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Role of GPS2 in the regulation of adipocyte fate and function: a multi-omics approach

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Cover illustration: microscopy pictures of mature adipocytes (pink) in a GPS2 mask

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Role of GPS2 in the regulation of adipocyte fate and function: a multi-omics approach

Thesis for Doctoral Degree (Ph.D.)

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To my beloved parents

Rosella & Giuseppe

ABSTRACT

The escalating prevalence of obesity and its association with comorbidities like insulin resistance and type 2 diabetes have raised the interest in adipose tissue biology and its therapeutic potential. Adipose tissue remodeling during the development of obesity is an important regulator of systemic metabolic homeostasis, and dysfunctional adipose tissue is linked to the risk of developing type 2 diabetes. Adipocyte differentiation and function are orchestrated by a complex network of transcription factors and coregulators that transduce regulatory signals into epigenome alterations and transcriptional responses. While the role of transcription factors involved in adipogenic pathways is well established, the role of their associated coregulators remains poorly understood. Of particular interest is G protein pathway suppressor 2 (GPS2), a core subunit of the HDAC3 corepressor complex, which is downregulated in humans with obesity and implicated in regulating metabolic and anti-inflammatory pathways in various tissues. The overall aim of this thesis was to identify hitherto unknown functions of GPS2 in the adipose tissue, with a particular emphasis on mechanisms underlying adipocyte dysfunction in the context of obesity and type 2 diabetes.

In **Paper I,** by generating a unique loss-of-function model using human multipotent adipose-derived stem cells, we showed that loss of GPS2 triggers the commitment of fibroblast-like progenitors towards the adipogenic lineage and induces hypertrophy of mature adipocytes associated with a deep remodeling of the adipocyte lipidome. Furthermore, we demonstrated that adipocyte hypertrophy was likely the consequence of the increased expression of ATP-binding cassette subfamily G member 1 (*ABCG1*) that mediates sphingomyelin efflux from adipocytes and modulates the activity of lipoprotein lipase (LPL). We validated the cell-derived findings by gene expression analysis of an obese cohort, where *GPS2* is downregulated in diabetic patients and negatively correlated with the expression of *ABCG1*.

In **Paper II**, by characterizing adipocyte-specific *Gps2* knockout mice, we discovered a hitherto unknown function of GPS2 in the induction of adipocyte hypertrophy, inflammation and mitochondrial dysfunction. The knockout phenotype was driven by over-activation of the transcription factor HIF1A that orchestrates an inadequate white adipose tissue remodeling and disrupts mitochondrial activity. The validation of the experimental mouse data in a human cohort of non-obese and obese individuals with or without diagnosed type 2 diabetes showed a negative correlation between the expression of *GPS2* and *HIF1A*, adipocyte hypertrophy and insulin resistance.

In **Paper III**, we found that the expression of *GPS2* in the white adipose tissue of humans was strongly correlated with the insulin secretion rate. The causality of this relationship was confirmed using adipocyte-specific *Gps2* knockout mice, in which adipocyte dysfunction caused by the loss of GPS2 triggered the secretion of factors that provoked pancreatic islet inflammation and impaired beta cell function.

In conclusion, the research within this thesis revealed novel insights into the multifaceted regulatory roles of GPS2 in altering the epigenome and the transcriptome linked to adipose

tissue metabolism and inflammation. These discoveries increase our understanding of the mechanisms underlying the development of obesity and its link with type 2 diabetes, and they may help to define novel potential targets for treating these metabolic diseases.

LIST OF SCIENTIFIC PAPERS

I. Serena Barilla*, Ning Liang, Enrichetta Mileti, Raphaëlle Ballaire, Marie Lhomme, Maharajah Ponnaiah, Sophie Lemoine, Antoine Soprani, Jean-Francois Gautier, Ez-Zoubir Amri, Wilfried Le Goff, Nicolas Venteclef, Eckardt Treuter*. Loss of G protein pathway suppressor 2 in human adipocytes triggers lipid remodeling through upregulation of ATP binding cassette subfamily G member 1. Molecular Metabolism. 2020 Aug 11; https://doi.org/10.1016/j.molmet.2020.101066

II. Karima Drareni, Raphaëlle Ballaire, Serena Barilla, Mano J. Mathew, Amine Toubal, Rongrong Fan, Ning Liang, Catherine Chollet, Zhiqiang Huang, Maria Kondili, Fabienne Foufelle, Antoine Soprani, Ronan Roussel, Jean-Francois Gautier, Fawaz Alzaid, Eckardt Treuter*, Nicolas Venteclef*. GPS2 deficiency triggers maladaptive white adipose tissue expansion in obesity via HIF1A activation. Cell Reports. 2018 Sep 11; 24(11):2957-2971

III. Karima Drareni*, Raphaëlle Ballaire, Fawaz Alzaid, Andreia Goncalves, Catherine Chollet, Serena Barilla, Jean-Louis Nguewa, Karine Dias, Sophie Lemoine, Riveline Jean-Pierre, Ronan Roussel, Elise Dalmas, Gilberto Velho, Eckardt Treuter, Jean-François Gautier, Nicolas Venteclef*. Adipocyte reprogramming by the transcriptional coregulator GPS2 impacts beta cell insulin secretion. Cell Reports. 2020 Sep 15; 32(11):108141

Related publications not included in this thesis

- Ning Liang, Anastasius Damdimopoulos, Saioa Goñi, Zhiqiang Huang, Lise-Lotte Vedin, Tomas Jakobsson, Marco Giudici, Osman Ahmed, Matteo Pedrelli, Serena Barilla, Fawaz Alzaid, Arturo Mendoza, Tarja Schröder, Raoul Kuiper, Paolo Parini, Anthony Hollenberg, Philippe Lefebvre, Sven Francque, Luc Van Gaal, Bart Staels,Nicolas Venteclef, Eckardt Treuter* & Rongrong Fan* (2019). Hepatocyte-specific loss of GPS2 in mice reduces non-alcoholic steatohepatitis via activation of PPARα. Nature communications. 2019 Apr 11; 10(1):1684.

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LIST OF ABBREVIATIONS

ABCG1	ATP-binding cassette transporter G1
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BMI	Body mass index
BMP	Bone morphogenetic protein
C/EBPα	CCAAT/enhancer-binding protein alfa
C/EBPβ	CCAAT/enhancer-binding protein beta
C/EBPδ	CCAAT/enhancer-binding protein delta
cAMP	cyclin adenosine monophosphate
ChIP-seq	Chromatin immunoprecipitation coupled with next-generation sequencing
CREB	cAMP-responsive element-binding protein
FFA	Free fatty acid
GPS2	G protein pathway suppressor 2
GR	Glucocorticoid receptor
H3K27ac	Histone H3 lysine K27 acetylation
H3K4me	Histone H3 lysine K4 methylation
HDAC3	Histone deacetylase 3
HIF1A	Hypoxia inducible factor 1 subunit alfa
hMADS	Human multipotent adipose-derived stem
HSL	Hormone-sensitive lipase
IL6	Interleukin 6
KDM	Lysine (K)-specific demethylase
LPL	Lipoprotein lipase
LXR	Liver X receptor
MCP1	Monocyte chemoattractant protein 1, alias CCL2
NCoR	Nuclear receptor corepressor, alias NCOR1
PGC-1α	PPARG coactivator 1 alfa
PPARγ	Peroxisome proliferator-activated receptor gamma
PRDM16	PR/SET domain 16
SMRT	Silencing mediator of retinoid acid and thyroid hormone receptor, alias NCOR2
SREBP-1	Sterol regulatory element-binding protein 1

STAT5A	Signal transducer and activator of transcription 5A
TBL1	Transducin beta-like protein 1
TBLR1	TBL-related protein 1
TF	Transcription factor
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alfa
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WHO	World Health Organization

1 INTRODUCTION

1.1 Obesity

Obesity is a metabolic disease, the prevalence of which has exponentially increased in the last thirty years. In 2016 the World Health Organization (WHO) estimated that more than 1.9 billion people worldwide were overweight and among those more than 650 million were obese[1]. This pathological condition represents one of the major health issues correlated with high cost for the public health care system, in particular because it is a major risk factor for the development of comorbidities associated with metabolic syndrome, such as diabetes, cardiovascular disease, hypertension, dyslipidemia, coronary heart disease, and certain types of cancer[2].

Obesity is characterized by an excess of body fat accumulation, an outcome of the imbalance between energy intake and energy expenditure. While a small number of obese cases result from monogenic alterations, the common form of obesity is the outcome of a complex interplay of multiple factors like obesogenic environment and genetics. These factors can contribute to a dynamic alteration of the epigenetic signature resulting in a 'personalized' epigenome, which influences the fat distribution and the susceptibility and progression of this pathology[3] (**Figure 1**).

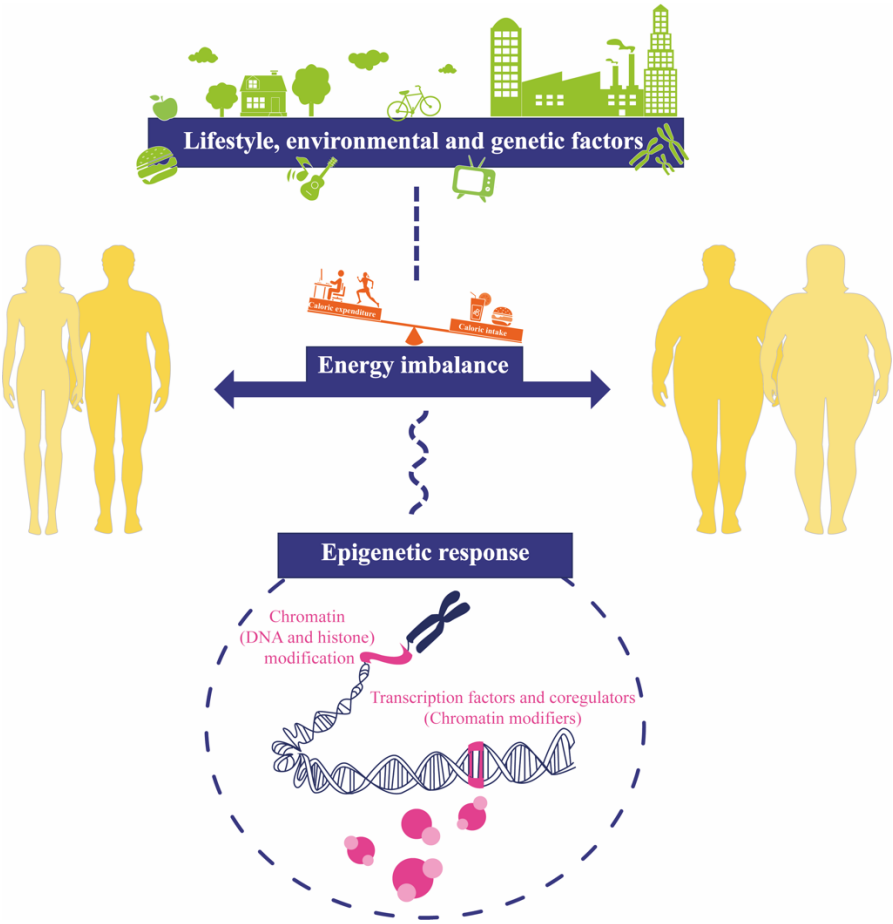

Figure 1. Components of epigenetic responses that influence obesity.

Several candidate genes have been linked to obesity, including genes that regulate energy homeostasis. For example, mutations in the genes encoding leptin or leptin receptor lead to severe obesity[4,5]; leptin is an adipocyte-specific hormone which acts on the hypothalamus suppressing food intake. However, these mutations are rarely observed in obese human subjects, and paradoxically the majority of cases are leptin-resistant with high levels of circulating leptin in proportion to the fat mass[6]. Genome-wide association studies have identified a cluster of single nucleotide polymorphisms (SNPs) located within the *Fat Mass and Obesity Associated* (*FTO*) gene, which are associated with the susceptibility of being affected by obesity[7]. Also, the environment has a major impact on the risk of developing obesity. Energy expenditure depends on the physical activity and it has been shown that watching TV for more than 5 hours per day increases the risk of childhood obesity[8]. Likewise, energy intake depends on the composition of macronutrients in the diet that have different effects on satiety. Nowadays it is easy to find food that is rich in fats which, when compared to proteins, have a reduced effect on inducing satiety[9]. Moreover, multiple evidences suggest that fetal nutrition influences a long-term programming of genetic expression that can determine the later onset of obesity and type 2 diabetes independently from genetic inheritance[10].

In clinical practice, obesity is estimated by the body mass index (BMI), calculated as weight in kilograms divided by height in meter squared (kg/m^2). The classification of overweight and obesity proposed by the WHO applies to both men and women and to all adult age groups. Individuals with a BMI of 18.5 to 24.9 are considered normal weight, those with a BMI between 25 and 29.9 are considered overweight, and those with a BMI over 30 are considered obese (**Table 1**)[11].

Classification	BMI	Risk of comorbidities
Underweight	< 18,50	Low (but risk of other clinical problems increased)
Normal range	18,50 – 24,99	Average
Overweight	≥ 25,00	
Preobese	25,00 -29.99	Increased
Obese class I	30,00 – 39,99	Moderate
Obese class II	35,00 – 39,99	Severe
Obese class III	≥ 40,00	Very severe

Table1. Classification of obesity according to BMI and associated risk of comorbidity.

Even if BMI is accepted as a strong predictor of overall mortality, several studies reported an inverse association between BMI and mortality in patients with different disease situations. Metabolically obese normal-weight subjects (MONW, introduced by Ruderman), have a normal BMI but suffer from metabolic complication typically found in obese subject[12]. On the other hand, metabolically healthy obese (MHO) have a BMI above 30 kg/m^3 but don't show insulin resistance or dyslipidemia. It has been estimated that in the United States about

10% of adults have obese BMI and are metabolically healthy, while 8% have a normal BMI and are metabolically unhealthy[13]. The BMI classification presents some limitations, such as that it does not distinguish between muscle and fat accumulation and it does not accurately measure the distribution of visceral and subcutaneous fat. Additionally, it has been shown that most of MONW have a relatively low BMI but significantly more visceral adipose tissue, while MHO have a high BMI with less visceral adipose tissue[14].

Currently, the main therapeutic methods to control energy balance and reduce body weight are caloric restriction, exercise and bariatric surgery. However, calorie-reducing diet and exercise demand persistent efforts and considerable willpower from affected individuals and they are often unsuccessful for long-lasting results. Bariatric surgery reduces food intake by reconstructing the digestive system and induces significant weight loss. Nevertheless, it comes with the risk of side-effects such as dumping syndrome, reflux and nutritional malabsorption, which makes this treatment nun suitable for many obese individuals[15]. Therefore, there is a need to better characterize adipose tissue biology and its crosstalk with other organs to develop new treatment strategies for obesity and its comorbidities.

1.2 Adipose tissue

In mammals, adipose tissue is composed of two different tissues, the white adipose tissue (WAT) and the brown adipose tissue (BAT). The WAT is classified as visceral fat, located around internal organs in the intra-abdominal cavity, and as subcutaneous fat, situated under the epidermis in the lower and upper part of the body[16]. The belief that the BAT is present only in human newborns has been recently changed by evidence showing its presence above the clavicle and in the subscapular region in human adults[17].

WAT is mainly composed of unilocular adipocytes and has an extensive role in storage and release of free fatty acids based on energetic request of the organism (**Figure 2**). It shows a high level of plasticity and it expands in response to caloric excess via hyperplasia, increased number of adipocytes, or hypertrophy, increased adipocyte size, or both[16]. It communicates with metabolically relevant organs by secreting adipokines, which can act locally as paracrine factors or have a long-range effects and act on the feeding centers in the hypothalamus[18].

BAT, like WAT, accumulates and stores lipids in multilocular adipocytes. The main function of BAT is to maintain the core body temperature in response to cold stress by generating heat, a process known as thermogenesis. BAT is more vascularized compared to WAT. Furthermore, brown adipocytes are differentiated from white adipocytes due to their mitochondria content and enrichment with uncoupling protein 1 (UCP1) (**Figure 2**). They are massively innervated by the sympathetic nervous system, which regulates the expression of UCP1 through activation of the β3-adrenergic receptor. During cold exposure the body temperature is stabilized by non-shivering thermogenesis in which UCP1 uncouples the oxidative phosphorylation from ATP production generating heat[19].

UCP1-expressing thermogenic adipocytes can also be found in WAT upon prolonged cold stimulation or activation of pathways that increase intracellular cyclic AMP (cAMP); these

cells are called “beige” or “brite” adipocytes. The beige adipocytes have a higher mitochondrial density compared to the white ones, with a more oxidative phenotype that promotes energy consumption[20] (**Figure 2**).

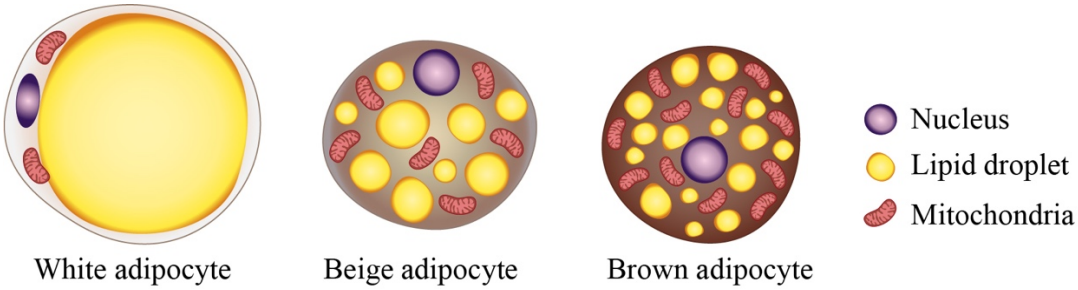

Figure 2. Morphological differences between the three types of adipocytes.

1.3 Physiological function of white adipocytes

The primary function of white adipocytes is to maintain the whole metabolic balance of the body by being the major site for storage and release of fatty acids. The energy surplus is stored in adipocytes through the lipogenic process in the form of triglyceride-containing lipid droplets. However, during energy expenditure or starvation, the lipid reserves are released through lipolysis, where triglycerides are broken down into glycerol and fatty acids. The released fatty acids are then transported to the muscle, liver and BAT where they are oxidized for the modulation of the body energy balance[21]. Lipogenesis and lipolysis are sensitive to nutrition and multiple hormone-sensitive enzymes that regulate the balance between lipid influx and efflux into/from the adipocytes to maintain the systemic energy homeostasis and insulin sensitivity.

1.3.1 Lipogenesis and lipolysis

During the feeding state, high blood glucose levels stimulate the release of insulin from the beta cells of the pancreas which promotes glucose uptake in the adipocytes, and its metabolization into acetyl coenzyme A, an important substrate for the synthesis of endogenous fatty acids through *de novo* lipogenesis. However, in adipocytes glucose is not the main source for the production of fatty acids but is the main source to produce glycerol for their esterification. Under normal conditions, the fatty acids used for triglyceride production in adipocytes come from circulating triglycerides[22]. During energy surplus, circulating triglycerides are synthetized in the form of lipoprotein by the liver and intestine and delivered to the WAT. The triglycerides are hydrolyzed into free fatty acids (FFAs)[23] by the endothelial cells of the adipose tissue, through the action of the key enzyme lipoprotein lipase (LPL). The FFAs are then transported by scavenger receptor CD36 or fatty acid binding protein (FABPs) through the endothelial lumen into the adipocytes[24,25]. Finally, the endogenous and exogenous fatty acids are esterified into triglycerides by the sequential actions of multiple enzymes including diacylglycerol acyltransferase (DGAT) which catalyzes the last step of triglyceride synthesis and plays a critical role in their deposition in the lipid droplets of the adipocytes[26] (**Figure 3**). Insulin is the predominant stimulus that

promotes lipogenesis. Besides stimulating glucose uptake and activation of glycolytic and lipogenic enzymes, insulin promotes fatty acid uptake and esterification increasing the expression and activity of the LPL[27,28], the translocation of fatty acid transport protein and their related gene expression[29]. Recently, the membrane ATP-binding cassette transporter G1 (ABCG1) has been identify as an important player in triglyceride storage in adipocytes[30]. Beyond the well-characterized role of ABCG1 in the regulation of cholesterol efflux in macrophages, new studies suggest it plays a role in the regulation of adiposity. *Abcg1* knockout mice showed protection from diet-induced obesity and reduced adipose tissue mass with smaller adipocytes[31]. Further, ABCG1 has been shown to mediate sphingomyelin efflux contributing to increased LPL activity and triglyceride storage in the adipocytes[32]

On the other hand, lipolysis is the process that induces the breakdown of triglycerides and supplies free fatty acids for oxidation to the liver, muscle and BAT, and glycerol for hepatic gluconeogenesis based on the energy need[33,34]. During fasting, the elevated levels of circulating glucagon and catecholamine released by the sympathetic nervous system stimulate the activation of the cAMP-dependent protein kinase A (PKA) pathway and consequent lipolysis in the adipocytes[35]. Triglycerides are broken down into di-, monoacylglycerides and finally into individual fatty acids by the action of three lipases: adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL)[36,37](**Figure 3**).

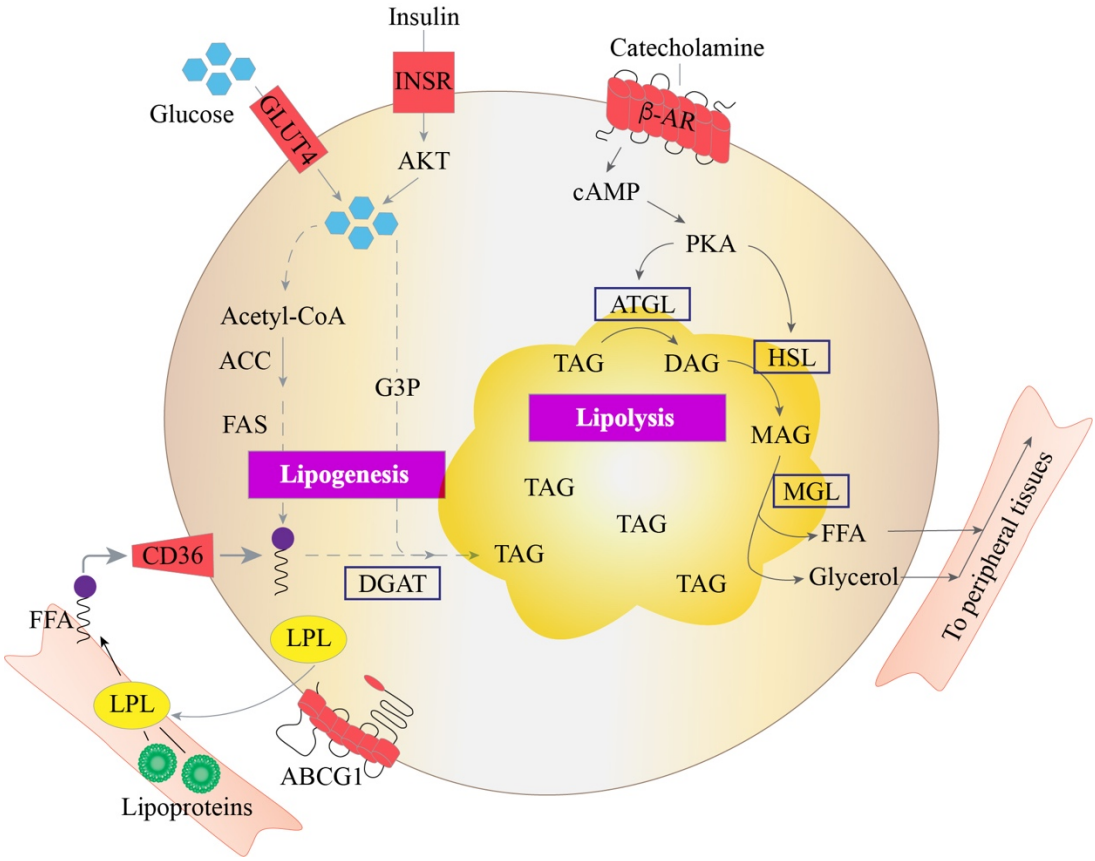

Figure 3. Schematic representation of lipogenesis and lipolysis in adipocytes.

1.3.2 Endocrine function of the adipocytes

In addition to energy storage, over the past 20 years the role of adipose tissue has been expanded to integrate its endocrine function that includes the production of a variety of factors such as metabolites, lipids and adipokines, that regulate systemic metabolism and inflammation[38,39]. The first discovered secreted adipokine was leptin[40], followed by adiponectin[41]. Later many other adipokines have been discovered including resistin and proinflammatory cytokines such as chemokine (C-C motif) ligand 2 (CCL2, also known as monocyte chemoattractant protein 1, MCP1), interleukin 6 (IL-6) and tumor necrosis factor alfa (TNFα)[39]. Interestingly, adipocyte-secreted factors have the ability to regulate a variety of biological process such as immune response, inflammation, glucose metabolism, insulin sensitivity, blood pressure, vascular growth and function, cell adhesion and lipid metabolism. Adipokines have the ability to crosstalk with central and peripheral cells and their dysregulated production or secretion is implicated in metabolic, inflammatory and cardiovascular diseases as well as cancer.

Leptin, popularly known as the satiety hormone, is encoded by the *obesity* (*ob*) gene and regulates energy balance by targeting the hypothalamus and suppressing appetite[40,42]. Mouse models with deficiency of leptin (*ob*/*ob*) or leptin receptor (*db*/*db*) show increased food intake and body weight gain with decreased of energy expenditure[40,43]. In line with this, the administration of recombinant leptin to rodents suppresses food intake and induces weight loss, however, this effect is not as strong when leptin is administrated to humans[44,45]. Apart from this central effect, leptin also regulates the function of peripheral organs such as the pancreas, modulating glucose homeostasis by acting on beta cell mass and expression and secretion of insulin[46]. Furthermore, leptin modulates the immune response[47] and vascular permeability through stimulating angiogenic factors such as fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF)[48].

Adiponectin targets several tissues and acts to regulate insulin sensitivity, being crucial in the regulation of energy homeostasis. Adiponectin acts on the liver, suppressing hepatic gluconeogenesis[49]. Moreover, adiponectin is able to improve insulin sensitivity, promoting adipogenesis and lipid accumulation[50], but also enhancing insulin secretion from the pancreatic beta cells[51].

Resistin is almost exclusively produced by adipocytes and induces insulin resistance and glucose intolerance[52]. This hormone is able to regulate beta cells function impairing glucose-stimulated insulin secretion[53]. In addition, resistin affects the blood perfusion of the pancreas and WAT[54] and, since adequate beta cell development depends on vascular perfusion[55], resistin may have a potential role in the development and expansion of the beta cells.

Adipocytes can also secrete inflammatory cytokines such as MCP1, IL-6 and TNFα. During obesity, the expression of these cytokines increases and contributes to the chronic inflammation of the adipose tissue characteristic of this pathology[56]. MCP1 is a potent chemotactic factor for monocytes which contributes to macrophage infiltration in adipose tissue and drives insulin resistance and hepatic steatosis during obesity[57]. TNFα is a pro-

inflammatory cytokine that activates the release of other pro-inflammatory cytokines such as IL-6[58]. Both TNFα and IL-6 have effects on adipocyte proliferation and differentiation, downregulating the expression of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα)[59,60]. These two cytokines also affect the insulin signaling pathway suppressing the secretion of adiponectin[60,61].

1.4 Adipose tissue remodeling

Fat mass remodeling is an adaptive response of adipocytes to the energetic body request. In a state of overnutrition the extra calories are stored in adipose tissue. To maintain the metabolic homeostasis, adipocytes enlarge to increase their storage capacity and store excess of lipids in a "safe" manner. When adipocytes fail to store excess lipids, they accumulate in "ectopic" places, such as liver, bone marrow and skeletal muscle, leading to lipotoxicity[16].

Adipose tissue expansion in adulthood is achieved mainly by two processes: hypertrophy, an increase in lipid accumulation and the size of existing adipocytes, as well as hyperplasia, the proliferation and differentiation of adipocyte precursors to produce new adipocytes, in a process known as adipogenesis. Adipose tissue expansion occurs in combination with extracellular matrix and vascular remodeling (**Figure 4**). The balance between hypertrophy and hyperplasia has a deep impact on metabolic health. Studies in rodents and humans have shown that adipocyte expansion proceeds until a given critical volume, and once adipocytes have reached the maximum size, the increase in cell number is initiated[62].

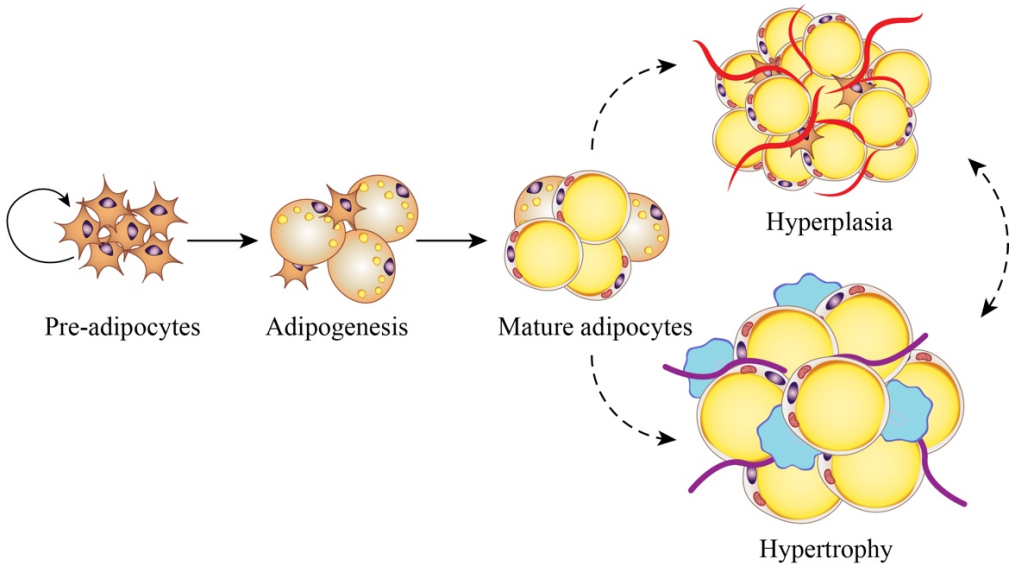

Figure 4. Mechanisms of adipose tissue remodeling.

Hyperplasia is considered a healthy adaptation mechanism of adipose tissue which is able to maintain proper vascularization and levels of beneficial adipokines like leptin and adiponectin[63]. In contrast, hypertrophy of adipose tissue is associated with increased hypoxia caused by the massively expanded adipocyte size limiting oxygen diffusion. Hypoxic adipose tissue induces, instead of normal vascularization, the expression of pro-fibrotic genes leading to tissue fibrosis[64]. Hypertrophic adipocytes experience increased mechanical stress from the

contact with neighboring cells that contributes, with the hypoxic stress, to adipose tissue inflammation (**Figure 4**). All these elements combined with adipose tissue dysfunction lead to elevated levels of nutrients in the blood and ectopic lipid deposition in non-adipose organs which contributes to the early onset of metabolic disease.

1.4.1 Adipogenesis

Adipocytes arise from pluripotent mesenchymal precursors that have the ability to differentiate into several cell types, including adipocytes, chondrocytes, osteocytes and myocytes. These progenitor cells are present in the stromal vascular fraction (SVF) of adipose tissue as well as in the bone marrow. Adipogenesis can be considered a two-step process: the commitment and the terminal differentiation. Under appropriate stimulation the fibroblast-like progenitor cells become committed to the adipocyte lineage, giving rise to adipocyte precursors. When induced, the preadipocytes undergo multiple rounds of mitosis (mitotic clonal expansion) and then accumulate triglycerides and differentiate into mature adipocytes.

White adipocyte precursors derive preferentially from the $MYF5^-$ lineage. However, the presence of $MYF5^+$ adipocyte precursors in WAT revealed that white adipocytes can derive from both $MYF5^+$ and $MYF5^-$ lineages[65]. BAT adipocyte precursors are derived only from the $MYF5^+$ lineage[66]. Both brown and white mature adipocytes can also originate from endothelial precursors[67], while brown mature adipocytes can additionally originate from stem-cell-like skeletal muscle satellite cells[68]. Beige adipocytes can originate either from WAT adipocyte precursors or directly from mature white adipocytes (**Figure 5**).

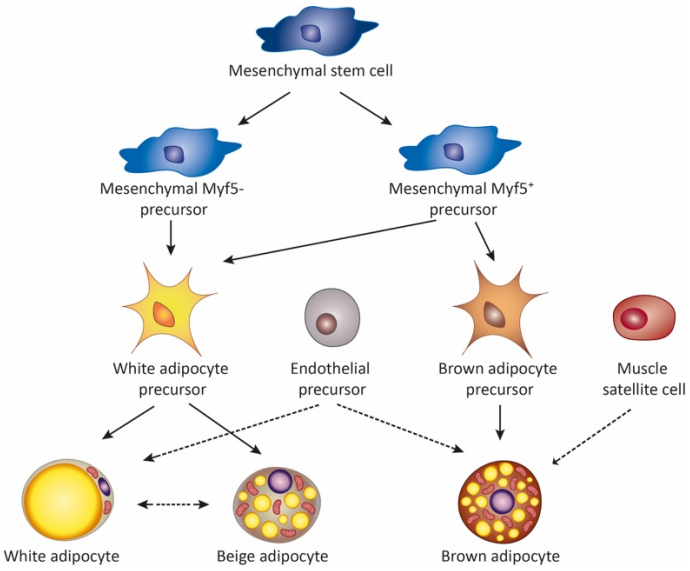

Figure 5. Origins of white, beige and brown adipocytes.

The commitment from pluripotent stem cells to the adipocytes lineage is induced by multiple factors, which include insulin, glucocorticoids, transforming-growth factor β (TGFβ) superfamily members, such as TGFβ and bone morphogenetic proteins (BMPs), and Wnt family members.
Insulin is essential for *in vitro* adipocyte differentiation through the activation of the

intracellular insulin-signaling pathway. The binding of insulin to the insulin receptor leads to the recruitment and phosphorylation of the insulin receptor substrate 1 (IRS1) that further activates the phosphoinositide 3-kinase (PI3K) and the downstream kinase AKT. This cascade leads to the activation of mTOR, cAMP-responsive element-binding protein (CREB) and the family of forkhead proteins (FOXOs)[69]. Disruption of the insulin signaling pathway leads to failure of adipogenesis[70,71]. Glucocorticoids, by activating the glucocorticoid receptor (GR), are also a crucial components for adipocyte differentiation, as they facilitate growth arrest[72] and contribute to the terminal differentiation upregulating the expression of C/EBP family members[73]. TGFβ inhibits adipocyte differentiation through the activation of SMAD3, which is able to bind to C/EBPβ and repress its transcriptional activity on the PPARγ promoter[74]. In contrast, BMP4 and BMP2 promote adipocyte differentiation. The exposure of multipotent stem cells, like C3H10T1/2, to BMP4 triggers the commitment of these cells to the adipocyte lineage[75]. Interestingly, BMP4 also has a function during the terminal phase of differentiation impairing the acquisition of a brown-like phenotype and inducing more a white-like phenotype of the mature adipocytes[76]. The action of BMP2 on the other hand is controversial and it seems to be dependent on its concentration. Low levels of BMP2 stimulate adipocyte differentiation while high levels of BMP2 stimulates the development of chondrocytes and osteoblasts [77,78]. Wnt signaling is an important switch in the regulation of adipogenesis. Interestingly, some Wnt family proteins can induce the commitment to the adipocyte lineage[79], while others can inhibit adipocyte differentiation in the late stages of adipogenesis[80]. For example, it has been shown that WNT10B prevents adipocyte differentiation by blocking the expression of PPARγ and C/EBPα[80] (**Figure 6**).

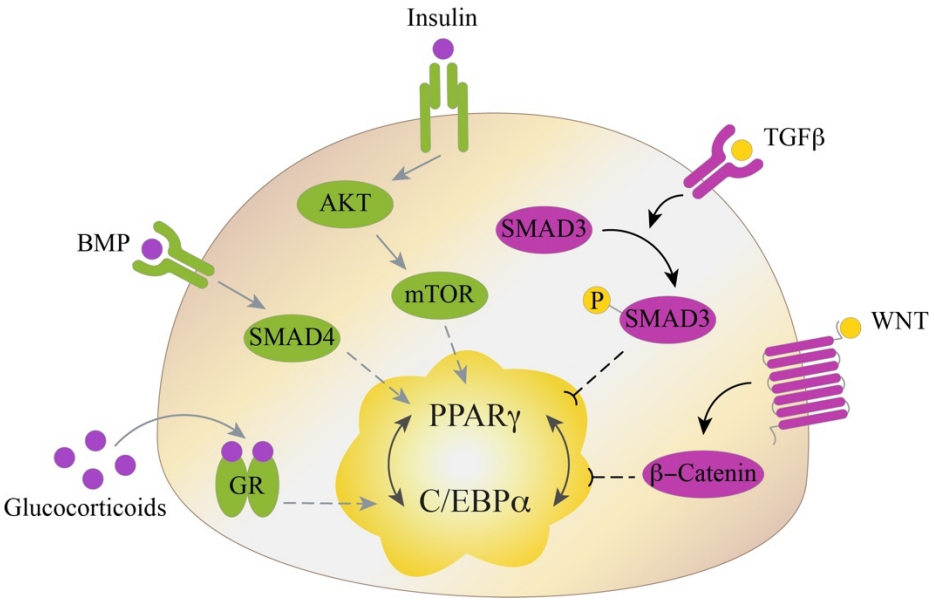

Figure 6. Regulation of adipogenesis by extracellular factors.

After induction, pre-adipocytes undergo clonal expansion, in which the cells synchronously enter into the S phase of the cell cycle and undergo one or two rounds of mitosis. Then the cells exit the cell cycle and begin to accumulate cytoplasmic triglycerides and to express genes involved in lipid metabolism and adipocyte functionality[81].

1.4.2 Transcription factors in adipogenesis

Although brown and white adipocytes have different embryonic origins and physiological functions, both have similar transcription cascades controlling their differentiation into mature adipocytes. There are three transcription factor (TF) families playing pivotal roles in adipogenesis, cooperating to induce a transcriptional cascade that leads to the stable differentiation of the adipocytes. These TFs are PPARγ, C/EBPα, C/EBPβ, C/EBPδ, and adipocytes determination and differentiation dependent factor 1 (ADD-1), also known as sterol regulatory element-binding protein 1 (SREBP-1)[16]. However, during the first stage of adipogenesis, other TFs have important functions in this process, including GR, signal transducer and activator of transcription 5A (STAT5A) and CREB (**Figure 7**).

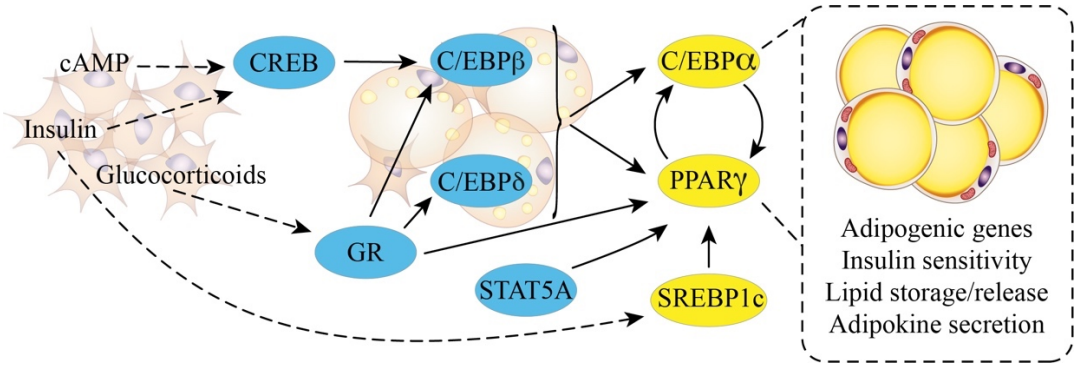

Figure 7. The transcriptional cascade controlling adipogenesis.

C/EBPβ and C/EBPδ are rapidly transcribed directly after the phosphorylation of CREB. Despite the rapid expression upon induction of differentiation, C/EBPβ is initially inactive and only becomes activated to bind DNA around 16 hours post induction. This is generally concomitant with the entry into the S phase and the mitotic clonal expansion[82]. 18-24 hours after the induction, C/EBPβ coordinately activates the expression of PPARγ and C/EBPα through C/EBP regulatory elements in the promoters of their respective genes. Subsequent to their expression, PPARγ and C/EBPα activate an extensive group of genes that trigger the adipocyte phenotype. Further, PPARγ and C/EBPα positively cross-activate each other through their respective C/EBP regulatory elements[83]. Once expressed, PPARγ and C/EBPα also stimulate SREBP1c, which is involved in the regulation of lipidogenesis[84]. The stimulation of thermogenic brown or beige phenotype during adipogenesis is, additionally, driven by specific factors like PR/SET domain 16 (PRDM16) and PPARγ coactivator 1 α (PGC-1α). PRDM16 is a zinc-finger protein that regulates brown fat cell fate by stimulating the expression of brown adipocyte-specific genes and suppressing white adipocyte-specific gene expression. In fact, it has been reported that PRDM16 represses the expression of resistin by interacting with the corepressor C-terminal binding protein-1 and 2 (CtBP1, CtBP2), while the recruitment of PGC-1α to PRDM16 displaces CtBP, allowing PRDM16 to activate the expression of brown fat genes[85]. Furthermore, it has been shown that the interaction of PRDM16 with C/EBPβ leads to brown fate adipocytes[86].

The ability to recruit new fat cells is a critical factor for healthy adipose tissue expansion and metabolic health during obesity. A high generation rate of adipocytes is associated with

hyperplastic adipose tissue and a better metabolic profile in obese individuals[87]. The generation of smaller adipocytes correlates with increased angiogenesis and reduced hypoxic stress and inflammation[88]. Long-term weight regain after bariatric surgery shows an increased number of fat cells and improved insulin sensitivity compared to body weight-matched control subjects[89]. In addition, diabetic patients treated with thiazolidinediones paradoxically experience weight gain, associated to subcutaneous adipocyte hyperplasia, but with a significantly improve metabolic profile[90].

1.4.3 Hypertrophic adipose tissue

During chronic overnutrition the number and size of adipocytes reaches a saturation point in which the adaptive homeostatic mechanisms of the structural remodeling of the adipose tissue fail. This leads to adipocyte hypertrophy and dysfunction characterized by sustained inflammation, impaired secretion of adipokines, exacerbated fibrosis and ectopic lipid accumulation which contribute to the pathogenesis of insulin resistance[91]. Hypertrophic adipocytes are a hallmark of a maladaptive mechanism of WAT enlargement and represent a risk factor for developing type 2 diabetes, in addition to the obese status associated risk[92]. Furthermore, hypertrophic adipocytes show an increased basal rate of lipolysis resulting in an overflow of FFAs and cholesterol to muscle and liver. In this context, the regional distribution and contribution to leakage of FFAs and cholesterol of the different fat pads is very important. It has been shown that adipocytes from visceral fat have bigger lipolytic activity compared to those from subcutaneous depots. Additionally, adipocytes derived from omental adipose tissue of obese individuals have a higher release of FFAs in response to β-adrenergic stimulation compared to controls[93]. FFAs released from visceral fat have direct access to the liver through the hepatic portal system, causing several metabolic disturbances such as impaired insulin metabolism and action, increased gluconeogenesis and release of glucose and altered lipoprotein profiles. Reduced hepatic clearance of insulin triggers hyperinsulinemia that causes downregulation of insulin receptors in skeletal muscle and reduction of glucose uptake. At the same time, the pancreatic beta cells produce more insulin to reduce the glycaemia but over time their degeneration leads to development of hyperglycaemia and type 2 diabetes onset. Furthermore, due to a relative vasculature deficiency, adipocyte hypertrophy induces a local adipose tissue hypoxia[94], which induces a significant change in adipokine production increasing the secretion of pro-inflammatory cytokines and reducing the secretion of leptin and adiponectin[95]. In comparison, the expansion of subcutaneous fat pads occurs by increasing the number of small cells, which are well vascularized and show a "healthy" phenotype[2].

1.5 Epigenetic and transcriptional regulation of adipose tissue plasticity

The definition of epigenetics was first introduced by Conrad Waddington in 1942 as "the branch of biology which studies the casual interactions between genes and their products which bring the phenotype into being"[96]. Since then this original definition has been modified repetitively and today epigenetics is more broadly understood as the study of heritable phenotype changes in the chromosome without alterations in the DNA sequence[97]. Dynamic gene expression, termed as the transcriptome, is important during development, cell and

tissue homeostasis and disease, and can be modulated by several regulatory components. During activation of gene transcription, for example, chromatin adopts a locally accessible and transcriptionally active form referred to as euchromatin, while highly condensed and transcriptionally less active genetic material is known as heterochromatin. Gene expression or repression can be controlled by the covalent DNA methylation or by post-translational modifications of histone tails, including acetylation and methylation. The histone modification status, termed as epigenome, is critical for the creation of the local chromatin environment that induce or repress the gene expression. The transcriptome can be directly regulated by epigenetic modification at cis-regulatory elements such as promoters, defined as proximal TF-binding regions close (<2 kb) to the transcription start site, and enhancers, defined as distal TF binding regions (up to several hundreds of bp away from the transcription start site) that communicate with promoters within topologically associating domains (TADs) to initiate transcription[98]. Additionally, the activity of TFs depends on the recruitment of coactivator or corepressor complexes, which often contain epigenetic chromatin-modifying enzymes as subunits[99,100]. The genome-wide chromatin binding of TFs and coregulators is described as the cistrome (**Figure 8**).

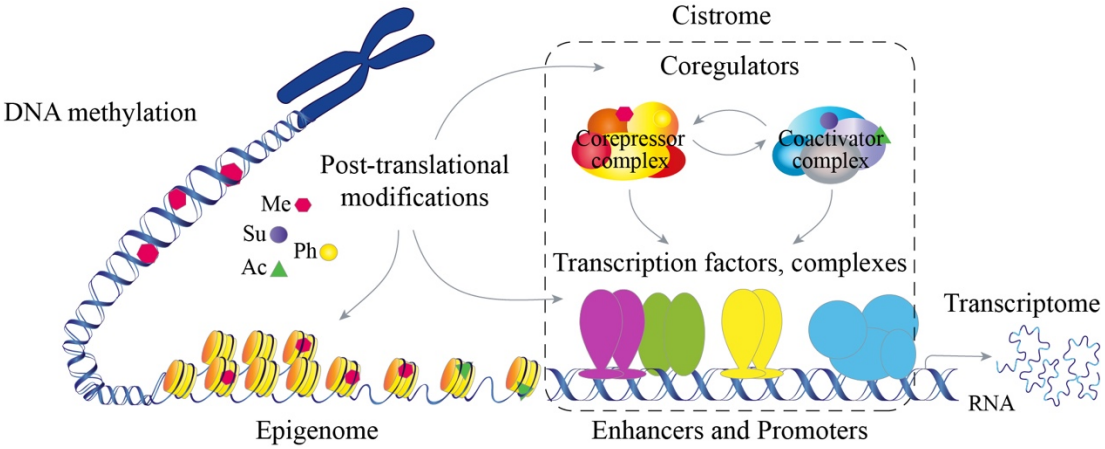

Figure 8. Regulatory components of gene expression.

DNA methylation is a modification that directly targets the DNA by adding a methyl group to the carbon-5 position of cytosine in CpG islands, usually associated with gene silencing. The summary of all genome-wide DNA methylation events in a given cell type can be referred to as the 'methylome'. DNA methylation can be a potential molecular mechanism that results from the interaction between genetics and environment, influences the metabolic homeostasis, and predisposes to metabolic disease[101]. It has been theorized that the nutritional status of the fetus "*in utero*" induces epigenetic reprogramming, preserved through generations, that can affect metabolic health later in life[102]. The children of mothers who were exposed to the Dutch famine during their pregnancy showed altered DNA methylation of the imprinted insulin like growth factor 2 gene[103] and were predisposed to develop obesity and metabolic disease later in life[104]. Moreover, DNA demethylation plays an important role in the lineage commitment of mesenchymal stem cells (MSCs). It has been demonstrated that the treatment of the murine mesenchymal cell line C3H10T1/2 with 5-azacytidine, a methyltransferase inhibitor, results in the demethylation of the *Bmp4* gene, which contributes to the commitment of MSCs to adipocyte lineage[105]. Different studies have further

investigated the association between obesity-related traits and DNA methylation in adipose tissue. It has been shown that omental and subcutaneous adipose tissue have differential methylation in response to gastric bypass before and after weight loss, which is correlated with changes in clinical trait like fasting glucose levels[106]. The DNA methylation comparison of isolated fat cells from women examined 2 years after gastric bypass with never obese weight-matched women showed that 27% of the differentially methylated CpG sites were mapped to genes important for adipogenesis, which could contribute to the adipose hyperplasia observed in post-obese woman[107]. Several lines of evidence indicate that different diets also have an impact on the human methylome. For example, a randomized trial showed that overfeeding with saturated fatty acids (SFAs) or polyunsaturated fatty acids (PUFAs) induces different DNA methylation patterns in human adipose tissue that can predict the weight increase in response to overfeeding[108].

The major covalent modifications of histone tails involve acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation. The summary of these histone modifications, along with DNA methylation, in a given cell type can be referred to as the 'epigenome'. As the epigenome is dynamically regulated and differs from cell type to cell type, one organism has multiple epigenomes despite having only one genome. A particular histone modification can have different effects on gene expression. For instance, histone lysine acetylation is linked to gene activation, while lysine methylation outcomes depends on the site of modification: histone H3 lysine 4 dimethylation or trimethylation is associated with active genes, while histone H3 lysine 9 dimethylation or trimethylation inactivates gene expression[109]. Histone modifications are added or removed by a large number of enzymes, that as part of coregulator complexes contribute to the complex regulation of epigenetic processes determining gene expression[110].

The stimulation of differentiation induces a dynamic modulation of the chromatin landscape. Whole-genome DNase I hypersensitive site (DHS) analysis, which identifies open chromatin regions, showed that in the early stages of adipogenesis C/EBPβ binds with other TFs, such as STAT5A and GR, to particular 'hotspots', inducing chromatin remodeling and expression and recruitment of late-acting adipogenic factors, such as PPARγ (**Figure 9**). Moreover, it has been shown that some of the DHS sites are detected only at day 2 after induction of adipogenesis, and not in mature adipocytes, indicating some genes are induced only transitory. These transient sites are involved in the regulation of the cell cycle and are enriched in C/EBPβ and GR binding sites[111]. When PPARγ is highly activated at the end of adipocyte differentiation, its expression can be regulated via an autoregulatory loop in cooperation with C/EBPα and coregulator complexes[112](**Figure 9**). Another study, using chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq) analysis, showed that the expression of adipogenic genes is related to increased marks of open chromatin, such as histone H3 lysine K4 methylation (H3K4me) and histone H3 lysine K27 acetylation (H3K27ac), in the distal region of these genes[113]. The comparison between mouse and human models during adipogenesis allowed the identification of cis-regulatory elements, i.e. enhancers, which are different between the two models because of the evolutionary turnover of transcription factors sites that is facilitated by the presence of distal regulatory elements at adipocyte-dependent loci[113]. These events are important for both WAT

and BAT, but how the different adipocyte lineages are specifically established remains still not completely understood. It has been shown that around 55% of PPARγ-binding site profiles of adipocytes from epididymal WAT, inguinal WAT and interscapular BAT are overlapping[114]. However, the genome-wide comparison between interscapular BAT and epidydimal WAT shows that 10% of PPARγ-binding sites are specific to BAT[115]. This suggests that the BAT and the WAT-selective gene transcriptional programs are driven by a selective chromatin structure that drives selective PPARγ and C/EBPs recruitment.

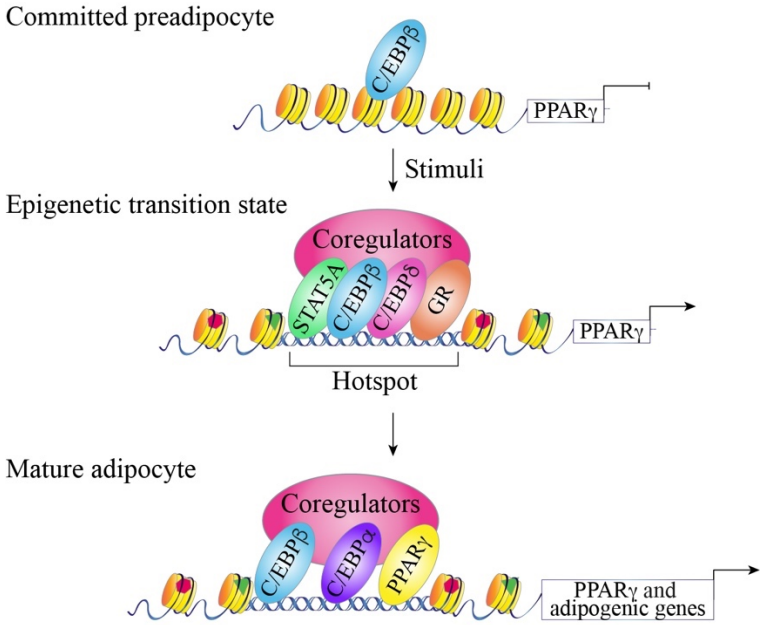

Figure 9. Dynamic modulation of the chromatin landscape during adipogenesis.

1.5.1 Transcriptional coregulators involved in adipocyte biology

Many TF-associated proteins, often acting in multi-protein complexes, have been identified to play a critical role as coactivators or corepressors in determining the activity of TFs at specific gene loci. In general, gene expression is inactive when TFs are not bound to DNA or when DNA-bound TFs are silenced by corepressor complexes. During signal-dependent activation, some TFs can be transcriptionally induced (e.g. C/EBPs), and most TFs undergo post-translational modifications and conformational changes. These alterations then either induce DNA-binding (e.g. in case of GR), or trigger the exchange of corepressors for coactivators (e.g. in case of PPARγ) to subsequently activate the transcription of target gene.

Various coactivators have been characterized to play significant roles in adipocyte biology. The two homologous proteins CREB-binding protein (CBP, gene name *Crebbp*) and p300 regulate adipocyte differentiation and fat accumulation. CBP and p300 have a histone acetyltransferase activity and are considered crucial coactivators that function beyond CREB with a multitude of TFs. Because of these features, they are predominantly found at enhancers to acetylate histone H3 lysine K27, a key histone modification linked to gene activation. Heterozygous *Crebbp* knockout mice showed lipodystrophy and are protected from weight gain induced by high fat diet without affecting the functionality of other

organs[116]. Embryonic fibroblasts from heterozygous *Crebbp* knockout mice had impaired capacity to differentiate to adipocytes due to the reduce activation of C/EBPβ and PPARγ and inhibition of SREBP-mediated lipogenesis. In addition it has been demonstrated that p300 is required for adipocyte differentiation[117].
The thyroid hormone receptor-associated protein (TRAP) complex, today referred to as the mediator (MED) complex, is a multiprotein complex that connects TFs to the basal transcriptional machinery assembled by RNA polymerase II (Pol II). The subunit 1 of the mediator complex, known as MED1 or TRAP220, is a coactivator of PPARγ and important for adipocyte differentiation. Depletion of MED1 in embryonic fibroblasts induces the loss of their adipogenic potential[118]. Importantly, it has been demonstrated that the recruitment of MED1 and p300 proteins is important for the establishment of promoter-enhancer loops at specific target genes and activation of their transcription during adipocyte differentiation[119].

Amongst the different corepressor complexes, of particular interest for adipose tissue remodeling and functionality is the 'histone deacetylase 3 (HDAC3) corepressor complex'. This complex contains the core subunit HDAC3, nuclear receptor corepressor (NCoR, alias NCOR1), silencing mediator of retinoid and thyroid hormone receptors (SMRT, alias NCOR2), transducin β-like protein 1 (TBL1 alias TBL1X), TBL-related 1 (TBLR1, alias TBL1X) and G protein pathway suppressor 2 (GPS2). A variety of transgenic mouse models have increased our understanding of the function of these core subunits in adipose tissue. Adipocyte-specific *Ncor1* knockout mice display overactivation of PPARγ with increased adiposity but optimal insulin sensitivity[120]. On contrast, mutant *Nocr2* mice, lacking the nuclear-receptor binding domain 1 of SMRT (NCOR2), show hypertrophic adipocytes and insulin resistance upon diet-induced obesity[121,122]. Interestingly, the hypertrophic phenotype of these mice was driven by mitochondrial dysfunction due to decreased mitochondrial biogenesis and fatty acid oxidation. A genome-wide DNA-binding profiling study using mouse 3T3-L1 cells showed that SMRT has a role as gatekeeper of adipogenesis[123], consistent with earlier data indicating that the corepressor activity of NCoR and SMRT on PPARγ was crucial for the inhibition of adipogenesis[124]. Two independent adipocyte-specific *Hdac3* knockout models revealed opposite effects on the browning of WAT. Emmett et al. demonstrated that the adipocyte-specific loss of HDAC3 was inducing a deficient WAT remodeling and browning. They suggested HDAC3 to modulate the activity of the coactivator PGC-1α important for *Ucp1* gene transcription[125]. In contrast, Ferrari et al. showed that specific loss of HDAC3 in adipocytes was leading to browning of WAT and increasing the expression of *Ucp1*[126]. Mice lacking TBLR1 in adipocytes showed increased adiposity and insulin resistance likely due to impaired fasting-induced lipolysis[127]. Finally, as discussed in the next chapter, several studies have especially pinpointed the involvement of the subunit GPS2 in adipocyte biology.

1.5.2 GPS2 functions in metabolism and inflammation

GPS2 was originally cloned in 1996 as a human cDNA encoding a potential human suppressor of the G protein pathway[128] and independently identified in yeast two-hybrid screening using PPARα as bait[129]. GPS2 was then biochemically purified and found to be a subunit of the HDAC3 corepressor complex and suggested to inhibit gene repression via

interfering with JNK signaling[130]. GPS2 is a highly conserved and ubiquitously expressed 37 kDa protein, containing 327 amino acids (aa) in mice and humans. Structure analysis has revealed that the N-terminal coiled-coil domain (aa 1-90) of GPS2 forms a three-way corepressor complex core structure with SMRT and TBL1[131]. The C-terminal GPS2 domain (aa 100-327) is suspected to be unstructured and has been demonstrated to function as binding site for TFs, including PPARs[132,133], liver X receptors (LXRs)[134,135] and c-Jun[136]. Both *in vivo* and *in vitro* studies indicate that GPS2 is implicated in the (epi-)genomic modulation of metabolic and inflammatory pathways in different organs like liver, adipose tissue and macrophages[133,136,137]. Some of these studies have provided evidence that GPS2 acts along with the corepressor complex, while others suggest independent roles as gene- and cell type-selective coactivator or as regulator of nongenomic signaling.

In hepatocytes, GPS2 is necessary for the anti-inflammatory action of LRH1 and LXRβ (termed 'trans-repression') of the hepatic acute phase response (APR). It has been reported to act like a bridge between SUMOylated nuclear receptors and the NCoR-containing corepressor complex on acute phase gene promoters. This GPS2-SUMO-nuclear receptor complex is able to stay bound to the promoters even upon inflammatory stimulation, thereby repressing the APR response[134](**Figure 10A**). The hepatic function of GPS2 has also been explored using hepatocyte-specific knockout mouse models. Loss of GPS2 in hepatocytes induced PPARα-dependent lipid catabolism, increasing the expression of fatty acid oxidation genes and thereby protecting the mice from the development of non-alcoholic fatty liver disease (**Figure 10B**). Liver gene expression data from human patients validated the conserved hepatic function of GPS2 in mice and humans where the expression levels of GPS2 were correlated with the expression of fibrogenic and inflammatory genes[138].

Another study demonstrated that GPS2 is an important player in the regulation of cholesterol efflux, coordinating the expression of *ABCG1* in human hepatocytes and macrophages[135]. This study unraveled an unusual mechanism of action of GPS2 as 'pioneer' factor to promote chromatin access of TFs. Upon treatment with LXR agonists, GPS2 cooperates at the *ABCG1* locus with histone lysine demethylases (KDMs) to trigger H3K9 demethylation, LXR DNA-binding and promoter-enhancer communication (**Figure 10C**). Notably, a highly related mechanism was afterwards identified for the recruitment of PPARγ to the promoters of *Atgl* and *Hsl* in 3T3-L1 adipocytes[139]. In this case, GPS2 was demonstrated to inhibit the E2 ubiquitin ligase RNF8, thereby stabilizing the histone demethylase KDM4A and creating the right chromatin environment for the binding of PPARγ to the promoters of selective target genes (**Figure 10D**).

A recent study reported a role for GPS2 in the epigenomic control of macrophage activation during metabolic stress. Macrophage-specific *Gps2*-knockout mice display a pro-inflammatory profile with elevated systemic and adipose tissue inflammation, and reduced insulin sensitivity. The analysis of cistrome, epigenome and transcriptome showed that GPS2 occupies H3K27ac-marked enhancers at regulated genes, and upon removal of GPS2 the response of these genes to inflammatory stimuli was enhanced[136] (**Figure 10E**).

The role of GPS2 in adipose tissue biology is still poorly understood and subject of debate. It has been shown that the expression of GPS2 was down-regulated in the adipocytes of humans with obesity, thereby contributing to chromatin remodeling and transcriptional activation of key inflammatory genes including *IL-6*, *IL-8* and *CCL2*, favoring the inflammation of adipose tissue[137]. The role of GPS2 in controlling the inflammatory response in adipocytes was recapitulated *in vivo* using aP2-GPS2 transgenic mice[140]. In addition, GPS2 may also have a cytoplasmic role preventing the hyper-stimulation of TNFα-induced gene program[140] or regulating the insulin signaling pathway via AKT ubiquitination[141].

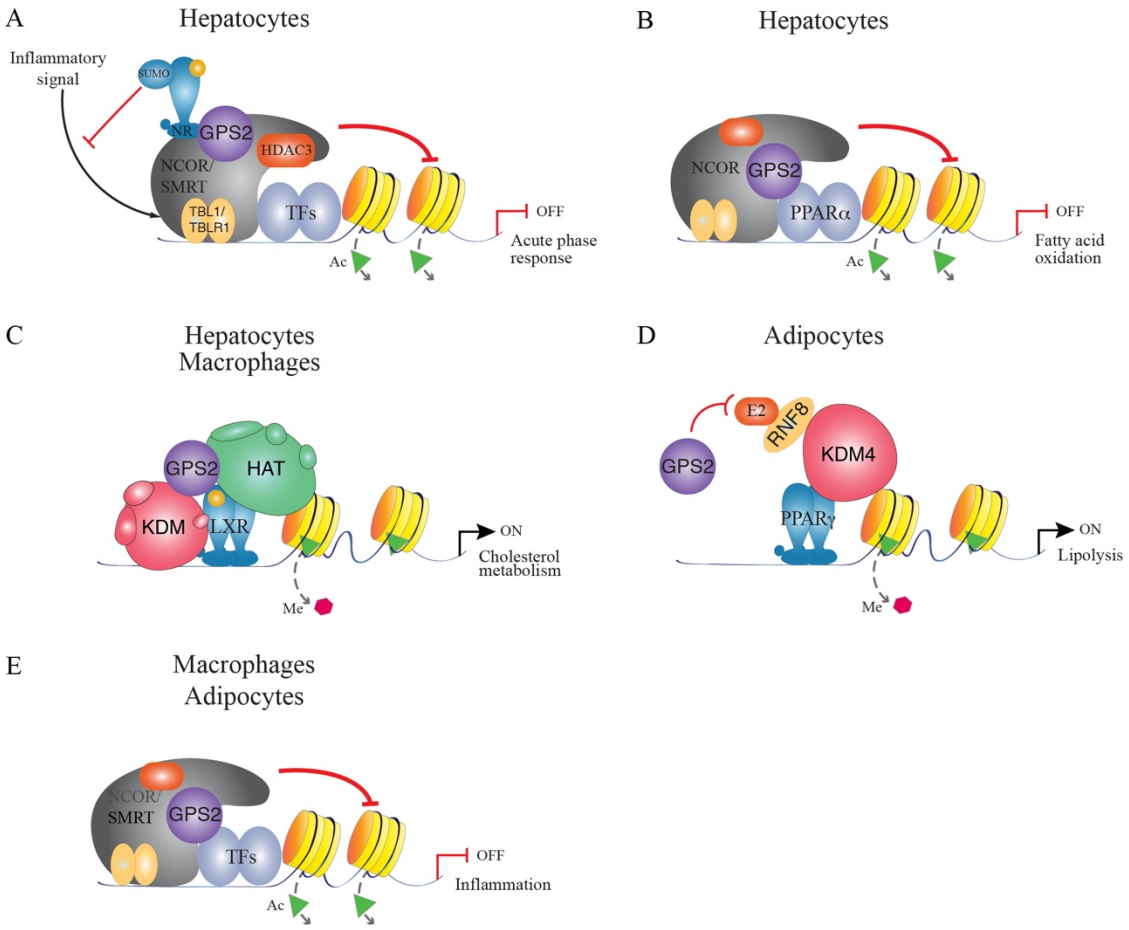

Figure 10. Multiple function of GPS2 in metabolism and inflammation.

2 AIMS OF THE THESIS

The overall aim of this thesis was to extend the current knowledge about the role of GPS2 in adipose tissue biology, with a particular emphasis on mechanisms underlying adipocyte dysfunction in the context of obesity and type 2 diabetes.

The specific aims were:

Paper I

- To explore which pathways are regulated by GPS2 during the *in vitro* differentiation of human adipocytes.

Paper II

- To investigate the mechanisms by which GPS2 controls adipose tissue remodeling during energetic surplus in mice and humans.

Paper III

- To characterize how GPS2-dependent reprogramming of adipose tissue during the progression of type 2 diabetes influences glucose homeostasis.

3 METHODOLOGICAL CONSIDERATIONS

The aim of this chapter is to highlight interesting aspects of key methods. A detailed description of material and methods is provided in each study.

3.1 Patients

All studies were conducted in accordance with the guidelines of the Helsinki Declaration and registered in the public trial registry, Clinicaltrials.gov, under the number NCT02368704. The Ethics Committee of CPP Ile-de-France approved the clinical investigations and written informed consent was obtained from all individuals. Visceral and subcutaneous adipose tissue (VAT and SAT) biopsies were obtained from different populations admitted to the Lariboisière and Geoffroy Saint-Hilaire hospital, Paris, France. VAT and SAT samples from non-obese subjects were obtained after local surgery, while VAT and SAT from obese subjects were obtained during bariatric surgery. The clinical and anthropometric variables of the different populations used for the papers were described in detail in each study.

3.2 Mouse models

Although the complete (conventional) knockout of a gene is of great importance for studying the overall effects of the loss of a specific protein in the whole animal, we decided to generate tissue-specific (conditional) knockout mice using the Cre-lox system for two reasons: firstly because the total body *Gps2* knockout mice are embryonically lethal[132], and secondly because the main question of the research presented in this thesis was to specifically evaluate the effects of the loss of GPS2 in adipocytes and to evaluate the consequences of this disturbance on the cross-talk of the adipose tissue with other organs.

Gps2$^{flox/flox}$ mice were generated at Ozgene Pty, Ltd. (Bentley DC, Australia) using a targeting construct, which contained loxP sites flanking exons 2 and 5 of *Gps2* gene, followed by a FRT site and a neomycin cassette inserted between exons 5 and 6[136]. The targeting vector was electroporated into C57BL/6 Bruce4 embryonic stem (ES) cells. The correctly recombined ES colony was then injected into C57BL/6 blastocysts. Male chimeras were mated with female C57BL/6 mice to get mice with a targeted *Gps2* allele. The mice were crossbred with C57BL/6 flp-recombinase mice to remove the neomycin cassette and to create heterozygous *Gps2*$^{flox/+}$ mice. These mice were then crossbred with C57BL/6 mice for nine generations before being bred with heterozygous *Gps2*$^{flox/+}$ mice to finally obtain the *Gps2*$^{flox/flox}$ mice. *Gps2*$^{flox/flox}$ mice were crossed with *adiponectin*-Cre mice (B6; FVB-Tg(*Adipoq*-Cre)1Evdr/J; Jackson Laboratory stock no. 010803) in order to generate adipocyte-specific *Gps2* knockout mice (AKO). *Gps2*$^{flox/flox}$ mice littermates were used as control (labeled as wild-type, WT).

The use of conditional gene targeting to create mouse models is often matter of debate about the tissue specificity and the efficacy of the gene inactivation. For example, to generate adipocyte-specific knockout mice there are two different Cre lines, the transgenic Cre line

driven by the adiponectin (*Adipoq*) gene promoter, and the transgenic Cre line driven by the adipocyte protein 2 (*aP2*) gene promoter. We decided to use the *Adipoq*-Cre since it has been demonstrated to have a higher tissue specificity compared to the *aP2*-Cre, that has shown recombination in endothelial and nonendothelial cells of heart and skeletal muscle[142].

3.3 *In vitro* models

The usage of *in vivo* mouse models has the advantage of allowing the study of a factor in the physiological context involving crosstalk between multiple tissues and signaling pathways. However, to elucidate specific molecular mechanisms in a particular cell type, it is necessary to use *in vitro* cell-based models. One advantage is that such models often increase the data reproducibility, particular for genomic experiments, and allow better to dissect cell type-specific pathways. Additionally, given possible differences between human and mouse biology, the use of human models is necessary to support human relevance. Finally, cell models are crucial to implement the 3R principle in animal research (replace, reduce, refine).

3.3.1 Human multipotent adipose-derived stem cells

The majority of the experiments in Paper I were made using human multipotent adipose-derived stem (hMADS) cells isolated from prepubic fat pad of a 4-month-old healthy male[143,144]. hMADS cells have a normal karyotype and were used between passages 12 and 24. hMADS cells represent a particularly relevant human cell model as they retain many molecular and functional properties of human white adipocytes, including browning, have a high self-renewal capacity, and can differentiate into mature adipocytes and osteocytes[145]. Adipogenesis from preadipocytes to mature adipocytes does not require the presence of rosiglitazone during the process, a great advantage to study the effect of a factor without the interference of the PPARγ agonist. Unfortunately, hMADS cells proliferate slowly even with the addition of FGF2, and their complete differentiation into mature adipocytes takes more than 14 days.

3.3.2 Mouse 3T3-L1 pre-adipocyte cell line

The 3T3-L1 cell line is a commercially available immortalized pre-adipocyte cell line originally derived from Swiss 3T3 mouse embryos[146]. The main advantage of this model is that they grow rapidly and differentiate into mature adipocytes in only 6 days. 3T3-L1 cells are highly expandable which is advantageous when a sizeable number of cells with low degree of biological variation is necessary. Unlike the hMADS cells, 3T3-L1 are already pre-committed to the adipogenic lineage. Although some key components (e.g. PPARγ, C/EBPs) and pathways relevant for human adipogenesis are likely to be conserved in 3T3-L1 cells, others may not be conserved. This applies in particular to mouse vs. human differences regarding the regulation of gene expression, largely due to genomic (e.g. location of genes) and epigenomic differences (e.g. chromatin modifications, location of cis-regulatory elements such as enhancers).

3.4 RNA interference

RNA interference (RNAi) is a simple, rapid and powerful method to study the cellular function of a specific gene product. The silencing of a specific gene can be achieved by using small interfering RNA (siRNA) or short hairpin RNA (shRNA). Once transfected into a specific cell, the synthetic double-stranded shRNAs are cut into small fragments (siRNA) by the Dicer enzyme. The siRNA is then associated with the RNA-induced silencing complex (RISC) that recognize the corresponding mRNA, cleaves it and causes its subsequent degradation. In the papers presented in this thesis we used either siRNA or shRNA delivered by adenovirus or lentivirus. Since adipocytes have a low transfection rate for siRNAs, the virus-based shRNAs were in some cases a better option for the silencing of target genes. Additionally, siRNAs mediate a transient silencing of the gene of interest, while lentivirus-delivered shRNAs induce stable gene silencing that is more appropriate for the study of long-time treatment or when a large amount of starting material is needed.

3.5 Multi-omics approaches to determine transcriptome, cistrome, epigenome, and lipidome

3.5.1 RNA-sequencing

RNA-sequencing (RNA-seq) is a method that uses next-generation sequencing technology to deeply evaluate the transcriptome profile. RNA-seq allows the evaluation of dynamic changes in expression of known and unknown genes, which cannot be spotted through the use of probe-based microarray methods. In addition, this technique is able to identify unique transcripts such as single-nucleotide polymorphisms or alternative splice variants, that can enhance the understanding of the mechanisms that underlie physiological and pathological conditions. This method has a simple general workflow but the sequence annotation and data interpretation, despite the fact that several bioinformatic programs are available, can be challenging.

3.5.2 Chromatin immunoprecipitation coupled with next-generation sequencing

ChIP-seq is a powerful tool to investigate the regulation of the transcription via mapping protein-DNA binding (cistrome) and epigenetic histone modifications (epigenome) at the genome-wide scale. The protocol for ChIP is composed of several different steps and some of them are critical to obtain a high-quality ChIP-seq profile. For example, in our ChIP we decided to add an additional fixative, the protein-protein cross-linker disuccinimidyl glutarate (DSG), before the standard DNA-protein crosslinker formaldehyde (FA), to increase the efficiency of the ChIP. This may be critical especially for GPS2 that as a coregulator does not directly bind to DNA but requires protein-protein interactions with DNA-bound TFs. Among the most critical factors to obtain a high-quality ChIP is the selection of a high-quality antibody (i.e. excellent target affinity and specificity). In this thesis we used verified (e.g. by ENCODE) commercially available antibodies for histone marks, H3K27ac and H3K4me3, while for GPS2 we used our own custom-made rabbit polyclonal antibodies raised against the N-terminus and the C-terminus[135].

3.5.3 Lipidomics

Lipidomics is an emerging tool that allows for the identification and quantification of cellular lipid molecule species. There are different methods to analyze the lipidome of cells, in the study presented in this thesis we used a mass spectrometry-based technique. The lipidome of eukaryotic cells is important for the cell membrane dynamics and is linked to their biological functions such as storing energy or suppling precursors for bioactive metabolites[147]. The study of the perturbation of the composition of different lipid species finds an interesting application for understanding the mechanisms under lipid metabolism and trafficking in healthy and disease conditions. However, the lipid species annotation and data interpretation remain still very challenging.

4 RESULTS AND DISCUSSION

4.1 Paper I: Loss of G protein pathway suppressor 2 in human adipocytes triggers lipid remodeling through upregulation of ATP binding cassette subfamily G member 1

Adipogenesis plays a critical role in adipose tissue remodeling and is an important influencing factor in the development of obesity. Multiple studies have elucidated the mechanisms by which TFs orchestrate adipogenic pathways, but it is still poorly understood how coregulators transduce regulatory signals into epigenome alterations and transcriptional responses. In this study, we combined different methodologies to investigate the contribution of the coregulator GPS2 to human adipocyte differentiation.

GPS2 depletion increases adipogenic commitment

To explore the function of GPS2 during adipogenesis, we generated an *in vitro* model using human multipotent adipose-derived stem (hMADS) cells. We depleted GPS2 using RNA interference and followed the effect of this depletion along the differentiation process. Transcriptome analysis of the early event of differentiation revealed that the loss of GPS2 increased the transcription of genes important for the commitment towards the adipogenic lineage, such as BMP4, and inflammatory genes. Interestingly, the transcriptome changes were correlated with the epigenome changes, i.e. H3K27ac levels were higher in shGPS2-upregulated genes and lower in shGPS2-downregulated genes. In addition, the cistrome analysis revealed enrichment of putative DNA-binding motifs for AP-1 and C/EBP family members at GPS2-occupied chromatin regions. Furthermore, in absence of GPS2 the cells had a higher response to the induction phase of differentiation to adipocytes.

Loss of GPS2 increases expression of genes implicated in lipid metabolism

To further investigate the changes upon loss of GPS2 in fully differentiated adipocytes, we analyzed the transcriptome, epigenome and cistrome of the lipid-filled adipocytes. GPS2 depletion increased the expression of adipocyte marker genes, including *PPARG*, *CEBPA* and *FABP4*. The pathway analysis of the upregulated genes in shGPS2 adipocytes showed enrichment for different metabolic processes such as fatty acid or steroid metabolism. The integrated analysis of the GPS2-dependent transcriptome and epigenome showed that their changes were highly correlated. Additionally, the TF DNA-binding motif analysis revealed that binding sites for C/EBPα, PPARγ and TWIST1 were among the top motifs enriched at GPS2-occupied chromatin regions.

GPS2 depletion induces adipocyte hypertrophy and changes in phospholipid composition

To explore the phenotype of mature adipocytes upon depletion of GPS2, we stained neutral lipids with Oil Red O and BODIPY. We discovered that the loss of GPS2 in preadipocytes significantly increased triglyceride accumulation within adipocytes, hypertrophy and the percentage of differentiated cells. Lipidome analysis showed that the removal of GPS2 induced a depletion of sphingomyelin and an enrichment of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), important lipids for membrane remodeling. Noteworthy,

shGPS2 adipocytes showed a reduction of phospholipids containing PUFAs and enrichment of monosaturated PC and PE species. The phospholipid phenotype was further confirmed by the transcriptome analysis. We found that GPS2 depletion increased the expression of genes encoding for enzymes involved in the synthesis of PC and PE, while it decreased the expression of genes encoding for enzymes involved in the synthesis of sphingolipids.

ABCG1 and *LPL* as targets of GPS2 in adipocytes

Previous studies have demonstrated that the loss of sphingomyelin is modulated by ABCG1[32] and that ABCG1 plays a critical role in lipid homeostasis[148,149]. Since we observed a reduction of sphingomyelin in our shGPS2 cells, we investigated the effect of GPS2 depletion on the expression and activity of ABCG1. The loss of GPS2 significantly increased ABCG1 mRNA and protein levels starting from day 6 of differentiation. Moreover, we found increased H3K27ac levels at the *ABCG1* promoter and enhancer regions upon removal of GPS2, these altered regulatory regions were co-occupied by GPS2, C/EBPα and PPARγ. Interestingly, we found that the increased expression of *ABCG1* was observed not only when GPS2 was depleted in preadipocytes but also when we depleted GPS2 in mature adipocytes. The presence of sphingomyelin on the plasma membrane has been also reported to modulate the LPL activity which is important for the triglyceride accumulation. We found that the loss of GPS2 significantly increased the expression of *LPL*, the H3K27ac levels on the promoter and enhancer regions of the *LPL* locus and that these altered regulatory regions were also co-occupied by GPS2, C/EBPα and PPARγ. Furthermore, we observed increased intracellular and extracellular LPL activity upon removal of GPS2 (**Figure 11**).

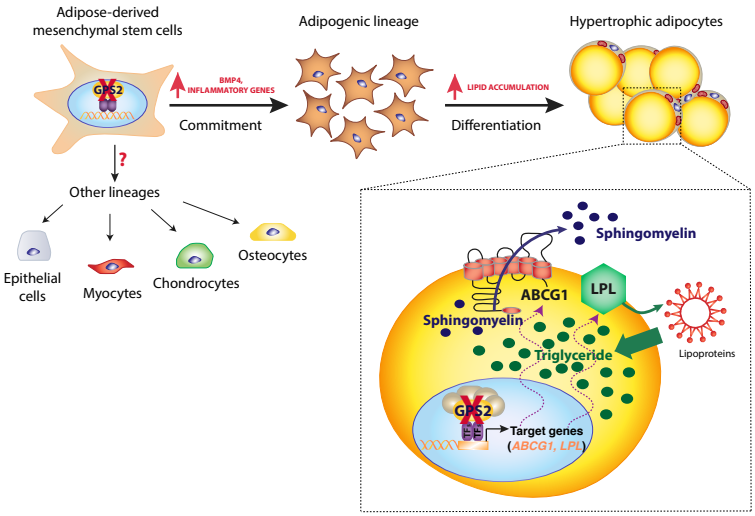

Figure 11. Graphical abstract of Paper I. Reprinted from Barilla et al. Molecular Metabolism (2020)

GPS2 expression correlates with ABCG1 expression and diabetic status

To explore whether our *in vitro* findings are relevant for human disease, we analyzed the transcriptome of the omental adipose tissue of obese individuals with or without type 2 diabetes. We found that the expression levels of *GPS2* were lower in diabetic obese compared to the non-diabetic obese individuals. The comparison between the upregulated genes in diabetic versus non-diabetic obese with the upregulated genes in shGPS2 versus shGFP

hMADS cells showed that 165 genes were overlapping in these two conditions. These common genes belonged to inflammatory pathways as well as lipid metabolism. Remarkably, we found that the expression of *ABCG1* was higher in the diabetic condition compared to the non-diabetic one and was inversely correlated with the expression of *GPS2,* independently from the diabetic status. Therefore, we conclude that GPS2 modulates the chromatin landscape and gene expression during differentiation of human adipocytes. In addition, we identify a hitherto unknown GPS2-ABCG1 axis that is potentially linked to adipocyte hypertrophy in humans.

4.2 Paper II: GPS2 deficiency triggers maladaptive white adipose tissue expansion in obesity via HIF1A activation

Obesity is a complex metabolic disease strongly associated with the development of type 2 diabetes. White adipose tissue remodeling in response to nutritional status plays an important role in maintaining the systemic metabolic homeostasis. It has been shown that hypertrophic adipocytes are often linked to dysfunctional adipose tissue and are a predisposing factor for the development of obesity comorbidities. However, the molecular mechanisms that predispose to hypertrophic adipose tissue are poorly understood. Several studies proposed that the alteration of the epigenome, orchestrated by transcription factors and coregulators, may have a critical role in the remodeling of adipose tissue.

To explore the role of GPS2 in adipose tissue remodeling during energetic surplus, we generated adipocyte-specific *Gps2* knockout (AKO) mice and challenged them with high-fat diet. AKO mice displayed increased adipocyte size and unhealthy adipose tissue expansion, characterized by impairment of insulin sensitivity, higher inflammation and collagen deposition. The transcriptome analysis also revealed that the genes highly expressed in AKO mice belonged to HIF1A-dependent pathways, in particular we observed increased expression of *Hif1a* and its target genes *Vegfa* and *Angptl4*. Next, we demonstrated that GPS2 was bound to the promoter of the aforementioned genes and that the loss of GPS2 increased H3K4me3 levels, marker of active transcription, on *Hif1a* promoter. Concomitantly, primary adipocytes isolated from AKO mice under hypoxic condition showed increased expression of hypoxia-response genes.

Increased activity of HIF1A is known to provoke mitochondrial dysfunction, that leads to impairment of fatty acid oxidation and contributes to adipocyte hypertrophy[150,151]. The transcriptome analysis showed a significant downregulation in the expression of genes involved in mitochondrial function and biogenesis in WAT of AKO mice. This result was confirmed by the quantification of the mitochondria staining within the WAT. To further explore if mitochondrial dysfunction was a consequence of GPS2 depletion we challenged the mice with a β_3-adrenergic receptor agonist or cold exposure. WAT from WT mice responded to the treatment with increased mitochondrial biogenesis and adipose tissue browning characterized by UCP1 staining. In contrast, this response was strongly impaired in the WAT from AKO mice. Alongside, we observed a significant decrease of body temperature and oxygen consumption in AKO mice compared to the WT littermates. Interestingly, the treatment with a pharmacologic inhibitor of HIF1A reversed the pro-

diabetic phenotype of AKO mice and restored the response to the β_3-adrenergic receptor stimulation.

To investigate the human relevance of our mouse discoveries, we measured the mRNA level of GPS2 and HIF1A in the subcutaneous and visceral adipose tissue of non-obese and obese subjects with or without diagnosed type 2 diabetes. We found that the expression of *GPS2* was significantly reduced in non-obese and obese subjects with type 2 diabetes while the expression of *HIF1A* was significantly increased in diabetic subjects. Moreover, we found that in obese individuals the expression of *GPS2* was inversely correlated with the average adipocyte size and this correlation was independent from the diabetic status. Hence, we can propose that the loss of GPS2 associated with the development of obesity predisposes to a maladaptive WAT expansion and a pro-diabetic phenotype in mice and humans (**Figure 12**).

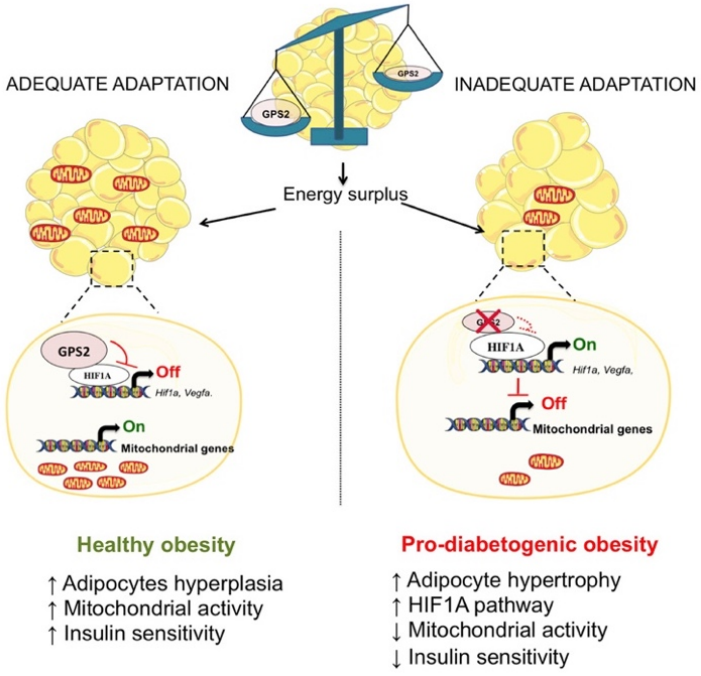

Figure 12. Graphical abstract of Paper II. Reprinted from Drareni et al. Cell Reports (2018)

4.3 Paper III: Adipocyte reprogramming by the transcriptional coregulator GPS2 impacts beta cell insulin secretion

Glucose homeostasis is regulated by a coordinated organ crosstalk that controls the secretion of insulin and glucagon to maintain a narrow physiological level of blood glucose. During type 2 diabetes, this coordinated regulation is altered, leading to inappropriate insulin secretion by the beta cells of the pancreas and glucose intolerance. The reprogramming of white adipose tissue during type 2 diabetes progression is an important factor that can affect the function of the beta cells. Adipose tissue-secreted factors play a pivotal role maintaining glucose homeostasis, but the critical regulatory components modulating their secretion remain poorly characterized.

To investigate the impact of GPS2 action in WAT on insulin secretion rates in humans, we measured the mRNA levels of *GPS2* in subcutaneous WAT of different subjects presenting normal glucose tolerance or type 2 diabetes, which were subjected to graded glucose infusion in order to measure insulin secretion rates. We found that participants with high expression of *GPS2* in the adipose tissue had more pronounced insulin secretion in response to glucose infusion compared to the subjects with low expression. However, we did not find any correlation between *GPS2* levels in WAT and glucagon secretion. In accordance, we found similar association in adipocyte-specific *Gps2* knockout mice (AKO) under high-fat diet.

To explore the mechanism underlying the inadequate insulin secretion triggered by the loss of GPS2 in adipose tissue, we evaluated the size and the number of pancreatic islets in WT and AKO mice. We observed a decreased size of the pancreatic islets in AKO mice under high-fat diet in compared to WT animals. This phenotype was accompanied by increased macrophage infiltration, increased beta cell apoptosis and decreased beta cell proliferation. We therefore considered that secreted factors from WAT of AKO mice were influencing islet function.

The transcriptome analysis of different adipose depots revealed that the most up-regulated genes in the WAT from AKO mice were involved in immune response and insulin secretion. This pathologic gene signature was then confirmed by the analysis of the protein level of adipokines and inflammatory cytokines in serum and WAT explant media. Serum and WAT explant media from AKO mice showed decreased level of adiponectin and increased levels of leptin, resistin and inflammatory cytokines such as IL-6 and CCL2/MCP-1 compared to those from WT mice. Thus, our data suggest that GPS2 controls adipose tissue remodeling which influences pancreatic islet function and insulin secretion in mice and humans (**Figure 13**).

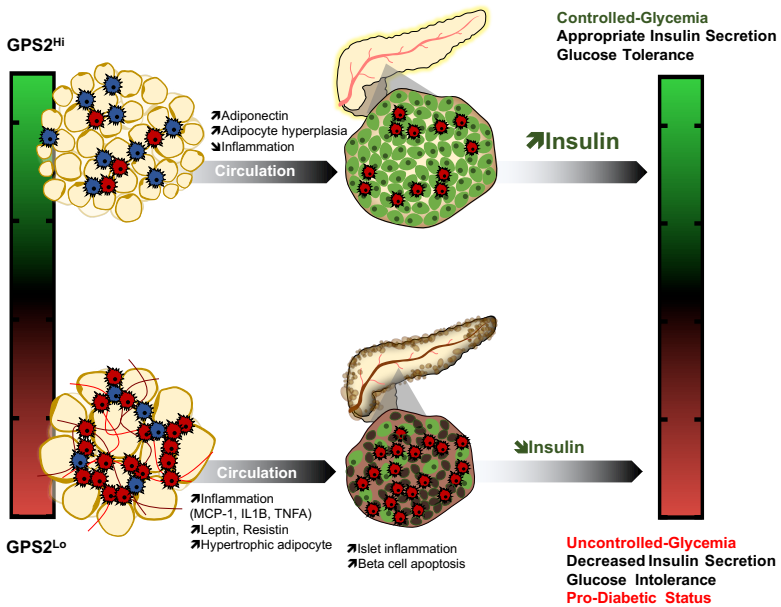

Figure 13. Graphical abstract of Paper III. Reprinted from Drareni et al. Cell Reports (2018)

5 CONCLUSIONS AND PROSPECTIVES

The three studies included in this thesis focused on the role of GPS2 in adipocyte function and metabolism during obesity and associated type 2 diabetes. We used *in vitro* and *in vivo* models to investigate cell type-specific mechanisms by which GPS2 potentially links epigenome alterations to metabolic diseases.

Paper I

- We demonstrated that the loss of GPS2 in hMADS cells leads to coordinated changes of the chromatin landscape and gene expression during adipocyte differentiation.
- The depletion of GPS2 in hMADS cells increases the expression of genes, including BMP4, that trigger adipogenic commitment but also upregulates ABCG1 and LPL expression in mature adipocytes contributing to adipocyte hypertrophy and lipidome remodeling including sphingomyelin depletion.
- *GPS2* and *ABCG1* levels in omental adipose tissue inversely correlate with type 2 diabetes in obese humans.

In summary, we propose a model in which loss of GPS2 in hMADS cells triggers, in the early stage of differentiation, the commitment of fibroblast-like progenitors towards the adipogenic lineage and, in the late stage of adipogenesis, adipocyte hypertrophy with a deep remodeling of their lipidome. The hypertrophic phenotype, linked to increased triglyceride accumulation, was triggered by increased expression of *ABCG1* and *LPL*, likely via de-repression of their promoters and enhancers, and increased LPL activity, likely via sphingomyelin depletion.

This study provides novel mechanistic insights into the transcriptional and epigenetic regulation of adipogenesis and adipocyte hypertrophy in humans, and into the changes that occur in the WAT during the development of obesity and type 2 diabetes. In particular, the identified GPS2-ABCG1 pathway emphasizes that the network of coregulators and TFs is often cell type-selective and may offer possibilities for the future therapeutic intervention against adipocyte hypertrophy in humans.

Paper II

- We have established that the specific loss of GPS2 in mouse adipocytes predisposes to maladaptive adipose tissue expansion during energy surplus characterized by adipocyte hypertrophy and alteration of the HIF1A pathways.
- The dysregulation of the GPS2-HIF1A interplay induces increased expression of *Hif1a* and its target genes. Consequently, this provokes disrupted mitochondrial activity that favors inadequate adipose tissue expansion and a pro-diabetic status.
- Correlation analysis confirms a causal relationship between adipose tissue *GPS2* levels, hypertrophic adipocytes and *HIF1A* in humans.

In summary, we propose a mechanism by which GPS2 acts as a corepressor of HIF1A to control adipose tissue expansion that predisposes to the pro-diabetic status. The de-repression

of HIF1A induced a mitochondrial dysfunction that can be corrected by the pharmacological inhibition of this TF.

This study highlights how alterations of a single regulatory factor, the corepressor GPS2, triggers adipocyte transcriptional reprogramming determining the individual pathophysiological responses, such as adipocyte hypertrophy, to a common disease environment like obesity and type 2 diabetes. Additionally, our study advances the understanding of HIF1A activity control, which is already known to be important for therapeutic possibilities. The specific modulation of the GPS2-HIF1A pathway in adipocytes, could be used therapeutically to modulate adipose expansion during obesity.

Paper III

- We have shown that the expression of *GPS2* mRNA in adipose tissue is positively associated with the insulin secretion rate in humans.
- The use of adipocyte-specific *Gps2* knockout mice allowed us to demonstrate that the loss of GPS2 in adipocytes has an impact on insulin secretion upon diet-induced obesity. Moreover, this model allowed us to demonstrate that the deregulation of beta cell function, observed in *Gps2* knockout mice, was mediated by adipokines released by the adipose tissue that increased islet inflammation and cell apoptosis.

Our findings suggest that GPS2 is an important regulator of the transcriptional signature of adipokines and inflammatory genes in adipose tissue. The depletion of GPS2 leads to coordinated changes of transcription in adipocytes that influences the functionality of the beta cells in the pancreas. Our study thus specifically advances the understanding of crosstalk between adipose tissue and pancreatic beta cells that predisposes to inadequate insulin secretion and the progression towards a pro-diabetic status. Importantly, our work implies that the underlying mechanisms are conserved between mice and humans, suggesting therapeutic possibilities to modulate insulin secretion via targeting GPS2 pathways in adipose tissue.

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