchored protein biosynthesis at the endoplasmic reticulum: the same but different. *Biochem Soc Trans* **32**, 659-62 (2004).
111. Huang, S. & Czech, M. P. The GLUT4 glucose transporter. *Cell Metab* **5**, 237-52 (2007).
112. Sinha, M. K. et al. Adipose tissue glucose transporters in NIDDM. Decreased levels of muscle/fat isoform. *Diabetes* **40**, 472-7 (1991).
113. Ren, J. M., Semenkovich, C. F., Gulve, E. A., Gao, J. & Holloszy, J. O. Exercise induces rapid increases in GLUT4 expression, glucose transport

capacity, and insulin-stimulated glycogen storage in muscle. *J Biol Chem* **269**, 14396-401 (1994).
114. Thong, F. S., Dugani, C. B. & Klip, A. Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. *Physiology (Bethesda)* **20**, 271-84 (2005).
115. Ishiki, M. & Klip, A. Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. *Endocrinology* **146**, 5071-8 (2005).
116. Kelemen, B. R., Hsiao, K. & Goueli, S. A. Selective in vivo inhibition of mitogen-activated protein kinase activation using cell-permeable peptides. *J Biol Chem* **277**, 8741-8 (2002).
117. Kawanishi, M. et al. Role of SNAP23 in insulin-induced translocation of GLUT4 in 3T3-L1 adipocytes. Mediation of complex formation between syntaxin4 and VAMP2. *J Biol Chem* **275**, 8240-7 (2000).
118. Nakamura, N., Banno, Y. & Tamiya-Koizumi, K. Arf1-dependent PLD1 is localized to oleic acid-induced lipid droplets in NIH3T3 cells. *Biochem Biophys Res Commun* **335**, 117-23 (2005).
119. Kooijman, E. E., Chupin, V., de Kruijff, B. & Burger, K. N. Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. *Traffic* **4**, 162-74 (2003).
120. Huang, P., Altshuller, Y. M., Hou, J. C., Pessin, J. E. & Frohman, M. A. Insulin-stimulated plasma membrane fusion of Glut4 glucose transporter-containing vesicles is regulated by phospholipase D1. *Mol Biol Cell* **16**, 2614-23 (2005).
121. Honda, A. et al. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521-32 (1999).

122. Avruch, J. Insulin signal transduction through protein kinase cascades. *Mol Cell Biochem* **182**, 31-48 (1998).
123. Bartz, R. et al. Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. *J Lipid Res* **48**, 837-47 (2007).
124. Bartz, R. et al. Dynamic Activity of Lipid Droplets: Protein Phosphorylation and GTP-Mediated Protein Translocation. *J Proteome Res* **6**, 3256-65 (2007).
125. Cermelli, S., Guo, Y., Gross, S. P. & Welte, M. A. The lipid-droplet proteome reveals that droplets are a protein-storage depot. *Curr Biol* **16**, 1783-95 (2006).
126. Jahn, R. & Scheller, R. H. SNAREs--engines for membrane fusion. *Nat Rev Mol Cell Biol* **7**, 631-43 (2006).
127. Araki, S. et al. Inhibition of the binding of SNAP-23 to syntaxin 4 by Munc18c. *Biochem Biophys Res Commun* **234**, 257-62 (1997).
128. Tamori, Y. et al. Inhibition of insulin-induced GLUT4 translocation by Munc18c through interaction with syntaxin4 in 3T3-L1 adipocytes. *J Biol Chem* **273**, 19740-6 (1998).
129. Kanda, H. et al. Adipocytes from Munc18c-null mice show increased sensitivity to insulin-stimulated GLUT4 externalization. *J Clin Invest* **115**, 291-301 (2005).
130. Schlaepfer, I. R. et al. Increased expression of the SNARE accessory protein Munc18c in lipid-mediated insulin resistance. *J Lipid Res* **44**, 1174-81 (2003).
131. Dey, D., Basu, D., Roy, S. S., Bandyopadhyay, A. & Bhattacharya, S. Involvement of novel PKC isoforms in FFA induced defects in insulin signaling. *Mol Cell Endocrinol* **246**, 60-4 (2006).
132. Polgar, J., Lane, W. S., Chung, S. H., Houng, A. K. & Reed, G. L. Phosphorylation of SNAP-23 in

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Paper I

Paper II

Paper III