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**The transcription factor Prep1  
regulates adipose tissue functions**

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*To my family*



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## **LIST OF PUBLICATIONS**

This dissertation is based upon the following publications:

Oriente F, Cabaro S, **Liotti A**, Longo M, Parrillo L, Pagano TB, Raciti GA, Penkov D, Paciello O, Miele C, Formisano P, Blasi F, Beguinot F. *PREP1 deficiency downregulates hepatic lipogenesis and attenuates steatohepatitis in mice*. Diabetologia. 2013 Dec;56(12):2713-22.

Valentino R, D'Esposito V, Passaretti F, **Liotti A**, Cabaro S, Longo M, Perruolo G, Oriente F, Beguinot F, Formisano P. *Bisphenol-A Impairs Insulin Action and Up-Regulates Inflammatory Pathways in Human Subcutaneous Adipocytes and 3T3-L1 Cells*. PLoS One. 2013 Dec 9;8(12):e82099.





## **LIST OF ABBREVIATIONS**

ACC: acetyl CoA carboxylase	LIP: liver-enriched inhibitory protein
AMPK: AMP-activated protein kinase	LKB1: liver kinase B1
aP2/FABP4: adipocyte protein 2/fatty acid binding protein 4	LPL: lipoprotein lipase
BAT: brown adipose tissue	LPS: lipopolysaccharide
C/EBPs: CCAAT/enhancer-binding proteins	MAP kinase: mitogen-activated protein kinase
DXM: Dexamethasone	MIX: methylisobutylxanthine
ERK: extracellular-signal-regulated kinases	MSC: mesenchymal stem cell
FA: fatty acid	mTOR: mammalian target of rapamycin
FAS: fatty acid synthase	NASH: Non Alcoholic Steatohepatitis
FATPs: fatty acid transport proteins	p160MBP: p160 Myb-binding protein
FFA: free fatty acid	Pbx1: pre-B cell leukemia transcription factor
GC: glucocorticoid	PGC-1 $\alpha$ : PPAR $\gamma$ co-activator 1
GLUT4: glucose transporter type 4	PI3-K: phosphatidylinositol 3-kinase
HSL: hormone-sensitive lipase	PKA: protein kinase A
IL6: interleukin 6	PKB/Akt: protein kinase B
IR: insulin receptor	PKC: protein kinase C
IRS1-4: insulin receptor substrate 1-4	PPARs: peroxisome proliferator-activated receptors
LAP: liver-enriched transcriptional activator protein	

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*LIST OF ABBREVIATIONS*

Prep1: Pbx regulating protein 1

PTEN: phosphatase and tensin homolog deleted on chromosome ten

SCD1: stearyl CoA desaturase

SHIP2: SH2-containing inositol 5'-phosphatase 2

SHP1: SH-PTP1 Src homology domain tyrosine phosphatase

SREBP: sterol regulatory element-binding protein

SVC: stromal vascular cells

SYP: SH-PTP2 Src homology domain tyrosine phosphatase

T2D: type 2 Diabetes

TALE: three-amino acid loop extension

TG: triglyceride

TNF $\alpha$ : tumor necrosis factor alpha

UEF-3: urokinase enhancer factor-3

uPA: urokinase plasminogen activator

WAT: white adipose tissue

**ABSTRACT**

Adipose tissue is crucial for maintaining energy and metabolic homeostasis and its functionality is closely related to the adipocytes differentiation state. Adipogenesis is regulated by several transcription factors. *Prepl* is an homeodomain transcription factor belonging to the TALE proteins, which plays an important role in hematopoiesis, organogenesis and development. Previous studies have indicated that *Prepl* hypomorphic heterozygous (*Prepl*<sup>i/+</sup>) mice, which express only 55-57% of protein, have a complex metabolic phenotype. In fact, these mice present smaller but otherwise normally structured islets with reduced fasting and post-loading plasma insulin levels and increased insulin sensitivity in skeletal muscle and in liver which is accompanied by protection from streptozotocin-induced diabetes. In addition, *Prepl* deficiency in mice induces a reduction of hepatic triglycerides synthesis and a protection from methionine and choline-deficient diet-induced steatohepatitis.

In this study, I focused my attention on the role of *Prepl* on the regulation of adipocyte differentiation and on the adipose tissue functionality.

To understand the possible role of *Prepl* in adipose tissue, I first evaluated the features of adipose tissue of *Prepl*<sup>i/+</sup> and WT mice. *Prepl*<sup>i/+</sup> mice show a reduction of adipose tissue weight, a reduction of the area but no change in the number of adipocytes. In addition, expression of adipogenic markers, C/EBP $\alpha$ , GLUT4 and FABP4, is increased in adipose tissue of *Prepl*<sup>i/+</sup> mice, while PPAR $\gamma$  does not change. Consistent with these data, upon insulin stimulation, insulin receptor (IR), AKT and MAPK phosphorylation is increased in adipose tissue of *Prepl* hypomorphic heterozygous mice. In addition, basal and insulin-stimulated glucose-uptake is increased in adipocytes isolated from adipose tissue of *Prepl*<sup>i/+</sup> mice compared to the adipocytes from WT mice. The increased basal uptake is fully consistent with the higher expression of GLUT4 on the plasma membrane of adipocytes of *Prepl*<sup>i/+</sup> mice compared with that of control animals.

To further study the function of *Prepl* on adipocyte differentiation, I have analyzed *Prepl* expression during different steps of adipogenesis in 3T3L1 murine cells. Levels of Prepl are progressively reduced during the conversion from 3T3L1 preadipocytes to adipocytes. Moreover, 3T3L1 adipocytes stably transfected with *Prepl* cDNA display reduced lipid accumulation, and expression of C/EBP $\alpha$ , GLUT4 and FABP4. Interestingly, insulin molecular signaling pathway is less activated in presence of *Prepl*.

All together these data suggest that *Prepl* regulates adipocyte differentiation, giving a rationale to investigate *Prepl* as possible new therapeutic agents in preventing adipose tissue dysfunctions.

## SOMMARIO

Il tessuto adiposo è un organo che regola l'omeostasi energetica e metabolica e la sua funzionalità è strettamente correlata allo stato differenziativo degli adipociti.

Prep1 è un fattore di trascrizione ad omeodominio appartenente alla famiglia delle proteine TALE (Three Aminoacid Loop Extension), i cui membri sono coinvolti nella formazione e nello sviluppo di diversi organi. Studi recenti hanno dimostrato che i topi eterozigoti ipomorfi per *Prep1* (*Prep1<sup>i/+</sup>*), che esprimono tra il 55-57% della proteina, hanno un fenotipo metabolico particolare. Infatti, tali topi mostrano una riduzione delle isole pancreatiche associata alla riduzione dei livelli plasmatici di insulina e all'aumento della sensibilità insulinica nel muscolo e nel fegato. In aggiunta, i ridotti livelli di espressione nel topo *Prep1<sup>i/+</sup>* inducono la riduzione della lipogenesi epatica e la protezione dalla steatoepatite indotta da dieta carente in metionina e colina. In questo progetto, ho focalizzato la mia attenzione sullo studio del ruolo del fattore di trascrizione *Prep1* nella regolazione del differenziamento adipocitario e nella funzionalità del tessuto adiposo.

Al fine di comprendere il possibile coinvolgimento di *Prep1*, ho valutato alcune caratteristiche del tessuto adiposo di topi *Prep1<sup>i/+</sup>* e di controllo. I topi *Prep1<sup>i/+</sup>* mostrano la riduzione del tessuto adiposo epididimale e dell'area adipocitaria. In aggiunta, l'espressione dei markers di differenziamento adipocitario, C/EBP $\alpha$ , GLUT4 e FABP4 è aumentata nel tessuto adiposo dei topi eterozigoti per *Prep1*, invece l'espressione di PPAR $\gamma$  non varia. Coerentemente a questi dati, in seguito a somministrazione intraperitoneale di insulina, la fosforilazione del recettore dell'insulina (IR), di AKT e MAPK è aumentata nel tessuto adiposo dei topi *Prep1<sup>i/+</sup>*. In aggiunta, la captazione di glucosio basale e insulino stimolata è aumentata in adipociti maturi isolati da tessuto adiposo di topi *Prep1* eterozigoti ipomorfi. L'aumento basale di captazione del glucosio è giustificato da un' aumentata espressione di GLUT4 sulla membrana plasmatica degli adipociti dei topi *Prep1<sup>i/+</sup>*. Per meglio caratterizzare il ruolo di *Prep1* nel differenziamento adipocitario, ho valutato il livello di espressione proteica di Prep1 durante il differenziamento adipocitario. Esperimenti di Western Blot mostrano la riduzione significativa del livello proteico di Prep1 durante la conversione di fibroblasti 3T3L1 in adipociti maturi. Inoltre, la sovrpressione di Prep1 in adipociti 3T3L1 causa una significativa riduzione dei vacuoli lipidici intracellulari dopo colorazione con Oil Red-O e dell'espressione di C/EBP $\alpha$ , GLUT4 e FABP4. In aggiunta, adipociti 3T3L1 iperesprimenti *Prep1* mostrano una ridotta capacità di risposta allo stimolo insulinico. Da questi dati è possibile supporre un ruolo di Prep1 nella regolazione dello stato differenziativo degli adipociti e nella funzionalità del tessuto adiposo.





# 1. BACKGROUND

## 1.1 Adipose Tissue

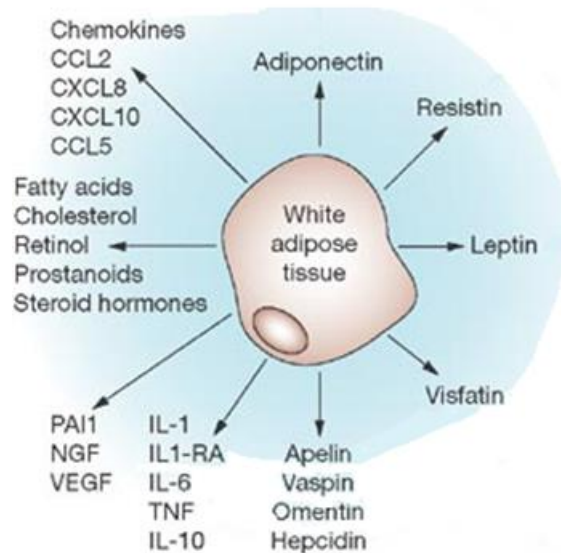
Adipose tissue is the most prevalent tissue in the human body. It is commonly found in subcutaneous loose connective tissue and also surrounds internal organs. Mature adipocytes constitute the majority of cells in adipose tissue. Beside adipocytes, fat tissue contains several other cells types, including stromal-vascular cells (SVC) such as fibroblasts, smooth muscle cells, pericytes, endothelial cells and adipogenic progenitor cells or preadipocytes. Recent research shows that adipose tissue plays a more dynamic role than previously recognized in physiological processes of the whole body (Niemelä et al. 2008).

Adipose tissue is divided into two subtypes: white and brown fat. White adipose tissue (WAT), the predominant type of adipose tissue in humans, is characterized by adipocytes with a single lipid inclusion and eccentrically located nucleus (Ahima & Flier 2000). It has long been recognized as the main site of storage of excess energy derived from food intake. White adipocytes store dietary energy in a highly concentrated form as triglyceride. By contrast, brown fat is specialized primarily for non-shivering thermogenesis. Brown adipocytes are characterized by multiple, smaller droplets of triglyceride, which are accessible for rapid hydrolysis and rapid oxidation of the fatty acids. Brown fat depots are present in human infants and recent evidences suggest that dispersed brown adipocytes might persist in adults (Guilherme et al. 2008).

White adipose tissue may be found in subcutaneous and visceral compartments and the respective adipocytes show different molecular and physiological features. In fact, increased visceral adipose tissue is associated with an increased risk of insulin resistance and cardiovascular disease, whereas increased subcutaneous adipose tissue is not. Adipocytes in visceral depots are sensitive to lipolytic stimuli, whereas adipocytes from structural depots (such as around the eyes and in the heel pads) do not easily release stored lipid. In addition, visceral adipose tissue accumulates more macrophages in the inflammatory status and secretes higher levels of pro-inflammatory cytokines (Rosen & MacDougald 2006).

Fat tissue plays an important role in numerous processes through its secretory products and endocrine functions. Adipocytes secrete several factors known to play a role in immunological responses, vascular diseases and appetite regulation. For example, leptin is a peptide hormone primarily made and secreted by mature adipocytes, and it has various biological activities, including appetite, food intake and body weight regulation, fertility,

reproduction and hematopoiesis (Niemelä et al. 2008). In addition, adipose tissue secretory products act in an autocrine and paracrine way to regulate adipocyte metabolism and growth, as well as to regulate energy homeostasis (Figure 1).



**Figure 1: The multiple functions of white adipose tissue include the synthesis and secretion of molecules.**

WAT produces more than 50 cytokines and other molecules. These adipokines engage, through endocrine, paracrine, autocrine or juxtacrine mechanisms of action, in a wide variety of physiological processes.

Adapted from Lago F et al. *Nature* 2007. 3: 716–724

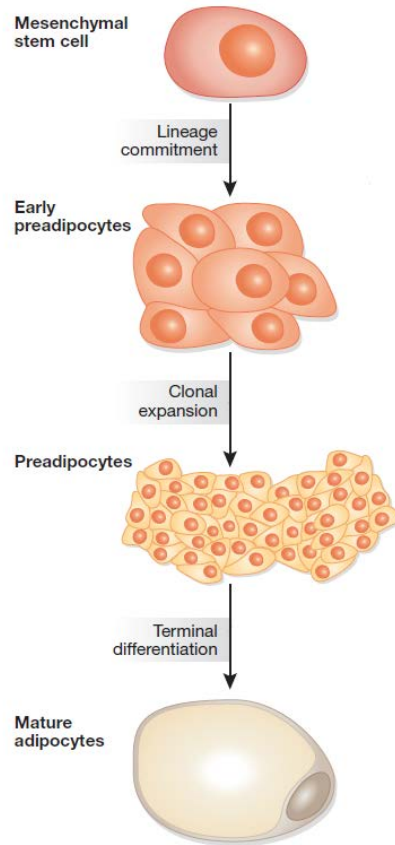
Despite the different function and distribution, adipocytes from the two types of white adipose tissue share many common differentiation features. For example, all adipocytes derive from stem cells in a process known as adipogenesis (Cristancho & Lazar 2011).

## 1.2 Adipogenesis

Adipocytes derive from pluripotent mesenchymal stem cells (MSCs) having the capacity to develop into several cell types, i.e., adipocytes, myocytes, chondrocytes and osteocytes. These stem cells reside in the vascular stroma of adipose tissue as well as in the bone marrow, and, when



appropriately stimulated, undergo a multistep process of commitment in which the progenitor cells become restricted to the adipocyte lineage. Recruitment to this lineage gives rise to preadipocytes that then differentiate into adipocytes (Tang & Lane 2012) (Figure 2).



**Figure 2: Stages of adipocytes differentiation.**

Multipotent stem cell with the capacity to differentiate along mesenchymal lineages of myoblast, chondroblast, osteoblast and adipocyte, gives rise to preadipocyte. When exposed to appropriate environmental and gene expression conditions, these cells undergo clonal expansion and subsequent terminal differentiation; cells enlarge in size while accumulating lipid vacuoles that coalesce and eventually fill the cells.

Adapted from Romao JM et al. *Exp Biol Med* 2011. 236(9):997-1004; Ricoult SJ & Manning BD. *EMBO Rep.* 2013. 14(3):242-51.

White adipose tissue compartments begin to develop in late gestation and the rate of adipogenesis rapidly surges in response to increased nutrient availability, leading to a marked postnatal expansion of adipose compartments. Whether adipogenesis occurs in the adult adipose tissue remains controversial. In animal studies, white adipocyte numbers increase through puberty but are

relatively steady in mature fat pad. However, within human adult WAT, adipocytes seem to undergo approximately a 10% annual turnover. Thus, adipogenesis takes place in adults to maintain the adipose compartment. (Cristancho & Lazar 2011). Adipogenesis can be divided into two phases: *determination* and *terminal differentiation*. *Determination* results in the conversion of the stem cell into a preadipocyte, which cannot be distinguished morphologically from its precursor cell but has lost the potential to differentiate into other cell types. In the second phase, the *terminal differentiation*, the preadipocyte takes on the characteristics of the mature adipocyte that acquires the machinery necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipocytes-specific proteins (Rosen & MacDougald 2006).

Moreover, the *terminal differentiation* can be further divided into several sequential steps that have been well characterized. Briefly, the sequence of events includes following: (1) Growth arrest at confluence, (2) clonal expansion which involves synchronous entry of all cells into S phase of the cell cycle, leading to one or two rounds of mitosis, (3) expression of late genes which is accompanied by an exit from the cell cycle and expression of adipocyte markers. All of these sequential steps are controlled by a network of interacting transcription factors operating to coordinate expression of many hundreds of proteins responsible for establishing the mature fat cell phenotype (Louet & O'Malley 2007).

The molecular regulation of *terminal differentiation* is more extensively characterized than *determination* because of the use of cell lines that have a restricted potential to differentiate into other cell types i.e., 3T3L1 and 3T3-F442A murine cells. Confluent 3T3L1 preadipocytes can be synchronously differentiated by a defined adipogenic cocktail. Maximal differentiation is achieved upon early hormonal induction for 48 h with a combination of insulin, glucocorticoids (GCs), and methylisobutylxanthine (MIX), which elevates intracellular cAMP levels, in the presence of fetal bovine serum. Dexamethasone (DXM), a synthetic GC agonist, is traditionally used to stimulate the GC receptor. After the first 48 h, insulin alone is required to continue the differentiation program (Moreno-Navarrete & Fernández-Real 2012).

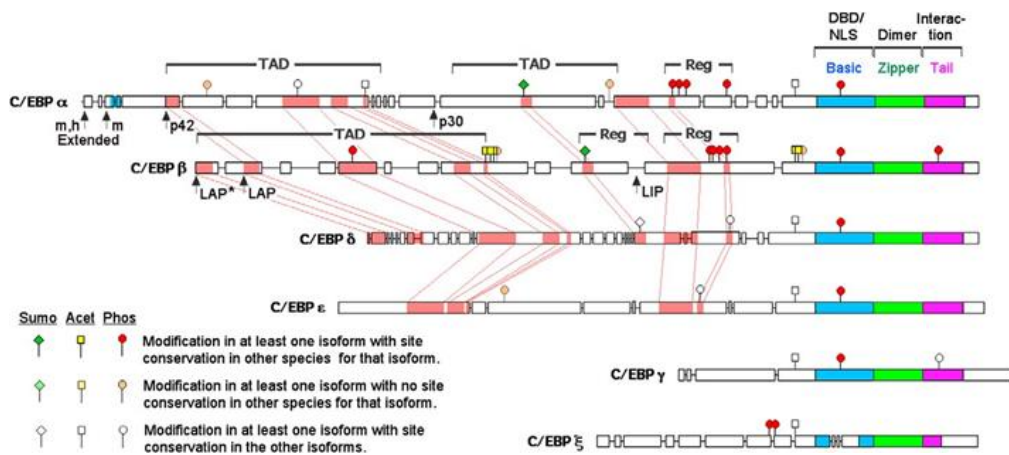
### **1.3 Molecular regulators of adipogenesis**

Adipocytes differentiation involves a temporally regulated set of gene-expression events, and understanding the underlying transcriptional networks is of fundamental importance. In particular, Peroxisome Proliferator-Activated Receptor gamma (PPAR $\gamma$ ) and CAAT-enhancer-binding protein (C/EBPs)

can be considered as principal regulators of adipogenesis (Gregoire et al. 1998).

### 1.3.1 C/EBP family of transcriptional factors

The CCAAT/enhancer-binding proteins (C/EBPs) encompass a family of six transcription factors with structural as well as functional homologies (Figure 3).



**Figure 3: Amino acid sequence relationships among C/EBP family members.**

The six gene-specific isoforms of C/EBP are presented as scaled graphics that display intra-isoform species homology (open boxes) as well as inter-isoform conservation (pink shaded regions with interconnecting lines revealing relative locations). Homology is not displayed for the bZIP regions, which are generally highly conserved (with the exception of the modifications associated with the basic DBD of C/EBP $\zeta$ ). Stick figures represent sites of post-translational modification, as indicated by the key. The m and h designate murine- and human-specific initiation sites for extended forms of C/EBP $\alpha$  that encode a nucleolar localization sequence (blue shading).

Adapted from Tsukada J et al. *Cytokine*. 2011.54(1):6-19.

Similarity between C/EBP family members suggests an evolutionary history of genetic duplications with subsequent pressure to diversify. The resulting family of proteins varies in tissue specificity and transactivating ability. The pleiotropic effects of C/EBPs are in part due to a tissue- and stage-specific expression, leaky ribosomal reading, post-transcriptional modifications, and variable DNA binding specificities. Several works using genetically altered mice to abolish expression of C/EBPs have identified the role that these factors play in normal tissue development and cellular function, cellular

proliferation and functional differentiation (Birkenmeier et al. 1989; Landschulz et al. 1989).

C/EBPs are modular proteins consisting of an activation domain, a DNA-binding basic region and a leucine-rich dimerization domain. The dimerization domain, known as leucine zipper, is a heptad of leucine repeats that intercalate with repeats of the dimer partner, forming a coiled coil of  $\alpha$ -helices in parallel orientation.

C/EBP $\alpha$  was the first member cloned. Expression patterns of C/EBP $\alpha$  mRNA are similar in the mouse and human with measurable levels in liver, adipose, intestine, lung, adrenal lung, peripheral blood mononuclear cells and placenta. In liver and adipose compartment, highest levels of C/EBP $\alpha$  mRNA are detected only in differentiated tissue. Two isoforms of C/EBP $\alpha$  are generated from its mRNA by ribosomal scanning mechanism. The full-length protein is 42 kDa and contains three transactivation domains (TEI-III). TEI and TEII mediate cooperative binding of C/EBP $\alpha$  to TBP (TATA box-binding protein) and TFIIB, two components of the RNA polymerase II basal transcriptional apparatus. TEIII contains a negative regulatory subdomain. The shorter 30 kDa C/EBP $\alpha$  isoform retains its dimerization and DNA-binding domains; however, it possesses an altered transactivation potential compared with the 42-kDa isoform.

Constitutive expression of C/EBP $\beta$  is highest in liver, intestine, lung and adipose tissue. Stimulation with lipopolysaccharide (LPS), IL-6, IL-1, dexamethasone and glucagon strongly induces C/EBP $\beta$  expression, suggesting a role in the mediation of the inflammatory response. Like C/EBP $\alpha$ , two C/EBP $\beta$  isoforms are generated from a single mRNA. The full-length 32-kDa protein, also termed LAP, encodes for the conserved activation domains found in other C/EBPs proteins, as well as two regulatory domains, RD1 and RD2, which confer DNA binding inhibition in a cell type-specific manner. The truncated protein, LIP, translated from the third, in-frame AUG, possesses only the DNA-binding and leucine zipper domains.

C/EBP $\gamma$  is a short, intronless gene, whose mRNA is ubiquitously expressed with highest levels found in non-differentiated, progenitor cells.

C/EBP $\delta$  is expressed in intestines, adipose and lung, with high levels of expression in all tissues following LPS stimulation. The 269-amino acid protein encodes a leucine zipper dimerization domain and DNA-binding region.

C/EBP $\epsilon$  was originally identified from a rat genomic library. Four mRNA isoforms, expressed primarily in myeloid and lymphoid cells, are generated by the use of alternative promoters combined with differential splicing. The highest level of expression is detected in promyelocyte and late myeloblast-like cell lines.

C/EBP $\zeta$ , which is induced by DNA damage, possesses a leucine zipper dimerization domain and DNA-binding region. This transcriptional factor

functions as a dominant negative inhibitor of C/EBPs transcriptional activation (Lekstrom-Himes & Xanthopoulos 1998).

In the recent years, it has been demonstrated that C/EBPs appear to have different roles in regulation of adipocytes differentiation. After treatment of preadipocytes cell lines (i.e., 3T3L1 and 3T3-F442A) with inducers of differentiation, it is possible to observed a rapid and transient increase in transcription and expression of C/EBP $\beta$  and C/EBP $\delta$ . After induction of differentiation, preadipocytes undergo approximately two rounds of cell division, and subsequently cell proliferation ceases coincident with the transcriptional activation of C/EBP $\alpha$ . Induction of C/EBP $\alpha$  is followed by transcriptional activation of many genes encoding proteins involved in creating the adipocyte phenotype.

Embryonic fibroblast from mice lacking both C/EBP $\beta$  and C/EBP $\delta$  expression are unable to differentiate in response to hormonal stimulation. However, ectopic expression of C/EBP $\beta$ , but no C/EBP $\delta$ , in 3T3L1 preadipocytes is sufficient to cause differentiation in the absence of DEX and MIX.

Works from a several of investigators have suggested a link between expression of C/EBP $\alpha$  and some adipose-specific genes. For example, promoters of adipocytes genes such as *GLUT4*, *SCD1*, *leptin* and *ap2/FABP4* are transactivated by C/EBPs, including C/EBP $\alpha$ . In the absence of C/EBP $\alpha$ , adipose-specific genes are not expressed and triacylglycerol accumulation was not detected. Also, the conditional expression of C/EBP $\alpha$  in stably transfected clones of 3T3L1 preadipocytes is sufficient to bring about differentiation as measured by the cytoplasmic accumulation of lipid and the expression of *GLUT4*, *ap2/FABP4* and endogenous C/EBP $\alpha$  (Darlington et al. 1998).

Animals having a homozygous deletion of C/EBP $\alpha$  gene feature dramatically reduced fat accumulation in WAT and BAT pads. However, these mice succumb to hypoglycemia within the first week of life as a result of a failure to activate gluconeogenesis in the liver (Rosen & Spiegelman 2000).

### 1.3.2 PPAR $\gamma$

PPARs are members of the nuclear receptor superfamily and are characterized as adopted orphan receptor, that can be activated by a variety of fatty acids and their derivatives such as prostaglandins. This receptor family is comprised of three different subtypes (PPAR $\alpha$ ,  $\beta/\delta$ ,  $\gamma$ ), all of which are important regulators of lipid and glucose metabolism in many different tissues including skeletal muscle, liver, adipose tissue and gut. PPAR $\gamma$  is found in two different isoforms termed PPAR $\gamma$ 1 and PPAR $\gamma$ 2. The latter is almost exclusively expressed in adipose tissue, whereas PPAR $\gamma$ 1 is more ubiquitously expressed. PPARs, like other nuclear receptors, are comprised of a non-conserved N-terminal A/B domain containing the activating function 1 (AF-1),

a highly conserved DNA-binding domain (DBD), a hinge region (D domain), and a C-terminal ligand-binding domain (LBD) containing the activating function 2 (AF-2). The DBD anchors PPARs to their binding sites on the DNA template through interactions between the two zinc fingers in this domain and the major groove of the DNA double helix. PPARs bind as obligate heterodimers with the retinoic X receptor (RXR) to direct hexanucleotide repeats spaced by 1 nucleotide (DR-1) termed PPAR response elements (PPREs) (Siersbæk et al. 2010) (Figure 4).



**Figure 4: PPAR $\gamma$  structure.**

PPARs present different functional domain: 1- the activation function-1 region (AF-1); 2- the DNA binding domain (DBD) binds specific DNA sequences in the PPREs found in the promoter regions of genes regulated by PPARs; 3- the ligand binding domain (LBD) contributes to the dimerization of the receptor with RXR and binds coactivator and corepressor proteins here.

Adapted from Wadosky KM & Willis MS *Am J Physiol Heart Circ Physiol* 2012. 302(3):H515-26; Ahmadian M et al. *Nature* 2013. 19(5):557-66.

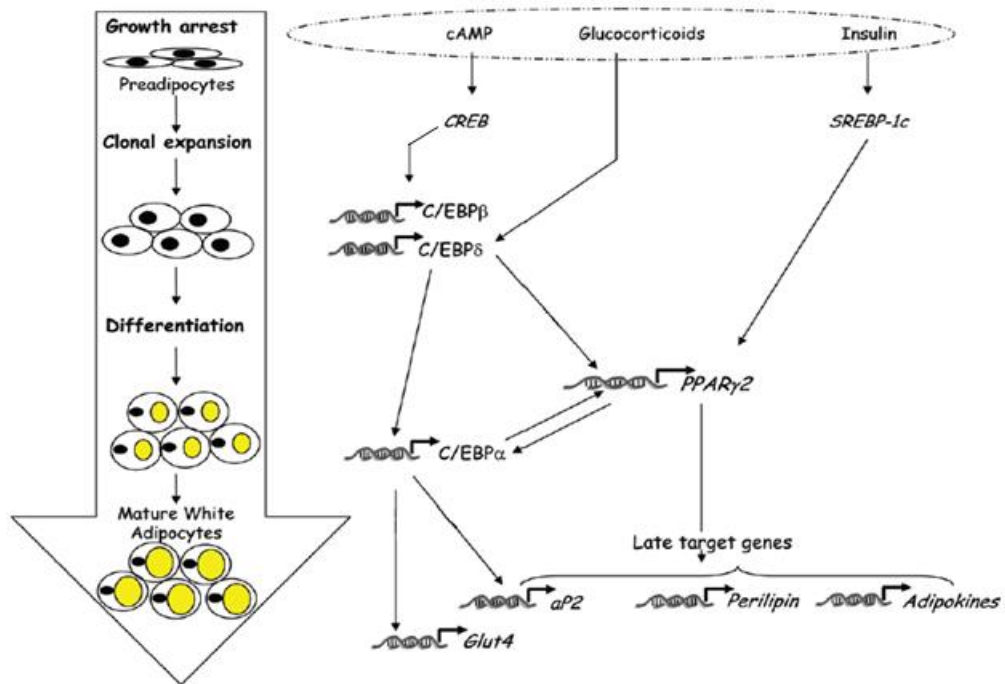
There are more evidences that show that PPAR $\gamma$  is a prime mover of fat cell differentiation. This was shown first in gain-of-function experiments that expressed PPAR $\gamma$  in non-adipogenic, fibroblastic cells using retroviral vectors, causing a strong differentiation response. The use of high-affinity selective PPAR $\gamma$  agonists such as thiazolidinediones (TZDs) has greatly improved on these observations. PPAR $\gamma$  agonist-mediated differentiation includes lipid accumulation and expression of many endogenous genes typical of the adipocyte. Interestingly, the ability of PPAR $\gamma$  to promote adipogenesis is not limited to fibroblastic cells. Indeed, myoblastic cell lines can also be converted to adipocytes, particularly when the cells co-express C/EBP $\alpha$  in addition to PPAR $\gamma$ .

The role of PPAR $\gamma$  in adipogenesis is also illustrated in studies that have deleted this gene in mice. The PPAR $\gamma$  homozygous null mutation is lethal relatively early in gestation (e 10-10.5), secondary to a defect in placental development. Interestingly, PPAR $\gamma$  heterozygous animals exhibit resistance to diet-induced obesity, although this results at least in part from elevated serum leptin levels and decreased food intake in these mice. In human, a role for PPAR $\gamma$  in adipogenesis is suggested by the results of clinical trials of PPAR $\gamma$  agonists in liposarcoma, a malignant tissue of adipose origin. These tumors exhibit a strong differentiative response to these agents; whether this leads to

improved patient outcome is currently the subject investigation (Rosen & Spiegelman 2000).

### 1.4 A transcriptional cascade regulates adipocytes differentiation

Adipogenesis, and in particular terminal differentiation, includes a series of transcriptional processes involving the sequential expression of several transcriptional factors, culminating in the activation of C/EBPs and PPAR $\gamma$  (Figure 5).



**Figure 5: A complex transcriptional cascade regulates adipogenesis.**

The transcriptional control of adipogenesis involves the activation of a variety of transcription factors. These proteins are expressed in a cascade in which C/EBP $\beta$  and C/EBP $\delta$  are among the earliest seen. These two proteins induce the expression of PPAR $\gamma$ , which in turn activates C/EBP $\alpha$ . C/EBP $\alpha$  feeds back on PPAR $\gamma$  to maintain the differentiated state. ADD-1/SREBP-1 can activate PPAR $\gamma$  by inducing its expression as well as by promoting the production of an endogenous PPAR $\gamma$  ligand. All these factors contribute to the expression of genes that characterize the terminally differentiated phenotype.

Adapted from Louet & O'Malley *Cell Cycle* 2007. 6(20):2448-52.2006; Ahmadian M et al. *Nature* 2013. 19(5):557-66.

Initially, the transcriptional factor CREB (cAMP response element-binding protein) induces adipocytes differentiation increasing C/EBP $\beta$  expression (Reusch et al. 2000; Tang & Lane 2012). C/EBP $\beta$ , together with C/EBP $\delta$ , promote the expression of C/EBP $\alpha$  and PPAR $\gamma$ ; this induction is likely to be a direct transcriptional effect through C/EBP binding site in the genes promoter (Rosen & Spiegelman 2000).

Subsequently, PPAR $\gamma$  and C/EBP $\alpha$  initiate positive feedback to induce their own expression and also activate a large number of downstream target genes whose expression determines the adipocyte phenotype (Moreno-Navarrete & Fernández-Real 2012). Genetic proof of this relationship has been obtained from experiments in which homozygous PPAR $\gamma$  null cells (MEFs or ES cells) have been exposed to a prodifferentiative regimen. These cells do not become adipocytes and fail to express C/EBP $\alpha$  despite normal levels of C/EBP $\beta$  and  $\delta$ . Interestingly, fibroblasts derived from C/EBP $\alpha$ <sup>-/-</sup> embryos have reduced levels of PPAR $\gamma$  and do not form fat when exposed to hormonal-inducing agents in culture. When C/EBP $\alpha$  is added back to these cells with retroviral vector, the expression of PPAR $\gamma$  and the ability to differentiate is restored (Rosen & Spiegelman 2000).

The activation of these transcriptional factors determines the acquisition of mature fat phenotype. In fact, adipocyte cultures feature markedly increase of *de novo* lipogenesis and become sensitive to insulin. The role of adipocyte determination- and differentiation-dependent factor-1/sterol regulatory element-binding protein-1 (ADD-1/SREBP-1) in adipocyte differentiation has also been indicated in detail. ADD-1/SREBP-1 is induced during adipocyte differentiation stimulating the expression of many genes necessary for lipogenesis *in vivo*, such as fatty acid synthase (FAS) and adiponectin (Niemelä et al. 2008).

During the adipogenic process it is possible to observe an increase of enzymes involved in TG metabolism including adenosine triphosphate (ATP) citrate lyase, malic enzyme, glycerol-3-phosphate dehydrogenase and fatty acid synthase. Furthermore, adipocytes also synthesize other adipose tissue-specific products that are not directly related to lipid and glucose metabolism. These include FABP4/aP2, an adipocyte specific fatty acid-binding protein, and GLUT4 that have been considered as intermediate markers of adipocytes differentiation. In addition, adipocytes produce various secreted factors including adiponectin, angiotensin II and leptin that are regarded as late markers of adipocytes differentiation (Table 1). PPAR $\gamma$  and C/EBP $\alpha$  are involved in the coordinated activation of several of these genes (Niemelä et al. 2008).



Adipogenic transcription factors	Characterized target genes
C/EBP $\beta$	PPAR $\gamma$ , C/EBP $\alpha$ , adiponectin
C/EBP $\delta$	PPAR $\gamma$ , C/EBP $\alpha$
C/EBP $\alpha$	PPAR $\gamma$ , aP2, SCD, GLUT4, insulin receptor, leptin, adiponectin
SREBP-1	FAS, C/EBP $\beta$ , adiponectin
PPAR $\gamma$	aP2, C/EBP $\alpha$ , LPL, HSL, malic enzyme, acyl-CoA synthetase, FATP, FAT, SCD, perilipin, PEPCK, GLUT4, UCP1, adiponectin

**Table 1: Target genes of key adipocyte transcription factors.**

Adapted from White & Stephens *Adipose tissue in Health and Disease* 2010.

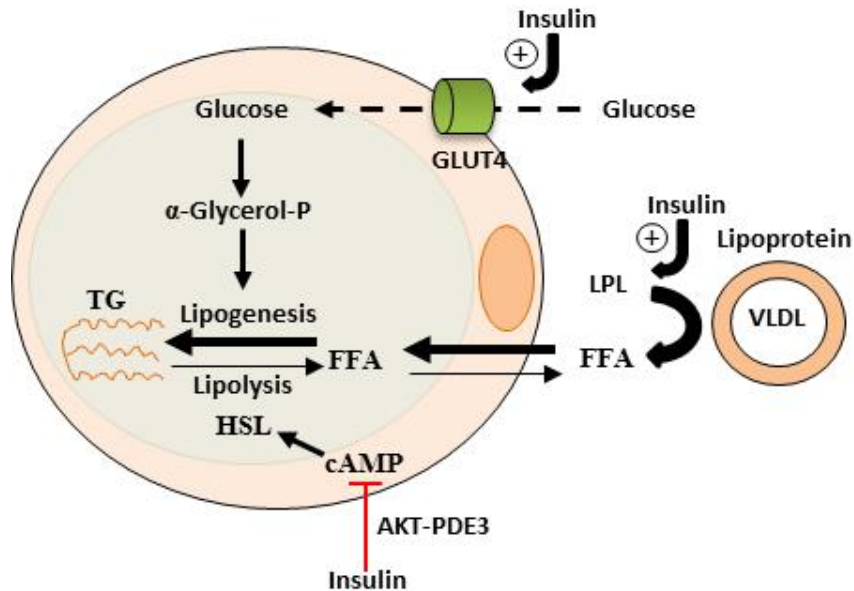
## 1.5 Adipocytes metabolism

Adipocytes control whole-body metabolism by regulating 95% of total body triglyceride (TG) concentration (Stahl et al. 2002). In fact, these cells store dietary energy in a highly concentrated form as TG, mostly in a single large lipid droplet. These structures are associated with a unique complement of proteins, which enable the sequestration or mobilization of lipids. In times of caloric need, triglycerides can be rapidly hydrolyzed by lipases (a process known as lipolysis) and the resulting fatty acids are transported to other tissues to be oxidized in mitochondria as an energy source. Critical to these functions is the capacity of the adipocytes to respond to insulin (Guilherme et al. 2008).

### 1.5.1 Insulin signaling

Increased plasma glucose levels induce insulin secretion from pancreatic  $\beta$  cells into the bloodstream where it increases fatty acid and glucose uptake in the liver, muscle and adipocytes and decreases gluconeogenesis in the liver (Badman & Flier 2005; Cheatham & Kahn 1995). In mature adipocytes insulin binds to the insulin receptor (IR), which then phosphorylates IR substrates (Miki et al. 2001; Zhou et al. 1999). This leads to the recruitment and activation of the phosphatidylinositol-3 kinase (PI3K) and Akt (Laviola et al. 2006). Akt signaling induces translocation of GLUT4 from

intracellular compartments to the plasma membrane where it facilitates transport of glucose into the cell (Watson et al.2004). Similarly, Akt also induces the translocation of fatty acid transport proteins (FATPs) 1 and 4 to the cell surface for import of fatty acids (Stahl et al. 2002). The increased concentrations of glucose and fatty acids that accumulate due to insulin stimulation rapidly induce synthesis of TG for long-term fat storage. Adipocyte precursor cells, positioned in the WAT, are also affected by insulin signaling. Here, proteins involved in cell proliferation and differentiation, especially the ERK and mTOR, are activated and initiate cell division and differentiation of precursor cells into adipocytes thereby increasing the storage capacity for fatty acids (Laviola et al. 2006) (Figure 7).



**Figure 7: Insulin signaling pathway.**

In adipocytes, insulin promotes free fatty acid (FFA) esterification into triglycerides (TG, schematically represented as the three-carbon glycerol backbone with three acyl fatty acid chains (wavy lines)) through stimulation of glucose transporter type-4 (GLUT4)-mediated glucose uptake. Glucose can be converted to  $\alpha$ -glycerol phosphate, the main source of the glycerol backbone of TG. Insulin signaling downregulates TG lipolysis through hormone-sensitive lipase (HSL). Insulin stimulation of the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway leads to activation of the enzyme phosphodiesterase-3 (PDE3). This enzyme catalyzes the breakdown of cyclic AMP (cAMP) which in turn reduces the activation of HSL.

Adapted from Guilherme *Nature* 2008 9(5):367-77.

Insulin regulates lipolysis, lipogenesis and glucose and fatty acids uptake. Lipogenesis depends on lipoprotein lipase (LPL) that is secreted from adipocytes and acts on the surface of endothelial cells in the WAT where it hydrolyzes the lipoprotein bound TG to FFAs which are then transported into adipocytes by passive diffusion (Hamilton & Kamp 1999) or by fatty acid transporter proteins (FATPs) (Abumrad et al. 1999). Inside the adipocyte, the fatty acids become chaperoned by adipocyte fatty acid binding protein 4 (FABP4/aP2) for re-esterification and conjugation to coenzyme A (coA) catalyzed by acyl CoA synthetases (ACS) (Fantuzzi & Mazzone 2007).

Lipolysis is due to the activity of the hormone-sensitive lipase (HSL), that catalyzes the hydrolysis of TG and is activated by protein kinase A (PKA) mediated-phosphorylation. Insulin inhibits lipolysis reducing adenylate cyclase activity and, in turn, PKA (Madsen & Kristiansen 2010).

In addition, insulin stimulates glucose uptake by inducing GLUT4 translocation. In the absence of insulin, only ~5% of the total transporter pool is found on the cell surface (Leto & Saltiel 2012). Upon binding to its own receptor, insulin induces GLUT4 recruitment to the plasma membrane to promote glucose uptake (Czech et al. 1993).

Insulin plays an important role, also, in adipocytes differentiation. In particular, study using IRS1<sup>-/-</sup> and IRS2<sup>-/-</sup> cells suggests that members of IRS family in adipocytes play a role in adipogenesis. In fact, cells lacking both IRS1 and IRS2 cannot undergo adipocytes differentiation and adipose tissue mass is dramatically reduced. Furthermore, PI3K and AKT1 are required for adipocytes differentiation *in vitro*. However, the molecular mechanism of insulin action on adipocytes differentiation is still under investigation (Madsen & Kristiansen 2010).

## 1.6 Adipose tissue and diseases

The classical perception of adipose tissue as a storage place of fatty acids has been replaced over the last years by the notion that adipose tissue has a central role in lipid and glucose metabolism and produces a large number of hormones and cytokines, including TNF $\alpha$ , IL-6, adiponectin and leptin (Hajer et al. 2008).

It is clear that adipose tissue is important to several normal processes of the human body, but it can be involved in human disease states such as obesity, type 2 diabetes mellitus (T2DM) and insulin resistance (IR).

Obesity is a chronic, multifactorial disorder that has reached epidemic proportions globally. It is primarily characterized by an increase of fat mass with development of different related disorders. Expansion of adipose organ, in particular of white adipose tissue, is caused by a combination of size increase of preexisting adipocytes (hypertrophy) and *de novo* adipocytes

differentiation (hyperplasia) (Tedesco et al. 2008). Adipogenesis has probably a role in the pathology of obesity; indeed, when animals are kept on a high-fat diet, adipocyte cell size initially increases, followed by an increase in fat cell number upon prolonged over-nutrition. In addition, short-term overfeeding studies in adults demonstrate increase in adipocyte cell number. By contrast, preclinical and human studies have shown that weight loss is associated with decreased adipocyte cell size but has no effect on adipocyte cell number (Cristancho & Lazar 2011). Adipocytes functionality is lost during obesity and has been related to adipocyte hypertrophy, disequilibrium between lipogenesis and lipolysis, impaired transcriptional regulation of the key factors that control adipogenesis and lack of sensitivity to external signals, as well as a failure in the signal transduction process. Thus, dysfunctional adipocytes contribute to abnormal utilization of fatty acids causing lipotoxicity in non-adipose tissue such as liver and pancreas, that is correlated with insulin resistance (Vázquez-Vela et al. 2008).

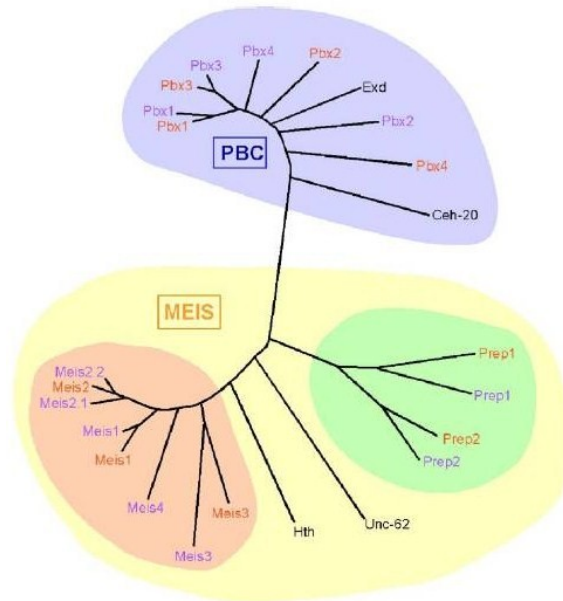
T2D accounts for 90% of all forms of diabetes and is most common in people older than 45 who are overweight. However, as a consequence of increased obesity among young people, it is becoming more common in children and young adults. T2D is a heterogeneous syndrome with many possible causes. This is due to the interaction of environmental factors with a genetic susceptibility to the disease, and it is becoming more evident that the relative contribution of genes and environment can differ considerably, even among individual whose clinical phenotype is similar (Diabetes Atlas 2006). The maintenance of normal glucose homeostasis depends on a precisely balanced and dynamic interaction between tissue sensitivity to insulin and insulin secretion. Type 2 diabetes develops because of defects in both insulin secretion and action, both of these with a genetic as well as an acquired component. Thus, T2D is made up of different forms each of which is characterized by variable degrees of insulin- resistance and beta cell dysfunction, and which together lead to hyperglycaemia. Insulin resistance, typically, is an early feature of T2D. It results from a genetically determined reduction in insulin sensitivity, compounded by exposure to the environmental factors, which further impair insulin action. Major sites of insulin resistance include liver and the peripheral tissues, skeletal muscle and fat. In muscle and fat, insulin resistance is manifested by decreased glucose uptake; in the liver, insulin resistance leads to failure of insulin to suppress hepatic glucose production, which is followed by glycogen breakdown and particularly by gluconeogenesis (Pickup & Williams 2005). The disease often remains asymptomatic and undetected for years. People with type 2 diabetes are not completely dependent on exogenous insulin, but may require the hormone for the control of blood glucose levels if this is not achieved with diet alone, regular exercise or with oral hypoglycaemic agents. While several environmental factors have been identified, discovery and characterization of

the genes involved in T2D has been an arduous task and has proceeded slowly. In the past 10 years, indeed, geneticists have devoted a large amount of effort to finding T2D genes. Recently, several works suggest that the family of homeodomain-containing transcription factors named TALE (Three Aminoacid Loop Extension) is strongly related to diabetes and insulin-resistance (Kim et al. 2002; Oriente et al. 2008; Oriente et al. 2011)

### **1.7 TALE proteins**

The three-amino acid loop extension (TALE) homeodomain proteins are recognized as transcription factors responsible for regulating growth and differentiation during vertebrate embryogenesis. The genes encoding these proteins are highly conserved and are present in the common ancestor of plants, fungi, and animals.

TALE proteins display a highly conserved DNA binding domain of approximately 60 amino acids called the homeodomain (Gehring et al. 1994). This region is composed of three alpha helices and a flexible N-terminal arm. The homeodomain interacts with the DNA through the third helix making base-specific contacts in the major groove of DNA and through the N-terminal arm which contacts the minor groove of DNA. Between the first and the second alpha helices of the homeodomain there is an extension of three amino acids, virtually represented by a proline (P) – tyrosine (Y) – proline (P) in position 24-26. This domain has been implicated in important protein–protein interactions that are required for fundamental aspects of development. The TALE homeodomain superclass is composed of two groups: the PBC and the MEIS families. The PBC subclass of the TALE homeodomain proteins, referring to the conserved PBC motif N-terminal to the TALE homeodomain (Burglin 1997; Burglin 1998), includes the vertebrate Pbx proteins, fly Extradenticle and worm Ceh-20. The MEIS class includes Homothorax (Hth) in flies and the Meis and Prep proteins in vertebrates (Figure 8) (Moens & Selleri 2006).



**Figure 8: Phylogeny of Hox cofactors.**

TALE homeodomain proteins are divided into two groups: the PBC family, including the vertebrate Pbx proteins, fly Extradenticle and worm Ceh-20, and the MEIS family, including vertebrate Meis and Prep, fly Homothorax (Hth) and worm Unc-62. Orange letters indicate mouse proteins, purple lettering indicates their zebrafish orthologs. Although in some cases the orthology assignments are not clear (as for *pbx2* and *pbx4*), genetic rescue experiments in zebrafish have suggested that the different *pbx* genes are functionally identical. Similar information is not yet available with regard to mammalian *Pbx* genes.

Cooperative function among TALE family members is critical for transcription regulation (Bischof et al. 1998; Chang et al. 1997; Knoepfler et al. 1997), and several members have been shown to function as essential contributors to Hox-mediated developmental programs (Chan et al. 1994; Mann and Chan 1996; Rauskolb and Wieschaus 1994).

### 1.7.1 Prep1 protein

Prep1 (Pbx regulating protein 1) is a 64 kDa ubiquitous homeodomain transcription factor of the three aminoacid loop extension (TALE) superclass of proteins mapping on the chromosome 21q.22.3. The Prep1 homeodomain is structurally related to the PBC homeodomain class and contains an isoleucine residue in the third position of the conserved WF\_N sequence in the homeodomain. Indeed, Prep1 forms DNA-independent dimeric complexes with the Pbx homeodomain transcription factor, enhancing target specificity and regulatory function. Prep1 shares homology with MEIS proteins within

the homeodomain and in two well-preserved amino terminal regions termed HR1 and HR2 (Homology Region). HR1 and HR2 bind the N-terminal region of Pbx1 (PBC-A) and are essential for Prep1-Pbx1 heterodimerization, which in turn is required for DNA binding (Berthelsen et al. 1998). Prep1 could be localized both in cytoplasm and in nucleus and the heterodimerization with Pbx1 appears to be essential to translocate Prep1 into the nucleus to bind DNA target. On the other hand, Prep1 dimerization prevents nuclear export and the proteasomal degradation of Pbx1 prolonging its half-life (Berthelsen et al. 1999). Formation of dimeric complex drastically increases binding affinity of Prep1/Pbx1 to the DNA and also broadens the DNA target selectivity of Pbx1 to include sequences containing the TGACAG sequence (Berthelsen et al. 1998). Together Prep1 and Pbx1 form the UEF-3 (Urokinase Enhancer Factor-3) transcription factor, which is important in regulating the activity of the urokinase plasminogen activator (uPA) gene enhancer. The UEF3 DNA target site is a regulatory element in the promoters of several growth factor and protease genes, such as interleukin-3 (IL-3), stromelysin and urokinase plasminogen activator (uPA) (Berthelsen et al. 1996). Prep1 and Pbx1 can form ternary complexes with PDX-1 to regulate somatostatin gene transcription (Goudet et al. 1999).

The role of Prep1 has been in part clarified by the generation of mutant mice. Nevertheless, *Prep1* null embryos results in early lethality before gastrulation (E7.5) (Fernandez-Diaz et al. 2010), precluding a study of the Prep1 role in later developmental processes. An insertion of a retroviral vector in the first intron of the *Prep1* gene results in a hypomorphic mutation (*Prep1<sup>i/i</sup>*) that exhibits variable penetrance. *Prep1<sup>i/i</sup>* embryos, expressing 5 to 7% of protein, die between E17.5 and P0 and present a profound alteration in the hematopoietic development with a deficient radioprotection activity of fetal liver (FL) progenitors and all colony-forming progenitors and profound anemia (Ferretti et al. 2006). However, a small percentage of the *Prep1<sup>i/i</sup>* embryos are born alive and subsequently live a normal length-life. The mice escaping embryonic lethality show T-cell development anomalies with a decreased number of circulating CD4<sup>+</sup> and CD8<sup>+</sup> T cells and increased apoptosis (Penkov et al. 2005). In addition, *Prep1<sup>i/i</sup>* feature an impairment of erythropoiesis and angiogenesis accompanied by liver hypoplasia, decreased hematocrit, anemia, and delayed erythroid differentiation together with a decrease in capillary formation (Ferretti et al. 2006). These mice show also alterations in eye development, in fact in most cases the size of the lens is strongly reduced, similar to the phenotype of *Pax6*-deficient mice. *Prep1* deficiency affects the expression of both TALE class partners *Pbx* and *Meis*, both required for embryonic hematopoiesis (Azcoitia et al 2005; Di Martino et al. 2001; Hisa et al. 2004). All of these effects are in part evident, although more weakly, also in a Prep1 heterozygous hypomorphic mice (*Prep1<sup>i/+</sup>*), expressing 55-57% of protein.

### 1.7.2 Prep1 and metabolism

In our laboratory we have recently evidenced an important role of Prep1 in pancreas development, glucose homeostasis, regulation of insulin-action in muscle and liver, and in the regulation of hepatic lipogenesis (Oriente et al. 2008; Oriente et al. 2011; Oriente et al. 2013).

*Prep1*-hypomorphic mice show smaller pancreatic islet but normal architecture. However, these mice feature islet hypoplasia, accompanied by significantly reduced absolute insulin levels (both basal and postloading). The *Prep1*<sup>hi</sup> pancreatic phenotype is characterized by also a strong reduction of pancreatic *Pbx1* expression, emphasizing the idea that Prep1 hierarchically acts upstream in the network regulating pancreas development by controlling the levels of Pbx1. Moreover, these mice exhibit protection from streptozotocin-induced diabetes and enhanced insulin sensitivity with improved glucose uptake and insulin-dependent glucose disposal by skeletal muscle. This muscular phenotype does not depend on the reduced expression of *Pbx1*, rather than on the reduced levels of the recently identified novel Prep1 interactor p160 Myb-binding protein (p160<sup>MBP</sup>), a molecule which is known to inhibit the PGC-1 $\alpha$ -mediated glucose transport and compete with Pbx1 to bind Prep1. Consistent with p160<sup>MBP</sup> decrease, these mice show a muscle-selective increase in mRNA and protein levels of PGC-1 $\alpha$ , accompanied by enhanced expression of the GLUT4 transporter and glucose uptake in muscle. Further experiments performed in cells overexpressing *Prep1* have confirmed these results and have indicated that Prep1 stabilizes p160<sup>MBP</sup>, inducing p160<sup>MBP</sup> escape from proteasomal and reduces the levels of PGC-1 $\alpha$  and GLUT4.

The mechanisms responsible for Prep1 action in liver differ from those in the skeletal muscle since Pbx1 and p160<sup>MBP</sup> are expressed at different levels. In fact, while there is a marked reduction of p160<sup>MBP</sup> in the skeletal muscle of the hypomorphic mice compared to the wild type animals, this difference has not detectable in the liver because in this organ the expression of p160<sup>MBP</sup> is very low. Thus, at variance with muscle, Prep1 major functional partner in liver appears to be Pbx1 rather than p160<sup>MBP</sup>.

*Prep1* deficiency in animal models improves insulin signaling in liver increasing hepatic glycogen content and decreasing glucose output and triglyceride levels. Analysis of the initial steps in insulin signaling in the *Prep1*-deficient mouse liver revealed increased tyrosine phosphorylation of both insulin receptor and the major IRSs present in these cells. These effects are paralleled by a significant reduction of expression of SYP and SHP1 tyrosine phosphatases. The functional relevance of Prep1 control of SHP1 versus SYP expression to hepatic sensitivity to insulin has been assessed in HepG2 liver cells stable transfected with *Prep1*. These cells feature a very significant reduction of insulin effect on glycogen accumulation. In particular,



expression of SHP1 in cellular models negatively correlates with insulin signaling. However, antisense silencing of *SHP1* rescued insulin effect in these cells, thus establishing SHP1 as the functionally relevant target of Prep1 in the liver. The regulatory region of *SHP1* gene contains several *Prep1* binding sites in the 4,000 base-pair region upstream *SHP1* transcription initiation site and one Prep1/Pbx1 binding site responding to the ectopic expression of Prep1 and Pbx1 in cotransfection experiments in vitro. In these assays, a fragment containing single Prep1/Pbx1 binding site displays a powerful enhancer function. Thus, Prep1-Pbx1 complex enhances *SHP1* gene transcription by acting on regulatory sequences upstream the ATG codon.

*Prep1* action in liver is not limited to the regulation of glucose metabolism but also on lipid synthesis (Oriente et al. 2013). In fact, serum triglyceride levels are strongly reduced in *Prep1* heterozygous hypomorphic mice, confirming previous data about the reduction of hepatic triglyceride content (Oriente et al. 2011). Indeed, FAS expression, an enzyme that regulates *de novo* hepatic lipogenesis, is significantly reduced in *Prep1* deficient mice. Consistent with these data, the molecular pathway controlling hepatic lipogenesis is downregulated. In fact, in *Prep1*<sup>i/+</sup> mice liver there is an increase of phosphorylation of PKCzeta, LKB1, AMPK and ACC, leading to an inhibition of TG synthesis. This regulation is due to the modulation of expression of SHIP2, a lipid phosphatase, known inhibitor of PI3Kinase/PKCzeta signaling. In the liver of *Prep1* deficient mice SHIP2, protein and mRNA expression, is strongly reduced. Accordingly with these data, HepG2 cells overexpressing *Prep1* display increased triglyceride levels and FAS expression, PKCzeta, LKB1, AMPK and ACC phosphorylation is strongly reduced, while SHIP2 levels are increased. Interestingly, overexpression of Pbx1 cDNA in HepG2 cells mimicks *Prep1*-induced triglyceride synthesis. At the opposite, *Prep1*<sub>HR1</sub> mutant, which is unable to bind Pbx1, fails to elicit these effects. ChIP and Re-ChIP experiments indicate that Prep1/Pbx1 complex can bind SHIP2 promoter region and regulate its expression. Treatment with a methionine and choline-deficient (MCD) diet induces steatosis in both *Prep1*<sup>i/+</sup> and non-hypomorphic control mice. However, alanine aminotransferase increase, intracellular TG content and histological evidence of liver steatosis, inflammation and necrosis is less evident in *Prep1*<sup>i/+</sup> mice, indicating that *Prep1* silencing protects mice from MCD diet –induced steatohepatitis (Oriente et al. 2013).

On the basis of the results, Prep1 might be considered as a new gene involved in the pathogenesis of type 2 diabetes, insulin-resistance and steatohepatitis.



## 2. AIM OF THE STUDY

The primary function of adipose tissue is to store energy in the form of triglycerides during period of energy excess and to release the energy during fasting as free fatty acids and glycerol. In addition, adipose tissue secretes a variety of peptides called adipocytokines that have endocrine, autocrine and paracrine effects on the brain, liver and skeletal muscle. These peptides play an important role in the regulation of energy homeostasis and intermediary metabolism. Dysfunction of adipose tissue may result in insulin resistance, type 2 diabetes mellitus and obesity (Garg 2006).

*Prepl* is an homeodomain transcription factor that regulates the overall size of the organism and individual organs as well as major developmental pathways. Data produced in our lab indicate that *Prepl* hypomorphic mice (*Prepl<sup>i/+</sup>*) have a complex metabolic phenotype characterized by increased insulin sensitivity accompanied by protection from streptozotocin-induced diabetes. In muscle tissue, *Prepl* deficiency positively modulates the intracellular pool of GLUT4 by enhancing the transcription of PGC-1 $\alpha$  gene. In the liver, the mechanism is independent on PGC-1 $\alpha$  and the improvement of insulin signaling is characterized by a reduction of tyrosine phosphatases SHP1, whose transcription is mediated by *Prepl* and by an increase of insulin-stimulated insulin receptor and IRS1/2 tyrosine phosphorylation. In addition, *Prepl* deficiency in mice induces a reduction of hepatic triglycerides synthesis and a protection from methionine and choline-deficient diet-induced steatohepatitis. In fact, in *Prepl<sup>i/+</sup>* mice liver there is an increase of phosphorylation of PKCzeta, LKB1, AMPK and ACC, leading to an inhibition of TG synthesis. This regulation is due to the modulation of expression of SHIP2, a lipid phosphatase, known to be an inhibitor of PI3Kinase/PKCzeta signaling.

Thus *Prepl* regulates several aspects of whole body metabolism (Oriente et al. 2008; Oriente et al. 2011; Oriente et al. 2013), but whether and how *Prepl* affects adipose compartment is still unknown. The aim of my project is to investigate the role of *Prepl* on adipogenesis and adipose tissue function, by analyzing the molecular mechanisms both “*in vivo*” and “*in vitro*”.



### 3. MATERIALS AND METHODS

#### 3.1 Materials

Media, sera, antibiotics for cell culture and the lipofectamine reagent were from Invitrogen (Grand Island, NY). The Prep1, actin, C/EBP $\alpha$ , FABP4, PPAR $\gamma$ , pAKT-Ser473 and MAPK antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The GLUT4 antibody was from Abcam, Inc. (Cambridge, MA). The p-IR-Tyr1146, IR, pMAPK, antibodies were from Cell Signaling Technology, Inc. The AKT antibody was from Millipore Corporation (Billerica, MA). Protein electrophoresis and Real Time PCR reagents were purchased from Bio-Rad (Hercules, CA), Western blotting and ECL reagents from Amersham Biosciences (Arlington Heights, IL). Oil Red O stain and all other chemicals were from Sigma (St. Louis, MO).

#### 3.2 Generation of Prep1 hypomorphic mice

*Prep1* targeted mice were generated by gene trapping by Lexikon Genetics, Inc. (The Woodlands, Texas) and have been previously described (Ferretti et al. 2006; Penkov et al. 2005; Fernandez-Diaz et al. 2010). In the experiments reported in this paper, heterozygous mice were backcrossed with wild-type (WT) C57BL/6J for 4 generations. All animal handling conformed to regulations of the Ethics Committee on Animal Use of H. S. Raffaele (IACUC permission number 207). Adipose tissue samples were collected and weighed after mice were sacrificed by pentobarbitone overdose. Tissues were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent western blotting and Real Time RT-PCR analysis.

#### 3.3 Adipose tissue histological and immunohistochemical analysis

For the histological examination, epididymal adipose tissue from killed mice were immediately incised and weighed. Small pieces were fixed in 10% formalin and stained with haematoxylin and eosin for histological analysis. Image-Pro Plus $\text{\textcircled{C}}$  program was used for area and cells number measurement. For cells number measurement, HPF means High Power Field that is the area visible under the maximum magnification power of the objective being used. For immunohistochemical examination, frozen sections (6 $\mu\text{m}$ ) were fixed in acetone at  $4^{\circ}\text{C}$  for 5 min, then blocked for endogenous peroxidase in 0.3%

H<sub>2</sub>O<sub>2</sub> in methanol for 20 min. Adipose sections were further incubated with mouse monoclonal antibody against GLUT4 (dilution 1:50) overnight at 4°C. Slides were washed three times with PBS, incubated with biotinylated anti-mouse and labeled streptavidin biotin (LSAB kit; DakoCytomation, Glostrup, Denmark) for 30 min, followed by incubation with streptavidin conjugated to horseradish peroxidase (LSAB Kit; DakoCytomation, Denmark). Color development was assessed following 5 min of diaminobenzidine treatment (DakoCytomation, Denmark) treatment. Sections were counterstained with Mayer's haematoxylin. In the corresponding negative control section, the primary antibody was either omitted or replaced with normal mouse serum.

### 3.4 Mouse mature adipocytes isolation and [U-<sup>14</sup>C]glucose uptake assay

Isolated mouse adipose cells of epididymal fat pads from male 6-months-old *Prepl*<sup>i/+</sup> and WT littermates were prepared by Type 1 collagenase digestion (1mg/ml) in KRBH-5% BSA buffer (Krebs Ringer Bicarbonate Hepes buffer, pH 7.4 containing 30mmol/L Hepes, 10mM NaHCO<sub>3</sub>, 0.12M NaCl, 4mM KH<sub>2</sub>PO<sub>4</sub>, 1mM MgSO<sub>4</sub>, 1mM CaCl<sub>2</sub>, 200nM adenosine and 5% BSA) at 37°C on shaking. After 2 hours of digestion, cells were filtered through a 250µm nylon mesh filter and then washed 3 times with KRBH-5% BSA buffer. After the final wash, cells were ready to use. Cells were counted using TC10 Automated cell counter (Biorad Laboratories, Inc. Hercules, CA) according to the manufacturer's instructions. Aliquots of adipose cells were incubated with or without 10KµU/ml (60nmol/L) insulin for 30 minutes before adding [U-<sup>14</sup>C]glucose (0.5µCi/ml) at 37°C on shaking. After 30 minutes aliquots of each samples were put in tubes with dinonylphthalate oil and spinned to separate the cells with aqueous phase. Scintillation liquid was added to the collected cells for the measurement of [U-<sup>14</sup>C]glucose on a beta counter.

### 3.5 Cell culture procedures and transfection

3T3L1 murine fibroblast cells were available in host laboratory. They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (FCS), 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2% L-glutamine in a 5% humidified CO<sub>2</sub> incubator. 3T3L1 cells were differentiated as previously described (Engelman et al. 1998). In brief, after 2 days from confluence (Day 0), the DMEM 10% FCS was changed and new DMEM 10% FBS was added to the cells with the addition of 174nM insulin, 10mM dexamethasone and 0.5mM 3-isobutyl-1-methylxanthine. After 2 days (Day 2), this medium was replaced with fresh DMEM 10% FCS containing

only 174nM insulin. After another 2 days, the cells were then propagated in only 10% FCS medium.

3T3L1 stable transfection of *Prep1* and PRC/CMV plasmid cDNAs were performed by the Lipofectamine reagent (Invitrogen) according to the manufacturer's instruction. For these studies, 60-80% confluent cells were washed twice with Optimem and incubated for 8h with 3-5  $\mu$ g of plasmid construct and 45-60  $\mu$ l of Lipofectamine reagent. In stably transfection individual G418-resistant clones were selected by the limiting dilution technique (G418 effective dose, 0.8 mg/ml).

### 3.6 Oil Red O-staining

To measure cellular neutral lipid droplet accumulation, 3T3L1 cells were stained by the Oil Red O method. *Prep1* stably transfected 3T3L1 cells were differentiated as described above. Mature adipocytes were washed three times with iced PBS and fixed with 4% paraformaldehyde for 30 minutes. After fixation, cells were washed three times and stained with Oil Red O solution (working solution, 0.5g Oil Red O powder Sigma dissolved in 60% ethanol) for 15 min at room temperature. Cells were washed again with phosphate-buffered saline (PBS) to remove unbound staining. The 3T3L1 adipocytes were examined under a light microscope, and the red oil droplets stained in the cells indicate TG accumulation. The ORO was extracted in 100% isopropanol and absorbance was evaluated at 510nm as previously described (Ramirez-Zacarias et al. 1992).

### 3.7 Western blot analysis

Tissue samples were homogenized in a Polytron (Brinkman Instruments, N.Y.) in 20 ml T-PER reagent/gram of tissue according to manufacture (Pierce, IL). After centrifugation at 10,000 rpm for 5 minutes, supernatant was collected. Cells were solubilized in lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 10 mmol/L EDTA, 10 mmol/l Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 100 mmol/L NaF, 10% glycerol, 1% Triton X-100, 1 mmol/L PMSF, 10 mg/ml aprotinin) for 1 h at 4C and lysates were centrifuged at 5,000g for 20 min. Total homogenates were separated by SDS-PAGE and transferred on 0.45 m Immobilon-P membranes. Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions.

### 3.8 Real-Time RT-PCR analysis

Total cellular RNA was isolated from adipose tissue and 3T3L1 cells by using the RNeasy Lipid kit (QIAGEN Sciences, Germany), according to manufacturer's instructions. 1 µg of tissue or cell RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen, CA). PCR reactions were analyzed using SYBR Green mix (Bio-Rad, Hercules, CA). Reactions were performed using Platinum SYBR Green qPCR Super-UDG using an iCycler IQ multicolor Real Time PCR Detection System (Bio-rad, Hercules, CA). All reactions were performed in triplicate and β-actin was used as an internal standard. Primer sequences used were as follows:

C/EBPα	F: 5' TTACAACAGGCCAGGTTTCC 3' R: 5' CTCTGGGATGGATCGATTGT 3'
GLUT4	F: 5' CAGAAGGTGATTGAACAGAG 3' R: 5' AATGATGCCAATGAGAAAGG 3'
FABP4	F: 5' AATCACCGCAGACGACAG 3' R: 5' ACGCCTTTCATAACACATTCC 3'
PPARγ	F: 5' TGGTGCCTTCGCTCATGC 3' R: 5' CTGTGGTAAAGGGCTTGATGTC 3'
PREP1	F: 5' CCTGGGACAATTAGGATCCAGAACT 3' R: 5' TCCTCTGTTGGGTAGGGATGCC 3'
β-ACTIN	F: 5' CGCCCTAGGCACCAGGGTGTG 3' R: 5' TCGGTGAGCAGCACAGGGTG 3'

### 3.9 Statistical procedures

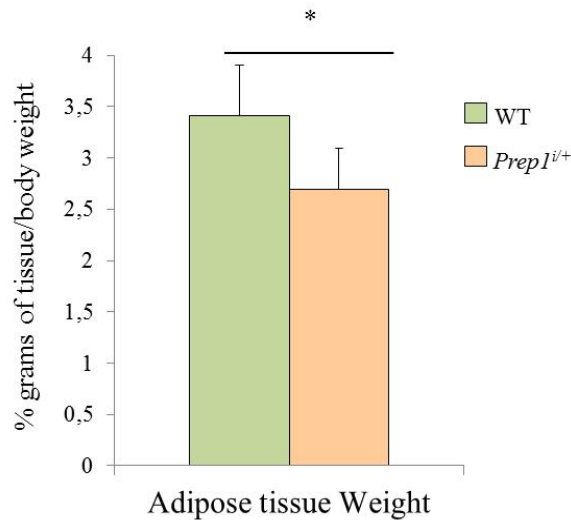
Data were analysed with the Statview software (Abacus-concepts) by two-factor analysis of variance. P values of less than 0.05 were considered statistically significant.



## 4. RESULTS

### 4.1 Characterization of *Prepl* hypomorphic heterozygous mice adipose tissue

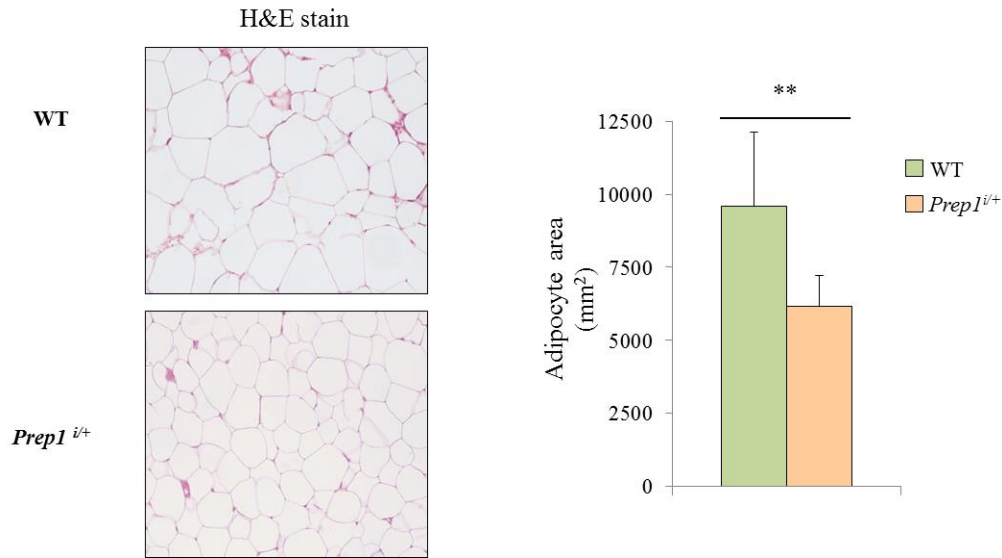
To investigate the impact of *Prepl* deficiency in adipose compartment, I first evaluated the epididymal adipose tissue weight of wild-type (WT) and *Prepl*<sup>i/+</sup> mice. As shown in figure 9, a reduction of epididymal fat pad weight in *Prepl* hypomorphic heterozygous mice was observed.



**Figure 9: Epididymal adipose tissue weight of *Prepl*<sup>i/+</sup> and WT mice.**

Adipose tissue of *Prepl*<sup>i/+</sup> and WT mice were dissected and weighed. The graphs show percent of epididymal adipose tissue weight/total body weight. Values are means  $\pm$ SD of six mice for groups of five different evaluation. (\*P < 0.05).

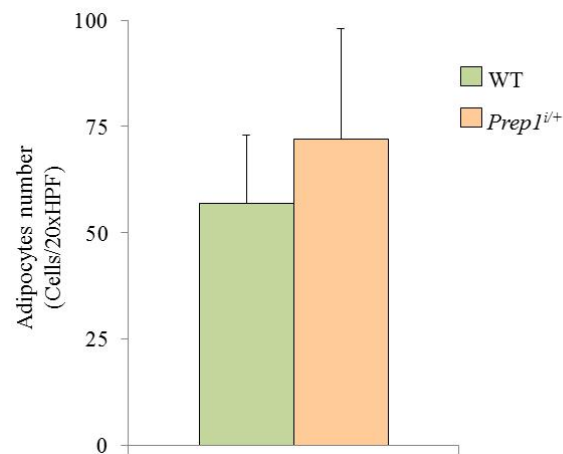
Next, I performed an histological analysis of adipose tissue of *Prepl*<sup>i/+</sup> and WT mice. Hematoxylin-eosin staining revealed that *Prepl* hypomorphic heterozygous adipocytes had a significantly reduced cell area ( $6149.25 \pm 1070.18 \mu\text{m}^2$ ) compared to the adipocytes of the WT mice ( $9605.94 \pm 2522.07 \mu\text{m}^2$ ) (figure 10).



**Figure 10: Histological analysis of adipose tissue from *Prep1<sup>i/+</sup>* and WT mice.**

Sections from epididymal adipose tissue from 6-months-old *Prep1<sup>i/+</sup>* and WT littermates were stained with hematoxylin-eosin (original magnification 40X). The microphotographs shown are representative of findings observation in five *Prep1<sup>i/+</sup>* and seven control animals. Image-Pro Plus© program was used for area measurement. Values were obtained from eight different observation in five *Prep1<sup>i/+</sup>* and seven control animals. (\*\* $P < 0.01$ ).

Despite the reduced cell area, there was no significant difference in cell number between WT ( $57 \pm 16$  cells/HPF) and *Prep1<sup>i/+</sup>* ( $72 \pm 26$  cells/HPF) adipocytes (figure 11).



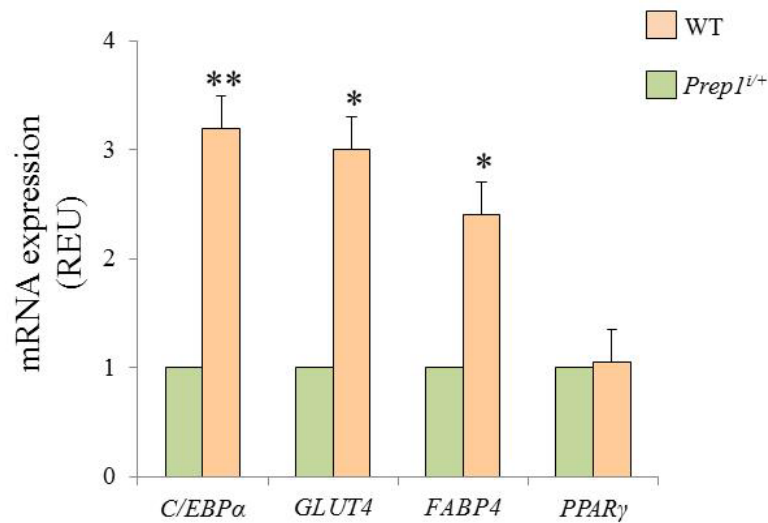
**Figure 11: Adipocytes number of *Prep1<sup>i/+</sup>* mice.**

Sections from epididymal adipose tissue from 6-months-old *Prep1<sup>i/+</sup>* and WT littermates were stained with hematoxylin-eosin. Image-Pro Plus© program was used for cells number

measurement. Values were obtained from eight different observation in five *Prep1<sup>i/+</sup>* and seven control animals

#### 4.2 *Prep1* deficiency leads to increased expression of adipogenic markers

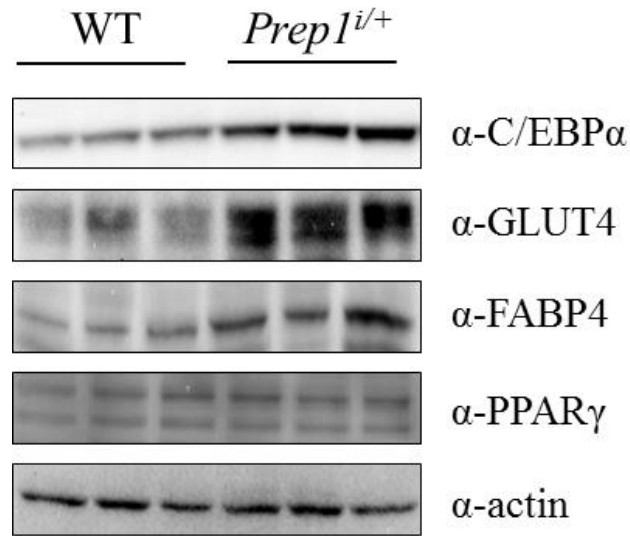
To further study the function of *Prep1*, I evaluated the expression of some markers of adipocytes differentiation, including *C/EBP $\alpha$* , *GLUT4*, *FABP4* and *PPAR $\gamma$*  in *Prep1* hypomorphic heterozygous and WT mice. In adipose tissue of *Prep1<sup>i/+</sup>* mice there was a significant increase of *C/EBP $\alpha$* , *GLUT4* and *FABP4* mRNA expression, while *PPAR $\gamma$*  expression level did not change (figure 12).



**Figure 12: *C/EBP $\alpha$* , *GLUT4*, *FABP4* and *PPAR $\gamma$*  mRNA levels in *Prep1<sup>i/+</sup>* mice.**

The abundance of *C/EBP $\alpha$* , *GLUT4*, *FABP4* and *PPAR $\gamma$*  mRNAs were determined by real-time RT-PCR analysis of total RNA isolated from the adipose tissue of heterozygous and control mice, using  *$\beta$ -actin* as internal standard. Bar represents the mean  $\pm$  SD of four independent experiments, in each of which reactions were performed in triplicate using total RNAs obtained from eight mice per genotype. Asterisks denote statistically significant differences (\* $P$ <0.05; \*\* $P$ < 0.01).

Consistent with these data, protein expression of *C/EBP $\alpha$* , *GLUT4* and *FABP4* was increased in the adipose tissue of *Prep1<sup>i/+</sup>* mice, while *PPAR $\gamma$*  protein levels were unchanged (figure 13).

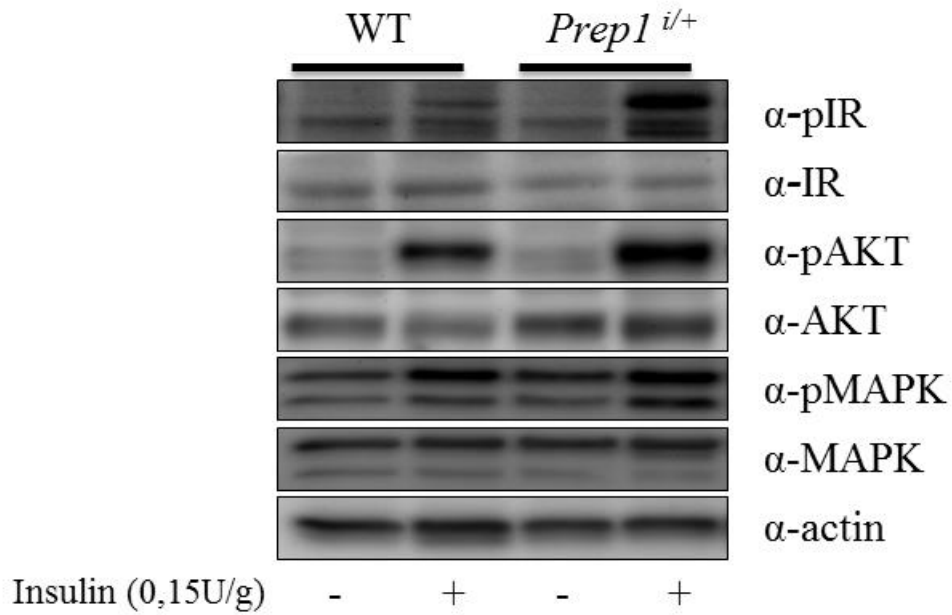


**Figure 13: C/EBP $\alpha$ , GLUT4, FABP4 and PPAR $\gamma$  protein levels in *Prep1<sup>i/+</sup>* mice.**

Tissues from *Prep1* hypomorphic heterozygous and control mice were dissected, solubilized, and protein samples analyzed by Western blot with C/EBP $\alpha$ , GLUT4, FABP4 and PPAR $\gamma$  antibodies. Actin antibody was used for normalization. Blot were revealed by ECL and autoradiograph is representative of three independent experiments.

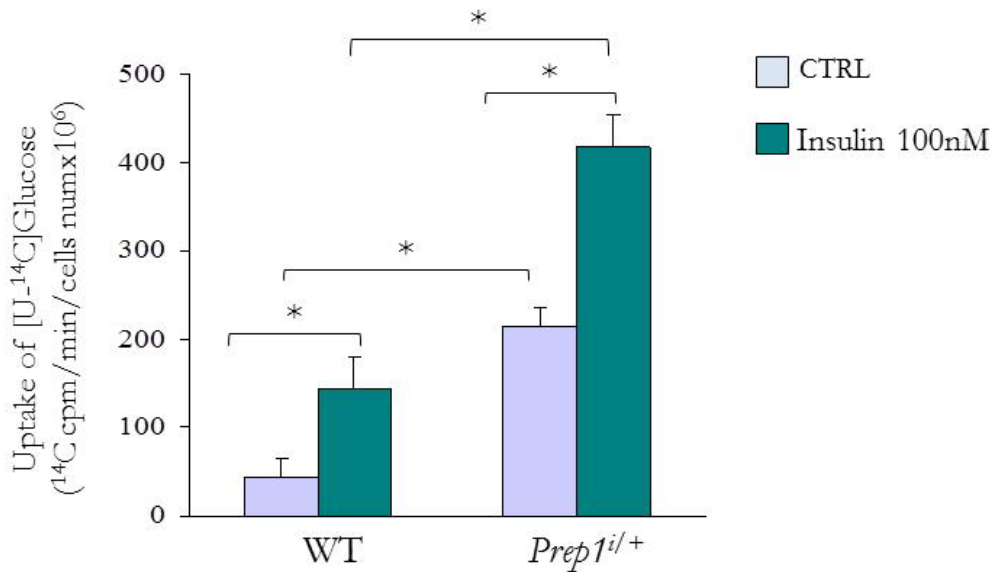
#### **4.3 Insulin signaling and [U-<sup>14</sup>C]glucose uptake in adipose tissue of *Prep1*-deficient mice**

To investigate the role of *Prep1* in adipose tissue function, WT and *Prep1<sup>i/+</sup>* mice were intraperitoneally injected with insulin (0,15U/g) and the activation of the downstream molecular effectors were analyzed by Western blot. As shown in figure 14, in adipose tissue of *Prep1* hypomorphic heterozygous mice I observed a significant increase of insulin receptor (IR)-tyrosine phosphorylation, upon insulin stimulation, while protein level did not change. Both the downstream effectors, AKT and MAP kinases, were also more activated in the *Prep1<sup>i/+</sup>* mice, with no difference in their expression.



**Figure 14: Insulin receptor, AKT and MAP kinase phosphorylation in *Prep1*<sup>i/+</sup> mice.** Tissues from *Prep1* hypomorphic heterozygous and control mice were dissected, solubilized, and protein samples analyzed by Western blot with pIR-Tyr1146, IR, pAKT –Ser473, AKT, pMAPK, MAPK antibodies. Actin antibody was used for normalization. Blot were revealed by ECL and autoradiograph is representative of three independent experiments.

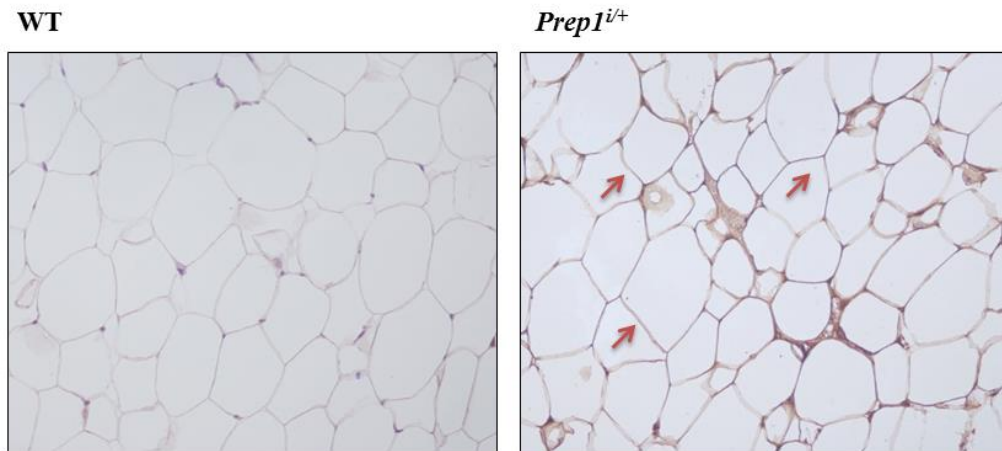
Next, I measured the insulin-stimulated [U-<sup>14</sup>C]glucose uptake of mature adipocytes isolated from *Prep1*<sup>i/+</sup> and WT mice adipose tissue. The graph (figure 15) shows a 3- and 2-fold increase of [U-<sup>14</sup>C]glucose uptake of *Prep1*<sup>i/+</sup> adipocytes at basal and insulin-stimulated level, respectively.



**Figure 15: [U-<sup>14</sup>C]glucose uptake of adipocytes isolated from adipose tissue of WT and *Prep1<sup>i/+</sup>* mice.**

Mature adipocytes were isolated as described in Material and Methods. Then were counted and aliquots were incubated in KRBH-5% BSA with or without 100nM insulin for 30 minutes before adding [U-<sup>14</sup>C]glucose (0.5 $\mu$ Ci/ml) at 37° C on shaking. After 30 minutes aliquots of each samples were put in scintillation liquid for measurement of [U-<sup>14</sup>C]glucose on  $\beta$ -counter. Bar represents the mean  $\pm$ SD of four independent experiments, in each of which reactions were performed in quintuple using adipocytes obtained from two mice for genotype (\*P<0.05).

To explain the increase of [U-<sup>14</sup>C]glucose uptake in *Prep1* hypomorphic heterozygous adipocytes, I performed immunochemistry for GLUT4 in adipose tissue of *Prep1<sup>i/+</sup>* and WT mice. Staining with specific antibody revealed significantly higher expression of GLUT4 on the plasma membrane of adipocytes of *Prep1<sup>i/+</sup>* mice compared with that of control animals (figure 16). This data was also consistent with the increased GLUT4 expression as showed in figure 12-13.

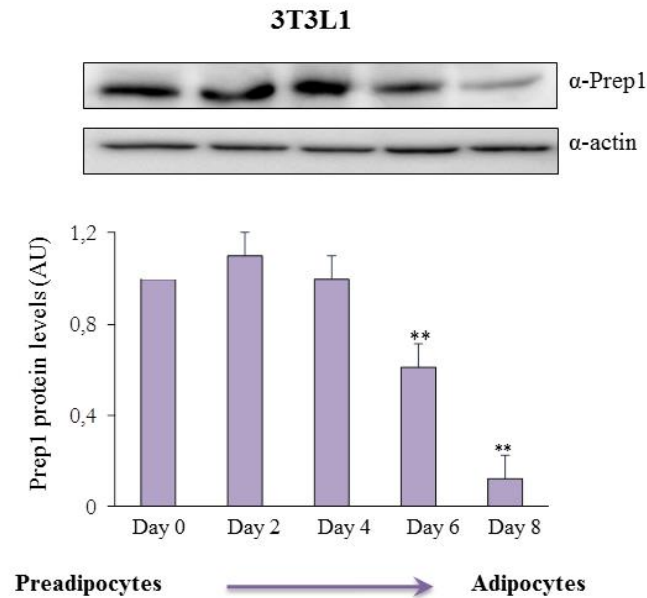


**Figure 16: Immunohistochemical analysis of adipose tissue from *Prep1*<sup>i/+</sup> and WT mice.** Sections from adipose tissue from 6-months-old *Prep1*<sup>i/+</sup> and WT littermates were stained with hematoxylin-eosin and immunostained with GLUT4 antibody (original magnification 40X). The microphotographs shown are representative of findings observation in six *Prep1*<sup>i/+</sup> and five control animals.

All together these data suggest that *Prep1* could regulate adipocyte differentiation and adipose tissue functions.

#### 4.4 *Prep1* protein expression is decreased during 3T3L1 adipocyte differentiation

To better understand the *Prep1* involvement in the regulation of adipogenesis, I evaluated in 3T3L1 cells, an established cell line of murine origin, whether the expression levels of *Prep1* may change during adipocyte differentiation. To this aim, I induced differentiation in this cell line after reaching the state of confluence and I evaluated the protein expression of *Prep1* during the different days of the differentiation process. As shown in figure 17, *Prep1* protein levels were progressively reduced during the conversion from 3T3L1 preadipocytes to adipocytes (from day 0 to day 8), indicating that *Prep1* down-regulation could be important for the differentiation process.



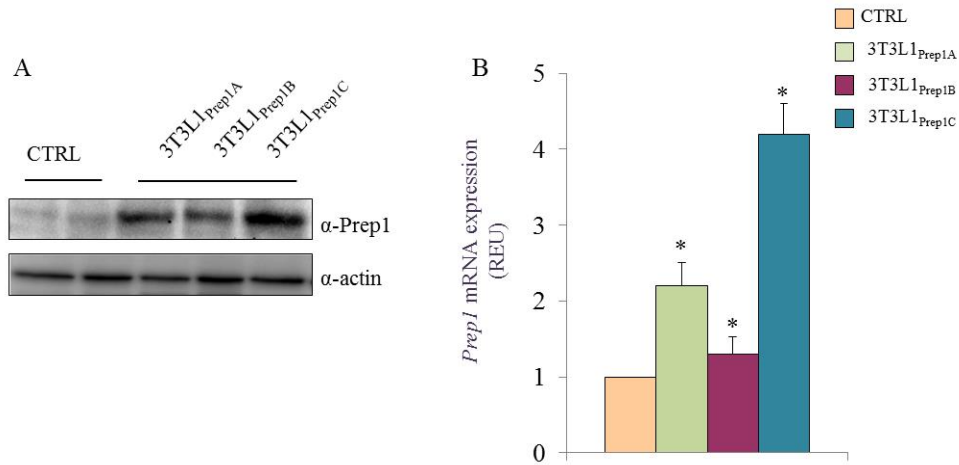
**Figure 17: Prep1 protein expression during 3T3L1 adipocytes differentiation.**

3T3L1 cells were solubilized, lysed and analyzed by immunoblotting with Prep1 antibodies and autoradiography. Actin antibodies were used for normalization. Blot were revealed by ECL and autoradiograph subjected to densitometric analysis. The autoradiograph shown on the top of the graphic is representative of four independent experiments. Asterisks denote statistically differences (\*\*P < 0.01).

#### 4.5 *Prep1* stable transfection and Oil Red-O staining in 3T3L1 cells

The data obtained in the *Prep1*<sup>i/+</sup> mice were further investigated in stably transfecting *Prep1* cDNA (PRC/CMV *Prep1*) or empty vector (PRC/CMV) in 3T3L1 cells. Cells were incubated with DMEM 10% FCS containing the antibiotic G418 (0.8 mg/mL) and several clones were obtained. Three of those, expressing increasing levels of Prep1, were selected and further characterized (figure 18A-B) and the 3T3L1<sub>Prep1c</sub> clone overexpressing Prep1 by 4-fold was used for the subsequent experiments.

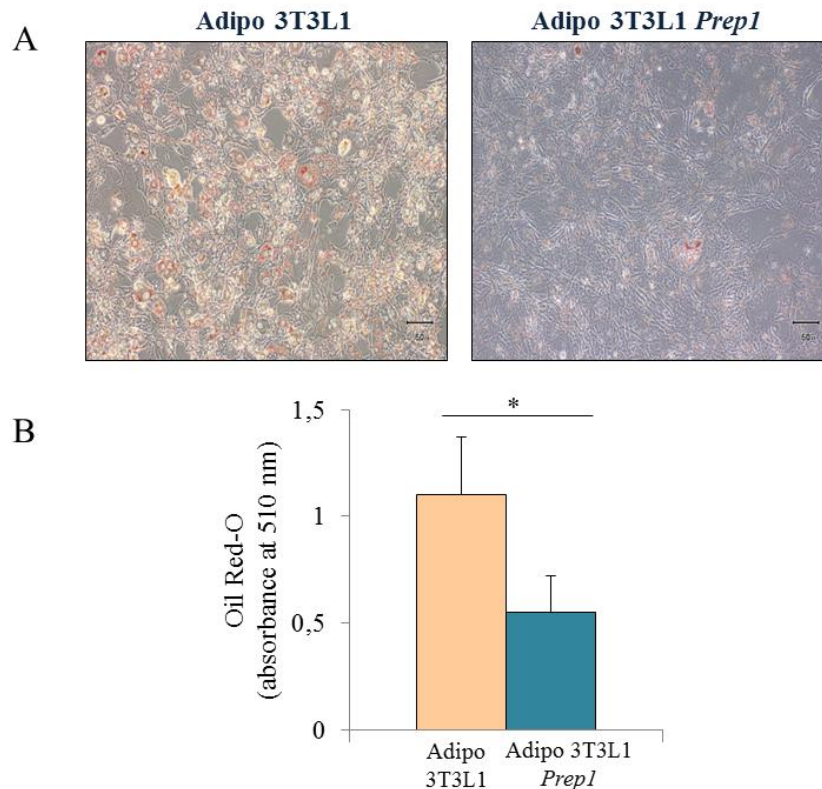




**Figure 18: *Prep1* stable transfection in 3T3L1 cells.**

A: 3T3L1 cells were stable transfected with *Prep1* cDNA. Cells were then solubilized and lysates were analyzed by immunoblotting with *Prep1* antibodies and autoradiography to verify the transfection. Actin antibodies were used for normalization. The autoradiograph shown is representative of three different experiments. B: The levels of *Prep1* mRNA was quantitated by real-time RT-PCR analysis, using  $\beta$ -actin as internal standard. Bars represent the mean  $\pm$  SD of four independent experiments. Asterisks denote statistically significant differences (\* $P < 0.05$ ).

Thus, I evaluated the effect of *Prep1* overexpression on lipid accumulation as marker of adipocytes differentiation. Control (CTRL) and overexpressing *Prep1* 3T3L1 adipocytes were analyzed by microscopy after staining with Oil Red-O (ORO). In control 3T3L1 adipocytes, lipid droplets were clearly observed with light red cytoplasmic staining, as also shows by the ORO amount (figure 19A), while in 3T3L1 adipocytes overexpressing *Prep1* accumulation lipid droplets was barely detected (figure 19B).

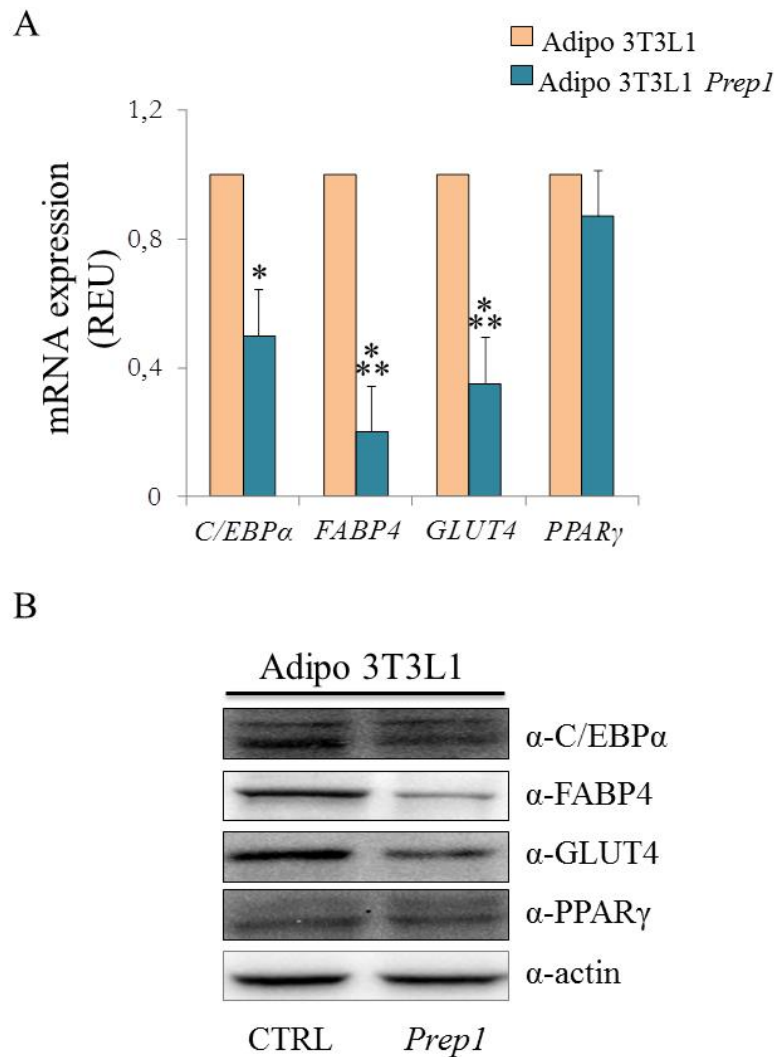


**Figure 19: Effect of *Prep1* overexpression on lipid accumulation in 3T3L1 adipocytes.**

A: Control and stably overexpressing *Prep1* 3T3L1 adipocytes were stained with Oil Red-O as described in Materials and Methods. The photographs shown are representative of five independent experiments. B: After microscopic study, the ORO was extracted in 100% isopropanol and absorbance was evaluated at 510nm.

#### **4.6 Adipogenic markers expression and insulin signaling in 3T3L1 adipocytes stably overexpressing *Prep1***

To further examine the role of *Prep1* on adipogenesis, I evaluated the mRNA and the protein expression of adipogenesis markers genes C/EBP $\alpha$ , PPAR $\gamma$ , GLUT4 and FABP4 in control and overexpressing *Prep1* adipocytes. As shown in figure 20, the overexpression of *Prep1* in 3T3L1 adipocytes induced a significant reduction of mRNA (figure 20A) and protein (figure 20B) expression of C/EBP $\alpha$ , GLUT4 and FABP4 compared to the control 3T3L1 adipocytes. PPAR $\gamma$  levels did not change between control and overexpressing *Prep1* adipocytes.

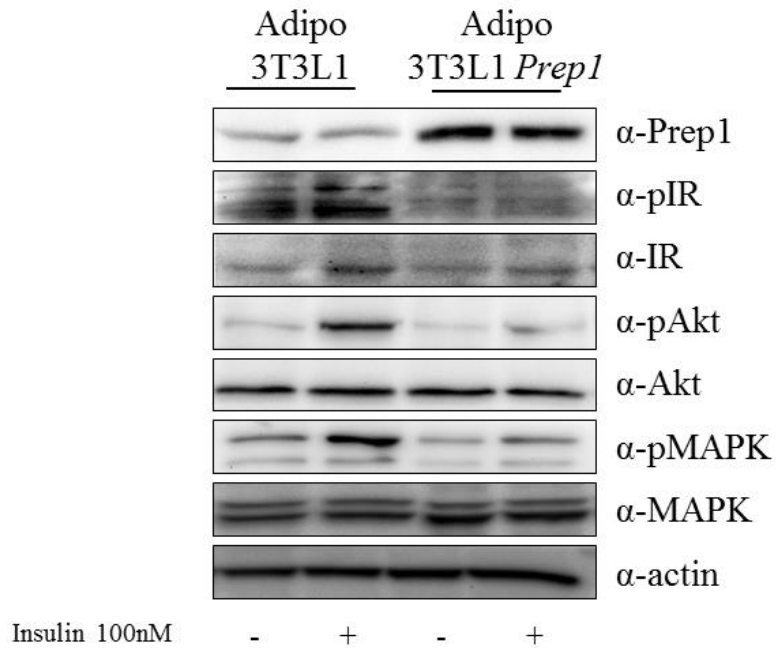


**Figure 20: Adipogenic markers in *Prep1* stably overexpressing 3T3L1 adipocytes.**

A: Control and stably overexpressing *Prep1* 3T3L1 cells were differentiated as described in Material and Methods. The levels of *C/EBPα*, *FABP4*, *GLUT4* and *PPARγ* mRNA were quantitated by real-time RT-PCR analysis, using  $\beta$ -actin as internal standard. Bars represent the mean  $\pm$ SD of four independent experiments. Asterisks denote statistically significant differences (\* $P < 0.05$ ; \*\*\* $P < 0.001$ ). B: Cells were solubilized and lysates were analyzed by immunoblotting with *C/EBPα*, *FABP4*, *GLUT4* and *PPARγ* antibodies and autoradiography. Actin antibodies were used for normalization. The autoradiograph shown is representative of three different experiments.

Next, I analyzed the insulin pathway in 3T3L1 adipocytes. As showed by Western blot experiments (figure 21), in control 3T3L1 adipocytes,

stimulation with insulin 100nM for 10 minutes induced the phosphorylation of IR-Tyr1146, AKT-Ser473, MAPK, while in adipocytes overexpressing *Prep1* insulin failed to elicit this effect.



**Figure 21: Insulin signaling in 3T3L1 adipocytes overexpressing *Prep1***

Cells were solubilized and lysates were analyzed by immunoblotting with Prep1, pIR-Tyr1146, IR, pAKT-Ser473, AKT, pMAPK, MAPK antibodies and autoradiography. Actin antibodies were used for normalization. The autoradiograph shown is representative of four different experiments.

These data confirm the role of *Prep1* in regulating both the adipose tissue differentiation and insulin action.

## 5. DISCUSSION

In a modern lifestyle, people are chronically exposed to elevated amounts of lipids and nutrients potentially leading to tissue dysfunction and disease. The adipose tissue is highly involved in the development of metabolic disorders such as cardiovascular disease (CVD), obesity and type 2 diabetes mellitus (T2DM). It is the body's largest storage site for triglycerides, and normally, fat storage takes place during food intake to be replaced by fat mobilization processes during fasting or situations with elevated energy demand. Adipocytes produce a number of endocrine hormones that contribute to the regulation of these mechanisms and of whole body homeostasis (Bjørndal et al. 2011). Knowledge of adipocyte biology is crucial for understanding the pathophysiological basis of obesity and type 2 diabetes.

Previous studies have identified *Prepl* as a physiologic regulator of glucose and lipid metabolisms (Oriente et al. 2008; Oriente et al. 2011; Oriente et al. 2013). In *Prepl*-deficient mice, muscle sensitivity to insulin action on glucose disposal is significantly increased, due to down-regulation of an important *Prepl* partner p160<sup>MBP</sup> and induction of the *GLUT4* gene activator PGC-1 $\alpha$ . In the liver, *Prepl* silencing enhances insulin signaling, thereby increasing glucose storage and reducing glucose output. Indeed, *Prepl* hypomorphic mice show increased hepatic glycogen content and decreased triglyceride levels. The potential significance of these findings to insulin-resistance and diabetes mellitus is further underlined by our experiments in the L6 skeletal muscle and HepG2 human hepatoma cell lines evidencing that overexpression of *Prepl* induces resistance to insulin action. In addition, *Prepl* deficiency decreases triglyceride synthesis in liver and this effect is due to an increase of PKC $\zeta$ , LKB1, AMPK and ACC phosphorylation, which determines the reduction of *FAS* expression. These effects are paralleled by a significant decrease of the lipid phosphatase SHIP2, that negatively regulates this pathway. Consistent with these data, *Prepl* gene silencing protects mice from the TG accumulation and hepatocyte damage caused by a methionine-choline deficient diet administration.

It is well established that *Prepl* regulates several aspects of whole body homeostasis, even though its impact on adipose compartment is still unknown. In the present work, I show that *Prepl* deficiency induces a reduction of adipose mass in mice, with a decrease of adipocyte cell area without variations of cell number. Adipocytes are dynamic cells and their expansion is critical for insulin sensitivity and overall metabolic health. Furthermore, adipose tissue functionality is closely related to the adipocytes differentiation state, I focused my attention on adipogenesis. The development of fully differentiated mature adipocytes from precursor cells is an elegant progression involving the sequential activation of several transcription factors (Stephens 2012). The activation of these transcription factors leads to expression of protein

necessary to established adipocyte phenotype. Early events of the adipogenic process include the activation of C/EBP $\alpha$  and PPAR $\gamma$ , critical pro-adipogenic transcription factors, that determine the expression of proteins involved in glucose and lipid metabolism, such as GLUT4 and FABP4; all these genes are usually considered adipogenic markers of differentiation. My results show that the mRNA and protein expression of C/EBP $\alpha$ , GLUT4 and FABP4 are increased in adipose tissue of *Prepl*<sup>i/+</sup> mice. Interestingly, expression levels of PPAR $\gamma$  are unchanged in *Prepl* hypomorphic heterozygous mice. It has been demonstrated that mice in which PPAR $\gamma$  was ablated in adipocytes became lipodystrophic (He et al. 2003), but on the other hand, PPAR $\gamma$ <sup>-/+</sup> mice were protected from adipocytes hypertrophy and insulin resistance under high-fat diet treatment (Kubota et al. 1999). It is clear that PPAR $\gamma$  plays an important role in adipose tissue homeostasis and it is plausible that in adipose tissue of *Prepl*<sup>i/+</sup> mice several compensatory mechanisms act to preserve PPAR $\gamma$  expression and so fat compartment.

Critical to adipocyte functions is the capacity to respond to insulin, and this feature is acquired during adipogenesis when mature cells arise from preadipocytes. In addition, it has well been demonstrated that *Prepl* hypomorphic heterozygous mice show better insulin sensitivity. So, upon intraperitoneal insulin injection, my results demonstrate that *Prepl*<sup>i/+</sup> mice show a significant increase of IR tyrosine phosphorylation, in parallel with an increase activation of the downstream effectors AKT and MAPK. This higher capacity to respond to insulin stimulation is accompanied by an increase of basal and insulin-stimulated [U-<sup>14</sup>C]glucose uptake of mature adipocytes isolated from *Prepl*<sup>i/+</sup> mice adipose tissue. The increase of [U-<sup>14</sup>C]glucose uptake in these mice is most likely due to higher persistence of GLUT4 on the plasma membrane of adipocytes. These data demonstrate that *Prepl* may play a role also in adipose tissue functions.

To address the hypothesis that *Prepl* can regulate adipose tissue functions through its capacity to affects adipocytes differentiation, I evaluated Prepl protein expression during adipogenesis in a cellular model, murine 3T3L1. 3T3L1 preadipocytes are fibroblastic cells determined to the adipocyte lineage and fully differentiated 3T3L1 adipocytes possess most of the morphological, biochemical and hormonal response characteristics of adipocytes *in vivo* (Sadowski et al. 1992). My results show that Prepl protein abundance decreases during the conversion of 3T3L1 preadipocytes to adipocytes, in particular at the last days of adipogenic process. This data indicates that the absence of Prepl could be important for adipocytes differentiation. To further investigate my hypothesis, I stably transfected 3T3L1 preadipocytes with full-length *Prepl* cDNA. The 3T3L1 adipocytes overexpressing *Prepl*, after Oil Red-O staining, show a reduction of lipid accumulation. In addition, at the opposite to the adipose tissue of *Prepl*<sup>i/+</sup> mice, the overexpression of *Prepl* significantly reduces the mRNA and protein expression of the adipogenic

markers C/EBP $\alpha$ , GLUT4 and FABP4, while PPAR $\gamma$  levels does not change. These data indicate that *Prep1* affects adipocyte differentiation. In addition, *Prep1* overexpression in 3T3L1 adipocytes induces a reduction of insulin receptor, AKT and MAPK phosphorylation, indicating that *Prep1* impact on adipogenesis is also paralleled by an impairment of 3T3L1 adipocytes functions. Further experiments will be needed to clarify the molecular mechanisms by which *Prep1* regulates expression of adipogenic genes and insulin pathway.

In summary, the major finding of the present study is that *Prep1* induces adipose tissue dysfunction probably through an impairment of adipocyte differentiation. Although the precise molecular mechanisms and signaling pathways regulated by *Prep1* remain to be better clarified, suppression of this transcription factor may represent a beneficial strategy in the treatment of obesity and thereby prevent type 2 diabetes. Nevertheless, as my data have been obtained in murine models, another important point which I would like to study in the future is the clinical relevance of *Prep1* in humans. We have preliminary data showing that first-degree relatives of type 2 diabetics (these individuals have an high risk of type 2 diabetes) feature 3.5-fold higher *Prep1* levels compared to control individuals with no family history of diabetes. In addition, in these first-degree relatives, *Prep1* expression levels negatively correlate with insulin sensitivity. These subjects have a very high risk of diabetes and are known to be insulin-resistant, suggesting that *Prep1* overexpression may provide an early contribution to diabetes progression in these individuals. However, whether *Prep1* overexpression induces insulin-resistance in human and whether it is sufficient to impair glucose tolerance is still unknown and will be the subject of my future studies.





## 6. CONCLUSIONS

Human obesity is characterized by an excess of adipose tissue mass that has potential adverse health consequences and may finally result in a reduced quality of life and life expectancy. Among the many complications that are associated with obesity, disturbances of glucose metabolism and cardiovascular system are of particular relevance. Excess body fat promotes the development of type 2 diabetes mellitus and dyslipidemia, favors other cardiovascular risk factors such as hypertension and impaired fibrinolysis and may therefore result in an increased incidence of cardiovascular complications. The precise causal relationship between obesity and these disturbances is only poorly understood, but there is growing evidence that alterations in fat cell function may directly or indirectly contribute to the development of these unfavorable health hazards (Hauner & Skurk 2001).

In the present work I have focused my attention on *Prepl* effects on adipose compartment. I have described that *Prepl*<sup>i/+</sup> mice show a reduction of epididymal adipose tissue weight, a reduction of the adipocyte area but not changes in the number of adipocytes. In addition, expression of adipogenic markers, C/EBP $\alpha$ , GLUT4 and FABP4, is increased in adipose tissue of *Prepl*<sup>i/+</sup> mice, while PPAR $\gamma$  does not change. These effects are paralleled by increased phosphorylation of insulin receptor (IR), AKT and MAPK in adipose tissue of *Prepl*<sup>i/+</sup> mice. In addition, basal and insulin- stimulated glucose-uptake is increased in adipocytes isolated from adipose tissue of *Prepl*<sup>i/+</sup> mice compared to the adipocytes from WT mice. This increase is accompanied by the higher expression of GLUT4 on the plasma membrane of adipocytes of *Prepl*<sup>i/+</sup> mice.

In conclusion, these findings have contributed to underline the link between *Prepl* and adipose tissue compartment and to clarify its role in the regulation of whole body metabolisms.



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...Vittoria, il tuo amore e la tua passione per la ricerca sono per me ogni giorno di stimolo per migliorare...da te ho sempre da imparare e i tuoi consigli mi hanno aiutato ad affrontare al meglio questa esperienza...

...Serena, le parole non bastano per descrivere quanto è stata importante la tua presenza in questi anni...solo tu sei in grado di rischiarare le mie insicurezze...il tuo starmi accanto mi ha regalato i ricordi più preziosi di questi tre anni...

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