STK25 as a regulator of lipid accumulation

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Cover illustration: “A mysterious life of lipid droplets” by Urszula Chursa. Image of differentiated 3T3-L1 cells, generated with Deep Dream Generator.

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Nic dwa razy

Nic dwa razy się nie zdarza
i nie zdarzy. Z tej przyczyny
zrodziliśmy się bez wprawy
i pomrzemy bez rutyny.

Choćbyśmy uczniami byli
najtępszymi w szkole świata,
nie będziemy repetować
żadnej zimy ani lata.

Żaden dzień się nie powtórzy,
nie ma dwóch podobnych nocy,
dwóch tych samych pocałunków,
dwóch jednakich spojrzeń w oczy.

Wczoraj, kiedy twoje imię
ktoś wymówił przy mnie głośno,
tak mi było, jakby róża
przez otwarte wpadła okno.

Dziś, kiedy jesteśmy razem,
odwróciłam twarz ku ścianie.
Róża? Jak wygląda róża?
Czy to kwiat? A może kamień?

Czemu ty się, zła godzino,
z niepotrzebnym mieszasz lękiem?
Jesteś - a więc musisz minąć.
Miniesz - a więc to jest piękne.

Uśmiechnięci, współobjęci
spróbujemy szukać zgody,
choć różnimy się od siebie
jak dwie krople czystej wody.

Wisława Szymborska

Nothing twice

Nothing can ever happen twice.
In consequence, the sorry fact is
that we arrive here improvised
and leave without the chance to
practice.

Even if there is no one dumber,
if you're the planet's biggest dunce,
you can't repeat the class in summer:
this course is only offered once.

No day copies yesterday,
no two nights will teach what bliss is
in precisely the same way,
with precisely the same kisses.

One day, perhaps some idle tongue
mentions your name by accident:
I feel as if a rose were flung
into the room, all hue and scent.

The next day, though you're here with
me,
I can't help looking at the clock:
A rose? A rose? What could that be?
Is it a flower or a rock?

Why do we treat the fleeting day
with so much needless fear and
sorrow?
It's in its nature not to stay:
Today is always gone tomorrow.

With smiles and kisses, we prefer
to seek accord beneath our star,
although we're different (we concur)
just as two drops of water are.

Translated by Clare Cavanagh and
Stanislaw Baranczak

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ABSTRACT

Type 2 diabetes mellitus (T2DM) and nonalcoholic steatohepatitis (NASH), a progressive form of nonalcoholic fatty liver disease (NAFLD), have become widespread metabolic disorders that have reached epidemic proportions. Obesity, with ectopic lipid accumulation, is the main factor for the development and subsequent progression of T2DM and NASH. To develop effective pharmacological treatment strategies against these metabolic diseases, it is important to understand molecular mechanisms that control ectopic lipid deposition and insulin resistance.

Previous findings demonstrate that inhibition of serine/threonine protein kinase (STK25) leads to protection against HFD-induced liver steatosis and improved whole-body glucose tolerance and insulin sensitivity. In contrast, STK25 overexpression results in aggravated steatosis, promoting NAFLD and driving NASH development. Based on these studies, we investigated STK25 in the development of diet-induced NASH, described in *Paper I*. In this project, we found that mice fed methionine-choline deficient (MCD) diet were protected against NASH development following depletion of STK25. However, STK25 overexpression led to development of a more severe NASH phenotype when mice were challenged with MCD diet.

In *Paper II*, we found that overexpression of STK25 leads to increased ectopic lipid storage, fibrosis and inflammation in skeletal muscle of

mice fed high-fat diet (HFD). Moreover, STK25-overexpressing mice had decreased *in vivo* insulin-stimulated glucose uptake, decreased endurance exercise performance and impairments in β-oxidation.

We also explored the role of STK25 in adipocyte lipid accumulation and maturation in *Paper III* using 3T3-L1 preadipocyte cells. We found that 3T3-L1 cells accumulated less lipid droplets and had several markers of adipocyte maturation reduced when STK25 was silenced prior to differentiation. Furthermore, we show a significant positive correlation between adipogenesis markers and STK25 expression in human adipose tissue.

Taken together, work in this thesis contributes to the concept that STK25 is a potential drug target for prevention and/or treatment of obesity-associated T2DM, NAFLD and NASH.

Keywords: STK25, T2DM, NAFLD, NASH, ectopic lipid accumulation, metabolism.

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SAMMANFATTNING PÅ SVENSKA

Typ 2 diabetes mellitus (T2DM) och non-alcoholic steatohepatitis (NASH), en progressiv form non-alcoholic fatty liver disease (NAFLD), har uppnått epidemiska proportioner och blivit ett globalt hot mot människors hälsa. Fetma, framförallt bukfetma, och leverförfettning är huvudfaktorerna i utvecklingen och progressionen av T2DM och NASH. För att utveckla effektiva farmakologiska behandlingsstrategier mot dessa metaboliska sjukdomar är det viktigt att förstå molekylära mekanismer som styr ektopisk lipidinlagring och insulinresistens.

Tidigare fynd visar att hämmandet av serin/threonin proteinkinas (STK25) leder till skydd mot leverförfettning orsakad av högfettsdiet, samt förbättrad glukostolerans och insulinkänslighet i hela kroppen. Däremot leder överuttryck av STK25 till ökad lipidinlagring i levern, som främjar NAFLD-utveckling och driver utvecklingen av NASH.

I denna avhandling fann vi att möss som saknar STK25 skyddades mot NASH-utveckling medan överuttryck av STK25 ledde till en snabbare utveckling av NASH. Vi har funnit att överuttryck av STK25 leder till ökad ektopisk lipidansamling, fibros och inflammation i skelettmuskulaturen hos möss som matas med högfettsdiet. Dessutom hade STK25-överuttryckande möss minskat sin insulinkänslighet, hade försämrad muskeluthållighet och nedsatt fettmetabolism. Vi har dessutom undersökt STK25 roll vid fettcellernas lipidansamling och mognad med hjälp av 3T3-L1 celler. Vi fann att 3T3-L1-celler utvecklade mindre lipiddroppar och hade reducerade markörer av fettcell-mognad när STK25 tystades före cell differentieringen. Vidare visar vi en signifikant positiv korrelation mellan fettcellernas differentieringsmarkörer och STK25 genuttryck i human fettvävnad.

Sammanfattningsvis visar vi att STK25 är ett potentiellt intressant protein för förebyggande och/eller behandling av T2DM, NAFLD och NASH och relaterade metabola komplikationer.

LIST OF PAPERS

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

I. Amrutkar M, **Chursa U**, Kern M, Nuñez-Durán E, Ståhlman M, Sütt S, Borén J, Marschall HU, Blüher M, Mahlapuu M. STK25 is a Critical Determinant in Nonalcoholic Steatohepatitis. *FASEB J. 2016, 30(10): 3628-3643*

II. **Chursa U**, Nuñez-Durán E, Cansby E, Amrutkar M, Sütt S, Ståhlman M, Olsson BM, Borén J, Johansson ME, Bäckhed F, Johansson BR, Sihlbom C, Mahlapuu M. Overexpression of Protein Kinase STK25 in Mice Exacerbates Ectopic Lipid Accumulation, Mitochondrial Dysfunction, and Insulin Resistance in Skeletal Muscle. *Diabetologia. 2017, 60(3): 553-567*

III. **Chursa U**, Hammarstedt A, Smith U. STK25 regulates lipid accumulation and maturation of 3T3-L1 cells. *Manuscript*

CONTENT

ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ALT	Alanine aminotransferase
AMPK	AMP-activated kinase
ATGL	Adipose triacylglycerol lipase
C/EBP	CCAAT/enhancer-binding protein
CAT	Catalase
CYPE21	Cytochrome P450 2E1
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
EDL	Extensor digitorum longus
EHC	Euglycemic-hyperinsulinemic clamp
ER	Endoplasmatic reticulum
FFA	Free fatty acid
GM130	Golgi matrix protein 130
GSH	Glutathione
HCC	Hepatocellular carcinoma
HFD	High fat diet
HSL	Hormone sensitive lipase
LCFA	Long-chain fatty acid

LD	Lipid droplet
LPL	Lipoprotein lipase
MAG	Monoacylglycerol
MCD	Methionine-choline deficient
MSC	Mesenchymal stem cells
MST	Mammalian Sterile20-like
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NEFA	Non-esterified free fatty acid
PPARγ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
STK25	Serine/threonine protein kinase 25
STRIPAK	Striatin-interacting phosphatase and kinase
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TBARS	Thiobarbituric acid-reactive substances
TEM	Transmission electron microscopy
VLDL	Very low-density lipoprotein
WAT	White adipose tissue

1 INTRODUCTION

Metabolic diseases and disorders are major challenges in the world today and include T2DM and NASH, both of which are related to ectopic fat accumulation including NAFLD and insulin resistance. Prevalence of T2DM is continuously rising. More than 400 million people were diagnosed with T2DM in year 2014 (World Health Organization). T2DM is highly prevalent in NAFLD (1). The main feature of it is hepatic steatosis, i.e.; liver triglyceride (TAG) accumulation (1). NAFLD progression to a more aggressive liver disorder, NASH, is described as a multiple hit mechanism (2). The first hit is considered to be metabolic insulin resistance, which promotes hepatic steatosis. Subsequent dysregulation of mitochondrial functions including *de novo* lipogenesis (DNL) and reduced ability of the liver to export fatty acids (FAs) promotes the second hit - accumulation of reactive oxygen species (ROS) causing oxidative stress. However, in these early stages of NASH, when mitochondrial dysfunction is not pronounced, mitochondrial adaptation in the liver is possible and the disorder pattern can reverse. This ability is unfortunately lost during NASH progression (3). When ectopic lipid accumulation is a fact and mitochondrial function is impaired, chronic oxidative stress mediates recruitment of inflammatory infiltrates, endoplasmatic reticulum (ER) stress and hepatocellular damage (4-6). Fibrosis, which is a predictor of cirrhosis, is an optional feature of NASH and does not always develop. However, approximately one fifth of NASH patients develop fibrosis, which further progresses to cirrhosis, hepatocellular carcinoma (HCC) and liver failure. A majority of obese T2DM patients has NAFLD and many of these develop NASH. Also, diabetic patients are at higher risk of developing hepatic fibrosis, HCC and other hepatic complications (7-9).

1.1 WAT

White adipose tissue (WAT) plays a fundamental role in energy homeostasis (10). Placed in various anatomical locations in the body, WAT functions as a lipid storage/release and endocrine organ that provides energy and insulation as well as mechanical protection. The subcutaneous depot is associated with protective metabolic effects and insulin sensitivity, whereas increased visceral adipose tissue is associated with metabolic disorders and insulin resistance (11; 12). WAT has remarkable expandability properties. By either increasing the pool of differentiating fat cells (hyperplasia) or by increasing the size of the fat cells (hypertrophy) it can meet increased demands for fat storage.

ADIPOGENESIS

Mesenchymal stem cells (MSC) are pluripotent cells present in adults and can differentiate to mature adipocytes as needed. Preadipocytes are committed to the lineage, proliferate and undergo growth arrest before mitotic expansion during the terminal differentiation event. All these phases are tightly regulated by a number of transcription factors. The commitment of MSC to preadipocytes involves dissociation of an inhibitory complex with zinc-finger protein 423 (ZFP423) and its translocation to the nucleus to activate peroxisome proliferator-activated receptor gamma (PPARγ) (13). PPARγ is the master regulator of adipogenesis and survival of mature adipocytes (14) and, together with several CCAAT/enhancer-binding proteins (C/EBPs), drives terminal differentiation (10; 13). Hyperplasia (expansion in adipose cell number), rather than hypertrophy (increase in adipocyte cell size) is considered to be metabolically beneficial when the excess dietary lipids need to be stored in adipose tissue (13; 15-17) and limited adipogenesis in the subcutaneous depot is associated with ectopic fat accumulation and insulin resistance (18; 19).

LIPID TURNOVER IN WAT

WAT stores fat as TAGs and during periods of energy needs, it makes the stored energy accessible through lipolysis, releasing free fatty acids (FFAs). In this process, TAGs are hydrolyzed to FFA and glycerol by lipases. There are three known lipases, acting in response to starvation or other signals, providing cells with energy from fat fuels (20). Monoacylglycerol lipase (MAL), hormone-sensitive lipase (HSL) and adipose-triglyceride lipase (ATGL), which hydrolyze monoacylglycerol (MAG), diacylglycerol (DAG) and triacylglycerol (TAG), respectively (21). Lipolysis stimulation through cyclic guanosine monophosphate (cGMP)- or cyclic adenosine monophosphate (cAMP) -dependent activation of protein kinase G (PKG) or protein kinase A (PKA), respectively, leads further to activation of ATGL and HSL (22). FFAs released through lipolysis are bound to albumin, oxidized by skeletal muscle and liver, and re-esterified by adipocytes. TAGs transported from intestine as chylomicrons and from liver as very low density (VLDL) particles are delivered to adipocytes by lipoprotein lipase (LPL) for TAG synthesis. LPL breaks down these TAGs into FFAs, which are further esterified to a glyceride-glycerol backbone. FFAs can also be taken up by adipocytes and used for TAG synthesis (11).

Postprandial inhibition of lipolysis is due to elevated insulin levels, where insulin mediates cAMP degradation and thereby inactivation of PKA and its downstream targets (23; 24). Lipid overload in hypertrophic adipocytes leads to local tissue inflammation that becomes chronic with time, leading to increased tumor necrosis-factor-α (TNFα), and other cytokines, secretion (25). TNFα is known to induce lipolysis and decrease TAG synthesis in adipocytes, leading to increased levels of circulating non-esterified FFAs (NEFAs). Inability to store excess lipids in the adipose tissue leads their deposition in other metabolically active organs, such as liver and skeletal muscle (26; 27). This ectopic lipid deposition induces lipotoxicity and enhances insulin resistance in these tissues (28; 29).

Obese and insulin-resistant subjects also have increased rate of non-stimulated lipolysis, which further promotes dyslipidaemia (30; 31).

1.2 HEPATIC GLUCOSE AND LIPID METABOLISM

As a central organ in glucose and fat homeostasis, liver not only stores glucose and lipids, it also provides skeletal muscle, heart and adipocytes with FFAs via very low-density lipoprotein (VLDL) particles. Moreover, carbohydrates delivered from the diet can be converted into FFAs following hepatic DNL. These FFAs are oxidized in the mitochondria or peroxisomes, esterified to TAGs and stored in small lipid droplets (LDs) or added to lipoprotein to form VLDLs for secretion. When FFAs are in abundance, ω-oxidation occurs in the ER.

Under control of insulin, glucose is stored in liver as glycogen. When blood glucose levels drop, pancreatic α-cells secrete glucagon signaling to the liver to enhance glycogenolysis and gluconeogenesis, in order to release glucose from glycogen or increase its synthesis from lactate and amino acids. This process keeps glucose levels stable, which is crucial for tissues with constant glucose demand, such as brain (5; 32-34). Excess glucose can also provide glycerol backbone, which can be coupled with esterified FFAs and stored as TAGs or secreted as VLDL.

LIVER IN NAFLD/NASH

The most prevalent cause of NAFLD is dysfunctional adipose tissue and obesity (35), where increased FFA secretion is caused by impairments in insulin-mediated suppression of lipolysis (36) and chronic adipose tissue inflammation. Skeletal muscle glucose uptake is reduced due to excessive lipid uptake and decreased insulin sensitivity leading to compensatory hyperinsulinemia. Liver dysfunction in NAFLD is characterized by liver steatosis, increased gluconeogenesis, decreased glycogen synthesis, and increased release of hepatokines (37-39). Progression to NASH is due to subsequent multiple hits, with oxidative stress and hepatocellular damage being persistent events.

OXIDATIVE STRESS IN NAFLD/NASH

ROS and reactive nitrogen species (RNS) generated by oxidative stress disrupt cellular processes by reacting with intracellular macromolecules, causing protein inactivation and denaturation, thereby changing signal transduction, causing DNA damage and lipid peroxidation (40; 41). Oxidative stress triggers inflammation, ER stress and reduced synthesis of endogenous antioxidants (42).

Mitochondrial electron transport chain and DNA are especially sensitive to reactive oxidative stress. Mitochondrial β-oxidation in NAFLD is affected due to progressive FFAs overflow, resulting in electron leakage and leading to a vicious cycle of mitochondrial ROS generation in hepatocytes and mitochondrial dysfunction during progression to NASH (3; 43).

LIVER INFLAMMATION AND HEPATOCELLULAR DAMAGE

It is not a single feature, but hepatic steatosis, inflammation, fibrosis and ballooning give a collective score to rate activity of NAFLD and its progression to NASH (44; 45). Oxidative stress, which becomes chronic with time, leads to hepatocellular damage and recruitment of inflammatory infiltrates in the pathogenesis of NAFLD/NASH (4-6).

1.3 SKELETAL MUSCLE

Skeletal muscles, with their high impact on systemic energy consumption, are responsible for the majority of the glucose disposal after a meal and, thus, play a key role in whole-body glucose homeostasis (46; 47). Glucose is taken up by skeletal muscle through insulin signaling pathways or contraction, both of which stimulate GLUT4 translocation to cell surface to take up glucose from the circulation (48).

Skeletal muscle is heterogeneous both in its function and structure. Composed of myofibrils gathered together into fibers, which in turn are enclosed in fasciculi, skeletal muscle is a powerful machinery to generate force and movement. Skeletal muscle also plays a major role as a metabolically active tissue. It is appreciated that there are four types of fibers with different contractile and metabolic properties. However, two main categories are recognized. Fibers with slow contractile properties, low fatigue, high oxidative capacity, use FFAs as the preferable substrate in order to generate the energy. Second main category is the extreme opposite, containing fibers that contract fast and have high level of fatigue, with glycolytic properties and glucose as a preferable substrate. The four types of fibers that fall into these two categories belong either to one of them, or have the mixed properties of both. Skeletal muscle fibers have a remarkable property to switch their metabolic abilities depending on systemic energy demand. For example a fast-to-slow fiber type switch increases oxidative capacity of the muscle (49-51).

ECTOPIC LIPID ACCUMULATION AND INSULIN RESISTANCE IN SKELETAL MUSCLE

Obesity-induced WAT spillover, as FFAs, is delivered to other metabolic tissues, such as skeletal muscle and liver for storage (52). This ectopic storage of fat, especially in skeletal muscle and liver, is strongly associated with insulin resistance (28; 53). In fact, agents that reduce the ectopic lipid content in skeletal muscle and liver, redirecting

it to subcutaneous adipose tissue, have shown improvement in insulin sensitivity (17; 54).

FFAs delivered to myocytes from circulation are stored as TAGs in LDs or directed to mitochondria for oxidation (55). FFAs were shown to play an important role in induction of insulin resistance and mitochondrial dysfunction in muscle cell culture (56; 57) and in human skeletal muscle (58; 59).

Increased number of LDs in the skeletal muscle is not always connected to a pathological situation. Oxidative fibers in skeletal muscles of marathon runners store more LDs than muscles from less active individuals. This phenomenon, called athlete's paradox, is positively correlated with insulin sensitivity (55; 60). However, excessive lipid storage in the glycolytic muscle fibers, at least in mice, has been shown to promote insulin resistance (61). In man, insulin resistance has been linked to increased intra-myocellular lipid content in a number of studies in past years (62-64). In obesity-associated insulin resistance, increase in intra-myocellular metabolites leads to quenched insulin action, mitochondrial dysfunction and oxidative stress, further worsening lipid accumulation and insulin resistance (55; 65; 66). Impairment in skeletal muscle mitochondrial function has been observed in obese and T2DM subjects (67).

1.4 SERINE/THREONINE KINASE 25 (STK25)

STK25 belongs to Sterile20 family of kinases, in the group of germinal center kinases (GCK). Sterile20 family of kinases contains a subfamily named Mammalian Sterile20-like (MST) family, which is further divided into subgroups: MST1, -2 and MST3/4/STK25. The second subgroup is a part of the striatin-interacting phosphatase and kinase (STRIPAK) complex; it has been shown to have a high impact on the regulation of the cytoskeleton and Golgi apparatus (68). Previous studies have demonstrated STK25 localization to the Golgi apparatus where it regulates cell migration and polarization. Moreover, interaction of STK25 and Golgi matrix protein 130 (GM130) promotes Golgi assembly (69). STK25 has a high expression in the brain (70) and has been widely described in neurodegenerative disorders (71-73), having, for example, a negative impact on neuronal cell migration (74). Several studies have shown oxidative stress influence on STK25 function, where STK25 translocates to the nucleus and induces apoptosis (70; 75; 76). Notably, a study by one of these groups has recently shown that hepatic STK25 is significantly activated in mice after HFD, compared to mice fed a normal chow diet (77).

It has been suggested that a central regulator of metabolism, AMP-activated kinase (AMPK), regulates expression of STK25 (78). This study has created a wave of extensive research on STK25 involvement in metabolic homeostasis, performed in our laboratory, exposing the STK25 impact on progress of diseases such as T2DM, NAFLD and NASH. Both *in vivo* and *in vitro* studies in various tissues and cell lines provided evidence that STK25 regulates metabolic balance between storing and oxidizing lipids, regulating whole body insulin and glucose homeostasis (79-85). Overexpression of STK25 in mice on high-fat diet has been shown to increase fasting plasma insulin and spontaneous activity, to reduce systemic glucose tolerance, insulin sensitivity and energy expenditure. Apart from adipocyte hypertrophy, overexpression of STK25 in mice leads to excessive accumulation of TAGs and increased expression of genes regulating lipid synthesis and repression of β-oxidation in liver and

muscle. In addition, liver glycogen levels were elevated in these mice, together with expression of genes regulating gluconeogenesis, glucose synthesis and FA synthesis (80; 82). Furthermore, there was a significant increase of STK25 mRNA expression in skeletal muscle of T2DM patients (86). On the other hand, mice lacking STK25 have opposite effects on almost all mentioned parameters, indicating beneficial aspects of low STK25 in challenged conditions (79; 85). Importantly, obese mice treated with *Stk25* anti-sense oligonucleotides have shown a beneficial effect on the phenotype associated with obesity and insulin resistance in wild-type mice fed a high-fat diet (87). A recent study also shows an effect of STK25 in the regulation of progression of cardiovascular disease (88).

A study in human hepatic cells has provided a suggested mechanism of STK25 function in lipid metabolism (Fig. 1). STK25 overexpression led to decreased β-oxidation and VLDL secretion in these cells and enhanced TAG synthesis. An inverse correlation was observed in human hepatic cells lacking STK25 expression. Moreover, there is a positive and significant correlation between *STK25* mRNA expression and hepatic fat (81).

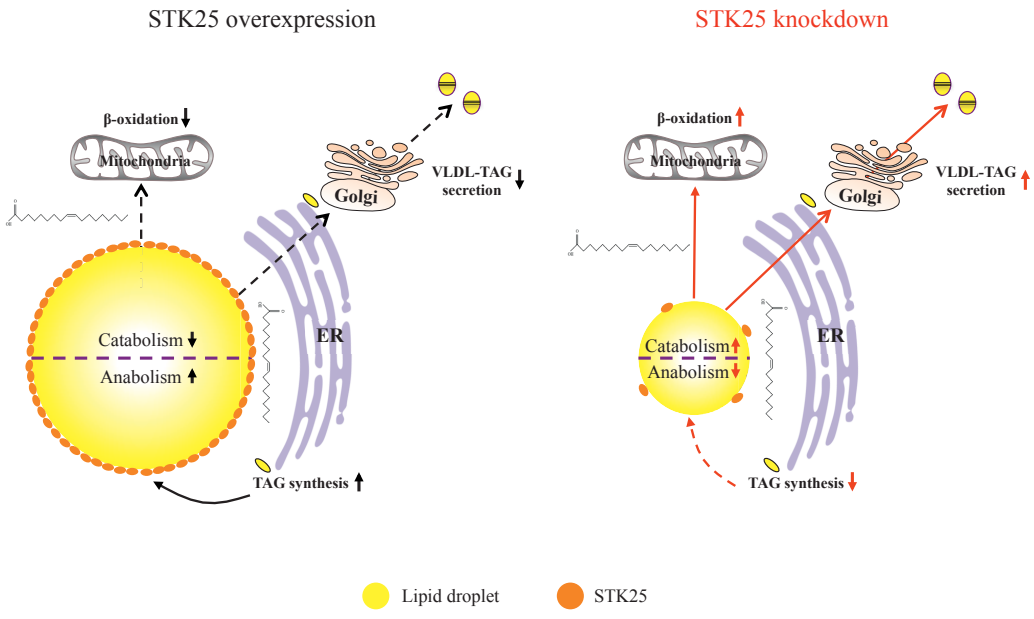

Figure 1. *Schematic illustration of proposed model of STK25 action in regulation of lipid accumulation in human hepatocytes. Overexpression of STK25 suppresses LD catabolism through repressed β-oxidation and VLDL-secretion, and increases TAG synthesis and thereby LD accumulation. On the other hand, STK25 knockdown leads to reciprocal effects with increased β-oxidation and VLDL-TAG secretion, and reduced TAG synthesis in the liver cells. Figure acquired from (81).*

2 AIM

The general aim of this thesis was to elucidate the metabolic impact of the kinase STK25 in the regulation of lipid metabolism, IR, T2DM and NAFLD/NASH progression.

The specific aims of the three papers included in thesis were:

Paper I. To investigate STK25 impact on NASH progression in short- term methionine and choline-deficient diet in *Stk25* transgenic and *Stk25* knockout mice.

Paper II. To understand the role of STK25 in ectopic fat accumulation and insulin resistance in skeletal muscle of high-fat-fed mice.

Paper III. To investigate the effect of STK25 on adipose cell maturation and lipid accumulation in 3T3-L1 cells.

3 EXPERIMENTAL PROCEDURES

For detailed information about the experimental procedures, please see corresponding Material and Methods sections for *Papers I*, *II* and *III.*

ETHICAL STATEMENT

The Ethical Committee of the University of Gothenburg approved the study design and written informed consent was received from study participants after the purpose and the potential risks of the study were explained. Approval from Local Ethics Committee for Animal Studies at the Administrative Court of Appeals in Gothenburg was received prior to performing any experiments in mice.

ANIMAL MODELS

The transgenic mouse model overexpressing STK25 on the whole-body level was generated, using C57BL/6N strain, by the Norwegian Transgenic Center in Oslo. For detailed information, see (82). One of the significant limitations of the animal model used in this study is the high expression of STK25 in transgenic animals. There are no reported cases in animals or humans with levels of STK25 expression being as high as in *Stk25* transgenic animals. This limits the translation of the current results to normal human pathophysiology and needs to be further examined and related to expression levels.

The whole-body *Stk25* knockout mice were a gift from Prof. B. Howell (Department of Neuroscience and Physiology, State University of New York Upstate Medical University, Syracruse, New York, USA), and were previously described in (74). Heterozygous *Stk25* knockout mice were backcrossed to a C57BL6/J genetic background as described in (79).

Since *Stk25* transgenic and *Stk25* knockout mice have different genetic backgrounds, C57BL6/N and C57BL6/J, respectively; it is problematic to directly compare these groups. Thus, both need to be compared to their respective wild-type littermates. This results in the need of excessive breeding of additional animals to have a direct comparison between *Stk25* transgenic, *Stk25* knockout and one wild-type group, but would have been beneficial in the interpretation of the results generated.

Mice were housed in temperature-controlled (21 °C) facility with 12-hour light-dark cycle, with free access to water and chow food. Mice were weaned at 3-5 weeks of age. Age-matched, male mice were used in the experiments and knockout and/or transgenic mice were compared to their corresponding wild-type littermates throughout this thesis. Mice were fed corresponding diets with start at 6 weeks of age with free access to food and water.

In Paper I, *Stk25* knockout and transgenic mice, and their respective wild-type littermates were fed pelleted methionine/choline-deficient (MCD) diet or pelleted control chow diet for 4 weeks. There are various animal models for induction of NAFLD, from genetic to dietary as well as a combination of both (5). We have chosen a diet-induced animal model, where mice received MCD diet during 4 weeks. Characteristic consequences of this diet include impairment in mitochondrial function, hepatic TAG accumulation, development of fibrosis, and NASH morphology. It is an effective and time-saving treatment. However, MCD diet is challenging for animals, making them cachectic. Animals have low levels of plasma TAG and no insulin resistance. These characteristics do not correspond to the typical NASH in humans (5).

In the experimental setup in *Paper II*, *Stk25* transgenic mice and their respective wild-type littermates were fed either pelleted HFD (45 kcal % fat) or pelleted control chow diet for 18 weeks.

EUGLYCEMIC-HYPERINSULINEMIC CLAMP

In order to evaluate degree of insulin sensitivity in metabolic tissues *in vivo*, a euglycemic-hyperinsulinemic clamp study was performed in conscious mice. Post high-fat diet *Stk25* transgenic mice were anesthetized. A catheter was inserted intravenously into the jugular vein and steadily connected to Vascular Access Button anchored to the neck area. Mice were given 3-4 days to recover from the surgery in a single-cage manner.

A catheter coupled directly to the circulatory system enabled constant and controlled administration of insulin and glucose intravenously. In the beginning of the experiment, a bolus of insulin was injected to suppress hepatic glucose production. Thereafter, insulin was administrated at a stable rate to maintain systemic insulin levels. As the hyperinsulinemic state was reached, glucose was infused to reach a steady-state rate at euglycemia. The infusion rate of the glucose has a direct correlation to how insulin sensitive the body is. However the systemic insulin sensitivity can give false information about organ-specific insulin sensitivity if the individual organs have differential insulin sensitivity. For that reason a bolus of radioactive-labeled glucose was given to measure tissue-specific glucose metabolism.

ENDURANCE EXERCISE

Aerobic exercise capacity was examined using treadmill-running test, where distance and total time were quantified. Thorough conducted acclimatization prior to the exercise test was performed to familiarize mice with the treadmill and reduce the stress of being handled. In the beginning of the test, mice started to run at established speed, which was increased continuously until the maximum speed adjusted for mice was reached. The test was terminated when the mice reached fatigue by spending 20 seconds or more at the base of the treadmill despite physical encouragement in form of mild poking. The test was performed at week 17 of the high-fat diet. Mice were allowed to recover 1 week prior to the termination.

TISSUE COLLECTION

Mice were terminated after 4 hours of food withdrawal, by overdose of anesthetics. Subsequently, blood was collected through heart puncture and stored frozen for analysis of plasma metabolites. Tissues were dissected and stored in liquid nitrogen for further analysis. Tissues for histological analysis were fixed in paraformaldehyde for 24 h and, thereafter, stored in ethanol until embedded in paraffin block, or embedded in optimal cutting temperature compound directly after dissection, slowly immersed in liquid nitrogen and stored at -80 °C.

Whole-body perfusion with glutaraldehyde, sodium cacodylate and sodium azide was performed in order to fixate gastrocnemius muscle and liver. Thereafter, the tissues were collected for transmission electron microscopy, and stored in fixatives.

Gastrocnemius muscles determined for proteomic and phosphoproteomic analysis were heat-stabilized immediately after dissection. Tissues naturally contain enzymes such as proteases, phosphatases and peptidases that induce biological changes to proteins post-dissection. Through rapid heat inactivation of the muscles, these enzymes are permanently inactivated and the proteins are preserved in their natal state, making proteome analysis more accurate.

CELL CULTURE

Following adherent cell lines have been used in this thesis – L6 myoblasts, HepG2 cells, immortalized human hepatocytes and 3T3-L1 cells.

HepG2 cells were originally isolated from liver biopsy of hepatocellular carcinoma cells found in a 15-year old male. IHH cells, on the other hand, are derived from a 59-year old male donor, suffering from colon cancer metastasis. Although both cell lines have similar lipoprotein

metabolism, HepG2 cells secrete LDL-like particles similar in size to plasma LDL, but different in their composition when compared to either LDL or VLDL particles found in plasma (89).

L6 cells are mononuclear myoblasts isolated from rat skeletal muscle. When differentiated, L6 cells fuse into multinuclear myotubes.

Mouse fibroblast cell line, 3T3-L1, effectively undergoes a pre-adipose to adipose-like conversion when stimulated with differentiation cocktail. Cells were grown to confluence and left intact for some days, in order to enter a resting state that is necessary for the differentiation process. Thereafter, differentiation medium was added, including pro-differentiator agents - insulin, dexamethasone and 3-isobutyl-1-methylxanthine. After few days of the differentiation, lipid accumulation was apparent. Differentiated cells are considered to be mature at day 9-12. Due to transient transfections, which were performed prior to cell cycle arrest, cells were used at days 4-6 after the differentiation.

Transient overexpression of myoblast- and hepatic cell lines was performed with pFLAG-*STK25* or an empty control plasmid. Small interfering RNA was used for transient *Stk25* knockdown in hepatic cell lines and the undifferentiated pre-adipocyte cell line. Efficiency of all transfections was facilitated using Lipofectamine reagents, consisting of specially designed cationic lipids. These liposomes and nucleic acid form a positively charged complex, mediating a fusion with negatively charged cell membrane possibly through endocytosis.

PROTEOMICS

Proteomics and phosphoproteomics have been applied in order to expose global changes in gastrocnemius muscle proteome between the genotypes. After purification, proteins were digested to peptides, eliminating the physico-chemical properties of the proteins and making the analysis more sensitive. Such peptides are rich in information and easier to interpret than full-length proteins, which might be processed and modified. Phosphorylation is an essential post-translational

protein modification, changing state of the protein activity. It is involved in all physiological and pathological processes. However, it is a rare modification making it difficult to detect in a total peptide sample. For that reason, samples determined for phosphoproteomic analysis were enriched in phosphopeptides using titanium dioxide that have a high affinity for phosphopeptides. Samples were marked with isobaric mass tagging reagents making it possible to directly compare between protein expression profiles in all the samples. Abundance of the total proteins and activity of the proteins through phosphorylation was analyzed with liquid chromatography mass spectrometry (LC-MS). Sample processing and analysis have been performed in collaboration with the Proteomics Core Facility at University of Gothenburg.

CARNITINES/ACYLCARNITINES MEASUREMENT

Carnitine can be either taken up from the diet, or synthesized from amino acids, mainly in the liver. Fatty acids are transported into mitochondria by carnitines, in order to generate energy in the process of β-oxidation. Mitochondria-rich tissues, such as skeletal muscle and heart, contain highest levels of carnitines and acylcarnitine. Acylcarnitine extraction and analysis was performed using hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS) (90) to detect impairments in FA β-oxidation . This method is simpler and more accurate than the conventional methods described previously in the literature, including more simple sample preparation that includes organic solvent for protein precipitation and labeled internal standards. Briefly, gastrocnemius muscles were homogenized in methanol. The samples were centrifuged and acetonitrile-methanol solution containing the deuterated reference standards was added to the sample supernatants. The analysis was made using multiple reaction monitoring and quantification was made against the internal standards. Sample processing and analysis have been performed in collaboration with Wallenberg Laboratory at the University of Gothenburg, Sahlgrenska University Hospital.

PROTEIN EXTRACTION AND WESTERN BLOT

Cells and tissues were mechanically homogenized in the presence of lysis buffer containing protease inhibitor cocktail, to prevent degradation of proteins. Proteins were purified through a series of centrifugation steps. Protein concentration was determined using colorimetric BCA assay, where a mixture of alkaline medium and cupric sulfate was added to the extracted protein sample. This caused reduction of the Cu^{+2} to Cu^{+1}, which bound to BCA creating a colored product that was quantified using spectrophotometer and compared to known protein standards.

Western blot is a widely used antibody-based technique for detection and analysis of proteins. Prior to electrophoresis, proteins tertiary and quarternary structures were disrupted with heat and β-mercaptoethanol; a reducing agent that breaks the disulfide bonds holding the structures. This makes the epitope more accessible for the primary antibody later in the detection process. Denatured proteins were thereafter loaded onto porous polyacrylamide gel to which voltage was applied. This enables proteins to separate through the gel and, depending on size, the proteins will migrate with different velocities. Proteins immobilized on the gel were relocated onto nitrocellulose membrane using semidry transfer, based on the same electro-mobility principle. To ensure that the proteins were transferred, membrane was stained with Ponceau S to visualize proteins. Membrane was incubated with non-fat milk solution to permanently cover the parts of the membrane not occupied by proteins, to reduce unspecific binding of the antibodies. Horseradish peroxidase (HRP)-conjugated secondary antibody was bound to a primary antibody, which was specifically bound to the epitope on the protein of interest. Chemiluminescence detection system was used to expose marked proteins. Briefly, HRP is an enzyme that catalyzes the oxidation of a luminol peroxide detection agent. This results in emission of light, which is further increased with chemicals in reaction known as enhanced chemiluminescence (ECL). Intensity of the signal is proportional to amount of the protein on the blot.

TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy exposes details and complexity of cellular components, which cannot be assessed through light microscopy. For ultra-structural preservation, tissue was fixated in glutaraldehyde and postfixated in osmium tetroxide. While glutaraldehyde rapidly preserves structure of proteins and other macromolecules bound to them, osmium tetroxide reacts slowly with proteins and additionally fixates double bonds of unsaturated lipids. Imagining of organic molecules in transmission electron microscopy depends in large scale on the differences in their electron density. Additionally to its fixative properties, osmium tetroxide reduces onto certain macromolecules enhancing contrast of electron-dense areas. After fixation, samples were dehydrated in ethanol to enable efficient embedding in plastic for ultrathin sectioning with a diamond knife in a microtome. Sections were lastly stained with other heavy metals – uranyl acetate and lead citrate, to further increase contrast of electron dense areas. Sample processing and analysis have been performed in collaboration with the Centre for Cellular Imaging Core Facility at the University of Gothenburg.

HISTOLOGY

One of the most traditional methods for histological evaluation is staining with hematoxylin and eosin. Hematoxylin stains acidic structures in the cell, such as nucleic acids, giving a blue-purple color. Eosin, on the other hand, is an acidic, negatively charged dye. Eosin reacts with basic structures, such as proteins, staining the cytoplasm pink.

Picrosirius red was used to stain collagen types I, II and III. However, it stains also other, non-collagen molecules. Picrosirius red is an elongated birefringent molecule and when bound to collagen it orients parallel to collagen fibrils enhancing their natural birefringence, and resulting in a bright red staining of collagen fibers when examined in a bright field microscope with polarized light.

Periodic acid-Schiff staining was used to identify glycogen deposition in the sections. Primarily periodic acid was added to a tissue section, oxidizing hydroxyl groups of carbohydrates, such as glycogen, to form aldehydes in the oxidation process. When Schiff reagent was added, it reacted with the aldehydes producing bright magenta color.

Lipids were visualized using Nile Red and Oil red O stainings. As the lipids are extinguished in the process of de-paraffinization of paraffin sections, frozen sections or formalin-fixated cells were used. Nile Red is a lipophilic dye. Being strongly fluorescent in hydrophobic environment, Nile Red detects membranes and lipid droplets, without disrupting them. Oil red O, on the other hand, is a fat-soluble diazole dye. It stains most hydrophobic and neutral lipids, such as DAGs, TAGs and cholesterol esters, leaving unstained polar lipids, such as ceramides, phospholipids and sphingolipids.

Mitochondria retain their enzymatic activity of the electron transport chain after dissecting the tissue. To access mitochondrial respiratory capacity in histological manners, we have stained mitochondria in frozen sections and live cells with MitoTracker Red stain and standard enzymatic stainings. MitoTracker Red staining contains thiol-reactive chloromethyl moiety, which accumulate in active mitochondria where it is oxidized leading to emission of fluorescent light.

Enzymatic activity staining was done for NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II) and Cytochrome c oxidase (complex IV). The principle of these stainings is to add an electron donor specific for respective complex in order to obtain a colored product. In the complex I staining, NADH serves as an electron donor. Nitro blue tetrazolium will catch the electron, causing its reduction to a blue-colored formazan product. As many other compartments of the muscle cell contain NADH-oxidizing enzymes, this stain is not exclusive for complex I. Conversely; complex II and IV stainings show high specificity for mitochondria. In the complex II staining, succinate is the electron donor. When added, succinate is oxidized to fumarate and releases

hydrogen. The hydrogen will reduce the nitro blue tetrazolium to form formazan, as in complex I stain. Brown color is produced when 3,3´-diaminobenzidine (DAB) donates electrons for cytochrome c. Reduced cytochrome c further transfers electrons to oxygen, a reaction that is coupled by complex IV of the electron transport chain.

Antibody based stainings were done to analyze proteins of interest using specific epitopes of these proteins. Either monoclonal or polyclonal unconjugated antibodies were used. Secondary antibody was always species-matched to the primary. Secondary antibody was conjugated either to a fluorochrome or to biotin. In the second case, an additional step was applied for the protein detection. Streptavidin conjugated DAB was added after incubation with the secondary antibody. DAB was oxidized when hydrogen peroxide was added, creating a brown color. Fluorochromes are small organic molecules holding one or more aromatic rings. Fluorochromes do not need any chemical activation as such; instead they absorb light energy and instantly emit light energy at a higher wavelength. A great advantage of the immunofluorescence is the possibility of detecting up to 5 fluorochromes, i.e. 5 different proteins, in the same staining. 4',6-diamidino-2-phenylindole dye was used as a nuclear stain. Its blue-fluorescence is enhanced greatly upon binding to AT-rich regions of double stranded DNA.

OXIDATIVE STRESS MEASUREMENT

Intracellular ROS functions contribute to physiological conditions but increased levels, known as oxidative stress, cause damage to the cell. In order to evaluate oxidative stress levels in Paper *II*, we have used a number of assays. Thiobarbituric acid-reactive substances (TBARS) display lipid peroxidation, indicating cellular injury through lipid peroxidation – an indicator of oxidative stress. Catalase (CAT) and glutathione (GSH) are responsible for H_2O_2 decomposition, thus low levels of these antioxidant enzymes indicate high oxidative stress, due to high amounts of ROS.

mRNA AND qRT-PCR

For gene expression analysis, total RNA extracted from tissue- or cell samples was transcribed into complementary DNA (cDNA) by a reverse transcription PCR (RT-PCR) reaction, as it is more stable than RNA. Thereafter, cDNA was used as a template for the quantitative real-time PCR reaction (qPCR). Ribosomal housekeeping gene, 18S, was used to normalize the gene expression data. TaqMan probes and assays for qPCR have been used and analyzed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (91).

STATISTICAL ANALYSES

The experimental results are presented as means ± the standard error of the mean in all *Papers*. Statistical significance between the groups was evaluated using unpaired two-tailed Student's *t* test and, between more than 2 groups, by analysis of two-way ANOVA followed by Turkey's (*Paper I, II, III*) or Games-Howell (*Paper III*) *post hoc* test. Correlation data in human studies was assessed by Spearman's rank correlation (*Paper I and III*) analysis after Kolmogorov-Smirnov test (*Paper I*) was performed to assess normality of the data. Statistics were calculated using IBM SPSS Statistics version 22 or Microsoft Excel. $P<0.05$ was considered statistically significant.

4 RESULTS AND DISCUSSION

In this section, the main results from *Paper I*, *II* and *III* are summarized. Details of the results can be found in the separate *Papers*.

4.1 PAPER I: STK25 IS A CRITICAL DETERMINANT IN NONALCOHOLIC STEATOHEPATITIS

The impact of STK25 on NASH progression was studied in short-term MCD diet-fed *Stk25* transgenic and *Stk25* knockout mice and also characterized in human liver biopsies.

We have previously seen increased hepatic steatosis in *Stk25* transgenic mice, and the opposite in *Stk25* knockout mice, on high-fat diet (79-82). Results from these studies indicate a negative impact on hepatic physiology caused by whole-body overexpression of STK25, and reciprocal benefits of the absence of STK25, supporting an important role of STK25 on hepatic lipid metabolism.

First hit in NASH includes hepatic steatosis together with mitochondrial dysregulation. Consistent with previous observations in high-fat-fed mice, we found that after challenge with a MCD diet, *Stk25* transgenic mice developed marked macro- and micro-vesicular hepatic steatosis with large lipid droplets, while hepatic lipid deposition in *Stk25* knockout mice resembled healthy liver of chow-fed wild-type mice, without steatosis. TEM analysis revealed abnormal mitochondria in *Stk25* transgenic mice, possibly indicating dysfunctional mitochondria in the livers of these animals, which was also supported by MitotrackerRed analysis of active mitochondria.

Second hit involves increased hepatic oxidative stress (3). We measured several markers of hepatic oxidative stress and antioxidant defense. Hepatic TBARS levels and CAT activity in *Stk25* knockout mice were equal to the respective wild-type mice on chow diet, indicating lack of oxidative stress and a proper antioxidant protection, respectively. Interestingly, a level of hepatic antioxidant enzyme, GSH, in Stk25 knockout livers was the highest among all the groups, including chow-diet mice. On the other hand, livers of *Stk25*

transgenic mice had elevated levels of 4-HNE, DHE and TBARS, indicating high lipid peroxidation and oxidative stress.

Furthermore, we measured hepatic protein expression of the fatty acid oxidation proteins acetyl-CoA carboxylase 1 (ACOX1) and cytochrome P450 2E1 (CYP2E1). Alterations in CYP2E1 levels are associated with microsomal lipid peroxidation in NASH (42; 92; 93). In contrast to CYP2E1, which is involved in microsomal ω-oxidation, ACOX1 functions in peroxisomal fatty acid β-oxidation pathway. No differences in CYP2E1 were observed between the groups. However, hepatic expression of ACOX1 in *Stk25* transgenic mice was significantly elevated.

Oxidative stress, which becomes chronic with time, leads to hepatocellular damage and recruitment of inflammatory infiltrates in the pathogenesis of NASH (4-6). The excessive liver cell apoptosis and severely damaged hepatocytes found in *Stk25* transgenic mice, and the absence of these features in the livers of *Stk25* knockout mice, have further confirmed the likely STK25 involvement in progression to NASH. *Stk25* transgenic mice also had increased alanine aminotransferase (ALT) levels in serum, further supporting hepatocellular injury. In contrast, serum levels of ALT in *Stk25* knockout mice were significantly lower compared to the respective controls fed MCD diet.

Excessive steatosis and oxidative damage increase risk of NASH. T2DM patients are at higher risk of developing advanced hepatic fibrosis, causing progression to cirrhosis and eventually hepatocellular carcinoma (7-9). Measurements of fibrosis by histological evaluation and biochemical assays revealed prominent hepatic fibrosis in *Stk25* transgenic mice while it was very restricted in livers of *Stk25* knockout mice.

In view of increased hepatocellular damage and fibrosis in *Stk25* transgenic mice, inflammatory cell infiltration was clearly expected. Immunostaining also revealed a markedly increased infiltration of

inflammatory cells in livers of *Stk25* transgenic mice. Particularly, the presence of Ly6C positive cells, known to be associated with chronic inflammation and fibrogenesis (94), indicated that *Stk25* transgenic mice have developed progression to NASH. As expected, levels of inflammatory infiltrates were minor in *Stk25* knockout livers. Also, characteristic aggregations of inflammatory cells were observed mainly in livers of *Stk25* transgenic mice. These characteristics of progressive inflammation were absent in *Stk25* knockout livers.

Previously, we have reported that *STK25* mRNA expression also correlates positively with human liver steatosis (81). In the present study, we found a significant positive correlation between *STK25* mRNA expression and markers of NASH in human liver biopsies supporting a role of STK25 in the progression of this disease in man.

Taken together, this extensive work in mouse models and human liver biopsies support an important role of STK25 in the progression of NAFLD to severe NASH (Fig. 2)

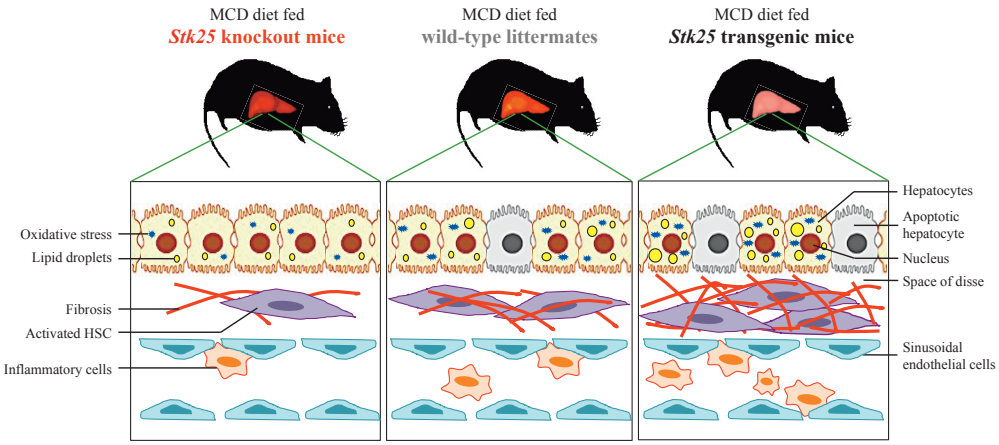

Figure 2. *Reciprocal responses to the MCD-diet-induced development of NASH in Stk25 knockout and Stk25 transgenic mice. Figure acquired from Paper I.*

4.2 PAPER II: OVEREXPRESSION OF PROTEIN KINASE STK25 IN MICE EXCARBATES ECTOPIC LIPID ACCUMULATION, MITOCHONDRIAL DYSFUNCTION, AND INSULIN RESISTANCE IN SKELETAL MUSCLE

In this study we aimed to evaluate involvement of STK25 in ectopic lipid deposition, insulin sensitivity and mitochondrial dysfunction in skeletal muscle.

In order to investigate the expression of STK25 in skeletal muscle, we performed protein analysis by western blot on white (glycolytic) and red (oxidative) muscle. Quantification of protein expression showed significantly higher STK25 in the white portion of the gastrocnemius muscle in wild-type mice fed HFD, compared to the red portion of the same muscle. Interestingly, there was a significant alteration in fiber diameter in the MHC2b fibers in the *Stk25* transgenic gastrocnemius, in comparison to wild-type littermates, indicating hypertrophy of the white portion of the fibers. However, expression pattern of STK25 assessed through immunofluorescence technique did not display any clear specificity for fiber types.

Through histopathological evaluation of H&E-stained sections and TEM images, we identified an extensive damage in *Stk25* transgenic muscle. Gastrocnemius muscle in *Stk25* transgenic mice had degenerating fibers, intracellular inclusions, focal necrosis, infiltration of mononuclear cells and adipocyte replacement. TEM images revealed disorganized sarcomere structure. Further muscle damage was indicated by increased fibrosis, assessed through Picrosirius Red staining and hydroxyproline content. It has been previously shown that degenerating or damaged muscles may undergo a fiber-type switch (95). To investigate if such a compositional change of fibers occurs in

Stk25 transgenic muscles, we stained and counted the myosin heavy chain (MHC) types. However, the results suggest that there was no fiber type switch.

The complex question of lipid accumulation in skeletal muscle causing insulin resistance has been discussed for many years (96; 97). Increased lipid droplet accumulation is present in individuals performing endurance exercise as well as in individuals with excessive calorie consumption. In contrast to the exercise-trained individuals, the obese individuals manifest metabolic dysregulation accompanied by inflammation, endoplasmatic reticulum stress and mitochondrial stress (65; 98; 99). Staining of red gastrocnemius muscle fiber types with Nile Red showed increased lipid accumulation in those fibers of *Stk25* transgenic mice. TEM image analysis revealed the same trend of increased lipid accumulation and also showed mitochondrial ultrastructure damage. Decrease in endurance exercise capacity in *Stk25* transgenic mice was most likely a consequence of damaged skeletal muscle and disrupted mitochondrial structure and function. Furthermore, mitochondrial respiration, assessed through Mitotracker Red staining, revealed decreased activity of the mitochondria in *Stk25* transgenic gastrocnemius muscle in all red fiber types. L6 myoblasts transfected to overexpress *Stk25* also showed excessive lipid accumulation and reduced mitochondrial respiration. Moving the perspective to a more defined system of the cell culture, the transfection experiments have shown a direct effect of STK25 on increased lipid accumulation and mitochondrial respiration.

In concordance with reduced mitochondrial respiratory capacity in *Stk25* transgenic gastrocnemius muscle and *Stk25*-overexpreesing L6 myoblasts, enzymatic staining for oxidative phosphorylation markers showed that *Stk25* transgenic gastrocnemius has reduced staining, and also likely activity, of NADH, succinate dehydrogenase and cytochrome c oxidase, which are three of the four complexes in the mitochondrial electron transport chain. This further confirms disturbance of mitochondrial function in skeletal muscle of *Stk25* transgenic mice. In agreement with reduced mitochondrial respiratory

capacity, β-oxidation was decreased in skeletal muscle of *Stk25* transgenic mice. *Stk25*-transfected L6 myoblasts showed the same trend in reduction of β-oxidation. Lipid overload and impaired mitochondria may lead to accumulation of fatty acid oxidation intermediates, such as acylcarnitines, due to incomplete β-oxidation, subsequently affecting insulin signaling (65; 100). However, despite reduced β-oxidation, *Stk25* transgenic muscle acylcarnitine levels were not changed.

Our previous studies have demonstrated impairment in whole-body insulin sensitivity in *Stk25* transgenic mice fed HFD (82). Organ-specific EHC revealed a decrease in *in vivo* insulin-stimulated glucose uptake in two major skeletal muscles of *Stk25* transgenic mice, quadriceps and gastrocnemius, and a similar tendency in soleus and EDL. Together, these results confirm the adverse consequences of lipid accumulation and mitochondrial damage coupled to STK25 overexpression (Fig. 3).

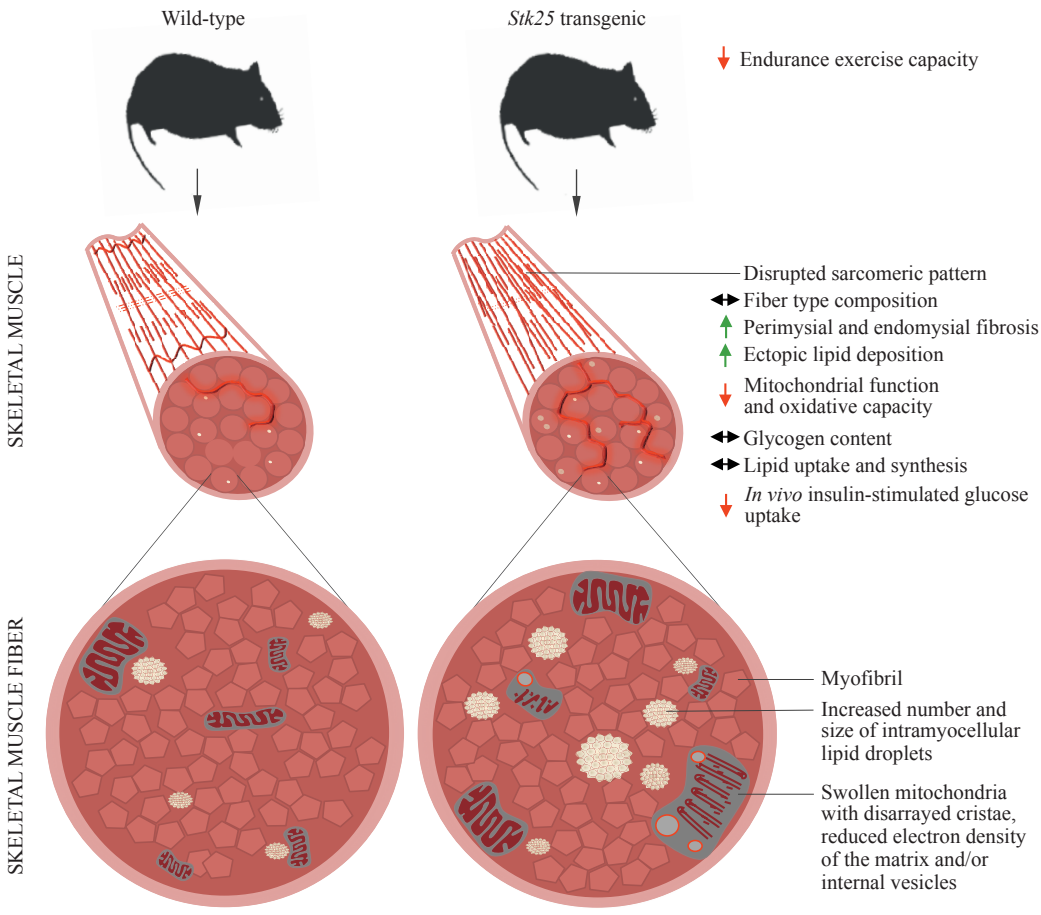

Figure 3. *Schematic illustration of the metabolic responses of Stk25 transgenic mice vs. wild-type littermates. Figure adapted from Paper II.*

In summary, this study, as well as previous studies on the role of STK25 in metabolism, has confirmed its importance in tissue lipid storage, mitochondrial function and insulin sensitivity during times of energy excess. Together, these data make STK25 antagonists an appealing potential target in the treatment of metabolic diseases, such as obesity-associated T2DM.

4.3 PAPER III: STK25 REGULATES LIPID ACCUMULATION AND MATURATION OF 3T3-L1 CELLS

Adipose tissue plays a key role in obesity and its metabolic disorders, such as T2DM and NAFLD/NASH (10; 28). Previous studies have shown a close connection between STK25 and ectopic lipid storage in peripheral tissues (79-84; 87; 101). The adipose tissue is the major organ to safely store lipids and, thus, it is essential to evaluate the effect of STK25 in adipocytes. For this, we have used murine 3T3-L1 pre-adipocytes and silenced STK25 prior to their differentiation. Differentiated 3T3-L1 cells, where STK25 was silenced, displayed a major reduction in lipid accumulation without any apparent shift in lipid droplet size. We have repeatedly observed decreased lipid accumulation with STK25 silencing and knockout, in several cell lines and tissues (79; 81; 87).

Decreased lipid accumulation can depend on several factors such as retardation of the differentiation process and/or more specific effects on lipid accumulation/release. To investigate if reduction in lipid accumulation was dependent on impaired differentiation, we examined expression of key transcription factors responsible for the adipocyte differentiation. During early differentiation, *Stk25* knockdown cells had small or no decrease in *Pparγ*, *C/ebpα* and *Srebp* expression, suggesting that the reduced lipid accumulation was unlikely to be fully attributable to defective differentiation process and early induction of *Pparγ*. However, activation of *Pparγ* is regulated by activating ligands, which apparently can be formed/secreted by 3T3-L1 cells in contrast to the situation in human cells, which are dependent on an exogenous ligand for differentiation (18).

Additional support for a positive effect of STK25 in the lipid accumulation process was provided by the data in human adipose tissue, where STK25 was positively and significantly correlated with a

large number of adipogenic markers. However, it should be emphasized that we used intact human adipose tissue biopsies rather than isolated adipose cells. Potential effects of other cells in the biopsies could contribute but it is unlikely that this would alter the conclusion of this more general effect of STK25 on lipid accumulation in different cells.

To examine if the decreased lipid content in *Stk25* knockdown 3T3-L1 cells was associated with an increased lipolysis, we stimulated the β-adrenergic receptors with isoproterenol. As evident by glycerol release and HSL phosphorylation status, lipolysis was reduced in *Stk25* knockdown.

Decrease in ACC has been linked to increased FA oxidation in several studies (102; 103), and it was also observed in livers of *Stk25* knockout mice and *Stk25* antisense oligonucleotide treated mice (79; 87). Consistently, the same observation was made in *Stk25* knockdown 3T3-L1 cells both at mRNA and protein levels at total and specific phosphorylation site of ACC. However, it is unlikely that this can explain the reduced cell lipid accumulation, as mitochondrial activity was not altered when STK25 was silenced in differentiated 3T3-L1 cells. As ACC is connected to FA oxidation and synthesis, further investigations such as evaluation of β-oxidation are needed.

LCFAs are proposed to be endogenous ligands for PPARs (104). This made us hypothesize that silencing of STK25 changes accessibility/release of endogenous ligands that activate PPARγ. Indeed, several of genes (*Gpr120*, *Glut4*, *Fabp4*, *Pgc1* and *Fasn*) and proteins (GLUT4, FABP4, PGC1, and FAS) regulated by *Ppar*γ and its ligands were down-regulated by *Stk25* knockdown.

Adding to decreased lipolysis in *Stk25* knockdown cells, protein expression of ATGL, which is the key lipolytic protein regulated by PPARγ (105), was also decreased. It has been shown that ATGL-induced lipolysis is crucial for the generation of endogenous PPARα ligands in heart and liver (106; 107). Importantly, treatment of *Stk25*

knockdown cells with a synthetic PPARγ ligand resulted in normalization of ATGL protein levels, improved stimulated lipolysis and a partial rescue in lipid accumulation. Also, expression of several genes and proteins regulating lipid and glucose homeostasis were increased following PPARγ ligand treatment of *Stk25* knockdown cells both at the early and late differentiation stage.

Taken together, this study further supports an important role of STK25 in lipid accumulation and metabolism. Our data also suggest that STK25 may contribute to the availability of lipid-derived endogenous ligands necessary for PPARγ activation and adipose cell maturation (Fig. 4).

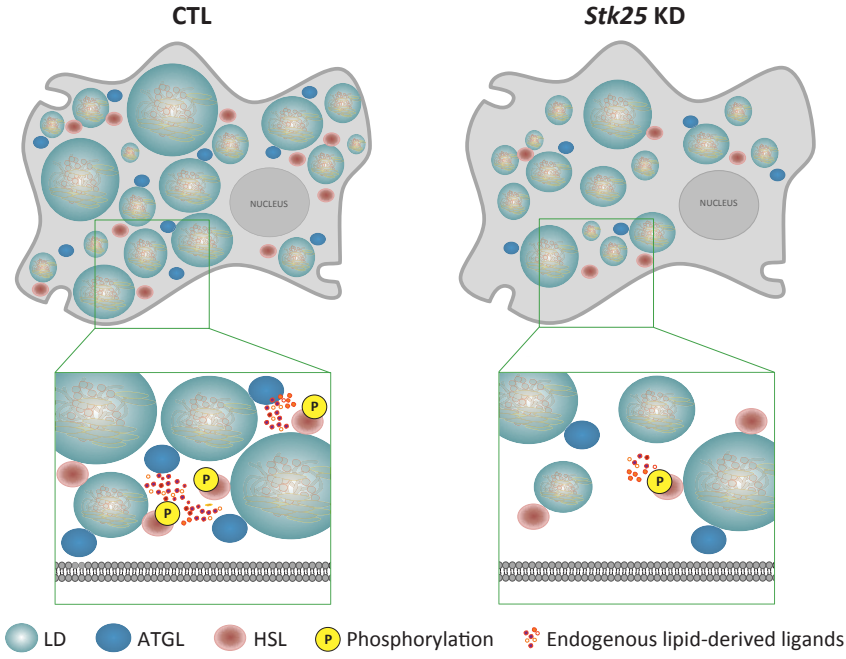

Figure 4. *Proposed mechanism of STK25 in 3T3-L1 cells. Low levels of STK25 reduces maturation of 3T3-L1 preadipocytes possibly through its regulation of lipid accumulation and lipolysis, attenuating secretion of endogenous PPARγ ligands.*

5 CONCLUSIONS

Taken together, the work in this thesis shows that STK25 plays an important role for lipid accumulation in three major tissues/cells; liver, skeletal muscles and adipose cells. Importantly, *STK25* mRNA is also positively associated with markers of NASH progression in human liver biopsies making it an interesting potential future target of therapy in this important human disease.

In *Paper I*, when mice where challenged with MCD diet to develop NASH, *Stk25* knockout mice were protected while *Stk25* transgenic mice progressed and developed severe steatosis, hepatocellular damage, inflammation and fibrosis. Also, *STK25* mRNA expression in human liver biopsies from overweight and obese subjects was positively correlated with NASH features.

Paper II also shows harmful effects of STK25 overexpression in skeletal muscle of mice chronically challenged with HFD. *Stk25* transgenic mice developed increased ectopic lipid accumulation, suppressed mitochondrial respiratory capacity and insulin resistance in major skeletal muscles.

Previous metabolic studies on STK25, as well as data in *Paper I*, show that deleting STK25 is beneficial in skeletal muscle and liver by reducing lipid overload, as well as improving systemic insulin sensitivity, in obese mice with ectopic lipid accumulation. Adipose tissue, on the other hand, is the primary and best site for lipid storage. In *Paper III,* using the murine preadipocyte 3T3-L1 cells, we found adipose cell maturation and lipid accumulation to be reduced during adipogenesis by silencing STK25. Although the mechanisms for this were not fully resolved, an interesting finding was that the addition of a synthetic PPARγ ligand essentially prevented the reduction in adipogenic maturation in this model. Thus, down-regulation of STK25 in adipose cells may lead to decreased production/release of endogenous lipid-derived ligands that serve as activators of *Pparγ* and adipocyte differentiation, resulting in decreased lipid accumulation and

maturation of these cells. The association between STK25 expression and adipose cell maturation and lipid accumulation is also supported by the positive correlations between *STK25* and several differentiation markers in human adipose tissue. However, no changes in adipocyte differentiation have been observed in STK25 knockout and transgenic mouse models (85). Thus, further studies are necessary to understand the role of STK25 in adipocyte function.

Based on the work in this thesis as well as previous studies on STK25 in relation to metabolic diseases, we conclude that STK25 is tightly integrated with lipid accumulation and ectopic lipid deposition in peripheral tissues in obesity. Its association with markers of NAFLD/NASH progression, in particular, makes it an attractive target for drug development in this disease.

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