From the Department of Medicine, Huddinge
Karolinska Institutet, Stockholm, Sweden

STUDIES ON NOVEL INTERACTORS IN HUMAN ADIPOSE TISSUE

Amanda T. Pettersson

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ABSTRACT

Obesity is associated with a mild inflammation and altered protein secretion in adipose tissue. These changes have been suggested to contribute to the development of obesity-associated disorders including, insulin resistance, dyslipidemia, type 2 diabetes and cardiovascular disease.

The aim of this thesis was to identify novel factors expressed in the adipose tissue that could be important in the development of obesity and obesity-associated disorders.

In **paper I** we studied the transcriptional control of the human *CIDEA* gene and the effects of TNFα. CIDEA has previously been shown to be differentially expressed in lean and obese subjects and repressed by tumor necrosis factor α (TNFα), however the human CIDEA promoter has not been investigated. Reporter assays demonstrated that the minimal transcriptional activity of the human CIDEA promoter was confined to a region 97 bp upstream of the transcriptional start site (TSS). TNFα attenuated the transcriptional activity of CIDEA and this regulation was confined to a region between 244 and 123 bp upstream of the TSS. Electrophoretic mobility shift assays and mutational analysis suggested that the regulation by TNFα is mediated by a nuclear factor (NF)-κB binding site.

In **paper II** and **III** we investigated the function of the *twist1* gene in human white adipocytes and its relationship with obesity. Twist1 is involved in various processes, such as bone development, cancer progression, inflammation and brown adipose tissue function, but the role in white adipose tissue is not known. In **paper II** we assessed the expression of twist1 in human white adipose tissue, liver, skeletal muscle and pancreas as well as in different cell types in adipose tissue. Twist1 mRNA expression was significantly higher in adipose tissue compared to the other organs and within adipose tissue; the expression was highest in the adipocyte fraction. *In vitro* silencing of twist1 by RNA interference was used to investigate the role of twist1 in lipid turnover and inflammation in human adipocytes. In contrast to mice where twist1 reduces fatty acid oxidation, reduction of twist1 expression in human *in vitro* differentiated adipocytes lead to a reduction in fatty acid oxidation. Furthermore, expression of the rate-limiting enzyme for fatty acid oxidation, carnitine palmitoyl transferase 1, was also reduced when twist1 was silenced, suggesting that twist1 is required for fatty acid oxidation in human white adipocytes. We were also able to demonstrate that twist1 regulates the expression of inflammatory proteins, including interleukin (IL) -6 and monocyte chemoattractant protein 1. Chromatin immunoprecipitation demonstrated direct binding to sequences in the promoter regions of these genes. In **paper III,** we further explored the expression of twist1 in non-obese and obese subjects and correlated the expression with different clinical parameters of insulin resistance. Interestingly and in contrast to studies in mice, low twist1 expression associated with a higher BMI and a more adverse clinical profile, characterized by higher HOMA-IR values, large adipocytes and an increased secretion of pro-inflammatory factors. We also assessed the effect of twist1 silencing on TNFα-induced cytokine and chemokine expression and secretion in human adipocytes. Twist1 knockdown accentuated the pro-inflammatory effects of TNFα- suggesting that twist1 may have a protective role in adipose inflammation.

In conclusion, we have defined the minimal promoter needed for transcription of human CIDEA and a region in the promoter regulated by TNFα. We have also demonstrated that twist1 is expressed in white adipocytes and that low expression of twist1 associates with high BMI, markers of insulin resistance and an increased secretion of pro-inflammatory factors. Additionally, silencing of twist1 in human *in vitro* differentiated adipocytes demonstrates a role for this transcription factor in fatty acid oxidation and inflammation. We hypothesize that low twist1 expression in obesity renders the adipose tissue more vulnerable to the pro-inflammatory effects of TNFα, which could be of importance in the development of obesity-associated insulin resistance and type 2 diabetes.

LIST OF PUBLICATIONS

I. **Amanda T Pettersson**, Jurga Laurencikiene, Elisabet Arvidsson Nordström, Britta M Stenson, Vanessa van Haremelen, Charlotte Murphy, Ingrid Dahlman, and Mikael Rydén. Characterization of the human CIDEA promoter in fat cells. International Journal of Obesity 2008; 32:1380-1387.

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LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
AMPK	5'AMP-activated protein kinase
ATGL	Adipose triacylglycerol lipase
ATP	Adenosine triphosphate
ATM	Adipose tissue macrophage
bHLH	Basic helix-loop-helix
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CGI-58	Comparative gene identification-58
cGMP	Cyclic guanosine monophosphate
ChIP	Chromatin immunoprecipitation
CIDEA	Cell death-inducing DNA fragmentation factor-α-like effector A
CNS	Central nervous system
DAG	Diglyceride/diacylglycerol
DEXA	Dual Energy X-ray Absorptiometry
DHAP	Dihydroxyacetone
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
EMSA	Electrophoretic mobility shift assay
FABP	Fatty acid binding protein
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
GLUT	Glucose transporter
Gi	Inhibitory guanine nucleotide-binding protein
Gs	Stimulatory guanine nucleotide-binding proteins
HIF	Hypoxia inducible factor
HOMA-IR	Homeostasis model assessment of insulin resistance
HSL	Hormone-sensitive lipase
IL-6	Interleukin 6
IR	Insulin receptor
IRS-1/-2	Insulin receptor substrate-1/-2
JNK	c-Jun N-terminal kinase
LEP-Rb	Leptin receptor b
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MAG	Monoglyceride/monoacylglycerol
MAGL	Monoacylglycerol lipase
MCP-1	Monocyte chemoattractant protein-1
MSC	Mesenchymal stem cells
NF-κB	Nuclear factor-κB
NEFA	Non-esterified fatty acid
NPR	Natriuretic peptide receptor
PDE3B	Phosphodiesterase 3B
PDK1	3-phosphoinositide dependent protein kinase-1

PGC-1	Peroxisome proliferator-activated receptor γ co-activator 1
PGE2	Prostaglandin E2
PH	Pleckstrin homology
PI3K	Phosphatidyl inositol 3-kinase
PIP3	Phosphatidylinositol (3,4,5) triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLIN	Perilipin
PPAR	Peroxisome proliferator-activated receptor
RIP	Receptor interacting protein
RNAi	RNA interference
RT-PCR	Reverse transcriptase polymerase chain reaction
Sc	Subcutaneous
SH	Src homology
siRNA	Small interfering RNA
SNP	Single-nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
TAG	Triglyceride/triacylglycerol
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
Vis	Visceral
VLDL	Very low density lipoprotein
WAT	White adipose tissue
WHO	World Health Organization

1 INTRODUCTION

The aim of this thesis was to investigate the function of two genes, twist1 and cell death-inducing DNA fragmentation factor (DFF)-α-like effector A (CIDEA), in human adipose tissue and the role of these two factors in obesity. In the following section I have summarized the most important concepts in the study of obesity and adipose tissue in the development of obesity-associated disorders like insulin resistance, hyperlipidemia, type 2-diabetes and cardiovascular disease.

1.1 OBESITY AND ASSOCIATED COMPLICATIONS

During the last decades the incidence of obesity has accelerated in the world, reaching epidemic proportions. In 2008, the World Health Organization (WHO) estimated that 1.5 billion adults in the world were overweight and approximately 500 million adults were obese. Along with obesity, the prevalence of insulin resistance and type 2 diabetes are also increasing leading to huge economical consequences. Moreover, obesity is associated with increased risk for hypertension, dyslipidemia, fatty liver disease, and cardiovascular disease as well as cancer. Given the vast number of pathologies linked to obesity there is a great need for understanding the association between obesity and its complications.

Body mass index (BMI) is defined as a subject's body weight (in kg) divided by the square of the subject's height (in m). It is an index which is used for classifying normal-, under-, and overweight. According to the World Health Organization (WHO), obesity is defined as a BMI equal to or greater than 30 kg/m^2 while a BMI equal to or above 25 kg/m^2 is defined as overweight. The BMI cut-off for obesity and overweight are based on epidemiological studies and the risk of developing cardiovascular disease[1-4]. The BMI classes are detailed in Table 1. Other methods for determining body fat and body composition include waist-to-hip ratio or waist circumference, which are fairly good and simple methods for assessing central (intra-abdominal) obesity. Bioelectrical impedance measurements estimate fat as well as the fat-free mass based on the conductivity of the body. Dual Energy X-ray Absorptiometry (DEXA) scanning gives an estimate of fat mass, muscle mass and bone mass. Underwater weighing provides information about fat mass and lean body mass (muscle and bone) and is considered the gold standard for assessment of body composition. However for obvious reasons it is rather complicated and time-consuming. Compared to DEXA, underwater weighing and bioelectrical impedance, BMI measurements have the advantage of being both simple and cheap. Therefore BMI together with waist-to-hip ratio and waist circumference are the most commonly employed techniques in routine clinical use. The limitation with BMI is that certain groups of patients, including children, elderly people, and highly trained athletes, cannot be categorized according to this index.

At its simplest level, obesity develops when energy intake exceeds energy expenditure. These processes are regulated by both genetic and environmental factors. Monogenic causes of obesity are rare, affecting less than 1% of the obese subjects and are often caused by mutations in genes expressed in the hypothalamus, which are involved in the

control of food intake. Such mutations are typically located in the coding region of these genes and cause dramatic effects on the gene function. Susceptibility genes increase the risk for developing common forms of obesity and are associated with single-nucleotide polymorphisms (SNPs) in both the non-coding and the coding region of genes. In contrast to the dramatic effects of mutations seen in monogenic obesity, obesity-susceptibility genes only explain a small percentage of the variation in body weight and genome-wide association studies of susceptibility genes have hitherto been somewhat disappointing. Furthermore, the rapid increase in obesity prevalence has made it clear that genetic factors cannot be the only determinants of adiposity. Nevertheless, from an evolutionary perspective, gene variants favoring energy storage have clearly conferred a survival advantage to mankind. However in the obesogenic environment of our modern society, with easy access to energy-rich food combined with a sedentary life style, these variants have become maladaptive.

Table1. BMI classes according to WHO.

Classification	BMI
Underweight	< 18.5
Normal weight	18.5 - 24.9
Overweight	≥ 25
Pre-obese	25.0 - 29.9
Obese	≥ 30
Obese class I	30 - 34.9
Obese class II	35.0 - 39.9
Obese class III	≥ 40

1.2 ADIPOSE TISSUE

White adipose tissue (WAT) is a loose connective tissue composed of adipocytes and a stroma-vascular fraction that contains adipocyte precursor cells, also termed preadipocytes; leukocytes, including adipose-tissue macrophages (ATM), lymphocytes, and mast cells; as well as fibroblasts and endothelial cells. WAT is found in numerous parts of the body but the largest depots in humans are the subcutaneous (sc), and the visceral (vis, subdivided in the omental and the mesenteric) depots. The two adipose depots display different properties, which will be discussed later. Another type of adipose tissue is the brown adipose tissue, primarily found in hibernating mammals, newborn infants and to a much lesser extent in human adults. In contrast to WAT, the main function of brown adipose tissue is to generate heat via lipid oxidation. During the last few years it has become clear that adult humans possess active brown fat and the possibility of activating this tissue has gained increasing attention in obesity research[5-7]. One area of particular interest is the conversion of white adipocytes into more brown-like adipocytes, which would favor energy expenditure instead of energy storage[8]. However, the studies presented in this thesis have exclusively been performed in WAT.

1.3 FUNCTION OF WHITE ADIPOSE TISSUE

The main functions of WAT are to insulate the body and store energy during periods of food abundance. In the adipocytes, energy is stored in the form of triacylglycerols (TAGs), composed of three fatty acids esterified to one molecule of glycerol. When the body needs energy, e.g. after an over-night fast or during prolonged exercise, TAGs can be hydrolyzed into non-esterified fatty acids (NEFAs) and released into the circulation. In the blood, NEFAs are bound to albumin and transported to target tissues, including skeletal muscle and liver, where they are used as energy substrates. Until the early-1990s, WAT was generally regarded as an energy-storing and insulating organ. Following the discovery of adipose-derived hormones like leptin, TNFα and many others, it has become clear that adipose tissue is an endocrine organ, which secretes factors collectively termed adipokines[9,10]. Adipokines can act on the adipose tissue itself in an auto- and/or paracrine fashion or be released into the circulation to regulate other organs including, skeletal muscle, liver and the central nervous system (CNS). This emphasizes the role of adipose tissue as an important regulator of whole body metabolism and also explains why an abnormal expansion of adipose tissue can have such detrimental effects on other metabolically important tissues.

In obesity, release of NEFAs and adipokines from the adipose tissue is altered, which leads to disturbances in metabolically important organs like skeletal muscle, pancreas and liver. Such alterations will eventually cause impaired whole-body insulin sensitivity which is manifested by reduced insulin-stimulated glucose uptake in skeletal muscle, reduced insulin secretion from pancreatic β-cells and increased lipid and glucose output from the liver[11].

In the following sections I will focus on the processes by which adipocytes release and synthesize TAGs, how these processes are regulated as well as some of the most important adipokines and concepts in the development of obesity-associated complications. In the final two sections I will introduce the two genes that have been the focus of the studies in this thesis.

1.4 LIPOGENESIS AND LIPOLYSIS

TAGs from the diet are hydrolyzed by pancreatic lipases into monoacylglycerols (MAGs) and NEFAs, which together with bile salts form micelles that are transported in the intestine to the absorptive cells. NEFAs and MAGs from the micelles are subsequently taken up, by passive diffusion or fatty acid transporters, into the enterocytes in the small intestine. Inside the enterocyte MAGs and NEFAs are re-esterified into TAGs and packaged with phospholipids and proteins, to form particles termed chylomicrons. Upon exit from the enterocytes, chylomicrons pass into lymphatic capillaries termed lacteals, subsequently into larger branches of the lymphatic systems and ultimately into the circulation that transport them to target tissues including WAT. At the adipocyte cell membrane, lipoprotein lipase (LPL) binds to chylomicrons and hydrolyzes TAGs into NEFAs that are taken up by the adipocyte through fatty acid transporters (FAT/FATP/FABP). LPL can also hydrolyze TAGs transported in circulating very low

density lipoprotein (VLDL) particles, which are produced in and released by the liver. Inside the adipocyte, NEFAs are activated by the addition of a coenzyme A (CoA) molecule, a process which is catalyzed by the enzyme fatty acyl–CoA synthetase (ACS). Three activated NEFAs are then coupled to one molecule of glycerol 3-phosphate in a process termed lipogenesis. Glycerol is synthesized from glucose that is taken up by glucose transporters (GLUT1 and -4) in the adipocyte cell membrane. Glucose is converted to dihydroxyacetone (DHAP) via the glycolytic pathway and DHAP is subsequently reduced to glycerol 3-phosphate by the enzyme glycerol phosphate dehydrogenase. Glycerol 3-phosphate can also be generated from circulating glycerol by the enzyme glycerol-3-kinase; however, this enzyme is expressed at very low levels in adipocytes. In addition, glucose intermediates, derived via glycolysis, can be used for the synthesis of NEFAs in a process termed *de novo* lipogenesis.

The regulated enzymatic hydrolysis of TAGs is termed lipolysis. In lipolysis, NEFAs are released from the glycerol backbone in a three-step process. The first step is catalyzed by an enzyme termed adipose triglyceride lipase (ATGL) or desnutrin[12], which generates one NEFA and a diacylglycerol (DAG) molecule. In the second step, DAG is hydrolyzed by hormone-sensitive lipase (HSL)[13] generating another NEFA molecule and a MAG molecule. HSL is the rate-limiting enzyme for human lipolysis and exhibits both DAG and TAG hydrolase activity although the activity towards DAG is approximately ten times higher than that on TAG[14]. This is in contrast to ATGL, which selectively uses TAGs as substrates[14,15]. In the final step, the remaining MAG is hydrolyzed by monoacylglycerol lipase (MAGL) into one NEFA and one glycerol molecule. NEFAs, hydrolyzed through lipolysis and released into the blood, are primarily taken up by skeletal muscle for fatty acid oxidation (β-oxidation) or by the liver for synthesis of TAGs and lipoprotein particles (e.g. VLDL). As previously mentioned, due to the low expression of glycerol-3-kinase, glycerol obtained from adipocyte lipolysis cannot be used by the adipocyte itself but is instead transported to the liver where it is phosphorylated by glycerol-3-kinase. Glycerol 3-phosphate is then used for TAG synthesis, glycolysis or gluconeogenesis in the liver. For a more comprehensive overview of lipolysis and lipogenesis see biochemistry books, e.g.[16,17], or the following reviews[18,19].

1.5 REGULATION OF LIPOLYSIS

In mammal adipocytes, there is a constant low level of lipolysis termed basal or spontaneous lipolysis. However, under the influence of specific hormones, lipolysis can be induced or inhibited to a very significant degree. The most important endogenous regulators are catecholamines (noradrenaline and adrenaline), natriuretic peptides and insulin (reviewed in[18,19]). The activity of HSL, and thus lipolysis, is tightly controlled by catecholamines, signaling through adrenergic receptors on the adipocyte cell membrane. There are four different adrenergic receptors on the human adipocyte, β1, β2, β3 and α2A. β1, β2 and α2A are the most important adrenergic receptors in human fat cells whereas β3 plays a more important role in rodents. The β-receptors are coupled to stimulatory GTP-binding proteins (G_s), which upon β-receptor ligand-binding, activate adenylate cyclase (AC) causing an increase in cyclic adenosine monophosphate (cAMP) formation and a subsequent activation of protein kinase A (PKA). PKA, in turn,

phosphorylates HSL which leads to its activation and a redistribution of HSL from the cytoplasm to the lipid droplet surface. In addition, PKA also phosphorylates the lipid-coating phosphoprotein perilipin A (PLIN)[20]. This changes the conformation of PLIN and leads to the recruitment of activated HSL to the lipid droplet surface for subsequent TAG hydrolysis[21]. Furthermore, PKA phosphorylation of PLIN releases another lipid-droplet coating protein termed comparative gene identification (CGI) -58. CGI-58 is a cofactor for ATGL and is needed for the full activation of ATGL[22,23]. In its unphosphorylated state, PLIN probably works as a barrier for TAG hydrolysis on the lipid droplet and inhibits CGI-58-dependent activation of ATGL. Taken together, the activation of PKA leads to an increase in lipolysis. In contrast to the β-receptors, the α2A-receptors are coupled to inhibitory G proteins (G_i) that inactivate AC, which reduces cAMP levels thereby causing a decrease in PKA activation and lipolysis. This regulation by α2A-receptors is specific for primates and provides another level of fine-tuning of the lipolytic response. Moreover, adrenaline has a higher affinity for α2A-receptors suggesting that these receptors might be especially prominent during situations of stress or after exercise when adrenaline levels are high. In conclusion, the net effect of catecholamines on lipolysis in human fat cells depends on the relative expression and function of α2A- versus β-receptors.

Apart from catecholamines, natriuretic peptides are also potent activators of lipolysis in humans and primates. They act by binding to the natriuretic peptide receptor A (NPR-A), which possesses guanylyl cyclase (GC) activity. Activation of GC increases the cyclic guanosine monophosphate (cGMP) concentrations in the cell and cGMP binds and activates protein kinase G (PKG). PKG in turn, phosphorylates HSL and PLIN, thus activating lipolysis.

The most potent anti-lipolytic agent is insulin. Insulin binding to the insulin receptor (IR) induces a conformational change that results in auto-phosphorylation of several tyrosine residues in the receptor (for reviews on insulin signaling, see[24,25]). The phosphorylated tyrosine residues in the IR provide docking sites for proteins with Src homology (SH) 2 domains, including insulin receptor substrates (IRS)-1 and -2, which are also activated by tyrosine phosphorylation. The tyrosine phosphorylated IRS-1 and -2 then activate a phosphatidylinositol (PI) 3-kinase complex that catalyzes the formation of phosphatidylinositol (3,4,5) triphosphate (PIP_3), which in turn activates Pleckstrin homology (PH) domain-containing proteins like 3-phosphoinositide dependent protein kinase-1 (PDK1). PDK1 activates the downstream effectors Akt or PKB and atypical isoforms of PKC (PKCζ and PKCλ), which results in enhanced translocation of GLUT4 to the plasma membrane and an increased glucose uptake. Moreover, Akt phosphorylates phosphodiesterase 3B (PDE3B) thereby increasing the breakdown of cAMP to 5'AMP and reducing the activity of PKA and HSL. In addition to these pathways, insulin also induces glycogen synthesis in skeletal muscle and liver, increases lipogenesis in liver and WAT, and increases protein synthesis, cell differentiation and proliferation[25].

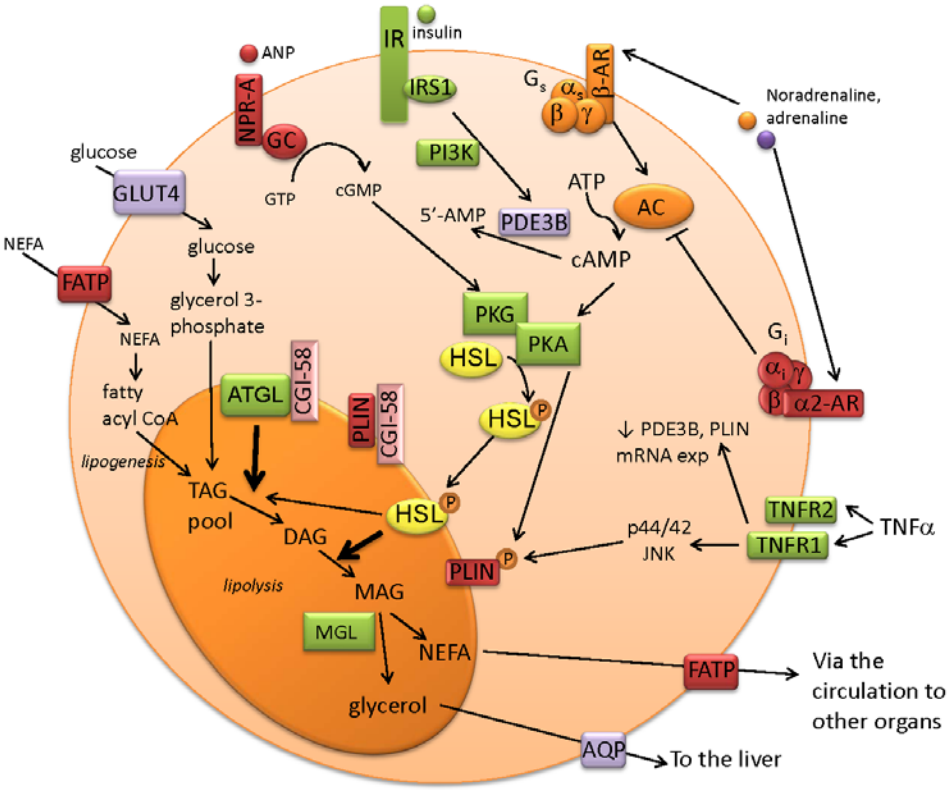


Figure1. Lipolysis regulation in human adipocytes. α2/β-AR, α2/β-adrenergic receptor; AC, adenylyl cyclase; AMP, adenosine monophosphate; ANP, atrial natriuretic peptide; ATGL, adipose triglyceride lipase; ATP, adenosine 5'-triphosphate; AQP, aquaporine; cAMP, cyclic AMP; CGI-58, comparative gene identification-58; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; FATP, fatty acid transport protein; $G_{i/s}$, inhibitory/stimulatory GTP-binding protein; GC, guanylyl cyclase; GLUT4, glucose transporter 4; GTP, guanosine triphosphate; HSL, hormone sensitive lipase; IR, insulin receptor; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; NEFA, non-esterified fatty acid; NPR-A, natriuretic peptide receptor A; PDE3B, phosphodiesterase3B; PLIN, perilipin A; PKA, protein kinase A; PKG, protein kinase G; TAG, triacylglycerol; TNFα, tumor necrosis factor α; TNFR, TNFα receptor.

There are several other regulators of lipolysis although the effects are smaller than for the above mentioned hormones. Prostaglandin E2 (PGE2) activates G_i and thus displays anti-lipolytic effects in adipocytes. Tumor necrosis factor α (TNFα) increases basal or spontaneous lipolysis in adipocytes by stimulating MAP kinases that among many other effects cause phosphorylation of PLIN (described in more detail below). Lipolysis regulation is summarized in Figure 1.

1.6 DYSREGULATION OF LIPOLYSIS IN OBESITY

Obesity is characterized by an increased basal but decreased stimulated lipolysis; however, there are regional differences in lipolysis. Basal lipolysis is increased in all adipose depots in obesity partly due to increased fat cell size and partly due to increased concentrations of TNFα[18]. In contrast; there are large variations in stimulated lipolysis in subcutaneous (sc) and visceral (vis) WAT. Thus, in obesity, stimulated lipolysis is

decreased in sc WAT. This is due to an increased expression of α2A receptors and a combined reduction in the expression of β2-receptors and HSL leading to a resistance to the lipolytic effect of catecholamines[18,26]. In contrast, in vis WAT, stimulated lipolysis is increased, which is due to an increased expression of β3-adrenoreceptors with a concomitant decrease in α2A-adrenoreceptors expression[27]. These regional differences in stimulated lipolysis lead to a redistribution of NEFAs during catecholamine stimulation so that NEFAs are primarily released from the vis depots. Although the vis adipose depots is significantly smaller than the sc depot the vis depot drains directly to the liver via the portal vein, which has detrimental consequences in obesity. NEFAs delivered to the liver are used for the synthesis of VLDL particles and to some extent glucose, which both contribute to impaired insulin action in peripheral organs. NEFAs released from the sc depots on the other hand can be transported via peripheral veins to skeletal muscle and pancreas. In skeletal muscle, NEFAs are preferentially taken up and used as energy substrate instead of glucose thereby reducing glucose uptake during insulin stimulation. In the pancreas, chronically elevated levels of circulating NEFAs lead to a reduced secretion of insulin. Altogether, the increased levels of NEFAs in the circulation impair insulin action in several organs leading to peripheral insulin resistance. Results in recent years have also demonstrated that NEFAs can act on insulin sensitivity mechanism by activating toll-like receptors (TLR)[28]. These receptors are involved in immunological responses and activation of TLRs induces an intracellular signaling cascade that activates pro-inflammatory cytokines (e.g. TNFα and interleukin (IL)-6)[28]. Moreover, NEFAs can also indirectly, by esterification to DAGs, activate isoforms of PKC, which in turn leads to phosphorylation of serine residues on IRS-1 and inhibition of insulin signaling[29].

1.7 ADIPOSE TISSUE INFLAMMATION

During the last decade it has been conclusively shown that obesity is associated with a chronic low-grade inflammation in WAT, which is tightly coupled to a functional dysregulation of the tissue[30,31]. It is now generally considered that the alterations in WAT function lead to changes in the release of adipokines as well as NEFAs, increasing the risk of developing obesity-associated complications[11,32-34].

ATMs are important regulators of WAT inflammation. In the WAT from lean, insulin-sensitive subjects, ATMs are probably involved in normal WAT turnover by clearing the tissue from apoptotic cells, lipids and cellular debris. These ATMs, primarily serving as scavenger cells, secrete cytokines with anti-inflammatory properties (e.g. IL-10) and have been termed M2-ATMs[35]. In contrast, in the obese state, monocytes are recruited from the circulation and accumulate in the expanding WAT where they, through unclear mechanisms, undergo polarization into a class of macrophages with pro-inflammatory properties termed M1-ATMs[36,37]. These cells release high levels of pro-inflammatory factors like TNFα, IL-6 and monocyte chemoattractant protein-1 (MCP-1), which attract more monocytes from the circulation and at the same time stimulate lipolysis and inhibit insulin signaling in the adipocytes.

1.8 OTHER PATHOGENIC MECHANISM FOR OBESITY-INDUCED INSULIN RESISTANCE

1.8.1 Adipose tissue morphology

In addition to adipokines and inflammation, the morphology of the adipose tissue has significant effects on the function of the tissue. As the adipose tissue enlarges with increasing obesity there is a need for re-organization of the tissue structure to permit adipocyte expansion. Adipose tissue can expand by an increase in adipocyte volume, adipocyte number or both. Recent studies have revealed that adipose tissue expands primarily by increasing adipocyte volume whereas adipocyte number, which is set early in life, remains relatively constant in adulthood[38]. However, approximately 10% of fat cells are renewed every year indicating that adipose tissue is indeed a plastic organ[38]. Increased adipocyte size correlates with plasma insulin concentrations and small and large fat cells display distinct gene expression and secretory patterns[39-41]. This suggests that adipocyte size is an important determinant for adipocyte function and metabolic parameters. Instead of adipocyte size, the morphology of the adipose tissue can be determined. An adipose morphology value can be calculated based on the difference between the observed and the expected adipocyte volume for any given body fat mass[42]. Based on the morphology value, adipose tissue can be divided into hypertrophic, characterized by few and large fat cells, or hyperplastic, characterized by many and small fat cells. Adipose morphology is influenced by neither sex nor obesity, however a hypertrophic adipose tissue associates with increased HOMA-IR values and increased fasting plasma insulin levels[42].

1.8.2 Adipose tissue vascularization and hypoxia

Adequate vascularization of the adipose tissue is crucial to allow for expansion and normal delivery of nutrients to the adipocytes[43,44]. A relatively new concept in the context of adipose tissue function and inflammation is hypoxia[44,45]. It has been suggested that hypoxia manifested by an insufficient supply of blood, and thus oxygen, to the expanding adipocytes can induce changes in adipocyte metabolism and initiate an inflammatory response. Reduction in oxygen levels can be caused by either inadequate vascularization of the expanding tissue, increased diffusion distances caused by increased adipocyte size or both. Low oxygen tension in adipocytes induces activation of the transcription factor hypoxia inducible factor (HIF)-1, which increases the transcription of IL-6, GLUT1, leptin and vascular endothelial growth factor (VEGF) and reduces the expression of adiponectin[46,47]. Hypoxia also leads to a reduction in mitochondrial oxidative phosphorylation and adenosine triphosphate (ATP) production forcing the cells to switch to anaerobic glycolysis and increased glucose uptake[45,48]. In addition, reduced oxygen tension inhibits preadipocyte differentiation and increases the inflammatory response of ATMs[49,50].

1.9 ADIPOKINES

As previously mentioned WAT is considered to be an endocrine organ, which secretes peptides collectively termed adipokines that act locally or in an endocrine fashion. The adipocyte proteome was recently characterized and comprises almost 1500 proteins whereas the adipocyte secretome comprises more than 300 specific peptides [51](and personal communication with Prof Juergen Eckel, Dusseldorf, Germany). In addition to adipocytes, other cells in WAT, including preadipocytes, inflammatory cells, endothelial cells as well as other cells can also release a number of adipokines which regulate diverse functions such as; inflammation, adipogenesis, proliferation, metabolism, stress and vascularization. A complete summary of all adipokines is out of the scope of this thesis and I have therefore chosen to focus on the adipokines with relevance for the papers presented herein.

1.9.1 Leptin

The first true adipokine to be identified was leptin, the product of the obese gene, which is almost exclusively produced by adipocytes[9,52]. Leptin is a 16 kD cytokine-like protein, which signals via a leptin receptor, LEP-R. Alternative splicing of the human leptin receptor gene generates at least six isoforms of the receptor (LEP-R a-f) some of which are soluble forms present in the circulation. However, only the LEP-Rb isoform contains the intracellular signaling domain needed for signal transduction and other isoforms likely function by regulating circulating levels of leptin.

Circulating levels of leptin are directly correlated with adipose tissue mass and thus increased in obese rodents and humans[9,53]**.** Leptin acts on cells in the arcuate nucleus in the hypothalamus where it increases energy consumption, reduces appetite and stimulates satiety by counteracting orexigenic and stimulating anorexigenic neuropeptides respectively[11,52]. Leptin functions as an adipostat that signals to the brain how much energy is stored in the body. Disruption of the leptin (ob/ob) or leptin receptor gene (db/db) in mice or defects in the corresponding human genes causes severe obesity[9,54]. Administration of recombinant leptin to leptin-deficient mice or humans restores body weight by reducing food intake and increasing metabolic rate[52,55]. However, apart from rare forms of congenital leptin deficiency, leptin treatment is not effective in treating common morbid obesity. This is probably due to the fact that, in non-congenital forms of obesity leptin levels are already high due to the increase in WAT mass. This implies that there may be a general resistance to leptin in common obesity.

1.9.2 Adiponectin

Adiponectin was discovered in 1995 as a 30 kD serum protein exclusively secreted by adipocytes[56,57]. Adiponectin signals via two membrane-bound receptors ADIPOR1 and ADIPOR2 expressed primarily in skeletal muscle and liver[33]. The secreted protein forms trimers, hexamers and high molecular weight forms (12-18mers), though the biologically active form has been debated. Adiponectin is detected at high levels in the circulation and in contrast to many other adipokines, circulating levels of adiponectin are decreased in

obesity[30,58]. Disruption of the adiponectin gene in mice has no effect on body weight but induces insulin resistance and an enhanced inflammatory response to vascular injury suggesting a role for adiponectin in insulin sensitivity and atherosclerosis[59]. In line with this, low adiponectin levels also correlates with coronary heart disease and type 2 diabetes in humans[60,61].

Ligand binding to the adiponectin receptors induces phosphporylation of AMPK, which in turns increases fatty acid oxidation and glucose uptake in skeletal muscle and reduces gluconeogenesis in the liver[62]. Adiponectin also inhibits the transformation of macrophages to lipid filled foam cells and acts as an anti-inflammatory factor by reducing TNFα-stimulated cytokine release and increasing release of the anti-inflammatory cytokine IL-10 from macrophages[62-66]. In addition to its action on macrophages adiponectin stimulates endothelial cell migration and differentiation further implicating adiponectin as an important link between adipose tissue and cardiovascular tissues[67,68].

1.9.3 TNFα

TNFα is a pleiotropic cytokine that regulates cellular and biological processes such as immune function, apoptosis, proliferation, differentiation and metabolism (for a review on TNFα see [69-71]). The protein is synthesized as a 26 kDa transmembrane protein that undergoes proteolytic cleavage to yield a secreted 17 kDa molecule. The 17 kDa TNFα monomers form trimers that bind to ubiquitously expressed transmembrane TNFα receptor 1 and 2 (TNFR1/-2). Like the ligand, TNFRs can be proteolytically cleaved generating soluble forms of the receptors. Both receptors activate distinct signaling pathways although less is known about the pathways regulated by TNFR2. Upon ligand binding, TNFR1 trimerizes and recruits receptor-interacting proteins that in turn convey the signal to intracellular key targets eventually leading to changes in gene transcription and/or phosphorylation.

TNFα is a potent stimulator of inflammatory pathways[69]. These signals are primarily attributed to NF-κB activation and increased gene transcription of pro-inflammatory genes. The role of TNFα in obesity and diabetes was first recognized in 1993 when Hotamisligil *et al* showed that WAT expression, as well as circulating levels of TNFα were increased in rodent models of obesity and diabetes[10]. Moreover, targeted null mutation in the gene encoding TNFα in mice ameliorates insulin resistance induced by a high fat diet or genetic obesity[72]. Increased expression and secretion of TNFα from WAT in obesity has subsequently been confirmed in humans[73]. However in contrast to rodents, plasma levels of TNFα are extremely low in humans and injection of TNFα blocking antibodies have failed to improve insulin sensitivity in type 2 diabetics[74,75]. These findings have led scientist to speculate that TNFα primarily functions as a para- and/or autocrine factor in human WAT. The main sources of TNFα in adipose tissue are the non-adipose cells in the stroma-vascular fraction, including macrophages[76].

At a cellular level, TNFα has been shown to reduce the expression of genes involved in insulin signaling, including GLUT4, IR and IRS-1 as well as the transcription factor peroxisome proliferator-activated receptor (PPAR)γ[77]. PPARγ is a central regulator of

adipogenesis and controls expression of genes with important functions in adipocytes. In addition, TNFα activates a set of different kinases that promotes phosphorylation of serine residues on the IR and IRS-1 thereby preventing insulin-induced tyrosine phosphorylation of IR and IRS-1 and ultimately insulin signaling[77].

TNFα effectively stimulates lipolysis in both human and rodent fat cells[78-80]. In human adipocytes, TNFα reduces the expression of PDE3B, which leads to an increase in cAMP levels, activation of PKA and phosphorylation of PLIN[81]. These effects are mediated via the MAP kinases p44/42 and c-Jun N-terminal kinase (JNK)[82]. NF-κB is also involved in TNFα-induced lipolysis, presumably by controlling transcriptional regulation of PLIN and HSL mRNA[83]. In rodent adipocytes, TNFα also induces lipolysis by a reduction in G_i expression, however this regulation has not been possible to demonstrate in human adipocytes[84]. In addition to lipolysis, TNFα also reduces fatty acid uptake and oxidation as well as expression of electron transport genes[85].

1.9.4 Interleukin 6 (IL-6)

IL-6 is a 21 kD pro-inflammatory adipokine produced by both macrophages and adipocytes. High plasma levels of IL-6 correlate with obesity and type 2 diabetes whereas weight-reduction reduces the plasma levels of this adipokine[86]. Adipose tissue contributes, at least in part, to the circulating levels of IL-6, as adipose tissue releases IL-6 to the circulation and IL-6 release is higher from omental compared to sc adipose tissue[86,87]. The exact role of IL-6 in insulin resistance is not clear. IL-6 stimulates lipolysis in human adipocytes in culture and impairs insulin-induced IR and IRS-1 phosphorylation in adipocytes and hepatocytes by activation of suppressor of cytokine signaling (SOCS) 3[88-92]. Unexpectedly, targeted deletion of the *IL-6* gene in mice induces mature-onset obesity, which can be partly reversed by IL-6 replacement[93]. In addition, chronic exposure of skeletal muscle to IL-6 induces insulin resistance although short-term treatment has beneficial effects on insulin sensitivity by activation of AMPK and by promoting fatty acid oxidation *in vivo* and *in vitro* in myotubes[94,95].

1.9.5 Monocyte chemoattractant protein 1 (MCP-1)

MCP-1, also termed CCL2, is an 11 kD secreted protein and as the name implies it is a potent attractant for monocytes. Increased secretion of this adipokine has been linked to enhanced infiltration of macrophages in obese adipose tissue[31,96,97]. Both diet-induced and genetically obese (ob/ob) mice as well as obese humans display increased expression of MCP-1 in adipose tissue[31,96-100]. Interestingly, disruption of the *mcp-1* gene in mice placed on a high-fat diet leads to reduced macrophage accumulation, improved insulin resistance and attenuation of hepatic steatosis[96]. Moreover, stimulation of murine fat cells with MCP-1 reduces insulin-stimulated glucose uptake[97]. These findings reveal MCP-1 as an important link between obesity and obesity-associated metabolic disturbances.

In WAT, MCP-1 is secreted by cells in the stroma-vascular fraction, e.g. resident macrophages (ATM), and by adipocytes, although secretion from isolated adipocytes is

significantly lower[31,96,99]. Whether MCP-1 is released from WAT into the circulation has been a matter of debate and at least in humans, MCP-1 primarily appears to work as a local factor[99]. Nevertheless, MCP-1 seems to be an important factor for recruitment of monocytes from the circulation and therefore for the regulation of adipose inflammation.

1.10 CIDEA

CIDEA was first identified as an apoptosis-inducing factor in the tumor cell lines 293T and MCF7[101]. It is highly homologous to CIDEB and CIDEC (the latter termed FSP27 in mice) however the three proteins show distinct tissue expression patterns[101,102]. CIDEB is most highly expressed in human liver and small intestine as well as in murine liver whereas CIDEC is most highly expressed in murine WAT, human heart, colon and small intestine[101-104]. CIDEA expression in mice is highest in brown adipose tissue and detected at lower levels in WAT[104,105]. In contrast, CIDEA is expressed in several human tissues, including heart, skeletal muscle, lymph node, brain, bone marrow and WAT.

CIDEA-deficient mice are lean and resistant to obesity and have an increased metabolic rate[104]. This phenotype is most probably due to increased AMPK protein levels and activity in brown adipose tissue as well as enhanced fatty acid oxidation in brown adipocytes[106]. In humans, CIDEA seems to have opposite effects. Expression of CIDEA in WAT is lower in obese compared to lean subjects and increases after diet-induced weight loss[107-109]. In addition, a coding SNP in the human gene has been associated with BMI in a Swedish cohort[110]. Moreover, low adipose CIDEA expression correlates with parameters of insulin resistance and increased CIDEA expression in WAT from cancer cachectic patients correlates with decreased fat mass[108,109,111,112]. At a cellular level, CIDEA silencing induces adipocyte lipolysis and TNFα secretion while CIDEA over-expression enhances FA-oxidation further implicating CIDEA as an important regulator in metabolism and obesity-associated inflammation and insulin resistance[109,111].

The cellular localization of CIDEA is a matter of controversy. Several groups have reported that endogenous and ectopically expressed CIDEA localizes to lipid droplets where it increases lipid storage[112-114]. On the other hand, others have found CIDEA localized to the endoplasmic reticulum in brown adipocytes whereas two studies have found ectopically expressed CIDEA localized both to the nucleus and the cytosol[106,115,116]. Clearly, there are differences in CIDEA cellular localization between cell lines, different tissues and different species although, in human adipocytes CIDEA appears to localize to the nucleus as well as to the cytoplasm, possibly to the lipid droplet[112,116].

The expression of murine CIDEA has been shown to be induced by PPARα, -γ and the PPARγ co-activator (PGC)-1α whereas receptor interacting protein (RIP)-140 negatively regulates the murine promoter[114,117]. Basal activity of the human promoter is regulated by Sp1 and Sp3 transcription factors whereas tissue- and cell-specific expression depends on CpG methylation[118].

1.11 TWIST1

Twist1 is a basic helix-loop-helix (bHLH) transcription factor first identified as a gene involved in determining dorsoventral pattern in *Drosophila melanogaster*[119]. It shares homology with twist2 and the two proteins have partly overlapping functions although twist1 has been more extensively studied. Null mutation in the murine *twist1* gene is embryonically lethal due to failure of the cranial neural tube to fuse[120-122]. In line with the murine data, mutations in the human gene lead to Saethre-Chotzen Syndrome characterized by facial and skeletal malformations and premature fusion of the skull bones (craniosynostosis)[120,123]. Moreover, twist1 has antiosteogenic functions *in vitro* further supporting an essential role of twist1 in bone formation[122,123]. Twist1 has also been shown to inhibit myogenesis by blocking MyoD and myocyte enhancer factor2 (MEF2), transcription factors needed for expression of muscle-specific genes[124-126]. In addition to its role in differentiation, twist1 can induce epithelial-to-mesenchymal transition (EMT) associated with cancer progression and metastasis[127,128].

Studies in non-adipose cells have shown that twist1 forms homo- or heterodimers with other bHLH proteins, which subsequently bind to conserved sequences, termed E-boxes (CANNTAG) in the genome. The composition of the twist1 dimer is central for its function and therefore twist1 action can vary depending on the dimerizing partner[120,125]. Another level of regulation of twist1 action comes from the phosphorylation status of twist1, which affects partner choice and E-box binding affinity[120,129,130].

In contrast to twist1 null mice, mice heterozygous for null mutations in the *twist1* and *-2* genes are viable, although severely growth retarded. These mice also display high levels of pro-inflammatory cytokines in the circulation suggesting a role for twist proteins in inflammatory processes[131]. Moreover, twist1 has been shown to inhibit transcription from the murine TNFα promoter by interfering with p65[131]. Twist1 also mediates suppression of inflammation in macrophages and Th1 cells suggesting that twist1 has important anti-inflammatory actions[132,133].

Recently, twist1 was shown to regulate brown adipose tissue metabolism in mice[134]. Interestingly, transgenic mice expressing twist1 in white and brown adipose tissue are prone to obesity induced by a high fat diet while heterozygous twist1 knockout mice are resistant to obesity. These phenotypes are explained by an altered metabolism in brown adipocytes where twist1 interacts with and suppresses the transcriptional activity of PGC-1α. In conclusion, twist1 regulates a range of cellular functions. The role in both inflammatory pathways and brown fat metabolism makes it tempting to speculate that twist1 might have important functions in WAT and obesity-associated inflammation as well.

2 AIMS OF THE PRESENT STUDIES

I. The aim of the first paper was to define the minimal promoter region needed for transcription of human CIDEA, and the effect of five SNPs in the promoter region as well as the effect of TNFα on transcriptional activity of the promoter.

II. In the second paper, the aim was to investigate the expression pattern of twist1 in human WAT as well as other metabolically important organs, and to assess the effect of twist1 *in vitro* silencing on fatty acid oxidation, lipolysis, secretion and mRNA expression in human white adipocytes.

III. The aim of the third paper was to investigate twist1 expression in human obesity and in relation to different clinical parameters as well as the effect of twist1 on TNFα-mediated inflammation in human white adipocytes.

3 COMMENTS ON MATERIALS AND METHODS USED IN THE STUDIES

The experimental methods have been described in detail in **paper I**, **II** and **III**. In the next section I will briefly discuss the methods and comment on the use of the method of choice.

3.1 ETHICAL APPROVAL

All studies were conducted in accordance with the guidelines of The Declaration of Helsinki and approved by the regional ethical committees. The studies were explained in detail to each subject and written informed consent was obtained.

3.2 SUBJECTS

Subjects in paper II and III are described in detail in the papers and summarized in Table 2.

Table 2. Description of subjects in cohorts from paper II and III. Values are given as mean±SD. WAT = white adipose tissue, VLCD = very low calorie diet, w = women, m = men.

Paper-cohort	Gender (w/m)	Age	BMI	Tissue/cell type	Study type
II-cohort 1				WAT, pancreas, liver and skeletal muscle	qRT-PCR
II-cohort 2	11/3	37.5±10	29.2±7	WAT and mature fat cells	qRT-PCR
II-cohort 3	10/0				qRT-PCR
III-cohort 1 non-obese	23/0	39±9	22±1	WAT	qRT-PCR
III-cohort 1 obese	107/0	39±9	39±5	WAT	qRT-PCR
III-cohort 2	10/3	39±7	40±6	WAT (before/after surgery)	qRT-PCR
III-cohort 3	3/9	45±10	38±5	WAT (before/during/after VLCD)	qRT-PCR
III-cohort 4 lean	12/2	41±13	24±2	Sc and om WAT	qRT-PCR
III-cohort 4 obese	23/1	42±10	44±4	Sc and om WAT	qRT-PCR

3.3 MODEL SYSTEMS AND CELL CULTURES

In vivo experiments using whole animals, e.g. mice, are of great importance when studying the overall effects of complete knock-down of genes. However, in many instances the research question might be more specific, concerning only one cell type or one specific mechanism. A common way of studying such cellular mechanism in research is by the use of cell cultures.

In all the present studies the majority of the *in vitro* experiments were performed in human white adipocytes differentiated *in vitro* from preadipocytes derived from the stroma-vascular fraction of human WAT. In **paper I** we also used adipocytes differentiated *in vitro* from human mesenchymal stem cell (MSC) derived from the stroma-vascular fraction and the commercially available murine cell line 3T3-L1.

Commercially available immortalized cell lines like, 3T3-L1, are cells that have changes in the cell cycle machinery which occurs either spontaneously, e.g. in tumor cells, or by manipulating the cell cycle regulation. The advantage of using such cells is that they grow rapidly; they are identical to previous passages and can be frozen and thawed whenever needed. Unfortunately, there is no commercially available human fat cell line and most studies on human adipocytes have been dependent on the use of primary cell cultures. On the other hand, because of changes in the cell cycle machinery, cell lines have a higher rate for spontaneous mutations and also an altered phenotype that do not reflect the *in vivo* situations as well as primary cells do. We have therefore tried to use human primary cell cultures whenever possible. In the case of some transfection experiments with concomitant TNFα stimulations we have chosen to use a murine cell line 3T3-L1 that is less vulnerable compared to primary cells.

As can be deduced from the introduction of this thesis there are several important differences in the metabolism of mice and humans. Although the important contributions from murine studies cannot be disregarded the relevance of such results for humans must always be verified. By using human cell system this problem can partly be avoided.

Due to the space limitation in journals the protocols for cell cultures are summarized very briefly in all the three papers. In the next section, I have therefore tried to explain in more detail how the different cell cultures were performed.

Isolation, culture and differentiation of preadipocytes and WAT-derived MSC have been described in detail before[135-137]. Briefly, WAT obtained from subjects undergoing bariatric or cosmetic surgery (liposuction) was washed, cut into small pieces using surgical scissors and digested with collagenase for 1 h at 37°C. The collagenase-treated cell suspension was centrifuged at 200×g for 10 min and the supernatant, containing mature adipocytes and collagenase solution, was removed. The remaining stroma-vascular fraction (containing both preadipocytes and MSCs) was resuspended in erythrocyte lysis buffer for 10 min, filtered through a nylon mesh and centrifuged as above. The supernatant was discarded and the pellet resuspended in an inoculation medium containing (Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum, 100 μg/mL penicillin-streptomycin) and subsequently filtered through a 70 μm pore size filter. The cells were plated at a density of 30000-50000 cells/cm^2 in inoculation medium to allow cell attachment. After 24 h the medium was changed to differentiation medium (DMEM/F12 supplemented with 15 mM Hepes, 100 μg/mL penicillin-streptomycin, 2.5 μg/mL amphotericin B, 66 nM human insulin, 1 nM triiodo-L-thyroine, 10 μg/mL human transferrin, 33 μM biotin, 17 μM panthotenate, 100 nM cortisol and 10 μM rosiglitazone (BRL49653)). Rosiglitazone was included the

first 3-6 days and then removed from the differentiation media. Medium was changed every 2-3 days.

For adipocytes derived from MSC, cells were plated at a density of 160000/cm^2 and allowed to proliferate in DMEM-low glucose supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution. In continued passages cells were plated at 4000/cm^2 and passaged at 60-70% confluency. Differentiation was induced by a differentiation medium containing DMEM-high glucose, 10% fetal calf serum, 50 μg/mL penicillin-streptomycin, 1.25 μg/mL amphotericin B, 15 mM Hepes, 1 nM triiodo-L-thyroine, 66 nM human insulin, 33 μM biotin, 17 μM panthotenate, 10 μg/mL human transferrin, 1 μg/mL dexamethasone, 10 μM rosiglitazone and 0.2 mM 3-isobutyl-1-1methylxanthine. Three days after induction of differentiation medium was changed to post-differentiation medium identical to the differentiation medium but without the addition of fetal calf serum, rosiglitazone and 3-isobutyl-1-methylxanthine. Medium was changed every 2-3 days and cells were differentiated for 16-20 days.

Proliferation and differentiation of 3T3-L1 cells were performed according to standard protocol with one exception. Differentiation of 3T3-L1 cells was performed in a typical adipogenic medium consisting of DMEM/F12 Glutamax1, 10% fetal bovine serum, 50 μg/mL penicillin-streptomycin, 1.25 μg/mL amphotericin B, 10 μg/mL bovine insulin, 1 μM dexamethasone, 0.2 mM 3-isobutyl-1-1methylxanthine with the addition of 10 μM rosiglitazone.

In addition to cell cultures we also used WAT explants in **paper III** to measure adipokine secretion. WAT pieces from a subset of cohort I in **paper III** were incubated in 4 ml Krebs Ringer buffer supplemented with 2% bovine serum albumin (BSA), 1 mg/mL glucose and 0.1 mg/mL ascorbic acid for 2 h at 37 °C and adipokine secretion into the media was measured by enzyme-linked immunosorbent assay (ELISA). This method gives information about the secretion from the WAT, which contains both adipocytes and inflammatory cells like macrophages. Another way of assessing adipokine secretion would be to use mature adipocyte isolated by collagenase treatment. However, standard collagenase-based isolation techniques for mature adipocyte or *in vitro* differentiated adipocytes as well as incubation with BSA have been shown to induce de-differentiation and increase expression of pro-inflammatory cytokines in adipocytes[138-141]. Such effects can be avoided by explant culture. Moreover, the short incubation time used in our experiments should also minimize the effect of albumin on adipocyte secretion. Although it is impossible to elucidate exactly which cells, in the tissue explants, that are responsible for the release of adipokines, explant cultures are devoid of the side-effects imposed by collagenase treatment and prolonged albumin incubation.

3.4 5’ RAPID AMPLIFICATION OF cDNA ENDS (5’RACE)

In **paper I** CIDEA transcriptional start site was identified by 5’ RACE. This is a method used to obtain full length sequence of RNA transcripts and to characterize the 5’ ends of mRNA:s. The same principle can be used for characterization of the 3’ ends of mRNA:s and is then referred to as 3’RACE. Messenger RNA:s can be used for the synthesis of a

cDNA libraries. Briefly, poly A-tail RNA is reverse transcribed to single strand cDNA using reverse transcriptase and a poly-T primer. The second cDNA strand is synthesized using DNA polymerase II. RNA is degraded with RNase H, followed by a T4 DNA polymerase treatment to generate blunt ends. An adaptor is subsequently ligated using T4 DNA ligase. In our experiments, we used a ready-made adaptor-ligated Human Adipocyte and Adipose Tissue Marathon-Ready cDNA pool. Amplification of CIDEA transcripts was performed using an adaptor-specific primer and a gene specific primer. The PCR products were gel purified and ligated into a pGEM T Easy vector and ligations were transformed into competent bacteria that were plated onto appropriate antibiotic-containing plates from which colonies were picked and inoculated. DNA from individual clones was isolated with a commercially available kit according to standard protocols and DNA sequencing was used to confirm the sequence.

3.5 REPORTER ASSAYS TO STUDY PROMOTER ACTIVITY

Luciferase reporter assay is a standard method used to assess promoter activity. In the first study we used luciferase reporter constructs to assay activity of the human CIDEA promoter. In **paper II**, we also used a luciferase reporter construct to assay activity of the human IL-6 promoter in the presence or absence of human twist1. Transfections were performed in human adipocytes differentiated from WAT-derived MSC or in murine 3T3-L1 cells using lipofection-based transfections agents as described in the papers.

Although reporter assays efficiently can demonstrate promoter activity there are some caveats to this method. First, small changes in promoter activity can be hard to detect using this method. Therefore it is possible that haplotype differences in the promoter regions might be missed because of lack of sensitivity of the assay. Second, reporter constructs lack DNA associated proteins like histones and the chromatin structure. Altogether, reporter assays should always be interpreted with caution although they offer a relatively straightforward promoter analysis when more advanced techniques like chromatin immunoprecipitation (ChIP) are not feasible.

3.6 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) AND ChIP

EMSA is a technique used for demonstrating interactions between DNA-binding proteins (transcription factors) and DNA. EMSA is performed on nuclear extracts or purified recombinant protein and oligonucleotides overlapping a putative binding site for the transcription factor of choice. Extracts or proteins are incubated with radioactively labeled oligonucleotides and then separated on non-denaturing polyacrylamide gels. Binding of proteins to the labeled oligonucleotide will cause a slower migration in the gel which can be detected by autoradiography.

ChIP is based on the interaction between DNA-binding proteins and chromatin, which are cross-linked using formaldehyde. The cross-linked DNA-protein complexes are subsequently isolated and sonicated into smaller fragments (100-1000 bp). These fragments are immunoprecipitated using an antibody directed against the protein of

interest. In the final step, DNA from the immunoprecipitated DNA-protein complexes is extracted and analyzed using conventional PCR (as in **paper II**), quantitative RT-PCR or DNA sequencing. In contrast to EMSA, which can detect putative interactions between DNA-binding proteins and oligonucleotides, ChIP is performed on chromatin instead of *in vitro* generated DNA oligonucleotides. This reflects more closely the *in vivo* environment in a cell. However, ChIP analysis is highly dependent on the protein-DNA interactions at the specific time-point when cells were cross-linked and is therefore highly dependent on the environment in the nucleus at the point of cross-link. Another caveat with ChIP is the requirement of large quantities of cells and the dependence on sensitive and specific antibodies.

In **paper II** we used ChIP to demonstrate binding of twist1 to regions in the human IL-6, MCP-1 and TNFα promoters.

3.7 QUANTITATIVE REVERSE TRANSCRIPTASE (RT)-PCR

Quantitative RT-PCR is used for measuring the mRNA levels of a specific gene of interest. Messenger RNA levels can be used as an indirect measure of the activity of a gene and mRNA levels are often (although not always) correlated with the protein levels of the same gene. In brief, total RNA from tissue or cells is extracted and reverse transcribed to complementary DNA (cDNA) using random or oligo dT primers and reverse transcriptase. The cDNA is subsequently used in a PCR reaction with a Taq polymerase and primers overlapping exon-exon boundaries of the gene of interest. In real time PCR, the PCR reaction is monitored in real-time using a PCR instrument which measures the amount of PCR product based on fluorescence in the reaction. With each PCR cycle more fluorescence is generated and this is directly proportional to the amount of PCR product in the reaction. SYBR Green and Taqman® are two commonly used technologies used for real time PCR.

SYBR Green is a non-specific dye for double stranded (ds) DNA that fluoresces when it is bound to dsDNA. The SYBR Green dye detects specific and unspecific products as well as primer-dimers equally well, which can generate false-positive results. This can be avoided by careful primer design and the use of melting curves and gel electrophoresis to confirm amplification of a single product.

In Taqman® assays, the primers are delivered by the manufacturer together with fluorescently-labeled probes that are complementary to a sequence in the target PCR product. The probes are labeled with a fluorophore and a quencher that absorbs the fluorescence when the probe has not bound. During elongation of the single stranded DNA the target-bound probe will be degraded by the Taq polymerase at the same time releasing the fluorophore from the quencher and fluorescence is emitted.

In both SYBR Green and Taqman® assays the result of the PCR run is a C_t value used for calculating the relative or the absolute target mRNA levels based on the $\Delta\Delta C_t$ method or a standard curve, respectively. The $\Delta\Delta C_t$ method, which is easier and faster, can be used if the amplification efficiency of the reference and target genes is close to 100%. Taqman® assays are guaranteed to give 100% amplification efficiency whereas SYBR Green assays need to be optimized using dilution series of cDNA to ensure that target and reference genes are amplified equally effective. In our analysis, we have used the $\Delta\Delta C_t$ method. For analyzes of large clinical samples we used SYBR Green assays and for evaluation of mRNA expression in *in vitro* experiments we used Taqman® assays. C_t values of the target genes were normalized to the reference gene GAPDH (for *in vitro* experiments), or 18S rRNA (clinical samples), which were amplified in parallel reactions. In addition another reference gene, LRP10, previously shown to be a reliable reference gene in WAT, was used to confirm all results[142]. The relative mRNA expression levels were calculated according to the formula $2^{-\Delta\Delta Ct}$ = arbitrary units (A.U.) where the target C_t is normalized to a reference and relative to a calibrator.

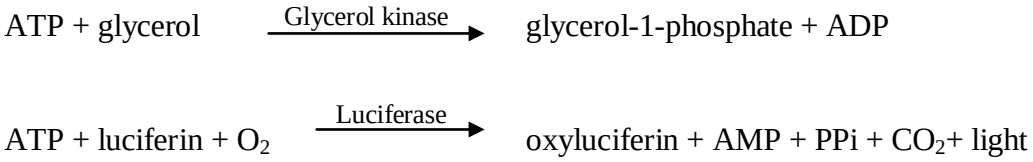
Other methods for measuring mRNA include Northern blot and *in situ* hydridization. None of these methods are as sensitive as RT-PCR and Northern blot is only semi-quantitative whereas *in situ* hydridization is qualitative.

3.8 RNA INTERFERENCE (RNAi)

To study the role of twist1 in human *in vitro* differentiated adipocytes we employed RNAi to silence twist1 using small interfering RNA (siRNA). This method was first described in *Caenorhabditis elegans* where introduction of small double stranded RNA was able to completely block the expression of the target mRNA:s that the siRNA was complementary to[143,144]. In **paper II** and **III** we have used a siRNA from Dharmacon, Thermo Fisher Scientific, CO, USA. This product consist of a pool of four different siRNA:s targeting different sequences in the same mRNA, in our case twist1. The exact protocol for siRNA treatment is described in the papers. The optimal transfection conditions were determined in separate titration experiments. To control for unspecific effects of siRNA treatment, control cells were treated with a negative control siRNA. These oligonucleotides have no known similarities to human sequences. Cells were incubated for 48 hours post-transfection and conditioned media was collected for analyzes of adipokine and glycerol release. Cells were then lysed for RNA or protein extraction as described below.

3.9 LIPOLYSIS MEASUREMENTS

The effect of twist1 silencing on lipolysis was assessed in **paper II**. Lipolysis, measured as glycerol release, was quantified using a bioluminescence method in conditioned media from human *in vitro* differentiated adipocytes[145,146]. In brief, this method is based on two competing reactions shown below.



When both enzymes (glycerol kinase and luciferase) and both substrates (glycerol and luciferin) are present the two reactions will compete for the available ATP. Increasing concentrations of glycerol will shift the reaction towards generation of more glycerol-1-phosphate. This in turn will lead to decreased generation of oxyluciferin and a decay in the emitted light. The decrease in light can be monitored and used as a measure of glycerol concentration in the solution.

As previously discussed, lipolysis generates one glycerol and three NEFA molecules per molecule of TAG. NEFAs can be reused in the synthesis of TAGs but glycerol cannot be converted to glycerol 3-phosphate in the adipocyte. Therefore, lipolysis is preferentially measured as the release of glycerol rather than the release of NEFA. On the other hand, by measuring both glycerol and NEFA release it is possible to determine the rate of NEFA re-esterification.

3.10 PROTEIN MEASUREMENTS

Western blot and ELISA are two qualitatively different methods to detect proteins in a sample. Western blot is performed on denatured proteins whereas ELISA is preformed on native proteins. The identity of the protein in Western blot is verified by antibody recognition as well as by the migration of the denatured protein compared to a known standard. In ELISA, the detection of protein is completely dependent on antibody-antigen interaction. On the other hand, competitors can be used in ELISA to verify the specificity of the antibody. Both methods are highly dependent on specific antibodies. ELISA is preferably used as a screening technique as this method is relatively more simple and faster than Western blot. Commercial ELISAs include a standard curve for the protein of interest thereby allowing a quantitative estimate of the concentration of the studied protein in an unknown sample.

In the papers presented here we used Western blot to detect protein expression in tissue or cell lysates whereas ELISA was used for detection of secreted proteins. Western blot was used for detection of proteins in protein lysates from human *in vitro* differentiated adipocytes treated with siRNA (**paper II**) or from WAT samples from non-obese and obese subjects in cohort I (**paper III**). The protocol has been described in the papers.

Secretion of adipokines into conditioned medium was measured using ELISA kits. Conditioned media was collected from human *in vitro* differentiated adipocytes treated with siRNA or from WAT samples. Assays were performed according to the manufacturer's instructions.

3.11 FATTY ACID OXIDATION/PALMITATE OXIDATION

Fatty acids are broken down in the mitochondria in a cyclic multi-step reaction where two carbons are released in the form of one acetyl-CoA. Each cycle in the fatty acid oxidation releases one molecule of acetyl-CoA until the whole fatty acid chain is broken down. The acetyl-CoA can be used in the citric acid cycle. The end product of the citric acid cycle is GTP, NADH, ubiquinol and carbon dioxide (CO_2).

In our assays fatty acid oxidation was assessed by the ability of adipocytes to oxidate palmitate added to the cell culture. The rate of palmitate oxidation is measured as the sum of released CO_2 and palmitate oxidation metabolites into the medium, as described in **paper II**. One of the rate-limiting enzymes in the fatty acid oxidation is carnitine palmitoyltransferase (CPT) 1, which transfers long-chain fatty acids over the outer mitochondrial membrane by binding them to carnitine. In our experimental set-up carnitine is added in excess to the incubation medium.

3.12 INSULIN SENSITIVITY

Insulin sensitivity was assessed by the homeostasis model of assessment of insulin resistance (HOMA-IR) in **paper III**. This insulin resistance test is calculated based on fasting insulin levels and blood glucose levels according to the formula: ((fasting plasma insulin [mU/L] * fasting plasma glucose [mM/L]) / 22.5). A normal HOMA value should be approximately 1.

The gold standard for determining insulin resistance is the hyperinsulinemic euglycemic clamp. However, this test is tedious and expensive and not suitable for routine clinical practice.

In addition to HOMA-IR and hyperinsulinemic euglycemic clamp, insulin resistance can be assessed using fasting insulin levels in the circulation or by a glucose tolerance test. During the glucose tolerance test the fasting subject receives 75 g glucose orally and blood glucose levels are measured under the following 2 hours. These methods are used in both experimental science and in routine clinical practice to determine insulin resistance. However, in our cohorts, insulin sensitivity was only assessed by the HOMA-IR.

4 RESULTS AND DISCUSSION

4.1 TRANSCRIPTIONAL CONTROL OF HUMAN CIDEA (PAPER I)

In the first paper we identified a single CIDEA transcript from an adipocyte and WAT cDNA library. This transcript corresponds to a previously published CIDEA transcript NM_001279 and has a 14 bp long 5'untranslated region (5'UTR). On the other hand, murine transcripts of CIDEA in liver has substantially longer 5'UTR:s, possibly indicating a species- or tissue-specific regulation of CIDEA[117]. Using bioinformatics we were able to identify an evolutionary conserved region between the rat, mouse and human CIDEA promoter. This conserved region also overlapped with a putative CpG island in both the murine and the human promoters. CpG islands are genomic regions with a high frequency of cytosine and guanine nucleotides in a linear sequence. The cytosine nucleotide can be methylated to form 5-mehylcytosine, a modification that is associated with inactive genes. CpG islands are typically found in the promoter region of genes and therefore play an important role in transcriptional regulation. Altogether, the conservation between species and the presence of putative CpG islands in both the murine and the human promoter suggest an important role for this region in the transcriptional control of CIDEA. During the preparation of the first paper a study on the transcriptional regulation of human *CIDEA* was published by Li *et al*[118]. In that study the authors showed that methylation of a CpG island is involved in the tissue-specific expression pattern of CIDEA in liver, spleen, stomach, heart and adipose tissue as well as in three different cell lines. The CpG island identified by us overlaps with the CpG island identified by Li *et al*.

To further assess the transcriptional control of human CIDEA we generated deletion fragments of the promoter. Using a luciferase-based reporter system we were able to identify the minimal promoter needed for transcription. This promoter overlapped with the minimal promoter identified by Li *et al*.

Results from our group has shown that a polymorphism in the coding region of CIDEA is associated with BMI in a Swedish cohort[110]. In the same study, several polymorphisms in the promoter region for CIDEA was also described[110]. In the present paper we investigated the effect of five SNPs located in the CIDEA promoter on the transcription of CIDEA. Using luciferase constructs of the human CIDEA promoter we could not detect any significant differences between the three haplotypes investigated. It is possible that the changes in transcriptional activity attributed by these SNPs are too small to be detected in our assays. In addition, we cannot exclude the possibility that these SNPs are important *in vivo* when linked to other SNPs and the chromatin structure.

TNFα attenuates the expression of CIDEA in human adipocytes[109]. To further explore this regulation we studied the transcriptional activity of deletion fragments in the presence or absence of TNFα. As expected, TNFα markedly reduced CIDEA promoter activity in both human and murine adipocytes. PPARγ has been shown to regulate transcription of CIDEA in murine liver. However, we were unable to detect any effect of a PPARγ agonist on the transcriptional activity of the human promoter in adipocytes, at least after a 24 h

incubation. Analysis of the activity of mutation fragments in the presence of TNFα showed that TNFα regulation was confined to a region between –244 and –123, relative the transcriptional start site, in the CIDEA promoter, which overlaps with a putative NF-κB binding site. Interestingly, mutation of this NF-κB binding site attenuated the transcriptional effect of TNFα. Moreover, TNFα induced binding between nuclear proteins and an oligonucleotide overlapping the NF-κB site in a gel-retardation assay. Incubation with nuclear extracts from adipocytes, treated with TNFα and an inhibitor of NF-κB, reduced the protein-oligonucleotide complexes whereas treatment of adipocytes with an inactive form of the NF-κB inhibitor had no effect on the interaction between nuclear proteins and the oligonucleotide. No interaction was observed when nuclear extracts were incubated with an oligonucleotide overlapping another region of the CIDEA promoter without any predicted NF-κB site.

In conclusion, in paper I we were able to define the minimal promoter needed for basal transcription of human CIDEA. In addition we defined a region in the promoter regulated by TNFα that overlaps an NF-κB binding site and probably contributes to the transcriptional repression of CIDEA by TNFα.

4.2 A POSSIBLE INFLAMMATORY ROLE OF TWIST1 IN HUMAN WHITE ADIPOCYTES (PAPER II)

Preliminary results from our laboratory, on WAT expression from lean and obese subjects showed that twist1 was significantly higher expressed in lean compared to obese subjects. Twist1 is also highly expressed in brown and white adipose tissue in mice and regulates brown fat function by interaction with PGC1α[134]. Therefore, in the second paper we set out to investigate the role of twist1 in human white adipocytes. We measured the expression levels in metabolically important organs, in different cells from human WAT as well as during *in vitro* differentiation of adipocytes. Compared to twist2, which was detectable in skeletal muscle, liver, pancreas and WAT, twist1 was selectively expressed in WAT. Twist1 expression was higher in adipocytes compared to adipose derived-endothelial cells and macrophages and increased during *in vitro* differentiation of human adipocytes. *In vitro* silencing of twist1 in human adipocytes reduced fatty acid oxidation and the expression of CPT1, a key enzyme involved in fatty acid oxidation. Furthermore expression of the brown fat cell regulator, PGC1α, was reduced whereas lipolysis as well as expression of PLIN and HSL were unaffected by twist1-silencing. Altogether, these results suggested that twist1 is needed to maintain fatty acid oxidation which is in contrast to results obtained in mice where twist1 impairs fatty acid oxidation and brown adipose tissue function by inhibiting PGC1α action. On the other hand, there is no previous study on the functional role of twist1 in murine or human WAT which makes it difficult to speculate whether the divergence in fatty acid oxidation is caused by tissue-specific or species-specific differences. In human WAT, fatty acid oxidation accounts for only a small percentage of the whole-body fatty acid oxidation. Therefore the impact of twist1 on WAT fatty acid oxidation probably is of less importance.

Previous work from twist1 and twist2 heterozygous knockout mice has indicated a role for these transcription factors in inflammatory pathways. Using ChIP and *in vitro* silencing of twist1 in cultured adipocytes we were able to demonstrate a role for twist1 in the regulation of the pro-inflammatory factors TNFα, IL-6 and MCP-1 but not of adiponectin. First; silencing of twist1 reduced the secretion and mRNA expression of IL-6 and MCP-1 and second; twist1 was able to bind to the promoters of TNFα, IL6 and MCP-1 suggesting that twist1 is needed for expression of several pro-inflammatory cytokines. In addition, over expression of human twist1 in murine 3T3-L1 cells reduced the transcriptional activity from a human IL-6 promoter construct, further supporting a role in the regulation of this cytokine. Curiously, our results in human adipocytes suggest that twist1 is needed for the expression of IL-6 and MCP-1 whereas the data from 3T3-L1 cells suggest that twist1 can reduce the transcriptional activity of the human IL-6 promoter. Previous studies show that twist1 is able to inhibit NF-κB signaling in COS cells by interacting with p65[131]. In light of these divergent data we believe that the correct interpretation is that twist1 can regulate the expression of pro-inflammatory cytokines and that the outcome of this regulation is highly dependent on the cell types, species and the present nuclear proteins at each specific time point. In *D. melanogaster* and in bone development the action of twist1 is tightly coupled to the protein levels of twist1 and even small changes in expression can alter the choice of hetero-dimerizing partners. Different dimers will recruit different transcription factors, which will modify the chromatin structure in different ways and thereby the expression of target genes[120]. Therefore it seems reasonable that the effect of twist1 in adipocytes is strongly correlated with the particular protein levels of twist1 and possibly with protein levels of its dimerizing partners.

Altogether, we have for the first time demonstrated a role for twist1 in human white adipocytes. Twist1 mRNA expression is highest in WAT compared to other metabolically important organs including liver, pancreas and skeletal muscle, possibly suggesting a role in human WAT. The expression of twist1 is also highest in the adipocytes compared to other cells in the tissue. In contrast to its function in murine brown adipose tissue twist1 represses neither fatty acid oxidation nor the expression of genes involved in fatty acid oxidation in human adipocytes. In fact, twist1 seems to be essential to maintain a normal fatty acid oxidation in human white adipocytes. Twist1 does however regulate the expression of pro-inflammatory cytokines, TNFα, IL-6 and MCP-1.

4.3 TWIST1 IN HUMAN WHITE ADIPOSE TISSUE AND OBESITY (PAPER III)

Because of its proposed role in brown fat function and inflammation we hypothesized that twist1 might be involved in obesity-associated inflammation. In **paper III** we measured twist1 mRNA expression in WAT from non-obese and obese subjects and correlated the expression levels with markers for insulin resistance and inflammation. Twist1 was significantly more highly expressed in non-obese subjects and the expression increased after weight reduction induced either by a diet or surgery. The expression was also higher

in WAT from the sc depot compared to omental depot and low expression of twist1 in sc WAT was associated with low insulin sensitivity, large adipocyte volume and low adiponectin expression. Furthermore, we divided our subjects into four different groups based on the obesity status and the morphology of the WAT, e.g. hyperplastic or hypertrophic WAT. Interestingly, twist1 expression was highest in the hyperplastic non-obese group and lowest in hypertrophic obese group. In addition, secretion of TNFα and MCP-1 from WAT pieces was significantly higher in subjects with low twist1 mRNA expression compared to a group of subjects with high twist1 expression. Altogether, these data show that twist1 expression is reduced in obesity and that low twist1 expression in sc WAT associates with a metabolically more unfavorable profile characterized by increased secretion pro-inflammatory markers.

Results from our previous work in human adipocytes (**paper II**) suggested that twist1 is needed for the expression of several pro-inflammatory genes, including IL-6 and MCP-1, and would imply that reduced twist1 expression in adipocyte could be beneficial to reduce inflammation[147]. At first glance, the previous results seemed to contradict our present results where twist1 associated with a lean and metabolically healthier profile. Therefore we set up an experiment where we tried to mimic the *in vivo* environment of WAT in obesity by reducing twist1 expression using siRNA and at the same time stimulating adipocytes with TNFα. Interestingly, in this context where twist1 expression was reduced and TNFα levels were high, twist1 down regulation augmented the effect of TNFα on MCP-1 expression and secretion. Moreover, twist1 silencing also accelerated the TNFα-induced increase in IL-6, RelA and TNFR1 mRNA expression. These results show that twist1 can attenuate the actions of TNFα in adipocytes and are therefore in accordance with our clinical data suggesting that low twist1 expression in an environment with elevated TNFα levels may have pathological rather than beneficial consequences. The repressive effect of twist1 on TNFα-induced gene expression is also in agreement with previous work in nonadipose cells[131]. Taken together we believe that twist1 regulates the effects of TNFα in at least two ways; 1) when TNFα levels are low, twist1 is needed to retain basal transcription of several cytokines and chemokines that may be necessary for normal tissue function, and 2) when TNFα levels are high, twist1 has protective effects on the TNFα-induced cyto- and chemokine expression.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As the title of this thesis implies, the aim of the presented work was to identify novel genes in WAT associated with obesity. In addition to obesity, the two genes studied in this thesis appear to play a role in WAT inflammation since they are either controlled by TNFα, regulate the action of TNFα or both.

CIDEA is a relatively unknown protein. The first data on the CIDE-family (CIDEA,CIDEB and CIDEC/FSP27) suggested that CIDE-proteins are involved in apoptosis[101]. The first indication that CIDEA might be involved in metabolic processes came from CIDEA null mice, which were lean and resistant to obesity, most likely due to an increased BAT function[104,106]. In contrast to these data, CIDEA expression is decreased in human WAT in obesity[109]. Moreover, CIDEA expression is increased in cancer cachexia and correlates with reduced fat mass[111]. Altogether data from mice and human suggest that CIDEA has species-specific functions on metabolism. Moreover, CIDEA expression is decreased by TNFα and *in vitro* silencing of CIDEA in human white adipocytes results in enhanced TNFα secretion indicating that CIDEA and TNFα cross-talk[109]. In **paper I**, we defined the minimal promoter region needed for transcription of CIDEA and defined a region in the promoter that is important for TNFα regulation. Together with previously published data these results confirm the regulation of CIDEA by TNFα and also suggest that high TNFα levels in obesity could be a contributing factor to the reduced CIDEA expression in human WAT.

Although several papers have been published on the function of CIDEA it still is not clear what the role of this protein is in adipocytes, where the protein is localized and what proteins CIDEA interacts with. Some studies have shown that CIDEA is localized to the lipid droplet and enhances lipid droplet size suggesting a role in the regulation lipid storage[112-114]. Interestingly, a recent study from our laboratory showed that CIDEA is localized both to the cytosol and the nucleus. Moreover, CIDEA was shown to bind to nuclear receptors suggesting that CIDEA might be involved in transcriptional regulation as well. On the other hand, the physiological outcome of this interaction has not been investigated.

It is possible that post-translational modifications of CIDEA can alter its cellular localization and thereby the function of the protein. Further studies investigating these mechanisms and the correlation between such modifications and the cellular localization of CIDEA, CIDEA function and potential interacting proteins would contribute to the understanding of the physiological function of CIDEA in adipocytes.

Twist1 is a transcription factor with well-described functions in developmental processes in *D. melanogaster*, differentiation, EMT as well as inflammation. However, the role in WAT has not been described before. In **paper II** and **III** we showed that twist1 is expressed in human WAT, primarily in the adipocytes, and that low twist1 expression

associates with a metabolically more unfavorable profile (high BMI, reduced insulin sensitivity, and increased MCP-1 and TNFα secretion). The association of twist1 with obesity has subsequently been confirmed in a French cohort comprising lean and obese subjects (unpublished data from Karine Clement's group, Paris, France). In contrast to murine data, twist1 seems to be needed to maintain normal fatty acid oxidation in human white adipocytes. Twist1 also regulates inflammatory pathways in human adipocytes and low expression of this transcription factor makes the adipocyte more sensitive to the pro-inflammatory effects of TNFα, one of the key regulators of obesity-related inflammation.

It is tempting to speculate that manipulation of twist1 expression in obese subjects to levels similar to those seen in non-obese subjects could have beneficial effects on WAT inflammation and perhaps also on fatty acid oxidation, which could influence whole-body metabolism. The first step in this approach would be to investigate the effect of *in vitro* over-expression of twist1 in our human adipocyte cell system. It is essential that such studies be performed in human cell systems and not in rodent cells, given that there are important species-specific differences in twist1 function in inflammation and metabolism. In fact, during the last period of my PhD studies we have set up a series of experiments where twist1 was over expressed in human *in vitro* differentiated adipocytes using an adenoviral construct. RNA from these experiments was subsequently analyzed by gene expression arrays and the data are now being processed for evaluation.

Another way of inducing twist1 expression would be to activate its expression. Acute TNFα stimulation has been shown to induce twist1 expression through the NF-κB signaling pathway in immortalized mouse fibroblasts. However, since TNFα is already high in the obese state this regulation is probably not functional during these circumstances and implies that chronic TNFα stimulation has different effects than acute stimulation[131]. Stimulation of macrophages with the anti-inflammatory cytokine interferon (IFN) α for two days also induces twist1 expression, via activation of a Tyro 3 receptor kinase termed Axl[133]. On the other hand, because of the delayed response in twist1 expression this activation probably involves other proteins and signaling pathways as well. It has been shown that insulin-like growth factor (IGF)-1, via activation of mitogen-activated protein kinases (MAPK), is able to induce the expression of twist1 in NIH-3T3 fibroblast whereas another study demonstrated that treatment with a PPARδ-agonist is able to stimulate twist1 expression in brown adipocytes[134,148]. In order to use any of these factors to stimulate twist1 expression the effects would first need to be confirmed in human adipocytes. It should also be noted that twist1 function is highly dependent on the choice of dimerizing partner and thus the identity of these proteins in human adipocytes needs to be assessed. Last but not least, since over expression of twist1 has been confirmed in several tumors and tumor cell lines the effect of over expression for EMT and tumor progression would need to be thoroughly assessed.

A further apparent and very relevant experiment is to perform ChIP sequencing on twist1 in human adipocytes to define target genes regulated by this transcription factor. Such experiment could be performed in cell cultures with or without over expression of twist1 or with or without *in vitro* silencing of twist1. A comparison of such ChIP sequencing data with gene expression data from experiments with *in vitro* over-expression of twist1

in adipocytes could provide novel insight into the function of twist1 in human adipocytes and also identify new target genes or pathways controlled by this transcription factor. It would also be interesting to compare twist1 target genes in the lean and obese state and with or without TNFα stimulation. Such experiments could also be used to examine the identity of the twist1 dimerizing proteins under the different conditions.

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