STK25 – a new key regulator of metabolic profile and a possible target for anti-diabetic drug

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Cover image: Immunofluorescence staining of primary human muscle cells with STK25 (green) and MitoTracker Red (red) by Urszula Chursa

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ABSTRACT

Type 2 diabetes (T2D) affects at least 285 million people worldwide and its prevalence is rapidly increasing. Understanding the molecular mechanisms controlling ectopic lipid deposition and insulin response in metabolic tissues is essential for developing new pharmacological strategies to effectively treat T2D. Obesity and overweigh are the main risk factors for developing T2D, but nonalcoholic fatty liver disease (NAFLD) also contributes to the pathogenesis of T2D. Today, achieving good glycemic control in T2D patients with the current treatment alternatives remains challenging and no specific therapy exists against NAFLD.

In this thesis, we describe protein kinase STK25 as a new key regulator of ectopic lipid deposition in skeletal muscle, liver and pancreas as well as whole-body metabolism. We have found that STK25 overexpression in mice challenged with a high-fat diet (HFD) results in an increased ectopic lipid deposition in skeletal muscle and pancreas, accompanied by an aggravated fibrosis and inflammation. The overexpression of STK25 also leads to impairments in β-oxidation and decrease in *in vivo* insulin-stimulated glucose uptake in skeletal muscle and reduced endurance exercise capacity in mice. The pancreas of *Stk25* transgenic animals shows a significant decrease in islet β/α-cell ratio and alterations in the islet architecture with an increased presence of α-cells within the islet core, together with an impaired insulin production during IPGTT after a HFD challenge. We also show that treatment with *Stk25* antisense oligonucleotides in obese mice protects against HFD-induced liver steatosis, glucose intolerance and insulin resistance. In addition, we found a significant positive correlation between nonalcoholic steatohepatitis (NASH) development and STK25 protein abundance in human liver biopsies. Furthermore, we have identified four

common non-linked SNPs in the human *STK25* gene that are associated with altered liver fat: two associated with increased hepatic fat levels and two associated with decreased levels.

Taken together, our studies suggest that pharmacological inhibition of STK25 potentially provides a new-in-class therapeutic strategy for the treatment of NAFLD, T2D and related metabolic complications.

Keywords: Type 2 diabetes, insulin resistance, ectopic lipid accumulation, β-cell dysfunction, NAFLD, NASH, antisense oligonucleotides.

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SAMMANFATTNING PÅ SVENSKA

Typ 2-diabetes (T2D) påverkar minst 285 miljoner människor världen över och prevalensen ökar snabbt. Övervikt och bukfetma är de väsentliga riskfaktorerna för att utveckla T2D. Leverförfettning som inte orsakas av överkonsumtion av alkohol (NAFLD) bidrar också till utvecklingen och progressionen av T2D. I dag är det fortfarande utmanande att uppnå tillfredställande resultat hos T2D-patienter med nuvarande behandlingsalternativ och det finns ingen behandling för NAFLD. Att förstå de molekylära mekanismerna bakom insulinresistens och lipidinlagring utanför fettvävnaden, som leder till T2D och NAFLD, är viktigt för att framställa nya farmakologiska strategier för att effektivt behandla dessa sjukdomar.

I denna avhandling beskriver vi enzymet STK25 som en ny nyckelregulator av lipidinlagring dels i skelettmuskulatur, lever och bukspottkörtel, samt av ämnesomsättningen i hela kroppen. Vi visar att överuttryck av STK25 i samband med högfettsdiet resulterar i en ökad lipidinlagring i skelettmuskeln och bukspottkörteln, tillsammans med en förvärrad fibros och inflammation. Överuttryck av STK25 leder också till försämringar i fettmetabolismen, förvärrad insulinkänslighet i skelettmuskel och minskad muskeluthållighet. I samband med överuttryck av STK25 uppvisar bukspottkörteln betydelsefulla förändringar i uppbyggnaden tillsammans med en försämrad insulinproduktion, samt minskning av förhållandet mellan beta- och alfa-celler i Langerhanska öar. Vi visar också att behandling med antisenseoligonukleotider, där uttrycket av Stk25 tystas, i samband med högfettsdieten skyddar mot lipidinlagringen i levern, samt glukosintolerans och insulinresistens. Dessutom fann vi en signifikant positiv korrelation mellan utveckling av icke-alkoholrelaterad steatohepatitis (NASH) och mängden av STK25-protein i leverbiopsier från människa. Vidare har vi identifierat fyra vanliga icke-länkade SNP i den humana STK25-genen som är associerade med förändrade nivåer av leverfett.

Sammantaget tyder våra studier på att farmakologisk inhibering av STK25 möjliggör en ny terapeutisk strategi för behandling av NAFLD, T2D 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. Urszula Chursa,* Esther Nuñez-Durán,* Emmelie Cansby, Manoj Amrutkar, Silva Sütt, Marcus Ståhlman, Britt-Marie Olsson, Jan Borén, Maria E. Johansson, Fredrik Bäckhed, Bengt R. Johansson, Carina Sihlbom, and Margit Mahlapuu. **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*

*Both authors contributed equally to this work

II. Esther Nuñez-Durán, Belén Chanclón, Silva Sütt, Joana Real, Hanns-Ulrich Marschall, Ingrid Wernstedt Asterholm, Emmelie Cansby, and Margit Mahlapuu. **Protein Kinase STK25 Aggravates the Severity of Non-Alcoholic Fatty Pancreas Disease in Mice.** *Journal of Endocrinology 2017, 234(1):15-27*

III. Esther Nuñez-Durán, Mariam Aghajan, Manoj Amrutkar, Silva Sütt, Emmelie Cansby, Sheri L. Booten, Andrew Watt, Marcus Ståhlman, Norbert Stefan, Hans-Ulrich Häring, Harald Staiger, Jan Borén, Hanns-Ulrich Marschall, and Margit Mahlapuu. ***Stk25* Antisense Oligonucleotide Treatment Reverses Glucose Intolerance, Insulin Resistance and Nonalcoholic Fatty Liver Disease in Mice.** *Hepatology Communications*. Under review

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ABBREVIATIONS

2-DOG	2-deoxy-D-glucose
ACC	Acetyl-CoA carboxylase
ASO	Antisense oligonucleotide
AMPK	AMP-activated protein kinase
ATGL	Adipose triacylglycerol lipase
cDNA	Complementary DNA
DAG	Diacylglycerol
EDL	extensor digitorum longus
EHC	Euglycemic-hyperinsulinemic clamp
FFA	Free fatty acid
H&E	Hematoxylin and eosin
HSL	Hormone sensitive lipase
IHC	Immunohistochemistry
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
IR	Insulin resistance
LPL	Lipoprotein lipase
LD	Lipid droplet
MCD	Methionine-choline deficient
MST	Mammalian sterile 20-like
NAFLD	Non-alcoholic fatty liver disease
NAFPD	Non-alcoholic fatty pancreas disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
qPCR	Quantitative PCR
ROS	Reactive oxygen species
siRNA	Small interfering RNA
STE20	Sterile 20
STK25	Serine/threonine protein kinase 25
T2D	Type 2 diabetes
TAG	Triacylglycerol
VLDL	Very low-density lipoprotein
WAT	White adipose tissue

1 INTRODUCTION

1.1 Obesity – a well established risk factor for type 2 diabetes

The spreading of obesity is one of the most worrisome problems worldwide. Data from 2004 reported that about 2.8 million of the world population died due to obesity and overweight and the registers in 2016 raised the numbers to 3.4 million [1]. Nowadays, overweight and obesity cause more deaths in the world compared to underweight, and the risk of children becoming obese has also increased [1-3]. Obesity occurs due to a chronic imbalance where energy intake exceeds energy expenditure. Obesity is a well-established risk factor for metabolic syndrome and several chronic diseases, such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease [4, 5]. However, there is growing evidence that obesity is not a homogeneous condition and obese individuals can remain healthy with no apparent signs of metabolic complications [6]. It has also been demonstrated that fat distribution plays a central role in determining the risk for obesity-associated complications [7, 8].

T2D is a complex metabolic disorder characterized by hyperglycemia and insulin resistance (IR), i.e. the reduced sensitivity of peripheral tissues to a physiological concentration of insulin [9-11]. Besides overweight and obesity, several other risk factors have been described for T2D, such as genetic background and environmental or lifestyle factors, including sedentary life, excess food intake, increased stress and sleep disruptions [9, 12, 13]. However obesity is considered the most common risk factor for developing T2D [14-16]. The relative risk for an obese subject to develop T2D is approximately 10-fold higher compared to a non-obese subject [5]. Untreated T2D is associated with a substantial increased risk of morbidity and mortality due to an increased risk of associated cardiovascular complications, renal disease, blindness, wound healing problems and its consequent risk for amputation [17]. Appropriate lifestyle changes can improve insulin sensitivity and also delay or prevent the onset and progression of T2D from the IR state [18, 19]. Furthermore, IR is also considered to be a predictor of T2D, independent of obesity [6, 7].

The WHO published in 2016 the *Global report on diabetes*, which demonstrates that the number of people with diabetes has almost quadrupled in the past four decades. This dramatic rise is mostly due to the increase in T2D and the factors driving it, primarily overweight and obesity [20]. In 2012 diabetes caused approximately 1.5 million deaths, and elevated blood glucose caused an additional 2.2 million deaths, by increasing the risks of cardiovascular disease and related complications [20]. The global health care expenditure for diabetes is suggested to rise by 30−40% between 2010 and 2035, becoming one of the major worldwide threats to human health in the current society [12, 21]. Taken together, there is an urgent need to understand the molecular mechanisms underlying the pathology in order to develop preventive measures and more efficient treatment strategies for T2D.

1.2 Insulin resistance and type 2 diabetes

Insulin is an important anabolic hormone produced by the β-cells in the islets of Langerhans in the pancreas and its major action is to lower circulating glucose levels by enhancing glucose uptake in the main metabolic tissues (Fig. 1). Insulin is released to the circulation as a response of high blood glucose levels, stimulating glucose uptake into skeletal muscle and adipose tissue, and inhibiting glucose output from the liver (gluconeogenesis). Glucose taken up by the liver and skeletal muscle is mainly stored as glycogen (9) and in the adipose tissue, glucose is converted to free fatty acids (FFAs) (lipogenesis) and further stored as triacylglycerols (TAGs) in lipid droplets (LDs) (Fig. 1). Insulin also inhibits lipolysis in the adipose tissue, decreasing circulating levels of FFAs. Increased plasma levels of FFAs have been shown to decrease glucose uptake in liver and skeletal muscle (10).

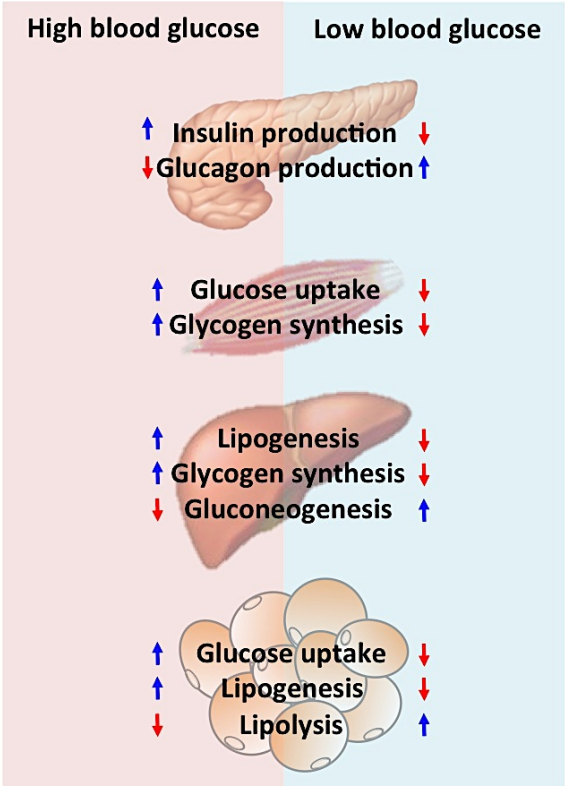

Figure 1. *Scheme of blood glucose and insulin effects in the main metabolic tissues. Blue or red arrows indicate up- or down-regulation respectively.*

Briefly, T2D can develop following IR, and IR is defined as the inability of metabolic tissues to respond to insulin and hence the uptake of glucose from the bloodstream is reduced [22]. As a result of IR the tissues present impairments in the intracellular insulin signaling pathway, glucose and lipid homeostasis, and the pro-inflammatory response [15, 23]. To compensate the IR state, the β-cells need to overproduce insulin, which eventually can result in hyperinsulinemia. As long as the β-cells are able to produce enough insulin to overcome the IR, blood glucose levels stay in the healthy range [24]. However, prolonged IR ultimately leads to β-cells failure, resulting in hyperglycemia and clinical onset of T2D [9, 25].

1.3 Obesity-induced adipose tissue dysfunction and insulin resistance

White adipose tissue (WAT) is the first tissue affected by the excess of calorie intake, and the whole-body energy homoeostasis critically depends on how well WAT remodels in response to overfeeding. WAT expansion can happen through two mechanisms: hypertrophy – the enlargement of existing adipocytes by lipid accumulation; or hyperplasia – the increase of adipocyte numbers by pre-adipocytes recruitment. Both mechanisms are involved in WAT expansion, however the hypertrophic expansion and the increase in fat cell size are closely associated with the risk of developing T2D [24]. WAT expansion is known to be associated with several harmful features, such as increased inflammation and fibrosis, dysregulated adipokine secretion, hypoxia and high production of reactive oxygen species (ROS), which might lead to mitochondrial impairments [24].

During hypertrophic obesity, adipose tissue fails to appropriately expand to store the excess energy, consequently the surplus lipids spillover to the circulation and non-adipose tissues, resulting in a dramatically increased level of TAGs and FFAs exceeding the metabolic capacity. At the whole-body level, this dysfunction results in ectopic fat deposition in non-adipose tissues, specially the liver, skeletal muscle and the endocrine pancreas [26]. Chronic exposure of the body to elevated levels of endogenous or exogenous lipids (lipotoxicity) subsequently causes impairments in cellular insulin signaling [24, 27]. Overall, adipose tissue dysfunction has a direct impact on the risk of developing systemic IR [28, 29].

In adipose tissue, insulin is not only important to promote the synthesis of lipids but also to shut down the breakdown of TAGs (Fig. 1). In states of IR, the breakdown of TAGs to glycerol and fatty acids cannot be properly inhibited, which contributes to increased circulating plasma lipid levels [30].

1.4 Ectopic fat storage and insulin resistance in skeletal muscle

Fatty acids spillover from WAT contributes to increased lipid content in skeletal muscle. When the oxidative capacity in WAT is compromised, the increased accumulation of lipids in skeletal muscle augments

intramyocellular lipid content [31]. A number of studies provide evidence that ectopic accumulation of intracellular lipids in skeletal muscle aggravates both organ-specific and systemic IR [32, 33].

In skeletal muscle, insulin facilitates glucose uptake though the translocation of the glucose transporter 4 (GLUT4) to the plasma membrane, lowering the plasma glucose levels [22, 34]. Skeletal muscle is responsible for 60−70% of the whole-body insulin-stimulated glucose uptake, and therefore, IR in the muscle plays a central role in whole-body IR [35]. Furthermore, skeletal muscle IR is considered as a predictor of T2D development and preservation of adequate muscle glucose disposal is considered to prevent T2D.

Increased lipid accumulation within skeletal muscle could occur either by diminished β-oxidation in muscle mitochondria or by increase in fatty acid uptake into muscle from the circulation. Several studies show that a decrease in mitochondrial fatty acid oxidation capacity due to mitochondrial dysfunction may affect intramyocellular lipid accumulation and IR in T2D patients [36-38]. However, increase in mitochondrial fatty acid oxidation in response to excess fuel has also been reported in connection to IR development in skeletal muscle [39].

Plasma FFAs play a key role in determining the rate of their own uptake by skeletal muscle; hence, higher plasma FFA levels could result in higher FFA uptake into muscle. Nevertheless, it has been shown that T2D patients may display reduced efficiency in plasma FFA uptake by skeletal muscle, despite their high disposal [40, 41].

1.5 Non-alcoholic fatty liver disease and insulin resistance

The liver plays a key role in energy metabolism, helping to maintain the systemic glucose homeostasis. In the liver, insulin stimulates glucose uptake and its conversion to glycogen by the activation of enzymes, such as glucokinase and glycogen synthase [15, 22]. Furthermore, insulin inhibits hepatic gluconeogenesis and glycogenolysis, resulting in suppressed hepatic glucose production, helping the blood glucose levels to get back to normal [42].

Hepatic IR is a complex metabolic disorder. It is known that hepatic fat accumulation, in particular the increased levels of hepatic TAG and diacylglycerol (DAG) concentrations are linked to IR [43, 44]. There are studies showing that the combination of increased levels of glucose and fatty acids in plasma, in the IR state, can lead to development of hepatic steatosis by enhancing hepatic fatty acid synthesis and disrupting β-oxidation [43, 45]. In contrast, it is has also been suggested that hepatic fat accumulation and IR can occur without the development of peripheral IR [45].

NAFLD is one of the most common chronic liver diseases. The hallmark of NAFLD is hepatic neutral lipid accumulation, in the absence of significant alcohol consumption, viral infection or other specific etiologies [46]. NAFLD contains a wide range of hepatic diseases, from simple steatosis to non-alcoholic steatohepatitis (NASH). In addition to fatty infiltration, liver injury in NASH is characterized by inflammation, fibrosis, and hepatocyte damage. The prevalence of NAFLD is increasing due to the obesity epidemic, with an estimated prevalence ranging from 20% to 30% in Europe and the Middle East, and one-third of the adult population in the USA [47]. While liver steatosis is clinically silent, NASH patients present a high risk of developing cirrhosis, liver failure and hepatocellular carcinoma (HCC) [14].

The development of NAFLD is strongly associated with hepatic IR; patients with NAFLD have both increased peripheral and hepatic IR [48]. Additionally, more than 90% of obese patients with T2D have NAFLD [14, 49, 50]. It has also been shown that patients presenting both T2D and NAFLD usually have worse glycemic control compared with patients who only have T2D [51-53]. Moreover, increased intrahepatic TAG content has been shown to be related to decreased production of insulin, which further deteriorates the whole-body glycemic control in T2D patients [54]. It is also known that obese individuals who develop ectopic lipid accumulation in the liver have a higher risk to develop the subsequent whole-body IR [44].

To date, the mechanisms controlling the progression from simple hepatic steatosis to the aggressive liver disease NASH, and the pathogenesis underlying the associated systemic IR with T2D, are not fully understood. As yet, no pharmacological treatment is available against NAFLD/NASH. Therefore, the characterization of the molecular mechanisms underlying the

disease and identifying novel treatment targets, are of outermost importance.

1.6 Non-alcoholic fatty pancreas disease

The islets of Langerhans are considered micro-organs, they are located in the pancreas and are composed of at least four types of endocrine cells. The α- and β-cells are the most abundant; they secrete the hormones glucagon and insulin, respectively. The correct function of the pancreas is crucial for maintaining the whole-body glucose homeostasis.

A major characteristic of T2D is the loss of the ability of pancreatic β-cells to increase insulin secretion to maintain normoglycemia in the context of IR [55]. Chronic exposition of β-cells to high levels of FFA could explain defects in β-cell function and decreased mass observed in T2D. Indeed, *in vitro* studies have shown that increased levels of circulating FFA are associated with a decrease in insulin expression, synthesis and processing [56, 57]. Another mechanism that can explain insulin secretion dysfunction in T2D is high FFA levels in islets inducing β-cell death [58].

Lipid accumulation within the pancreas or non-alcoholic fatty pancreas disease (NAFPD) is associated with reduced insulin secretion in humans with impaired glucose tolerance [59]. Furthermore, a recent study suggests that fatty pancreas exacerbate local inflammation in human pancreas [60]. NAFPD has become a growing health problem with an estimated prevalence ranging between 16 and 35% [61-63]. To date, the exact pathophysiology of NAFPD is still unclear and very poorly studied. As described above, obesity promotes ectopic fat accumulation in the pancreas (pancreatic steatosis). The pancreas is known to accumulate fat intracellularly, as well as through adipose tissue replacement of dead acinar cells and/or adipocyte infiltration in conditions of an excess of fuel [60, 64]. Even though there is evidence of metabolic crosstalk between fatty pancreas and fatty liver [60], the effect of steatosis in the pancreas has been less investigated than the liver [65]. Interestingly, several studies have shown a relationship between NAFPD and NAFLD [60, 66-68]. Individuals with NAFPD present higher frequency of NAFLD than healthy individuals [67, 69]. Both NAFLD and NAFPD are also strongly linked to obesity and visceral WAT lipid accumulation [59, 68].

Additionally, IR decreases insulin inhibition of lipolysis in WAT, leading to increased levels of circulating FFAs. The chronic exposure of β-cells to glucolipotoxicity (elevated glucose and FFAs levels) results in increased TAG accumulation, as well as reduced insulin gene expression and glucose-stimulated insulin secretion (GSIS), altogether increasing the risk of β-cell apoptosis [70, 71]. Consequently, there is accumulating evidence showing an intimate link between pancreatic steatosis, increased inflammation and β-cell dysfunction and apoptosis [60, 65, 72].

1.7 Serine/threonine protein kinase 25 (STK25)

1.7.1 STK25 – a kinase in STE20 superfamily

Serine/threonine protein kinase 25 (STK25; also referred to as YSK1 or SOK1) is a broadly expressed member of the sterile 20 (STE20) kinase superfamily. STK25 was first described in 1996 as a 426 amino acids long kinase that belongs to the germinal center of kinases III (GCKIII) subfamily of the STE20 proteins [73]. Studies by Pombo *et al*, as well as our research group, have shown that oxidative stress inducers, such as hydrogen peroxide (H_2O_2), the proinflammatory cytokine tumor necrosis factor-α (TNF-α) and menadione activate STK25 [73, 74]. STK25 has been shown to interact with cerebral cavernous malformation (CCM) 2 and 3 (also known as PDCD10), which are part of signaling pathways essential for vascular development and cell migration [75]. In several cell types, STK25 is localized to the Golgi apparatus, where it functions in regulating cell migration and polarization [75-78]. However, in response to chemical anoxia and oxidative stress, STK25 has been shown to dissociate from the Golgi complex, and translocate to the nucleus, where it induces apoptotic cell death [79, 80]. Matsuki *et al* showed that STK25 participates in neuronal cell polarity and migration by interacting with STRADα, an activator of LKB1. Depletion of STK25 hindered LKB1-STRADα-regulated epithelial cell polarization and anomalous neuronal migration, whereas its overexpression restored polarity defects observed in LKB1 knockdown neurons [78, 81].

Previous studies suggested that the expression of *Stk25* is regulated by AMP-activated protein kinase (AMPK) in skeletal muscle in mice [82]. AMPK is known as the central energy sensor in cells, and its activation increases glucose uptake and fat oxidation in the skeletal muscle, while inhibiting

glucose output and TAG synthesis in liver, altogether improving the main metabolic disturbances in the metabolic syndrome [83-85]. The findings of STK25 being regulated by the metabolic master-switch AMPK, led to the hypothesis that STK25 might also have a role in regulation of energy homeostasis. Indeed, our recent studies summarized below (Section 1.7.2) highlight the role of STK25 in regulation of lipid partitioning and glucose and insulin homeostasis. Interestingly, STK25 has also been suggested as a candidate for regulating high-density lipoprotein (HDL) levels in mice [86]. Recently, MST1, another member of the STE20 kinase family, was identified as a critical regulator of apoptotic β-cell death [87].

Our studies described below potentially open up new avenues of research to describe interactions of STE20 superfamily of kinases with known components in metabolic control network.

1.7.2 STK25 – a critical regulator of lipid partitioning and whole-body glucose and insulin homeostasis

To study *in vivo* function of STK25, our research group characterized the metabolic phenotype of STK25-overexpressing mice as well as the conventional *Stk25* knockout mice [74, 88-94]. We found that *Stk25* transgenic mice present impaired systemic glucose tolerance and insulin sensitivity compared to wild-type littermates when fed a HFD [93]. Reciprocally, *Stk25* knockout mice are protected against HFD-induced whole-body glucose intolerance and IR compared with their wild-type littermates [89]. To study the function of STK25 in the skeletal muscle, our research group studied the impact of STK25 inactivation in rat myoblast cell line L6 [74]. We found that partial depletion of *Stk25* in L6 by small interfering RNA (siRNA) increases lipid oxidation and improves insulin-stimulated glucose uptake [74]. Consistent with those findings, higher STK25 levels were observed in the skeletal muscle of patients with T2D, compared to individuals with normal glucose tolerance [74].

We further characterized the function of STK25 in regulation of liver lipid metabolism using the mouse model system, as well as human cultured hepatocytes. We observed increased lipid accumulation (steatosis) in liver samples of HFD-fed *Stk25* transgenic mice compared with wild-type [90].

Furthermore, we observed repressed lipolytic activity, β-oxidation and very-low-density lipoprotein (VLDL)-TAG secretion in *Stk25* transgenic livers, while lipid uptake and synthesis in hepatocytes of *Stk25* transgenic mice were similar to wild-type counterparts. We further studied the subcellular localization of STK25 by immunofluorescence, and found that STK25 coats hepatic lipid droplets (LDs), where it co-localizes with adipose triacylglycerol lipase (ADRP), the main LD-coating protein in mouse liver [90]. Furthermore, we found that the main liver lipase, adipose triglyceride lipase (ATGL), was displaced from its location around LDs to the cytoplasm in *Stk25* transgenic liver, which provides the likely mechanisms for the observed difference in lipolytic activity. Our *in vitro* studies in human hepatocyte cell lines (IHH and HepG2) are fully consistent with studies in mouse model systems and support a cell-specific role of STK25 in the regulation of metabolic balance of hepatic lipid usage *vs.* lipid storage (Fig 2) [92]. We also found a statistically significant positive correlation between *STK25* mRNA expression in human liver biopsies and hepatic fat content [92].

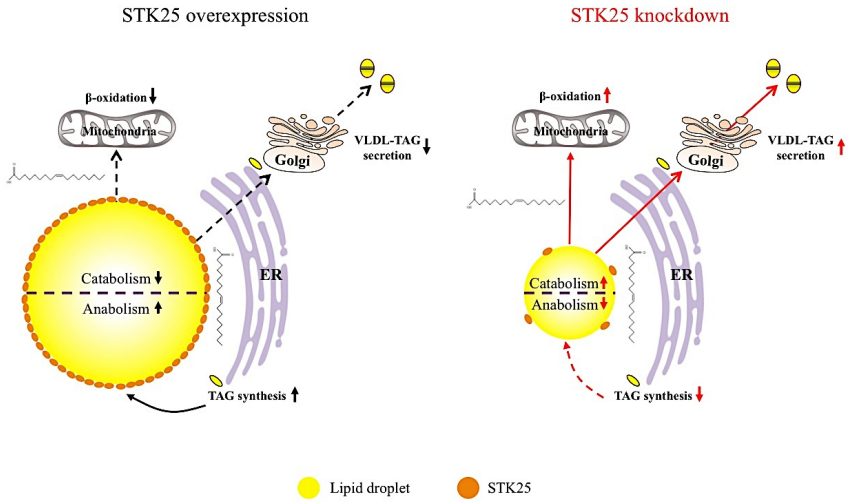

Figure 2. *Hypothetical model for STK25 function in regulating lipid accumulation in human hepatocytes. Overexpression of STK25 represses LD catabolism through suppressed β-oxidation and VLDL-TAG secretion, and promotes LD anabolism through enhanced TAG synthesis. Knockdown of STK25, conversely, increases β-oxidation and VLDL-TAG secretion and reduces TAG synthesis. Adapted from [92].*

Based on our findings of the role of STK25 in regulation of liver steatosis, we further studied the impact of STK25 on liver inflammation and fibrosis. For this, we challenged our mouse models with a methionine and choline deficient (MCD) diet to induce NASH. We observed that *Stk25* knockout mice showed a repression in lipid accumulation, oxidative stress, hepatocellular apoptosis, hepatic inflammation and fibrosis, compared with wild-type littermates. All these features of steatohepatitis were regulated in an opposite manner in the liver of *Stk25* transgenic mice (Fig 3) [88].

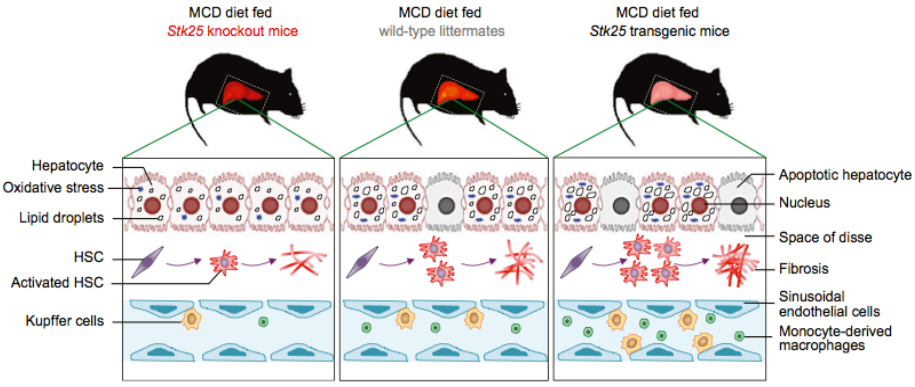

***Figure 3**. Reciprocal responses to the MCD-diet–induced development of NASH in Stk25-knockout and transgenic mice. Stk25 knockout mice fed an MCD diet displayed repressed lipid accumulation, reduced oxidative stress, and hepatocellular apoptosis, attenuation of hepatic inflammation, HSC activation and fibrosis, compared with wild-type littermates. All these features of steatohepatitis were regulated in an opposite manner in the liver of Stk25 transgenic mice. Adapted from [88].*

In summary, the previous studies by our research group have shown that STK25 is an important regulator of whole-body insulin sensitivity in peripheral metabolic tissues prone to diabetic damage, as well as in hepatic lipid partitioning and development/progression of NASH. The role of STK25 in skeletal muscle, liver and pancreas is further studied in this thesis.

2 AIMS

The general aim of this thesis was to elucidate the metabolic impact of the protein kinase STK25 in regulation of lipid metabolism, IR and T2D.

The specific aims of the three *Papers* included in this thesis were:

Paper I. Understanding the role of STK25 in control of ectopic fat storage and insulin response in skeletal muscle.

Paper II. Characterizing the role of STK25 in pancreatic β-cell function and NAFPD.

Paper III. Studying the pharmacological reduction of STK25 by antisense oligonucleotides (ASOs) as a possible strategy to revert diet-induced impairment in glucose and insulin homeostasis and progression of NAFLD.

3 METHODS

3.1 Ethical statement

All participants gave their written informed consent before taking part in the studies included in this thesis. All investigations were approved by the Ethics Committee of the University of Gothenburg, Sweden (Dnr. 1062-11) and were carried out in accordance with the Declaration of Helsinki. All animal experiments were performed after prior approval from the local Ethics Committee for Animal Studies at the Administrative Court of Appeals in Gothenburg, Sweden.

3.2 Experiments in human subjects

Paper III contains two studies performed in human cohorts; in the first one, the protein abundance of STK25 was measured in liver tissue samples obtained from 10 caucasian individuals, who fulfilled the following inclusion criteria: NAFL verified by CT and/or ultrasound, laboratory signs (elevated transaminases), and/or liver elastography findings (fibrosis 2-3) suggestive of NASH. A typical ultrasound-guided Menghini liver biopsy was performed, of which one part was transferred to ice-cold HEPES buffer, and stored at -80°C until further preparations. All liver biopsies were collected between 08:00 and 10:00 h after an overnight fast. The degree of NAFLD activity score (NAS) was assessed on liver sections by a certified pathologist, according to the Brunt scoring system [95]. These experiments were performed in collaboration with Hanns-Ulrich Marschall, Sahlgrenska University Hospital, Gothenburg. The second human study was done in the Tübingen Family (TÜF) study for T2D [96]. Liver fat was measured by ^{1}H-magnetic resonance spectroscopy in 430 healthy subjects with family history of diabetes, BMI $\geq$ 27, impaired fasting glycemia and/or previous gestational diabetes, and risk of developing T2D. The subjects had a mean age of 43 years (range: 18-69), a mean BMI of 30 kg/m² (range: 19-47), a mean liver fat content of 5.9% (range: 0.2-30.1), and 65 % were female. This study population was genotyped for 15 intronic tagging single nucleotide polymorphisms (SNPs) selected based on linkage disequilibrium information from the 1000 Genomes project covering 97% of the common genetic variation (minor

allele frequency ≥ 0.1) in the *STK25* gene. Genotyping was performed by mass spectrometry using the massARRAY system from Sequenom and the manufacturer's iPLEX software (Sequenom, Hamburg, Germany). Assay design for a 16th SNP necessary to cover 100 % of the common variation failed. Four of the 15 genotyped SNPs turned out to be not in Hardy-Weinberg equilibrium and, thus, were excluded from analyses. Our collaborator Harald Staiger, University of Tübingen, (Germany) performed these experiments.

3.3 Animal experiments

3.3.1 Mouse models

A transgenic mouse model overexpressing STK25 was created by the Norwegian Transgenic Center (Oslo, Norway) by pronuclear injection using C57BL/6N strain of mice. For further details, see [93]. *Stk25* knockout mice (a gift from Prof. B. Howell, Department of Neuroscience and Physiology, State University of New York Upstate Medical University, Syracuse, NY, USA) were generated by the deletion of exons 4 and 5, which causes a frameshift and a translational termination, as previously described [81].

Only male mice were used in the experiments included in this thesis. Briefly, mice were weaned at 3-4 weeks of age and housed 3-5 per cage in a temperature-controlled (21°C) facility with a 12-h light-dark cycle with free access to pelleted food and water. In all studies, transgenic and/or knockout mice, and corresponding age-matched wild-type littermates, were fed pelleted chow diet or high-fat diet (45 kcal% fat) starting from 6 weeks of age. The genetic background of transgenic and knockout mice is not identical, and therefore, each strain was only compared to their corresponding wild-type littermates.

3.3.2 Measurement of glucose tolerance and insulin sensitivity

In *Papers II* and *III*, intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) have been used to assess glucose tolerance and insulin sensitivity, respectively. Although both IPGTT and IPITT have limitations, these methods are widely used since they are easy to

use and are considered non-invasive. The mice were fasted 4 hours prior to all *in vivo* tests and blood sampling.

During IPGTT, a bolus dose of glucose (1 g/kg) was injected intraperitoneally and blood glucose and plasma insulin levels were monitored over a period of time. IPGTT determines the β-cell capacity of glucose stimulated insulin secretion, in combination with efficiency of insulin action in systemic glucose clearance [97].

During IPITT, a bolus dose of human recombinant insulin (1 U/kg) was injected intraperitoneally and blood glucose levels were monitored over a period of time. The decrease of blood glucose levels in response to insulin is an indicative of whole body insulin response and insulin sensitivity [97].

Saline solution (NaCl 9 mg/ml) was administrated at the end of these *in vivo* tests as a fluid replacement. The blood glucose and plasma insulin concentrations in IPGTT and IPITT were determined using an Accu-Chek glucometer and the Ultrasensitive Mouse Insulin ELISA kit, respectively.

3.3.3 Euglycemic-hyperinsulinemic clamp in conscious mice

Neither IPGTT nor IPITT determines which organs are responsible for the insulin resistant phenotype, a limitation that needs to be considered, because a different degree of IR may develop in different tissues. A more sensitive method to assess insulin sensitivity is the euglycemic-hyperinsulinemic clamp (EHC) technique. EHC is considered the "golden standard" method for the assessment of insulin sensitivity *in vivo* [98].

In *Paper I*, the EHC technique was used to assess insulin sensitivity in tissue-specific manner. Briefly, after 18 weeks on HFD, the mice underwent a microsurgery to implant a catheter in the left jugular vein, which was tunnulated subcutaneously to the neck, and connected to a vascular access button anchored in an incision behind the head. After surgery, the mice were single-caged for at least 3-4 days in order to recover before the experiment (se below).

After 4 hours of starvation, the systemic insulin concentration was raised by the administration of an intravenous bolus dose of insulin (62.25 mU/kg) and maintained by continuous intravenous infusion of insulin (7 mU/min/kg),

whereas glucose (30%) was simultaneously infused at a variable rate until mice reached a steady state (euglycemia). When the steady state remained stable over a period of time (*i.e.,* when blood glucose and GIR were stable for approx. 15-20 min), a bolus of [^{14}C]-labelled 2-deoxy-D-glucose (2-DOG) was administrated thought the jugular vein catheter. 2-DOG is converted to 2[^{14}C]-deoxyglucose-6-phosphate (2-DOG-6P) and trapped in the tissues, which enables to calculate the rate of insulin-stimulated glucose uptake in individual organs [99]. The glucose infusion rate (GIR) provides a measurement of the whole-body insulin action [100-102]. Insulin sensitive animals rapidly take up and utilize the glucose during the hyperinsulinemic condition, while glucose utilization and clearance are impaired in insulin resistant animals.

3.3.4 Systemic administration in mice

Different routs of systemic administration can be used in mice for acute and chronic treatment with hormones, pharmacological small-molecule compounds or ASOs. Subcutaneous (SC) injections are easy to use and rarely painful, however, the rate of absorption is lower compared to intraperitoneal (IP) injections. IP administration is the most widespread route, being easy to perform and allowing quite large volumes to be administrated, although it requires pH to be within a physiological range. Intravenous (IV) administration has many advantages. Solutions with high or low pH, as well as irritating substances, can be injected IV. However, this technique requires technical expertise and skill, and often involves anesthesia.

In this thesis, all administrations of glucose, insulin and ASOs to mice were performed via IP route except the EHC experiment in *Paper I*, where the administration of all substances was via IV though a jugular catheter.

3.3.5 Endurance exercise in mice

In *Paper I* endurance exercise capacity was assessed by treadmill running (Columbus Instruments) until the mice reached fatigue. This procedure starts with 3 days of acclimation, for the mice get used to the equipment and learn what they are expected to do. They also start getting accustomed to being handled, which reduces stress and minimizes variation during the actual exercise protocol. After that the mice are ready to perform the endurance exercise test. Initial speed was set to 10 m/min and the velocity was increased

by 2 m/min every 5 min. Once treadmill speed reached 22 m/min, this speed was maintained until fatigue. Fatigue was defined as mice spending ≥20 sec at the base of the treadmill despite manual encouragement.

3.3.6 Tissue collection

In *Papers I, II and III,* samples from different tissues have been collected for analysis of protein and gene expression, for histological assessment and biochemical measurements. Prior to tissue collection, mice were euthanized using 5% isofluorane with a mixture of air. Blood was collected by heart puncture for analysis of plasma metabolites. Tissue samples were dissected and snap frozen in liquid nitrogen and stored at -80ºC for Western blot, qPCR and/or biochemical analysis.

For histological analysis, tissue samples were fixated in 4% vol./vol. phosphate-buffered formaldehyde and embedded in paraffin, or embedded in optimal cutting temperature (OCT) mounting medium and frozen in in liquid nitrogen.

3.4 Histology

Hematoxylin and eosin (H&E) staining is one of the most frequently used stainings in histopathology. Nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. A limitation of hematoxylin staining is that it is incompatible with immunofluorescence. It is useful, however, to stain with H&E one-serial paraffin section from a tissue in which immunofluorescence will be performed. Hematoxylin, generally without eosin, is also used as a counterstain for immunohistochemical (IHC) stainings. In addition to H&E staining, tissue sections were also stained with Picrosirius Red in order to detect collagen fibers responsible for tissue fibrosis, and counterstained with Fast Green. Apoptotic cells were detected by TUNEL assay using the Apo-BrdU-IHC *In Situ* DNA Fragmentation Assay kit. Oil Red O (ORO), a diazo-based fat-soluble dye, was used for staining of neutral lipids and TAG on frozen tissue sections. For immunofluorescence, sections were incubated with specific antibodies, and counterstained with DAPI (4',6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to A-T rich regions in DNA. For detailed information about histology, IHC protocols and antibodies used for

immunofluerescence, please see corresponding Material and Methods sections for *Papers I, II and III.*

Ultrastructural analysis of muscle and pancreas was respectively performed in *Papers I and II* by transmission electron microscopy (TEM) in collaboration with the Centre for Cellular Imaging (CCI) Core Facility at the University of Gothenburg, as described [103].

All the quantifications were performed using the ImageJ 1.49v software.

3.5 Phosphoproteomic analysis

Global phosphoproteomics was used in *Paper* I to characterize the differences in the total and phosphoprotein abundance in *Stk25* transgenic mice *vs*. wild-type littermates. In brief, gastrocnemius muscles were carefully dissected and placed in a Maintainor® tissue card, vacuum packed and heat-stabilized by a 95ºC heat-shock during 21 sec in an Stabilizor^{TM} T1system. Heat stabilization is a revolutionary sample preservation technique that inactivates enzymes, such as kinases and phosphatases, which might interfere in the phosphorylation stage of the samples even after dissection, allowing for comparison of true biological variation. Conventional approaches such as inhibitor cocktails and pH changes are reversible and may interfere with the sample downstream analysis. In contrast, by using heat stabilization, enzymatic activity is permanently stopped.
The downstream analysis of total protein and phosphoprotein changes was performed by liquid chromatography mass spectrometry (LC-MS)/MS in collaboration with the Proteomic Core Facility (PCF) at the University of Gothenburg. For detailed information regarding to this technique see Material and Methods section and Supplementary Material in *Paper I.*

3.6 *Ex vivo* measurement of lipid metabolism

In *Paper I*, lipid anabolism and catabolism in skeletal muscle was studied by an *ex vivo* technique. The β-oxidation rate of palmitate was measured in quadriceps muscle homogenates as described previously [90]. Soleus and extensor digitorum longus (EDL) muscle strips were carefully dissected and

oleic acid uptake and TAG synthesis were measured as previously described [104, 105].

3.7 mRNA analysis and qPCR

Total RNA was first extracted from homogenized tissue samples, using commercially available kits and then transcribed into complementary DNA (cDNA) by a reverse transcription PCR reaction (RT-PCR). cDNA was used as a template for the quantitative real-time PCR (qPCR) reaction. TaqMan probes and assays for qPCR technique have been used and analyzed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [106] in all *Papers* of this thesis work. Gene expression of the ribosomal housekeeping gene 18S was used to normalize the data.

3.8 Western blot

Throughout this thesis work, protein expression has been evaluated using the western blot technique. Briefly, cells or homogenized tissue samples are lysed in lysis buffer optimized to maintain protein stability, to avoid protein degradation and inhibit phosphatase activity. Proteins were separated based on size using gel electrophoresis, and then transferred to a membrane. After the transfer of the proteins to the membrane, the membranes were stained using Ponceau. The proteins of interest were then identified using specific antibodies. In most analyses, hybridization with antibody against housekeeping protein, such as actin or glyceraldehyde 3-phosphate dehydrogenase, has been included, as endogenous control for equal loading and complete transfer.

3.9 Enzyme-linked immunosorbent assay (ELISA)

ELISA is used to quantify proteins in samples. There are different examples of ELISA, but most frequently a primary antibody binds to the protein of interest, followed by binding of a secondary antibody to the primary one. The secondary antibody carries an enzyme, and when a substrate for that enzyme

is added to the working solution, it will lead to a shift in color, which is detectable by a spectrophotometer. The color intensity is proportional to the amount of the protein of interest in the sample, and is analyzed in relation to a standard curve.

The fasting plasma insulin, C-peptide, leptin, lactate and glucagon concentrations were quantified using ELISA techniques as described in *Papers I, II and II.*

3.10 Statistical analyses

The experimental data are presented as means ± the standard error of the mean (SEM) in all *Papers*. In *Papers I and II,* statistical significance between the groups was evaluated using the unpaired 2-tailed Students t-test and in *Paper III*, Students t-test, or one-way ANOVA with t-test for post-hoc analysis were used. Statistics were calculated using IBM SPSS Statistics version 20 or Microsoft Excel. $P<0.05$ was considered statistically significant.

4 RESULTS AND DISCUSSION

In this section, the main results of *Paper I, II* and *III* are summarized and discussed. For further details, see the full *Papers* provided at the end of the thesis book.

Paper I

Overexpression of Protein Kinase STK25 in Mice Exacerbates Ectopic Lipid Accumulation, Mitochondrial Dysfunction, and Insulin Resistance in Skeletal Muscle

In this study, we have investigated the potential role of STK25 in control of ectopic lipid accumulation and insulin response in skeletal muscle.

Consistently with our previous investigations in the liver, we found that overexpression of STK25 in mice fed a HFD increases ectopic lipid accumulation in skeletal muscle [90]. This correlates to our previous findings in *Stk25* knockout mice, which showed a reduction in intramuscular lipid deposition [89]. Notably, the lipid levels were unchanged in transgenic mice fed a chow diet, suggesting that a HFD challenge is needed for STK25 overexpression to lead to metabolic alterations in the skeletal muscle.

Besides the increased ectopic lipid storage, we found a reduced β-oxidation in the skeletal muscle of *Stk25* transgenic mice, while the muscle lipid uptake and synthesis remained unaltered compared to wild-type mice. In line with these findings, we have previously reported reduced β-oxidation rate and mitochondrial activity in the liver of *Stk25* transgenic mice [90]. Reciprocally, previous studies by our group have shown that partial depletion of STK25 in the rat myoblast cell line L6 and in human hepatocytes by small interfering RNA (siRNA) increases β-oxidation [74, 92].

The histopathological evaluation of skeletal muscle from transgenic mice fed a HFD showed evidence of muscle damage, such as intracellular inclusions, small degenerating fibers, necrosis, infiltration of inflammatory cells and adipocyte replacement, while these features were rarely seen in wild-type muscle.

Ultrastructural analysis by TEM confirmed an increase in intramyocellular lipid droplets size and number, and showed also a disrupted sarcomere ultrastructure and abnormal mitochondria in *Stk25* transgenic muscle. We also found that overexpression of STK25 in HFD-fed mice resulted in an increase in muscle fibrosis.

We have previously reported a significant increase in acetyl-CoA carboxylase (ACC) protein levels in skeletal muscle of *Stk25* transgenic mice [93]. ACC synthesizes malonyl-CoA, which functions as a precursor for fatty acid synthesis, but also represses lipid oxidation through allosteric inhibition of mitochondrial fatty acid transporter carnitine palmitoyltransferase 1 (CPT1). It is possible that an increase in ACC, and the ultrastructural abnormalities in muscle mitochondria observed by TEM, contributed to the decrease in β-oxidation rate in transgenic muscle. However, no alteration in ACC was seen in skeletal muscle of *Stk25* knockout mice or in L6 cells where STK25 was depleted [74, 89].

Mitochondrial dysfunction and altered morphology is known to be involved in the pathogenesis of IR both in humans and mice [107, 108]. Previous studies using TEM have demonstrated alterations in mitochondrial morphology in skeletal muscle of humans and rodent models with IR and T2D [109, 110]. The exact mechanism linking mitochondrial dysfunction and IR is still unclear, although one probable hypothesis in humans is that skeletal muscle disposition to lipid accumulation is caused by impaired β-oxidation [111]. Based on this evidence, the impaired mitochondrial function observed in *Stk25* transgenic muscle probably contributed to increased myocellular lipid accumulation and development of skeletal muscle IR. Furthermore, previous studies by our group have shown that partial depletion of STK25 in the rat myoblast cell line L6 by siRNA increases β-oxidation [74].

The observation that STK25 overexpression in mice increased fat storage in the muscle prompted us to investigate whether these changes would affect skeletal muscle insulin sensitivity. To test this, we performed an EHC experiment, which showed a reduced *in vivo* insulin-stimulated glucose uptake in gastrocnemius and quadriceps muscles of *Stk25* transgenic mice compared to wild-type mice, with a similar tendency in EDL and soleus

muscles. Previous studies by our group have shown that partial depletion of STK25 L6 cells improves insulin-stimulated glucose uptake [74] and *Stk25* knockout mice display an improved *in vivo* insulin-stimulated glucose uptake in skeletal muscle [89]. Furthermore, the studies in transgenic mice with muscle- and liver-specific overexpression of lipoprotein lipase (LPL) suggest that the tissue-specific increase in lipid accumulation causes impairments on tissue-specific insulin-stimulated glucose uptake and IR [32]. In light of this evidence, it is likely that changes in intramyocellular lipid storage by STK25 contributed to impaired insulin sensitivity, although the underlying mechanism remains elusive.

We also found a markedly reduced endurance exercise capacity in *Stk25* transgenic mice, both in terms of running distance and time, which might be a consequence of the disorganized sarcomere structure and the increased fibrosis observed in *Stk25* transgenic muscle.

Global phosphoproteomic analysis revealed alterations in the total abundance and phosphorylation status of different target proteins located predominantly to mitochondria and sarcomere in *Stk25* transgenic mice, while no lipases where detected above the level of quantification.

Taken together, our findings suggest that overexpression of STK25, in conditions of excess dietary fuels, leads to a shift in the metabolic balance in skeletal muscle from lipid oxidation to lipid storage, resulting in IR and mitochondrial β-oxidation impairments in skeletal muscle (Fig. 4).

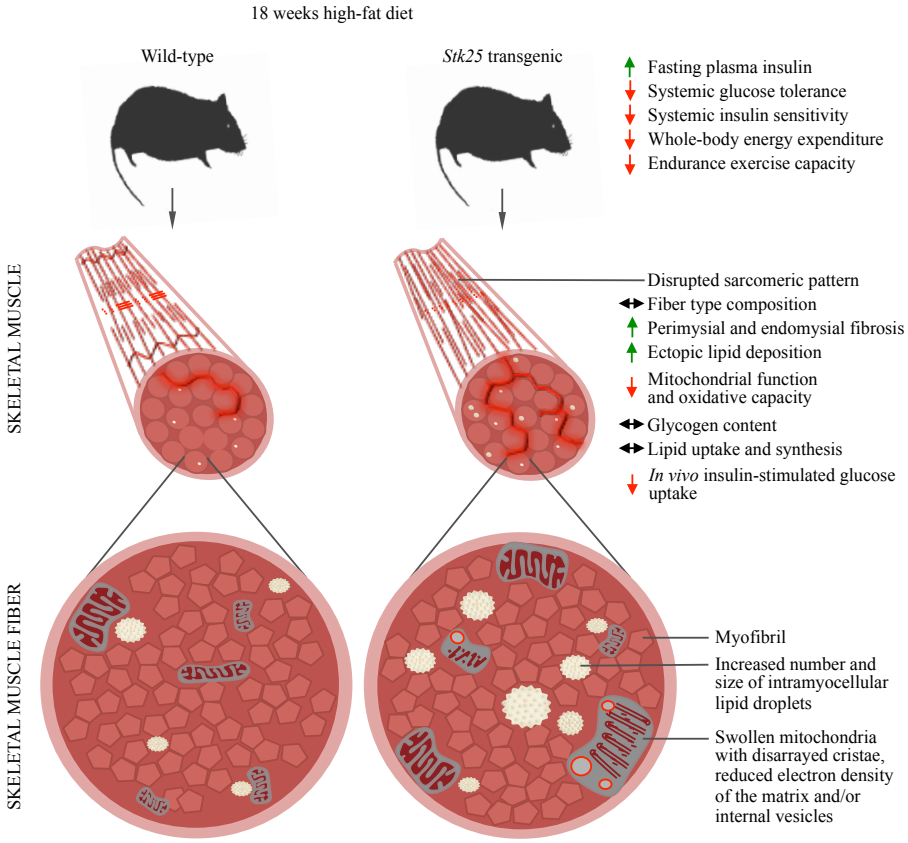

Figure 4. *Schematic illustration of the metabolic responses at the whole-body level, as well as in skeletal muscle fibers of Stk25 transgenic mice vs. wild-type littermates. Up- or down-regulation is indicated by green and red arrows, respectively. Figure adapted from Paper I.*

Paper II

Protein Kinase STK25 Aggravates the Severity of Non-Alcoholic Fatty Pancreas Disease in Mice

In this study we investigated the role of STK25 in control of progression of NAFPD in the context of chronic exposure to dietary lipids.

First, we investigated the overall pancreas morphology in tissue sections of *Stk25* transgenic and wild-type mice fed a HFD. We found no differences in the morphometric analysis, nor signs of compensatory hyperplasia as a result of previously described insulin resistance in *Stk25* transgenic mice compared with wild-type mice [93]. We found that pancreases of *Stk25* transgenic mice displayed a marked decrease in islet β/α-cell ratio and alteration in the islet architecture with an increased presence of α-cells within the islet core. Mouse β-cells are known to be located to the islet core while α-cells are arranged at the periphery of the islet. Islet dysmorphogenesis (i.e. presence of α-cells within the islet core) in mouse and humans has been related to impairments in insulin secretion [112-114]. It has been suggested that the changes in the islet morphology such as increased islet vascularization and infiltration of α-cells from the periphery to the islet core minimizes the β-cell-to-β-cell contact and suppresses the insulin output, which consequently contributes to the diabetes phenotype [112, 114]. We observed alterations in β/α-cell distribution in *Stk25* transgenic mice, but we did not see any changes in islet vascularization.

Next, we evaluated pancreas inflammation and found that *Stk25* transgenic mice presented an increased amount of mononuclear inflammatory cells aggregated on the periphery of the islet, defined as peri-insulitis. We also observed an aggravated degree of fibrosis in transgenic pancreas, which was evident, both in the exocrine and endocrine pancreas tissue. Additionally, we also found an increased number of activated pancreatic stellate cells (PSCs), which are the cells responsible for fibrosis, in the pancreas of *Stk25* transgenic mice. Interestingly, our preliminary unpublished investigations in the pancreas of *Stk25* knockout mice fed a HFD suggest that the level of peri-insulitis was reduced in *Stk25* knockout pancreas compared to the wild-type pancreas, reciprocal to our findings in *Stk25* transgenic pancreas (Fig. 5).

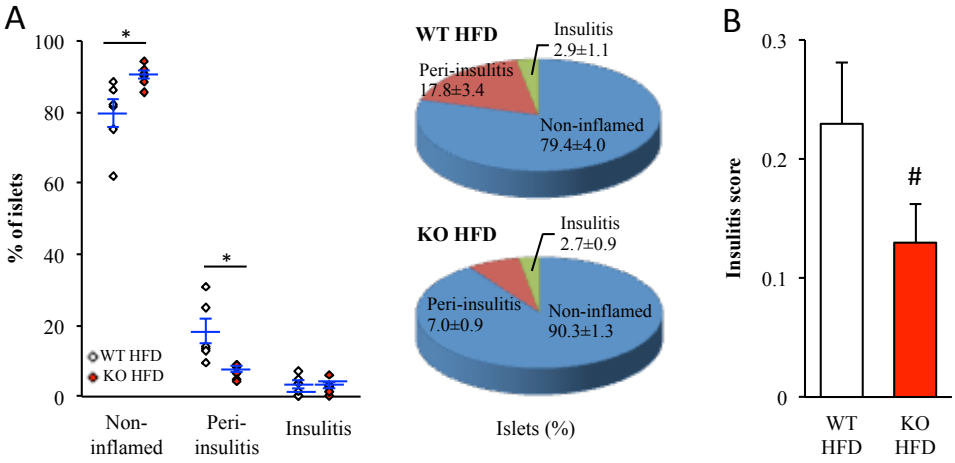

Figure 5. *Analysis of inflammatory cell infiltration in the pancreas of HFD-fed Stk25 knockout and wild-type mice. A: Quantification of non-inflamed islets, peri-insulitis and insulitis. Results are shown as a dot plot where each point represents one mouse and as a pie chart. B: The degree of insulitis as assessed using a semi-quantitative scoring system. Data are mean±SEM from 6 mice/genotype. *p<0.05; #p=0.07; HFD, high-fat diet; KO, knockout; WT, wild-type.*

The molecular mechanisms that cause pancreatic β-cell dysfunction in the context of obesity are not fully understood, but it is known that the long-term exposure to the combination of FFA and glucose (i.e. glucolipotoxicity) [71, 115-117] leads to β-cell apoptosis and to decreased insulin secretion. The pancreas of HFD-fed *Stk25* transgenic mice presented increased intracellular lipids visualized by ORO staining as well as increased TAG content measured in pancreas homogenates, which is in line with our findings in skeletal muscle presented in *Paper I*. Lipid overload in the β-cells is known to correlate with programmed cell death in both animal models and humans [118, 119]. We therefore examined the level of cell apoptosis by *in situ* DNA fragmentation within the pancreas tissue and observed an increased apoptosis in the pancreas of *Stk25* transgenic mice compared to wild-type mice, suggesting a role of STK25 in cell survival under HFD-induced lipotoxic conditions. Pancreatic β-cell apoptosis is a key event in diabetes progression [71, 120-122], which probably contributed to the lost ability to increase *in vivo* glucose-stimulated insulin secretion in *Stk25* transgenic mice. Interestingly, while the members of STE20 kinase family are generally not

described in relation to metabolic profiling, the STE20 protein MST1 (also known as STK4 or KRS2) was recently also identified as a critical regulator of apoptotic β-cell death [87].

To study whether the aggravated fat infiltration, inflammation and fibrosis in the pancreas of *Stk25* transgenic mice leads to an impairment of the *in vivo* glucose-stimulated insulin release, we performed an IPGTT. We found that blood glucose level was higher in *Stk25* transgenic mice over the whole time-course of the IPGTT, which is consistent with our previous observation based on a different cohort of mice [93], while insulin secretion was lower, suggesting a relative impairment in GSIS. The observed reduction in insulin output after the *in vivo* glucose stimulation might also be related to the described islet dysmorphogenesis observed in *Stk25* transgenic islets (Fig 6).

After the 18-week challenge with a HFD, we found lower levels of fasting plasma insulin and C-peptide in *Stk25* transgenic mice. In contrast, we have previously reported hyperinsulinemia in *Stk25* transgenic mice compared to wild-type mice after 16 and 17 weeks of HFD challenge [93]. Furthermore, higher levels of leptin were found in *Stk25* transgenic mice; however, we have previously reported no differences in body weight or food intake compared to wild-type mice [93], suggesting that *Stk25* transgenic mice could be leptin resistant. Furthermore higher circulating leptin has previously been connected to inflammatory processes in the pancreas [123]. Consequently, it is possible that increased circulating leptin levels contributed to the more severe pancreatic inflammation observed in *Stk25* transgenic mice.

Stk25 transgenic mice present global overexpression of *Stk25* gene [93], and to evaluate the direct cell-autonomous effect of STK25 in GSIS in mouse islets, we incubated isolated islets from HFD-fed mice *in vitro* with 5.5 or 16.5 mM glucose. However, there was no difference in GSIS comparing the islets of *Stk25* transgenic *vs.* wild-type mice, suggesting the potential contribution of systemic factors to regulation of GSIS by STK25.

In summary, we found that STK25 overexpression in HFD-fed *Stk25* transgenic mice increased lipid storage in pancreas, which was accompanied by exacerbated pancreatic inflammatory cell infiltration, stellate cell activation, fibrosis and apoptosis (Fig. 6). The GSIS was impaired in HFD-fed *Stk25* transgenic mice, despite of a higher net change in blood glucose

concentration compared with wild-type controls, suggesting a β-cell dysfunction (Fig. 6). Taken together, this study suggests a role for STK25 in determining the susceptibility to diet-induced NAFPD in mice.

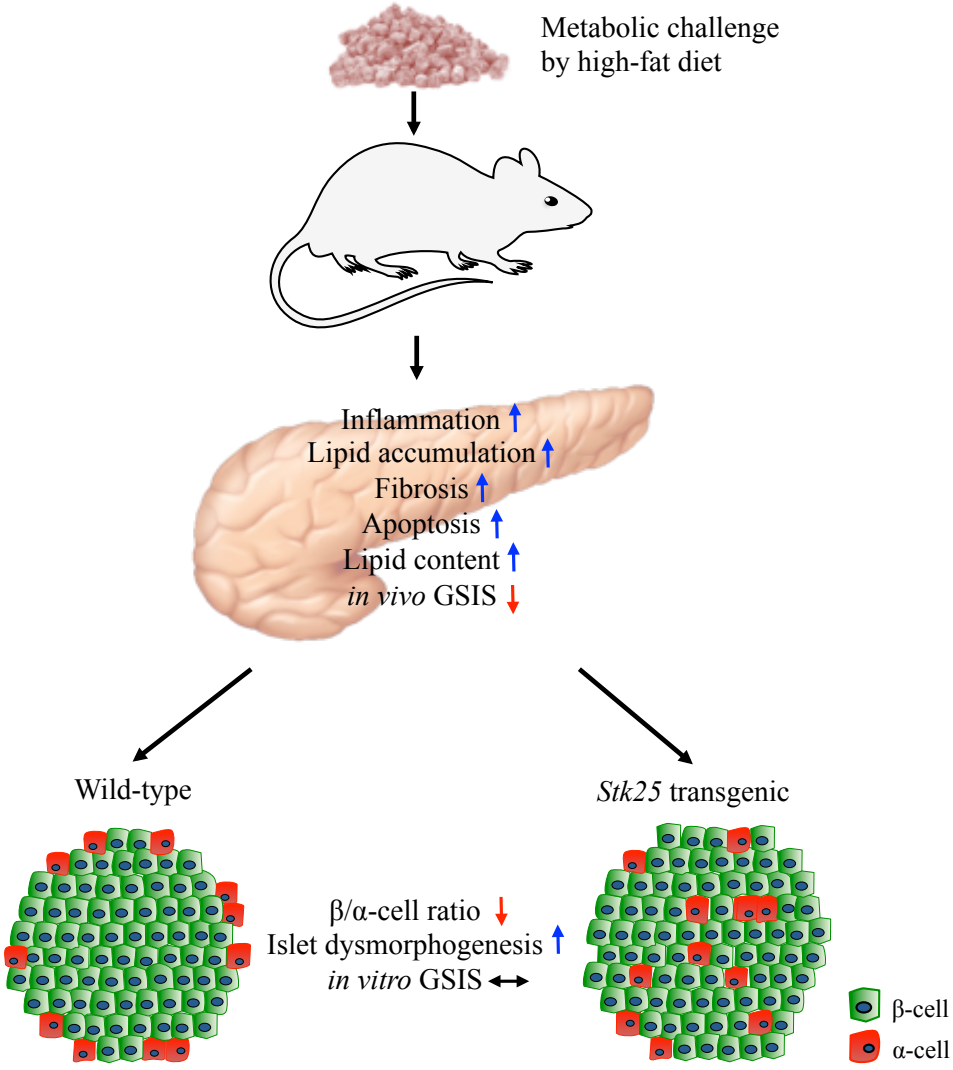

Figure 6. *Schematic illustration of metabolic alterations in pancreas and Langerhans islets of Stk25 transgenic animals vs. wild-type after a HFD challenge. Blue or red arrows indicate up- or down-regulation respectively.*

Paper III

Stk25 Antisense Oligonucleotide Treatment Reverses Glucose Intolerance, Insulin Resistance and Nonalcoholic Fatty Liver Disease in Mice

Chronic exposure to dietary lipids is known to promote ectopic lipid deposition in the liver [14]. Our previous findings revealed a key role of STK25 in control of liver lipid storage in mouse models and in human hepatocytes [88-90, 92, 93]. We have previously shown that *Stk25* knockout mice are protected against the development of HFD-induced liver steatosis and also against MCD diet-induced NASH progression [89-91]. The objective of this study was to address whether the treatment with anti-*Stk25* ASOs in mice enables to reverse the diet-induced impairment in glucose and insulin homeostasis and ameliorate liver steatosis, inflammation and fibrosis in the context of obesity.

The mice were fed a HFD for 21 weeks and treated with *Stk25* ASO#1 or ASO#2 or placebo (PBS) twice weekly for the last 6 weeks of the diet. We found that hepatic *Stk25* mRNA expression was lower in the groups treated with *Stk25* ASOs compared with the placebo group, whereas the protein abundance of STK25 was below the detection limit of Western blot in the livers of ASO-treated mice. The plasma insulin level was dramatically reduced in mice treated with ASOs, and consistently, the homeostasis model assessment of insulin resistance (HOMA-IR) was also lower throughout the ASO treatment. However, we have previously reported that HFD fed *Stk25* knockout mice did not completely restore the physiological levels of glucose and insulin after the dietary challenge to the levels observed in chow-fed controls, nor the HOMA-IR [89], suggesting that *Stk25* knockout did not fully ameliorate the progression to diet-induced T2D, but *Stk25* ASO treatment does.

The fasting levels of circulating glucose and insulin at the end of the treatment period with ASO#2 were significantly decreased compared to the values measured before the treatment started, and similar to the values before the HFD feeding was initiated, indicating that the diet-induced hyperglycemia and hyperinsulinemia were fully reversed by ASO#2. During the IPGTT experiment, blood glucose levels returned to normal more rapidly

in *Stk25* ASO-treated mice than in the placebo group, demonstrating a better glycemic control, and the glucose-stimulated insulin secretion during the test was also improved in both ASO-treated groups. The IPITT revealed an improvement in insulin sensitivity in both groups treated with *Stk25* ASOs.

We observed no changes in body weight during *Stk25* ASO-treatment. Consistent with this observation, we have previously reported that neither STK25 overexpression in transgenic mice nor *Stk25* depletion in knockout mice fed a HFD results in any changes in body weight, compared to their respective wild-type controls [89, 93]. These findings suggest that STK25 inhibition protects against the metabolic consequences of chronic exposure to dietary lipids independently of changes in body weight.

We found that the density and size of intrahepatocellular lipid droplets were dramatically reduced in *Stk25* ASO-treated mice, showing that *Stk25* ASO treatment repressed liver steatosis induced by the HFD challenge. In our previous studies in *Stk25* knockout mice, we also observed a reduction of hepatic lipid area [89], but this reduction is markedly greater in the ASO-treated mice. In this study, we have used generation 2.5 ASOs, which deplete their target in all peripheral tissues [124], even though we only observed efficient STK25 depletion in the liver. However, our most recent yet unpublished studies using highly hepatocyte-specific GalNAc-conjugated anti-*Stk25* ASOs also show that liver steatosis can efficiently be inhibited by selective repression of STK25 levels in hepatocytes, without any contribution from other cell types in the liver (Fig. 7).

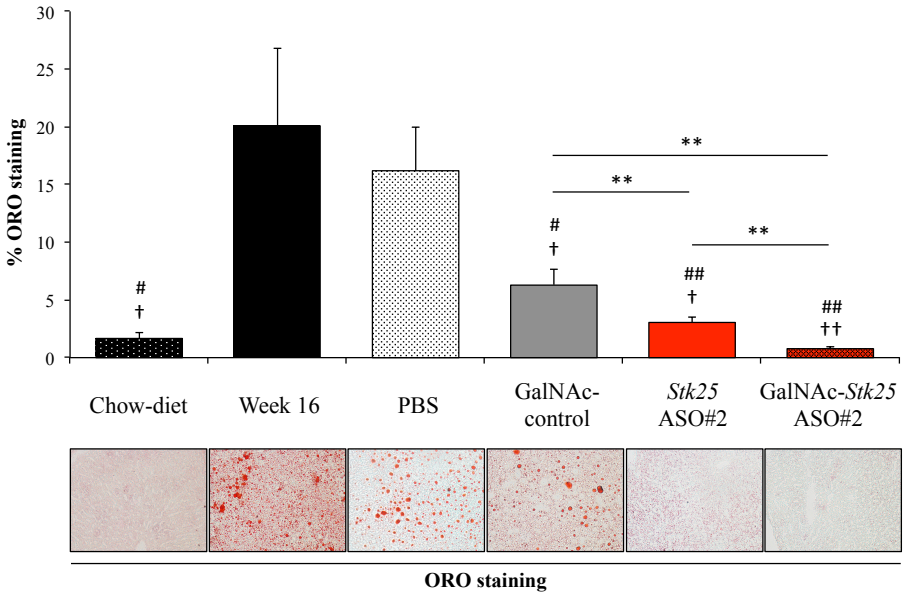

Figure 7. *Effects of GalNAc-Stk25 ASO treatment compared to ASO 2.5 in repression of diet-induced liver steatosis. Quantification of stained ORO area (%) and representative images of liver sections stained ORO. Data are mean±SEM from 3-6 mice/group. Significances are shown as* $^{**}p<0.01$*; significances to PBS are shown as* $^{\dagger\dagger}p<0.01$*;* $^{\dagger}p<0.05$*; significances to week 16 are shown as* $^{\#\#}p<0.01$*;* $^{\#}p<0.05$*.*

We also observed that *Stk25* ASO-treated mice were protected against HFD-induced liver fibrosis, both when assessed at histological evaluation and by measuring liver hydroxyproline content. Additionally, the inflammation level was approximately 2-fold reduced in the livers of *Stk25* ASO-treated mice. Moreover, mice treated with *Stk25* ASOs scored markedly lower both for NAS and fibrosis compared with the placebo group, based on the Kleiner/Brunt criteria adapted to rodents [125-129]. The findings of this study are consistent with our previously published results in *Stk25* knockout mice [89] and reciprocal to our data published in STK25 overexpressing transgenic mice [90] (Fig 8). Interestingly, we have shown that anti-*Stk25* ASO treatment improves insulin and glucose homeostasis and protects against liver steatosis to a better extent than the phenotype observer in the *Stk25* knockout model [89].

One of the main findings in *Paper III* was the reduction of ACC protein amount in the livers of ASO#2 treated mice, which is a key controller of both mitochondrial β-oxidation and fat synthesis. This result is consistent with our previous observations in HFD-fed *Stk25* knockout mice [89], and reciprocal to our studies in STK25 overexpressing transgenic mice [93] (Fig 8). This finding suggests that not only the liver phenotype, but also the possible mechanism underlying the repression of hepatic fat accumulation, are similar in *Stk25* ASO-treated and knockout mice. There is evidence that a diet-induced hepatic steatosis can be reversed in mice by reducing ACC expression, due to a decreased lipogenesis and an increased β-oxidation [130]. Interestingly, reduced liver steatosis, fibrosis and apoptosis have also been reported recently in a proof-of-concept trial in NASH patients who were given GS-0976 (a liver-specific ACC inhibitor) orally [131]. These results suggest that the repression of hepatic ACC levels in connection of STK25 inhibition, which we observed by *Stk25* ASO treatment or by genetic STK25 depletion [89], may not only constitute a key mechanism for this protein to reduce liver steatosis, but also to suppress hepatic fibrosis and hepatocyte damage.

We have previously reported that *STK25* mRNA correlates positively with liver fat [92] and NASH development in human liver biopsies [91]. Consistently, in this study we also found that STK25 protein correlates positively with NASH development in human liver biopsies. Furthermore, we found that several common non-linked SNPs in the human *STK25* gene are associated with altered liver fat, suggesting a critical role of STK25 in the pathogenesis of NAFLD in human (For details on SNP analyses, see *Paper III*).

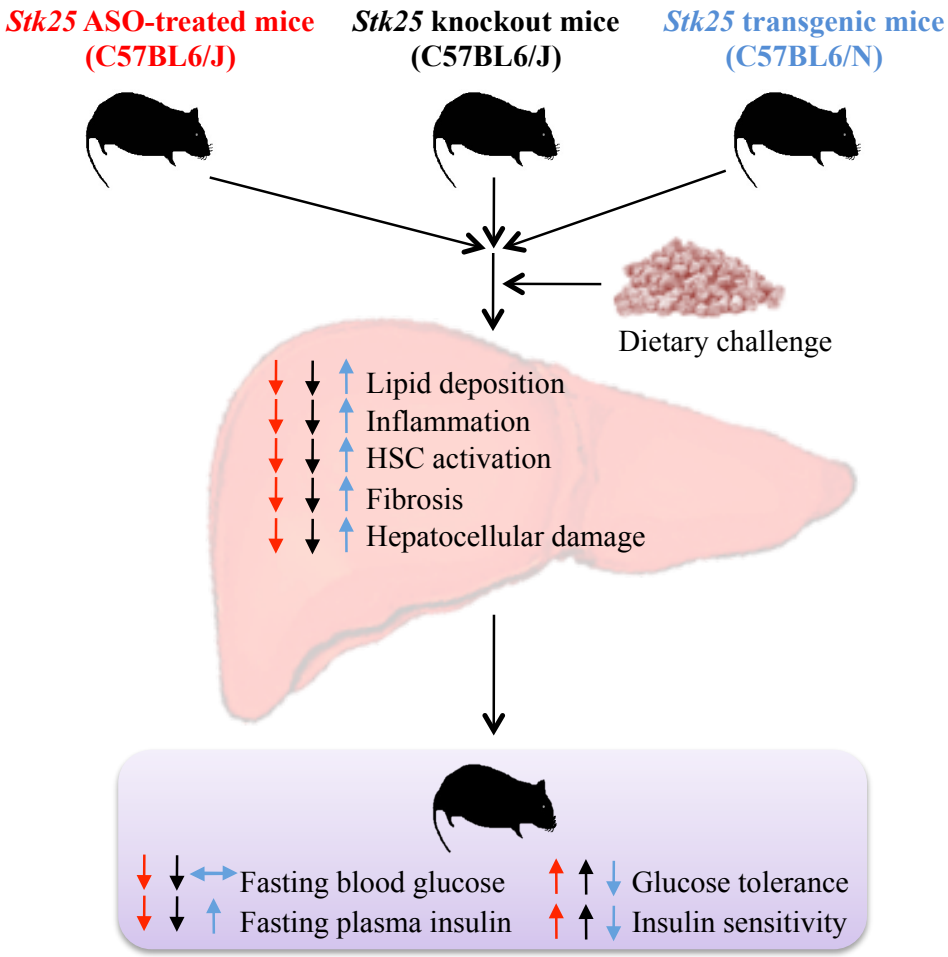

Figure 8: *Schematic illustration of the metabolic responses in the liver and at the whole-body level in mice with repressed STK25 function by either Stk25 ASO treatment or genetic disruption (indicated by red and black arrows, respectively) and transgenic mice overexpressing STK25 (indicated by blue arrows) compared with the corresponding control groups of mice. The phenotype of Stk25 knockout and transgenic mice has been described previously [89-91, 93]. Figure adapted from Paper III.*

In summary, the results of this study demonstrate that systemic administration of *Stk25* ASOs in mice significantly suppresses hepatic STK25 mRNA and protein abundance compared with the placebo-treated mice, which reverts HFD-induced systemic hyperglycemia and hyperinsulinemia, improves whole-body glucose tolerance and insulin sensitivity, and ameliorates steatosis, inflammatory infiltration, apoptosis, and nutritional fibrosis in the liver. The data of the current study demonstrate that inhibition of STK25 may provide new-in-class therapeutics for NAFLD, type 2 diabetes and related metabolic complications.

5 CONCLUSIONS

Paper I suggests STK25 as a new regulator of the complex interplay between lipid storage, mitochondrial energetics and IR in skeletal muscle in mice in connection to diet-induced obesity. STK25 overexpression results in a reduced endurance exercise capacity in mice challenged with a HFD.

Paper II shows a role for STK25 in determining the susceptibility to diet-induced NAFPD in mice in connection to diet-induced obesity. STK25 overexpression in HFD-fed mice results in a significant decrease in islet β/α-cell ratio and alterations in the islet architecture with an increased infiltration of α-cells within the islet core (islet dysmorphogenesis). Furthermore, STK25 overexpression also caused increased lipid storage in the pancreas and impairments in GSIS during IPGTT, suggesting a role of STK25 in islet β-cell dysfunction.

Paper III demonstrates that repressing STK25 in mice by ASO treatment effectively reverses the HFD-induced impairments in glucose and insulin homeostasis and ameliorates liver steatosis, inflammation and fibrosis in the context of obesity in mice. This finding warrants further investigations of the potential therapeutic benefit of pharmacological STK25 inhibitors as new-in-class drug candidates for NAFLD, T2D and related metabolic diseases in humans.

6 GENERAL DISCUSION

In this section, a brief general discussion on the findings of this thesis is provided.

We are the first, and to date the only research group, who has described a role of protein kinase STK25 in metabolic regulation [74, 88-90, 92, 93, 132, 133]. In this thesis, we describe STK25 as a new key regulator of whole-body metabolism. In *Paper I* and *II*, we have found that STK25 overexpression in mice challenged with a HFD results in an increased ectopic lipid deposition in skeletal muscle and pancreas accompanied by an increased fibrosis and inflammation, consistent with our previous findings in liver [90, 93, 132, 133]. In *Paper I* we have shown that the overexpression of STK25 also leads to impairments in β-oxidation and decreased *in vivo* insulin-stimulated glucose uptake in skeletal muscle and reduced endurance exercise capacity in mice [132]. In *Paper II* we have shown that the pancreas of *Stk25* transgenic animals shows higher degree of apoptosis, together with an impaired insulin production during IPGTT after a HFD challenge [94]. In *Paper III* we have shown that treatment with *Stk25* ASOs in obese mice protects against HFD-induced liver steatosis, glucose intolerance and IR. Furthermore, we found that the metabolic phenotype of *Stk25* ASO-treated mice was fully consistent with our observations of *Stk25* knockout mice [89, 91] (Fig. 8). This similarity between treatment with *Stk25* ASOs, which have most pronounced effect in the liver, and whole-body depletion of STK25 in knockout mice [89, 91], suggests that inhibition of hepatic expression of STK25 is sufficient for systemic metabolic effects of this mediator. Furthermore, our studies with *Stk25* ASOs demonstrate that STK25 repression is able not only to prevent, but also reverse, NAFLD and the metabolic impairments in systemic glucose and insulin homeostasis in mice after chronic exposure to dietary lipids.

One of the most important previous findings by our research group is that STK25 coats LDs in mouse liver [90], which has been further confirmed in hepatocytes of human origin [90]. Interestingly, our preliminary studies in adipocytes suggest that STK25 is also located to LDs in adipose tissue. However, in *Paper I* we have tried to address the subcellular localization of STK25 in skeletal muscle fibers, but, due to technical limitations of the assay, we were unable to conclude whether STK25 protein co-localizes to

LDs also in skeletal muscle. Based on our studies in mouse liver and human hepatocytes, we have proposed a mechanism of action of STK25 in liver; being associated with hepatic LDs, STK25 limits the association of liver lipase ATGL with the LDs, which results in reduced lipolytic activity, VLDL-TAG secretion and β-oxidation [90] (Fig. 2). Notably, alterations in lipid droplet proteins are increasingly recognized to be associated with metabolic abnormalities [134].

Several lines of evidence support that STK25 has a similar role in the pathogenesis of NAFLD in human liver as described in our mouse models. We found a significant positive correlation between *STK25* gene expression and liver fat content [92], as well as a positive correlation between NASH development and *STK25* mRNA [91] and protein abundance (*Paper III*) in human liver biopsies. Furthermore, in *Paper III* we have identified four common non-linked SNPs in the human *STK25* gene that are associated with altered liver fat: two associated with increased hepatic fat and two associated with a decreased. Even though these human genetic data need replication and deeper functional exploration, they are in line with our findings in mice. Furthermore, we have previously reported reduced lipid accumulation and improved insulin sensitivity, as well as reduction of NASH feature in human hepatocytes where STK25 is depleted by siRNA technology [91, 92].

In *Paper III* we show a reduction in hepatic ACC protein level by *Stk25* ASO treatment, which is consistent with our previous observations in *Stk25* knockout livers [89] (Fig 8). ACC is known to regulate both lipogenesis and β-oxidation though malonyl-CoA production [135]. Interestingly, in a recently reported initial proof-of-concept trial in NASH patients, an oral treatment with a liver-targeted ACC inhibitor GS-0976, was shown to significantly improve liver steatosis as well as circulating biomarkers of liver fibrosis and cell death [131]. These data suggest that the repression of hepatic ACC levels by *Stk25* ASOs that we found in this study, may not only constitute a key mechanism for the effect of *Stk25* ASOs on reducing liver steatosis, but also for suppressing fibrosis and hepatocellular damage.

In summary, there is an increasing body of evidence suggesting that ectopic lipid accumulation in peripheral tissues such as skeletal muscle, liver, and pancreas, is one of the main causes of development and aggravation of IR and T2D [27]. Therefore, understanding the molecular mechanisms

underlying the pathogenesis of ectopic lipid accumulation in the main metabolic tissues is of high clinical relevance. The existing pharmacological approaches for treating obesity largely act by suppressing food or caloric intake, whereas current antidiabetic therapies typically enhance insulin sensitivity and/or secretion. Although these agents have proved beneficial, there is still a huge unmet need for effective pharmacological interventions that are capable of more permanently reversing the diabetic phenotype with minimal side effects. With the findings presented in this thesis, STK25 is emerging as a key regulator controlling lipid accumulation not only in the liver but also in skeletal muscle and pancreas. This work also contributes to our overall understanding of the complex and integrated molecular networks connecting ectopic lipid deposition and mitochondrial impairments with IR, T2D and β-cell dysfunction. Furthermore, the strong results shown in *Paper III* provide preclinical *in vivo* proof-of-principle for the metabolic benefit of pharmacological STK25 inhibitors in conditions of excess dietary fuels in a mouse model and suggest that therapeutic intervention aimed at reducing STK25 function may provide a new strategy for the treatment of patients with NAFLD, T2D and related metabolic diseases.

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