# DOCTORAL DISSERTATION

# P66$^{\text{Shc}}$ Is Genetic Determinant Of Metabolic Syndrome

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*Devoted to all cancer victims*

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# PREFACE

# METABOLIC SYNDROME: A COMPLEX DISEASE

Metabolic syndrome (MS) is defined as a constellation of metabolic disorders in the same individual with frequency higher than it could be expected by chance.

MS is one of the most widespread diseases in the world. MS is evident in almost half of the population of specific age groups in developed countries.

The search for the genetic basis of complex pathological syndromes represents a challenge for experimental medicine and genetics. Indeed, the identification of single genes involved in such multifactorial diseases may be frustrating due to the intrinsic complexity of the processes involved and of their genetic determinants and because of the limitation of actual knowledge on gene functions and all the relative networks. Nevertheless, we believe that the investigation on crucial cellular processes, such as energetics, redox metabolism, mitochondria-nuclei crosstalk, which could be primarily involved in the onset of different multifactorial diseases, might allow the identification of few triggering genetic determinants.

# DEFINITION OF METABOLIC SYNDROME

Nowadays MS is mostly considered as a pathological condition where single metabolic disorders which are all dangerous heart attack risk factors have tendency to be present together and have common linking factor. This constellation of metabolic abnormalities increases the risk for different clinical states, primarily for cardiovascular diseases (CVD) and non insulin dependent diabetes mellitus (NIDDM) (1-3).

Even though the first description of MS in scientific literature appeared in 1923 (4), for a long time it was not widely distributed disease. Sharp increase in the prevalence of MS has happened just recently. That is why there is still a lot of confusion regarding the definition of MS. There are even different names for this pathological condition: MS, insulin resistance syndrome, the deadly quartet, beer-belly syndrome, Reaven's syndrome and others.

For simplification of clinical practice and research on MS, the leading world health organizations worked out their own definitions of this pathological condition. World Health Organization (WHO) (5), National Cholesterol Education Program's Adult Treatment Panel III (NCEP:ATPIII) (6), National Heart, Lung and Blood Institute/American Heart Association (NHLBI /AHA) (7) and American Association of Clinical Endocrinologists (AACE) (8) proposed their statements on MS.

These statements differ in proposed components, etiological factors of MS, criteria and their cutoffs for the diagnosis.

In 2005 International Diabetes Federation held Consensus meeting with the aim to overcome the problem of differences in the meaning of MS and proposed new MS definition for worldwide use in clinical and scientific investigations (9). Due to high correlation of abdominal obesity with other single components of MS, the IDF Consensus considered central obesity as a *sine qua non* for the manifestation of MS (9). Acccording to IDF, in addition to obesity, the diagnosis of MS required the presence of at least two of the following factors: dyslipidemia (presented as raised triacylglyceride (TAG) level or reduced level of high density lipoprotein (HDL) cholesterol), raised blood pressure, raised fasting plasma glucose (9). IDF also summarized other parameters related to MS which should be investigated for the necessity of their inclusion into a set of criteria in terms of improvement of current definition as a tool for prognosis of CVD/diabetes risk in a patient. These parameters are abnormal body fat distribution, atherogenic dyslipidemia (other than elevated TAG and low HDL cholesterol), dysglycaemia, insulin resistance (beyond elevated fasting glucose), vascular dysregulation (beyond elevated blood pressure), proinflammatory state, prothrombotic state and hormonal factors.

## METABOLIC SYNDROME AS RISK FACTOR FOR OTHER DISEASES

MS is associated with many diseases (Fig. 1). Two primarily clinical outcomes of MS are CVD and NIDDM, which both are leading diseases in the structure of the morbidity and mortality worldwide.

In fact, all individual components of MS are risk factors for CVD (10-15). MS implies even higher risk for coronary heart diseases (CHD) and CVD (11,14,16-18) and death from them (14,19) than individual abnormalities. Indeed, MS was shown to be the most important factor responsible for myocardial infarction in the population younger than 45 (20).

Similarly, individual components of MS (obesity, hypertension, low levels of HDL cholesterol, elevated TAG levels, and impaired fasting glucose) and MS *per se* are all significant predictors of NIDDM (3,18).

MS and its single components are tightly connected with the development of non-alcoholic fatty liver disease (21-23), which is one of the most distributed liver diseases in industrial countries (24-26).

MS is more prevalent in patients with polycystic ovary syndrome (PCOS) (27). The correlation was found between PCOS and single components of MS such as insulin resistance and obesity (28,29). Linking factor for both syndromes is considered to be insulin resistance (28).

Breast cancer is the main cause of death from cancer in women. It was shown, that individual components of MS increase risk for breast cancer (30-33).

MS is also associated with schizophrenia (34) and sleep apnea syndrome (35).
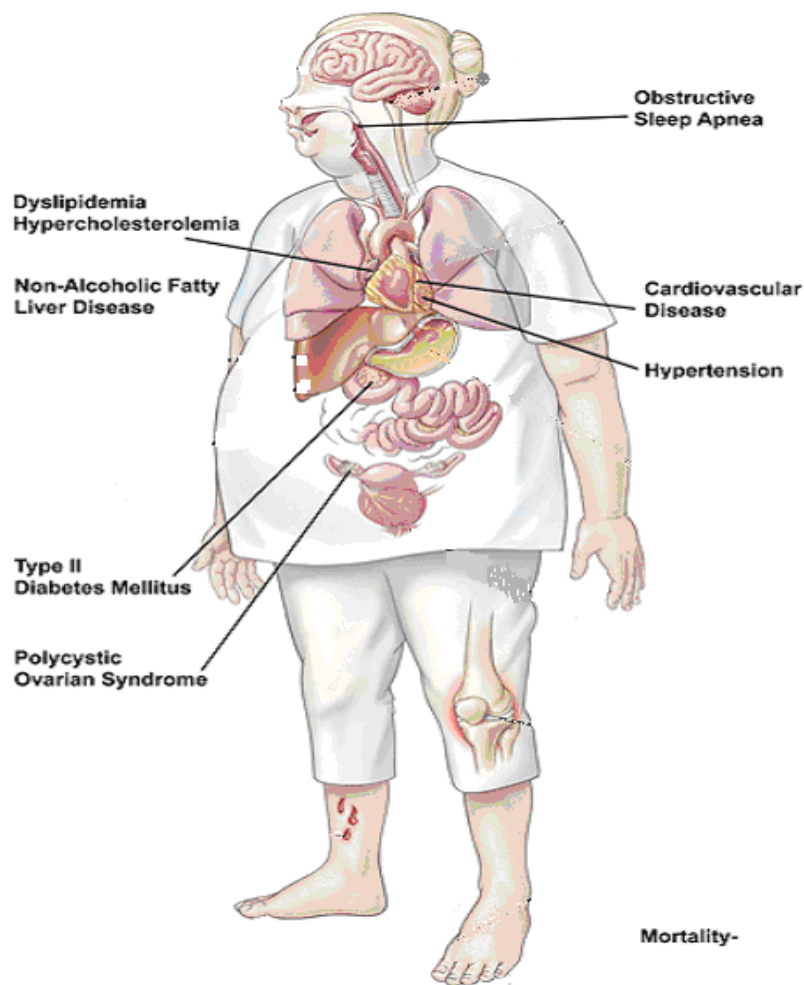


**Figure 1. Scheme reflecting diseases associated with MS.** MS is connected with sleep disturbances, non-alcoholic fatty liver disease, cardiovascular diseases, diabetes type II, polycystic ovary syndrome, hypertension, dyslipidemia and increases mortality from CVD.

# PREVALENCE OF METABOLIC SYNDROME

The main feature of the prevalence of MS is its extremely wide distribution. In overall it is estimated that from 15 % to 40% of adult population meet requirements for the diagnosis of MS (36). Since obesity is considered as one of the triggering factors for the onset of MS, high prevalence of MS may be explained due to pandemia of obesity in modern society. In fact, nowadays the number of overweight or obese individuals (around 1 billion) exceeds the number of underweight subjects (850 millions) (37). Having in mind that CVD and diabetes are primarily outcomes of MS, the increase in MS prevalence will also prompt their distribution. Indeed, the prevalence of NIDMM is expected to grow further and to be 300 million cases by 2025 (38). Since 80% of subjects with NIDMM die from CVD, MS could be considered as the driving force of a new CVD epidemics.

The frequency of MS differs between countries and is high where obesity has a wide distribution, like US (39). For instance, it is predicted that in Italian adult population 3.6 million women and 3 million men may have MS (40). In the U.S. the total amount of people with MS is one of the highest in the world and is estimated to 47-64 millions (39). (It should be noted that the population of Italy and the U.S. are around 60 and 300 millions, respectively). Some experts predict that at least half of persons over age 60 would meet the criteria for this syndrome in the U.S., whereas in Italy it is only 25% in the population older than 70 years (39,40). Additionally, MS is presented in the young US population: as many as 6.4% of US adolescents, age between 12 and 19, have the disorder (41). It seems therefore that local traditions of lifestyle and nutrition are important for the prevalence of MS.

# OBESITY PROMOTES METABOLIC SYNDROME

Etiology of MS is not well understood yet. Different factors may underlie the development of MS. Moreover, single components of MS could be independently regulated through their own mechanisms (by genetic and environmental factors). Both insulin resistance and obesity are considered as the most prominent etiological factors of MS. Nevertheless, it is difficult to distinguish their independent role on MS onset, since they are closely linked together.

Intensive studies in the last decades indicate, however, obesity as the original and significant predictor of other components of the cluster. Co-occurrence of obesity with other metabolic disorders was first shown by Vague. Notably, he paid particular attention to the fat topography as a parameter that has a better correlation with metabolic disorders than the excess of fatness *per se*

(42). Recent data proved that fat tissue distribution is important for MS onset and that namely increase in intra-abdominal fat (IAF) is the best predictor of other risk factors seen in MS (43,44).

In fact, it was shown that increase in IAF predicts dislipidemia, represented in a plasma profile with an increased triglycerides (45-51), increased low density lipoprotein (LDL) cholesterol, and apolipoprotein B levels (52) and with reduced concentration of HDL cholesterol (48-52), elevated level of uric acid (53) and elevated blood pressure (53-55).

Obesity also increases prothrombotic state of organism. PAI-1 is a key regulator of the fibrinolytic system. Its secretion by adipose tissue is augmented in obesity (56). Furthermore, obesity is in positive correlation with venous thromboembolis (57) and pulmonary embolism (58).

Obesity is associated with an increase of inflammatory state in the organism. In fact, in obesity state massive macrophage infiltration in adipose tissue was reported (59). Adipose tissue produces a variety of biologically active molecules involved in inflammation: TNFα, iNOS, IL-6, IL-1beta, monocyte chemotactic protein-1, leptin, macrophage migration inhibitory factor, nerve growth factor, vascular endothelial growth factor and haptoglobin [for review see (60)]. Expression of many of them is due to infiltrated macrophages (59). Adipose tissue secrets also C-reactive protein (CRP). Its level correlates with the inflammatory state of the organism (61) and is augmented in obese individuals (62).

IAF was shown to be also significant predictor of the insulin resistance and glucose intolerance (53,55,63-66).

Finally, in a study, specifically devoted to the determination of the differential contribution of insulin resistance and central body fat deposits to the development of MS it has been proven, that both insulin resistance and IAF were associated with the syndrome. However, IAF was independently associated with all five of MS criteria according to ATPIII's definition, whereas insulin resistance was independently associated with the criteria for HDL cholesterol, TAGs and fasting plasma glucose (67). Likewise, Phillips in the work devoted to identification of the underlying factor for MS found that visceral adipose tissue was higher predictor of components of MS than insulin level, which is one of insulin resistance parameters, in healthy men and women (43,44). Involvement of obesity in the pathophysiology of MS is indirectly supported by the fact that weight loss has beneficial effects on the treatment of all single metabolic components of the syndrome, including insulin resistance (68,69), and in fact is considered as principal treatment of MS (70). On the other hand, there is not a lot of data indicating that increase in insulin sensitivity gives the same result.

Thus, all these evidences strongly suggest that obesity is an important underlying factor of MS.

Adipose tissue can be involved in the regulation of MS through the secretion of a number of adipokines (cytokines secreted by adipocyte) (Fig. 2), which regulate the onset of other MS

components (71). In fact, fat tissue secretes angiotensinogen (72), which is further converted to angiotensin II, a known vasoconstrictor. Many adipokines, like resistin (73,74), TNF-α (75,76), retinol binding protein 4 (77) and leptin (78) exert profound effect on insulin sensitivity.
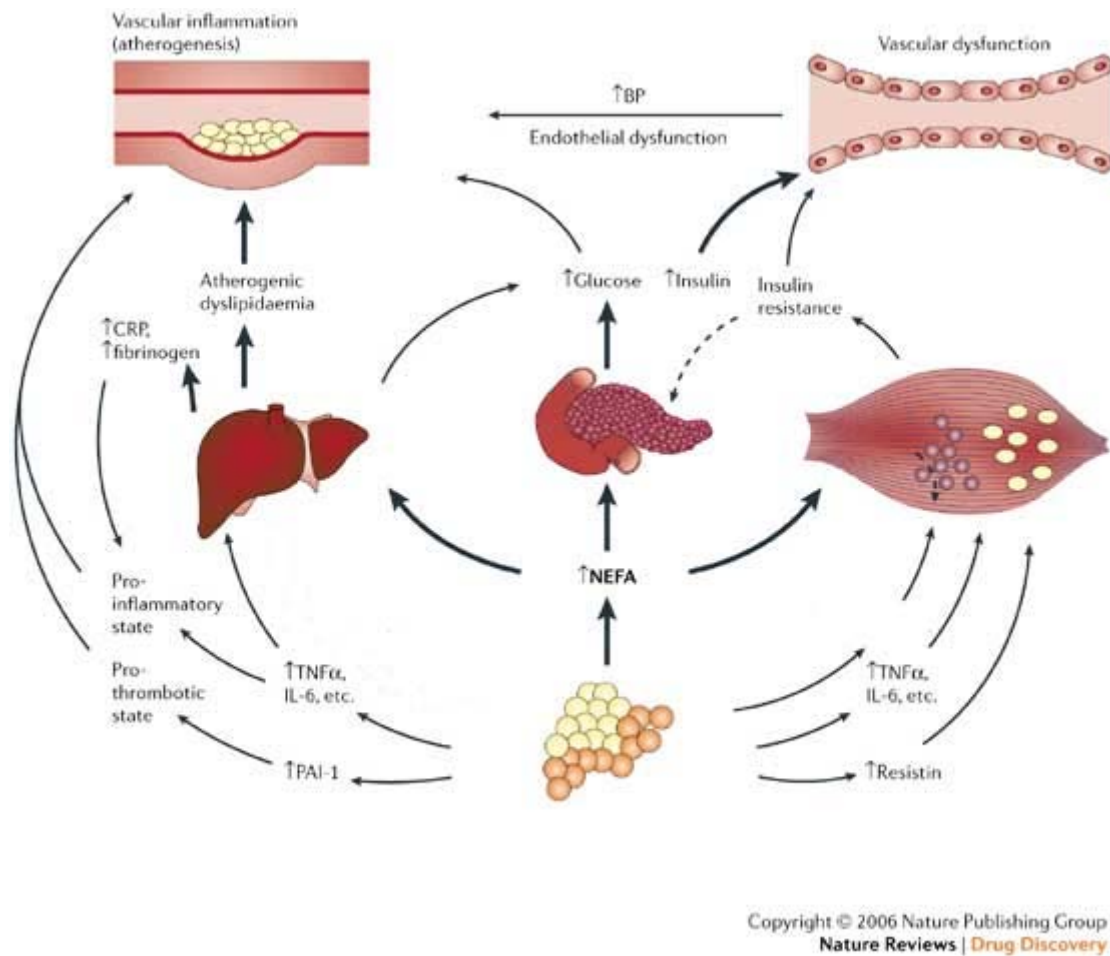
**Figure 2. Scheme, depicting how obesity is connected with the onset of metabolic abnormalities**. In obesity state secretion of TNFα, IL-6, PAI-1, resistin and fatty acids (NEFA) is increased. It leads to the development of **insulin resistance** in muscles, pancreas and liver. Also, it increases **proinflammatory and prothrombotic state**, and circulation of CRP and fibrinogen is increased. Proinflammatory and prothrombotic states as well as insulin resistance potentiate the development of **endothelial dysfunctions and atherosclerosis**. At the end the complex metabolic net of the organism is dysbalanced. *(Adapted from Grundy, 06).*

In obesity state fat tissue secretes augmented level of free fatty acids (FFA), which also participate in the onset of insulin resistance (79,80) and dislipidemia (81).

Another way in which obesity predisposes the development of metabolic abnormalities could be through the increase in the total body metabolic demands. In fact, obesity overloads cardiovascular system with work. It was shown that in obesity state activity is associated with higher workload on the heart (82,83).

Finally, obesity can predispose to MS through the increase in systemic oxidative steady state (see below).

## ROLE OF OXIDATIVE STRESS IN METABOLIC SYNDROME

Obesity can be involved in the onset of MS through the alteration of oxidative status of organism. In fact, increase in fat mass is associated with an increase in the oxidative stress both in human and rodents (84,85). Being a state with an elevated oxidative stress, obesity predisposes to the development of single components of MS and to MS itself. In fact, oxidative stress is tightly involved in the increase of blood pressure through the regulation of a variety of factors (86-88). More importantly, it is associated with the development of insulin resistance, another essential component of MS. In fact, oxidative stress induces insulin resistance in variety of cells which regulate systemic response to insulin: adipocytes (89,90), muscle (91,92) and liver cells (93). It has essential role in the development of systemic insulin resistance *in vivo*. Obese *db/db* and *ob/ob* mice develop severe insulin resistance in their early lives. The over-expression of the enzyme, which decreases oxidative stress (superoxide dismutase (SOD 1) in liver of *db/db* mice results in decreased oxidative steady state in liver and in increase in systemic insulin sensitivity (94). Antioxidant treatment of *ob/ob* mice also improved parameters of insulin sensitivity (75).

It has been proven recently, that oxidative stress has a causative role and is primarily in relation to the onset of insulin resistance (75). It was shown that inducers of insulin resistance, TNFα and dexamethasone, trigger the expression of genes involved in the regulation of the cellular oxidative status and augment oxidative steady state in cultures *in vitro* before the onset of detectable insulin resistance (75). Likewise, an *in vivo* study has demonstrated that oxidative stress is primarily in relation to the onset of insulin resistance in mice treated with high fat diet. In liver and fat tissue of these mice, reactions involved in the increase of oxidative state were activated before the establishment of insulin resistance through pathways specific for each organ (85).

Ultimately, oxidative stress has been shown to correlate with dislipidemia (high triacylglyceride concentration, low level of high-density lipoprotein cholesterol) and abdominal fat (95) in patients with MS and thus might be considered as a linking factor between the single components of MS.

# COMPLEX INTERACTIONS AMONG GENETIC AND ENVIRONMENTAL FACTORS ARE INVOLVED IN METABOLIC SYNDROME

Even though MS may be efficiently treated with changes in lifestyle, practically this therapeutic approach is difficult to apply in humans. That is why drug treatment is important. However, there is not much knowledge yet about the genetic determinants of MS, which could be possible targets for the drug therapy.

Indeed, genetic determination of MS exists. It was shown that the prevalence of MS was higher in the monozygotic (31,6 %) than in dizygotic pairs (6,3%) of twins (96). High level of the heretibility of MS was also shown in study of MS prevalence in Caribbean-Hispanic families (97).

MS is a complex disease and vast genomic studies are necessary for correct identification of its genetic determinants. Recent genetic association studies, however, gave some indications about possible genetic determinants of MS. These studies use single nucleotide polymorphism (SNP) markers across the genome. SNP variants are chosen among the candidate genes possibly involved in the pathogenesis. For this reason, genes regulating adipokines secretion, lipoprotein metabolism, inflammation, insulin sensitivity, pattern of fat distribution, and ROS metabolism were selected as candidate genes for such studies. In fact, genetic association studies revealed alleles associated with the risk of MS among such genes: *ADIPOQ*, *RSTN*, *APOC3*, *IL-6*, *TNFA, ZFP36* [for review see (98)], and *GPX1* (99) etc. It was shown that polymorphism of *PPARG* not only regulates the onset of metabolic abnormalities in response to the diet, but also controls beneficial effect of exercises for MS treatment (100,101).

In last few decades sharp increase in the prevalence of MS took place. Taking into consideration that human genome has not been changed significantly for this time, it brings to the suggestion that the changes in the lifestyle, such as decrease in physical activity and an atherogenic diet (rich in saturated fats, trans fatty acids and refined sugars) are responsible for the increase in the prevalence of obesity and, consequently, of MS (102).

Indeed, nutrients may regulate the establishment of metabolic abnormalities since they are direct participants of metabolic reactions. A diet rich in saturated fatty acids and refined sugar correlates with dislipidemia, whereas Mediterranean diet rich in virgin olive oil improves parameters of dislipidemia and hypertension (103). Augmented intake of betaine (trimethylglycine) has beneficial effect on stabilization of protein structure and improves vascular risk factors (104). Increased intake of vegetables and fruits decreases the risk of stroke (105). Consumption of refined sugars predisposes to insulin resistance, inflammatory state and obesity (106).

Diet also regulates gene expression and genome stability (107). The most impressive influence of food consumption on the organism is seen on the example of calorie restriction. Calorie restriction is the only laboratory way to prolong lifespan in a variety of species. It changes rate of metabolism and drastically changes gene expression. It was shown that effect of calorie restriction is effectuated through the activation of genes of sirtuins family (108). It was reported that not only availability of nutrients (109,110) but some specific nutrient treatment (fructose diet, resveratrol) also modifies sirtuins expression (111,112). Such genes, which are directly modulated by diet are of great interest since they could be the key elements in the genetic regulation of organism response to diet. In fact, Sirt1 was shown to be the regulator of fat mobilization upon calorie restriction (108). Thus sirtuins could be the main regulators of the onset of the diet induced metabolic disorders. In fact, transgenic mice over-expressing Sirt1 were protected from high fat diet induced metabolic abnormalities (113). Thus, the onset of MS and of its main underlying factor, obesity, is subjected to the regulation through the complex genetic and environmental interactions. Understanding of such mechanisms could shed a light on MS etiology and would help to find tools for its treatment.

# INTRODUCTION

# REGULATION OF FAT DEVELOPMENT

## Differentiation, lipolysis and lipogenesis in adipocytes

The increase in fat deposits is mostly dependent on fat cell size which is regulated by the rate of lipids accumulation. In adipocyte, accumulation of lipids in form of TAG depends on the balance between lipid synthesis (lipogenesis) and lipid oxidation (lipolysis). Efficiency of lipogenesis and lipolysis depends on the presence of proper enzymatic machinery involved in lipid metabolism. This enzymatic machinery is acquired by adipocytes during differentiation from precursor cells, preadipocytes [ as reviewd in (114)].

Adipose tissue in mammals is represented as two types. White adipose tissue (WAT) mainly participates in the storage of TAG and secretion of adipokines, which have profound effects on overall glucose/insulin metabolism, proinflammatory and prothrombotic state and fertility; brown adipose tissue (BAT) is primarily involved in heat generation, the so called thermogenesis. Because of different functions in organism, differentiation programme of white and brown adipocytes is slightly different, which allows obtaining of specific enzyme sets (described below).

During maturation adipose precursor cell changes its shape from fibroblastic into a spherical one, acquires sensitivity to insulin and undergoes changes in gene expression profile thus activating its competence on lipid metabolism. At early stages of differentiation of white adipocytes, up-regulation of three major transcriptional factors (TF) of differentiation happens: PPARγ, CCAAT/enhancer binding protein alpha (C/EBPα) and sterol regulatory element binding protein-1c (SREBP-1c). PPARγ is one of the most potent TF of differentiation. Many inducers of the differentiation of adipocytes, thiazolidinediones, 3-isobutyl-1- methylxanthine (IBMX), and dexamethasone exert their effect through the activation of PPARγ downstream pathways.

Both PPARγ and C/EBP (C/EBPβ and C/EBPδ) are involved in the differentiation of brown adipocytes. Brown adipocyte differentiation results in expression of genes involved in mitochondrial function and biogenesis: UCP1, necdin, vigilin, and metargidin (115). TFs forkhead box C2 (FOXC2), PPARγ co-activator-1α (PGC1α) (116), and others are important for maintenance of brown fat cell phenotype.

PPARγ has been suggested to be involved also in the regulation of lipogenesis in adipose tissue through the regulation of expression of enzymes involved in lipogenesis: fatty acid binding protein, lipoprotein lipase, acyl-CoA synthetase etc (117).

Lipolysis is the breakdown of TAG into diacyl and then monoacylglycerols with subsequent liberation of FA and glycerol. Hydrolysis of TAG is controlled by complicated set of enzymes

composed of lipases, which directly hydrolyze TAG and its derivatives, lipid droplet associated proteins, which regulate availability of TAG to lipases, carriers of fatty acids, which transport FA out and into adipocyte etc [for review see (118)].

In mammals, stimulation of lipolysis is associated with thermogenesis (heat generation) which enhances lipid oxidation. Thermogenesis takes place within mitochondria and is associated with uncoupling of energy of electrochemical gradient across inner membrane of mitochondria from ATP synthesis.

## Bioenergetics of adipocytes

In the cell all nutritive materials are subjected to the oxidation during redox reactions. Redox reactions involve the transfer of reducing equivalents (hydrogen atoms, electrons and hydrid-ions) from a donor to an acceptor. On the last stages of redox reactions, reducing equivalents are usually accepted by two major types of transporters: nicotinamid (NAD, NADP) and flavine (FAD, FMN) cofactors. They can be reoxidized via donation of reducing equivalents to the components of electron transport chain (ETC). ETC is a system of proteins located in the inner membrane of the mitochondria which are able pass electrons consequently from one to another due to strict sequence of their location in membrane according to the increase in redox potential. Redox potential is a measure of the ability of a substance to donate electrons. The higher the redox potential is the higher oxidative properties of the compound are. There are five complexes of ETC. Complex I, NADH dehydrogenase, accepts electrons from nicotinamide transporters. Complex II, succinate dehydrogenase, accepts electrons from flavine transporters. They pass electrons to coenzyme Q from which electrons are transferred to complex III (cytochrome $bc_1$ complex). Complex III transfers them to molecules of cytochrome $c$, a water-soluble electron carrier. Complex IV, cytochrome $c$ oxidase (COX), oxidizes cytochrome $c$ and transfers electrons to molecular oxygen. During electron transport in ETC, protons are pumped to the outer surface of membrane. Since the inner membrane of mitochondria is freely permeable only to water, to $CO_2$ and $O_2$, protons can not pass back to the matrix freely and their concentration on the outer side of inner membrane is increased. Thus an electrochemical gradient ($\Delta\psi$) is formed across the inner membrane of mitochondria. The flux of protons through impermeable membrane may happen only through ATP synthase (complex V of ETC). It couples the energy of $\Delta\psi$ liberated during the proton passage through ATP synthase with ATP synthesis. Respiration can be dissociated (uncoupled) from ATP synthesis. Altering permeability of membrane to protons, mitochondrial gradient is lost. Thus uncoupling of back flux of protons from ATP synthesis takes place. The energy of $\Delta\psi$ is dissipated in this case in the form of heat (Fig.4). The dissipation of $\Delta\psi$ induces ETC to restore it through the increased oxidation of

substrate during which protons are pumped again back out of matrix. This process increases respiration of mitochondria. There are few chemicals such as 2,4-dinitrophenol (DNP), carbonil-cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) etc. which being weak lipophilic acid (which can diffuse through the membrane) may bind protons on the acidic side and release them on the basic side of the inner membrane of mitochondria thus dissipating $\Delta\psi$.
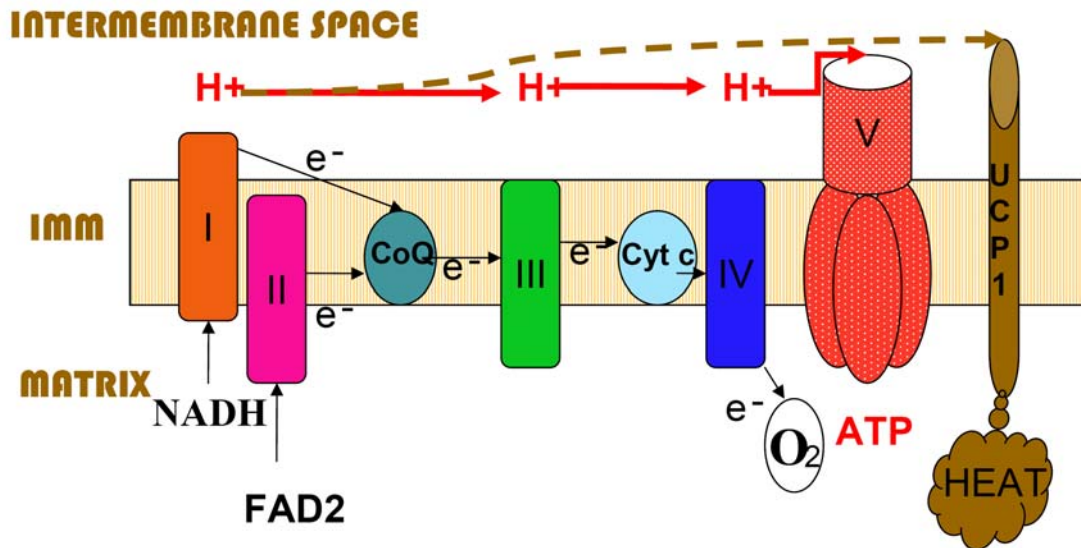


**Figure 3. Scheme depicts energy utilization in the inner membrane of brown adipocyte mitochondria.** NADH and FAD$_2$ pass electrons to the complexes I and II of ETC, respectively. They transfer electrons to coenzyme Q (CoQ), from where electrons through the complex III pass to cytochrome *c*. Complex IV oxidizes cytochrome *c* and reduces molecular oxygen till water. During the transport of electrons in ETC protons are pumped into intermembrane space of mitochondria and form electrochemical gradient. Protons come back into matrix or through the ATP synthase complex, what will be connected with ATP synthesis, or through the UCP1, in which case energy will be dissipated in the form of heat.

Back flux of protons through inner membrane of mitochondria can happen "physiologically" through the pathways mediated by uncoupling proteins (UCPs), naturally present in the inner membrane of mitochondria. UCP1 is abundant and specific protein of brown adipocyte mitochondria (119). It is associated with the leakage of protons back to matrix and dissipation of energy in form of heat. The flow of protons through UCP1 is inhibited by purine nucleotides (GDP, ADP) and activated by FFA (120),(121). It seems that the dissipation of $\Delta\psi$ is associated with the transport of FFA. UCP1 provides the transport of anions of FFA, meanwhile neutral FFA (which are associated with protons) are able cross back the lipid bilayer and thus carry the protons [for review see (119)]. Leakage of protons through UCP1, together with other properties of brown adipocyte mitochondria (low amount of ATP synthase, rich ETC etc.) allows heat generation in brown adipose tissue [for review see (119)]. This phenomenon is involved in the protection of mammals from the cold and maintenance

of body weight (non shivering thermogenesis) with UCP1 being indispensable for these phenomenon (122).

## Insulin control of adipogenesis

Many aspects of adipocyte physiology are regulated by insulin. Insulin is important factor of induction and enhancing of adipocyte differentiation. It was shown that insulin stimulates activity of TFs of differentiation (123). In some primary cultures only insulin is enough to trigger the differentiation. Insulin also regulates proliferation of preadipocytes and glucose transport, lipolysis, lipogenesis and thermogenesis in adipocytes. Final effect of insulin on fat cell is increase in TAG accumulation.

Insulin signaling starts from the activation of insulin receptors on the surface of adipocyte (Fig. 4). For signal propagation insulin receptor requires the recruitment of adaptor proteins. Among them IRS-1 is essential for most of the biological responses of the cell to insulin. It binds several SH2 containing effector proteins - for instance p85, the regulatory subunit of phosphatidylinositol (PI) 3-kinase, Grb2 etc.

Binding of Grb2 triggers mainly pathways involved in mitogenesis. Binding of p85 regulatory subunit of PI 3-kinase leads to the activation of the enzyme and its targeting to the plasma membrane. PI 3 kinase generates the lipid product phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$). PI 3-kinase activation is attenuated by $PIP_3$ dephosphorylation via $3'$ phosphatases such as PTEN or $5'$ phosphatases such as SHIP2 [for review see (124)]. Activated PI 3-kinase subsequently activates Akt, which regulates downstream responses like glucose uptake, protein synthesis and adipocyte differentiation. In particular, essential mechanism of Akt dependent regulation of adipocyte differentiation involves exclusion of anti-adipogenic transcriptional factors Forkhead factor 1 (FOXO1) from the nucleus (125). FOXO1 exclusion from the nucleus alters the expression of its dependent genes critically involved in adipogenesis (126).

One example of how insulin signaling regulates adipogenesis is represented by FIRKO (fat insulin receptor knock out) mice where insulin receptor is knocked out specifically in fat tissue (127). Mice, lacking insulin receptor in fat tissue did not activate insulin signaling pathways in adipocytes and their fat depots were significantly decreased (127). Interestingly, FIRKO mice showed increased cellular heterogeneity of adipose tissue. This fact was connected with altered expression profile of key adipocyte proteins (127,128). On the organism level fat specific knock out of insulin receptor protected mice from age-associated insulin resistance and glucose intolerance (127) and increased their life span (129).
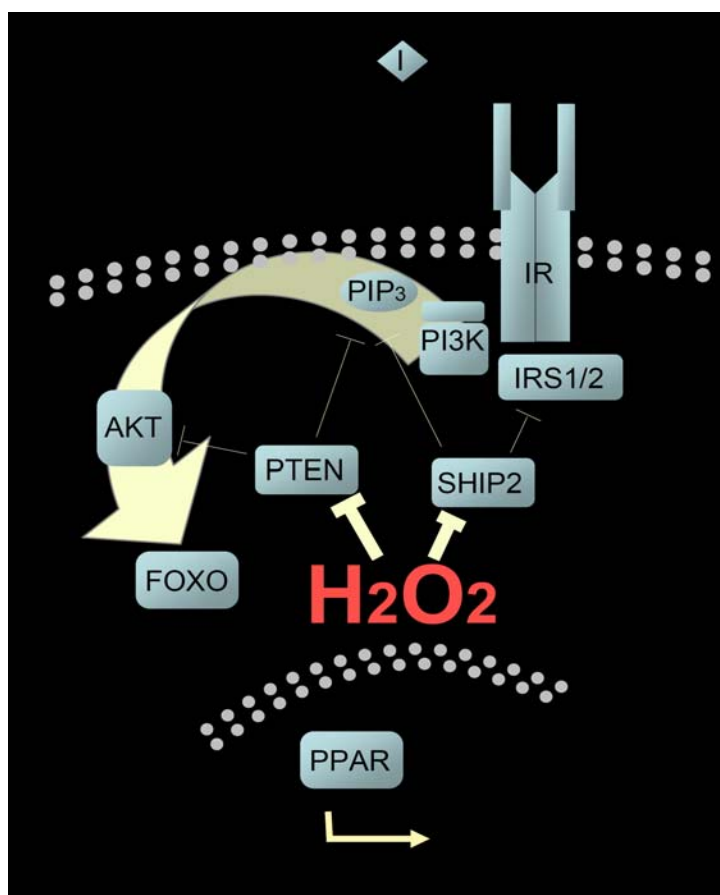
**Figure 4. Insulin regulates adipogenesis.** Insulin (I) stimulates insulin receptor (IR) which binds adaptor proteins IRS1/2. Activation of IRS1 leads to the activation of PI3K. PI3K generates lipid product PIP3. A key downstream effector of PIP3 is Akt. Akt induces relocalization of FOXO1 from the nucleus thus initiating expression of genes involved in adipogenesis. Insulin increases activity of PPARγ. PTPs PTEN and SHIP2 inhibit insulin signaling and are subjected themselves to the inhibition by $H_2O_2$.

# ROLE OF REDOX BALANCE IN FAT DEVELOPMENT

Reactive oxygen species, including ($O_2 \cdot^-$), hydroxyl radical (OH·) and hydrogen peroxide ($H_2O_2$), are partially reduced oxygen derivatives. They are extremely reactive due to their high oxidizing potential and can react practically with all biomolecules with reducing groups. ROS are formed in the cell as a product of aerobic metabolism. Around 0.1% to 2% of the oxygen consumed by the cell is converted to ROS. Major part of ROS is generated in mitochondria during oxidative phosphorylation.

ROS can be turned from one form into another. Upon acceptance of a single electron by molecular oxygen superoxide is produced:

$$O_2 + 1e = O_2^-$$

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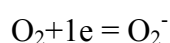
Superoxide is converted with special enzymatic systems (superoxide dismutases) into less reactive oxygen forms, like hydrogen peroxide:

$$2H^+ + O_2^- + O_2^- = H_2O_2 + O_2$$
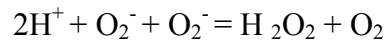
Hydrogen peroxide can be converted into hydroxyl radical and peroxide radical through Fenton reaction:

$$Fe^{2+} + H_2O_2 = Fe^{3+} + OH^{\cdot} + OH^-$$
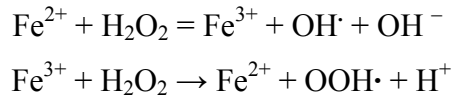$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\cdot} + H^+$$

Recently it has been shown, that ROS are produced not only spontaneously but also by specific enzymatic systems which exist in different cellular compartments such as mitochondria, cytoplasm, plasma membrane and other parts of the cell (p66$^{Shc}$, cyt P-450, NADPH oxidases, peroxisomal oxidases, amine oxidases, xanthine oxidase etc). Intracellular ROS are involved in the regulation of various cell processes (proliferation, differentiation, apoptosis). The contribution of different forms of ROS to the regulation of cellular processes varies. It seems that $H_2O_2$ has specific importance for the cell due to its membrane permeability as well as due to lower reactivity and higher life span compared to $OH^{\cdot}$ or $O_2^-$ [for review see (130)].

While physiological levels of ROS are important for normal cell function, elevated ROS levels (oxidative stress) result in increased modification of normal structure and properties of biomolecules and in cell damage. Depending on dose, overall effect of ROS on cell can become negative. Cell possesses special mechanisms for the deactivation of excessive amount of ROS. This is the so called scavenging system. Many non-enzymatic molecules such as glutathione, vitamins A, C, and E, and flavonoids, as well as enzymatic molecules such as superoxide dismutases, catalase, and glutathione peroxide have evolved to scavenge ROS (131).

$H_2O_2$ is tightly implicated in the regulation of processes in fat cell. Hydrogen peroxide might exert insulin-mimicking effect on adipocytes. In fact, in adipocytes exogenous $H_2O_2$ can mimic insulin induced glucose transport (132) and inhibit hormone-stimulated lipolysis (133).

Further, insulin stimulation of adipocytes elicits a burst of $H_2O_2$ (134). This event enhances tyrosine phosphorylation of the insulin receptor as well as distal signaling events in the insulin action cascade (135). It was demonstrated that this increase in $H_2O_2$ level was due to catalytic activity of the insulin-responsive system with characteristics of NAD(P)H oxidase (134). NAD(P)H oxidase catalyzes the reduction of molecular oxygen to superoxide, which undergoes subsequent dismutation to $H_2O_2$. It was shown, that insulin stimulated burst of $H_2O_2$ in adipocytes was attributed to Nox4 homologue of NADPH oxidase (136).

Other two important and abundant $H_2O_2$ generating enzymatic systems in adipocytes are monoamine oxidase (MAO) and semicarbazide-sensitive amine oxidase (SSAO). They belong to the family of amine oxidases, metabolizing amines with hydrogen peroxide as one of the final products (137,138). Activity of these enzymes exerts insulin-like effect on adipocytes. In fact, it stimulates Akt phosphorylation and glucose uptake in 3T3-F442A cells (139) and human adipocytes (140). Long-term treatment of 3T3-L1 (141) and 3T3-F442A (139) cells with substrates of MAO or SSAO results in their differentiation. Stimulating effect of MAO and SSAO on lipogenesis was shown in human (140) and rat adipocytes (142). Activation of these enzymes resulted in a huge promotion of lipid accumulation (139,143). These effects were inhibited by N-acetylcystein (NAc) treatment (143) or by catalase (141), suggesting involvement of $H_2O_2$.

Notably, there is evidence that ROS differentially affect two major downstream branches of the insulin signaling pathway. It stimulates PI 3'-kinase pathways but inhibits MAPK pathway in 3T3-L1 adipocytes (136).

The mechanisms through which ROS enhance and mimic insulin effect may be explained in several ways. ROS are able to modify protein structure and function through the direct reaction with amino acid residues.  It is known, that intracellular signal transduction is based on phosphorylation of proteins involved in signal propagation chain. Ratio between the processes of phosphorylation and dephosphorylation in the cell is regulated with protein tyrosine phosphatases (PTPs). ROS regulate the process of signal transduction through the direct reversible oxidation of cystein residues in the catalytic site of the PTPs thus lowering their activity.  In fact, PTP1B is a key PTP regulating insulin signaling (144). It was demonstrated that stimulation of cell with insulin is associated with a burst of intracellular $H_2O_2$ and subsequent reversible oxidative inhibition of up to 62% of overall cellular PTP activity and up to 88% of PTP1B activity in adipose cells (135). Other phosphatases, involved in the regulation of insulin signaling which are potential ROS targets include serine protein phosphatase PP2A (145) and PTEN (146).

Another mechanism of enhancing effect of ROS on insulin signaling is PTPs independent modulation of activity of insulin receptor. In fact, $H_2O_2$ treatment increases insulin receptor phosphorylation (132). It was shown, that optimal insulin responsiveness of insulin receptor may require a process of redox-dependent structural and functional alteration in the insulin receptor beta-chain (147).

However, the inhibitory effect of oxidative stress on insulin signaling in adipocytes was also reported. In cultures of adipocytes oxidative stress impairs glucose transport due to reduction in expression of GLUT 4 (89) and its impaired translocation to the plasma membrane (90), or through the modification of normal cellular localization of PI 3 kinase and IRS-1(148). Impaired insulin

stimulated glucose consumption can be restored with antioxidant treatment (149). Probably, the source and the amount of ROS play a role in the alteration of insulin sensitivity. In fact, the biological effect of $H_2O_2$ depends on its concentration [for review see (130)].

ROS are also implicated in fat tissue development *in vivo*. Thus, MAO and SSAO inhibition results in reduction of fat in obese Zucker rats (150) and in non-obese rats (151). Activity of SSAO is associated with the inhibition of lipolysis *in vivo* in obese mice (152). Antioxidant treatments with NAc or green tea have clear antiobesity effect (153,154).

Thus, ROS are enzymatically produced in adipocytes and are important for activation of insulin signaling. On organism level, ROS promote fat tissue development and predispose to obesity.


# P66[SHC] REGULATES REDOX BALANCE


P66[Shc] is an ubiquitous vertebrate protein, encoded by the Shc[A] locus. The modular structure of the protein is shown in Fig. 5.

P66[Shc] has been identified as a master regulator of intracellular amount of ROS. Indeed, all cells tested derived from p66[Shc-/-] mice have reduced ROS levels (immortalized fibroblasts (155), primary embryonic and adult fibroblasts (156), endothelial cells (157), lymphocytes (158), hepatocytes (159), brown preadipocytes (160) and total cell population from bone marrow (unpublished data). The loss of p66[Shc] is associated also with less intracellular and systemic oxidative damage (liver, spleen (156), vessels (161) and kidney (162).



**Figure 5.** Schematic modular structure of p66[Shc] protein. P66[Shc] has carboxy-terminal SH2 domain, followed by a proline-rich region (CH1) an amino-terminal phosphotyrosine binding domain (PTB) and amino-terminal proline-rich region, CH2, which contains a serine phosphorylation site (S36) and CB domain (cytocrome C binding region) which is involved in ROS regulation.

A fraction of p66$^{Shc}$ (about 20% in basal condition) exists within mitochondrial inter-membrane space (163) where it oxidizes cytochrome $c$ generating $H_2O_2$ (159). This fact demonstrates that the generation of ROS in mitochondria happens not only accidentally but also through a specific enzymatic system.

Mutations within p66$^{Shc}$ were engineered (p66Shc E132Q-E133Q, p66Shc qq), which prevented the binding of p66$^{Shc}$ to cytochrome $c$ and $H_2O_2$ generation (159).

The physiological role of p66$^{Shc}$ is still unknown. Mice lacking p66$^{Shc}$ are healthy and fertile. On the contrary, they show signs of retarded aging (162,164) and are protected from many degenerative diseases and have 30% increase in life span (165). In fact, wide spectrum of parameters of endothelium function was improved both in aged p66$^{Shc-/-}$ mice (164) and in p66$^{Shc-/-}$ mice subjected to hyperglycemia in streptozotocin induced diabetes (166). It was shown, that a number of factors beneficial for vasodilatation, existed in p66$^{Shc-/-}$ mice (up-regulation of eNOS and antioxidant enzymes, decreased superoxide production) (164,166).

P66$^{Shc-/-}$ mice are also less susceptible to atherosclerosis. They show decreased level of oxidized LDL, which are critically involved in the atherogenesis. P66$^{Shc-/-}$ mice also show decreased level of early atherosclerotic lesions with smaller content of foam cells and apoptotic vascular cells (161).

Also, p66$^{Shc}$ deletion protects from tissue damage following acute ischemia or ischemia/reperfusion (157).

In addition p66$^{Shc}$ deletion protects from renal diabetic complications. Streptozotocin-induced diabetic p66$^{Shc-/-}$ mice show fewer changes in renal function and structure, as indicated by the lower levels of proteinuria, albuminuria, glomerular sclerosis index, and glomerular/mesangial areas. Glomerular content of fibronectin and collagen IV is also lower in diabetic p66$^{Shc-/-}$ versus WT mice. Serum and renal tissue advanced glycation end products and plasma isoprostane 8-epi-prostaglandin F2$\alpha$ levels as measures of high-glucose damage are also lower in diabetic p66$^{Shc-/-}$ animals than in WT controls (162).

P66$^{Shc}$ is involved in the regulation of processes of aging, longevity and degenerative diseases onset, probably through its participation in ROS metabolism. Indeed, according to one popular theory, aging is linked to cumulative oxidative damage over a lifetime produced by ROS (167). In fact, markers of oxidative damage of proteins and DNA accumulate during age in mammals (168). On the contrary, antioxidant treatment or over-expression of ROS scavenging genes is associated with an increase in longevity in invertebrates (169).

# AIM OF THE STUDY

MS is a complex disease characterized by the co-existence of metabolic disorders like obesity, insulin resistance and glucose intolerance, raised blood pressure, dyslipidemia, prothrombotic and proinflammatory states etc. in their various combinations.  It is a world-wide spread pathological condition with CVD and diabetes being its primarily outcomes. Even though healthier lifestyle can successfully treat MS, this kind of treatment is difficult to apply in practice. That is why the identification of targets for drug therapy of MS as integrate clinical entity is of great importance. However, the problem is that up to now genetic determinants of MS are not well known. Nevertheless, there are indications that ROS underlie the etiology of MS and its single components. Since it is known, that ROS balance is subjected to the regulation by $p66^{Shc}$, we hypothesized a link between MS onset and $p66^{Shc}$.

Thus, the aim of my study was:

## TO INVESTIGATE THE ROLE OF $P66^{SHC}$ IN METABOLIC SYNDROME.

In particularity I studied:

*1) the role of $p66^{Shc}$ in fat tissue development,*
*2) the effect of $p66^{Shc}$ expression on metabolic abnormalities associated to  MS,*
*3) the interaction of  $p66^{Shc}$ with  a diet*

# MATERIALS AND METHODS

## REAGENTS AND MATERIALS

Antibodies against Akt, phosphorylated Akt, MAPK, phosphorylated MAPK, FOXO1 and phosphorylated FOXO1 were purchased from Cell Signaling Technology; anti-Shc, phosphorylated Shc from BD Transduction Laboratories, anti-PTEN anti-IRS-1, anti-p85a and anti-GLUT4 antibodies from Santa Cruz Biotechnology, human recombinant insulin from Roche, collagenase type IV and dispase from Invitrogene.

Hormones were measured by commercially available ELISA detection kits (insulin and leptin kits were purchased from Crystal Chem Inc., TNF-$\alpha$ from Biolegend). TMRM was obtained from Molecular Probes. Other materials were from Sigma, if it is not specified otherwise. The levels of blood cholesterol, triglycerides and free fatty acids were measured with Reflotron Plus system from Roche.

## *IN VIVO* STUDIES

### Animals

This study was performed according national law and following authority guidelines.

Experiments were carried out on p66$^{Shc-/-}$ mice and their WT controls. P66$^{Shc-/-}$ mice both in Sv/129 and C57Bl/6 backgrounds were previously produced in our group by gene targeting and homologous recombination in embryonic stem cells. Animals were maintained in a temperature-controlled room with a 12 h light/12 h dark cycle. Sacrification has been performed through cervical dislocation. Measurements of energy expenditure were made using a closed-circuit dual-gas respirometer (Micro-Oxymax System, Columbus Instruments). Body temperature was measured with rectal thermometer CHY502A (2Biological Instruments).

### High-fat diet treatment

High fat diet (5.2 kcal/gm) was purchased from Research Diets Inc (D12492). Eight weeks-old mice were divided into four groups. One group of male and another of female were fed with D12492 diet. Two other groups were used as control and fed with standard diet (3.3 kcal/gm) from Harlan Teklad (2018S). Mice were housed 5 per cage. Body weight and food consumption were controlled weekly. Upon 12 weeks of treatment with high fat diet mice were sacrificed and the weight of fat depots was measured. Serum was collected upon overnight starvation and used for the adipokine level

determination. In another experiment, C57Bl/6 and Sv/129 mice were fed high fat diet and tap water or water supplemented with 40mM of NAc and dynamics of body weight gain has been observed.

**Determination of calorie content in feces with bomb calorimetry**

Fecal samples were collected and stored at -20°C. Feces were grind in a mill and pelleted. The pellets were stored in a desiccator pending bombing. A Parr oxygen bomb calorimeter 8 (The Parr Plain Jacket Oxygen Bomb Calorimeter) was used for determination of the calorific value of the fecal pellets. Benzoic acid was used to calibrate the bomb. The pellet and ignition cup were weighed and set in the bomb in such a way that ignition wire was touching the pellet but not the cup. 1 ml of distilled water was put inside the bomb. The bomb was closed, filled with 25 atmospheres of oxygen which subsequently was released in order to eliminate nitrogen in the bomb. After this the bomb was filled again with 25 atmospheres of oxygen for the combustion. The water bucket had the same mass in each run. The water temperature was 19-21°C. The bomb was placed in the water bucket and given five minutes to equilibrate the temperature of the system. After equilibration, the temperature of the water bath was recorded every minute for five minutes to ensure the correct initial temperature of the system. After this, the bomb was ignited. After ignition, temperature readings were taken every 15 seconds for three minutes followed with reading every three minutes until there were seven equilibrium points (but no less than 25 minutes running time). Analysis of calorie content in feces has been done at University of Milan.

**Detection of p66$^{Shc}$ expression in fat upon diet treatment**

In one experiment, C57Bl/6 2 month-old male were used. At this age mice are already fertile, however they are still young and the influence of possible influence of age and age associated diseases on p66$^{Shc}$ expression is minimized. Mice were divided into three groups. One group has been treated with high fat diet for 10 days, another one has been subjected to normal diet for eight days with following 48 hours of starvation and another one has been left as control on normal diet. After sacrification perigonadal fat depots were isolated and used for p66$^{Shc}$ protein determination according to the standard procedure (see below).

In another experiment, analysis of p66$^{Shc}$ expression was performed in the same individual before and after treatment with diet. Before treatment two-month-old male in 129Sv background were anesthetized with IP injection of avertin (250 µg/g of body weight of 2,2,2-tribromoethanol dissolved in tert-amyl alcohol) and extraction of fat depot has been performed exactly as follows.

Mouse has been placed on her left side, and a small hole in the skin and peritoneum has been made. After that, correct positioning of fine forceps allowed hooking of right part of perigonadal deposit and drag it out from the hole in peritoneum. After that piece of fat was cut off. Extracted pieces have been snap frozen in liquid nitrogen and further used for analysis of expression of p66$^{Shc}$ on mRNA or protein levels. The hole in mouse skin was closed with clips. Mouse was left until awakes on the pad with temperature 37°C. After this, mouse was on recovery period for two weeks without disturbance. Mice survived procedure of fat extractions without any visible complications. After recovery period mice were treated with high fat or normal diet for ten days, or with normal diet for eight days with following 48 hours of starvation or left as control on normal diet. After this, mice again were anesthetized with avertin, and another symmetrical part of perigonadal depot was extracted in a similar way.

**Glucose tolerance test**

**Glucose tolerance tests** were performed on 3 and 24-months-old animals that had been fasted for 15 hours. Blood glucose levels were measured immediately before and 20, 40, 60 and 120 minutes after intra-peritoneal injection of D-glucose (2 g/kg of body weight).

**Insulin tolerance test**

**Insulin tolerance tests** were performed on 3 and 24-months-old mice after 15 hours starvation and blood glucose levels measured immediately before and 15, 30 and 60 minutes after IP injection of human recombinant insulin (0.2 U/kg of body weight in young and 0.5 U/kg in old mice). Blood glucose levels were measured using Accu-Chek active from Roche.

**Hormone levels determination**

For hormone determination blood from overnight starved animals was collected with cardiac puncture. Samples were left for one hour at room temperature to clook and then serum was obtained upon 15 minutes centrifugation at 4°C at 130000 $g$ and stored at -20°C for further use. The levels of hormones were measured with ELISA kits.

For hormone determination *in vitro*, cells were stimulated or not with insulin for differentiation according standard procedure. At day 3 upon the beginning of differentiation media was changed fo

a fresh one (0.5 ml per one well of 24 well dish) and left for 48 hours, after what media was collected and filtered through 0.45 μm filter and used for further analysis.

**Transplantation of brown adipocytes**

For transplantation of brown adipocytes, cells were isolated from WT and p66$^{Shc-/-}$ animals (see below for the protocol). 10 millions of brown adipocytes at passage one were subcutaneously injected in the sternum region of athymic nude mice. Developed fat mass at the place of injection was removed after one month post-transplantation, measured, fixed in formalin and used for preparation of histological sections.

**Preparation of histological sections**

Tissue samples were fixed with formalin. For the dehydratation of tissues and clearing of samples with consequent replacement of water with paraffin, samples were gradually passed through following solutions: 20 % ethanol for 20 minutes, 50, 70, 80, 90, 95 % ethanol for 40 minutes each, three times for 40 minutes in 100 % ethanol, 20 minutes in Bio-clear (Bio-Optica), other 20 minutes in Bio-clear at 60°C, 1 hour in paraffin at 60°C. After that samples were fully included in paraffin blocks.  Samples were finally cut with a microtome into sections around 5 μm thin.

**Eosin-hematoxylin staining**

Paraffin containing slides were deparaffinized and rehydrated. With this aim they were passed through the series of consequent incubations with various solutions. Blotting of excess of any solution was done before entering in a new one. Scheme of the order of incubation is presented below:
3 times for 3 minutes incubation with xylene, 3 times for 3 minutes with 100 % ethanol, 3 minutes with 95 % ethanol, 3 minutes with 80 % ethanol, 5 minutes with deionized water, 3 minutes with hematoxylin, 5 minutes with running tap water, 2 minutes with deionized water, 30 seconds with eosin, 3 times  for  5 minutes with 95 % ethanol, 3 times for 5 minutes with 100 % ethanol, 3 times for  15minutes  with xylene.
Slides were coverslipped with Eukitt (O.Kindler and Co) and left to dry overnight in the hood.

For cell size determination at least 10 fields/slide per each genotype (representing ~100 adipocytes)/slide were analyzed. Images were acquired using BX60 microscope (Olympus) and were analyzed using Image-Quant software.

**Electron microscopy**

Samples for electron microscopy were fixed with 2% paraformaldehyde and 2% glutaraldehyde in PBS for 1hour at room temperature followed by washing for 5 minutes with PBS. Further samples were incubated with 1% tetroxide of osmium for 1 hour, washed with water for 5 minutes, incubated with 1 % uranyl acetate for 1 hour, then with 70% ethanol for 3 minutes, with 95% ethanol for 3 minutes, with 100% ethanol three times for 5 minutes, with oxide of propylene three times for 15 minutes, with the mixture oxide of propylene : Epon (1:1) for one hour, with Epon two times for 1 hour. Finally samples were subjected to overnight incubation with Epon at room temperature, to overnight incubation with Epon at 40°C, to overnight incubation with Epon at 60°C.  Samples were analyzed with a microscope TEM CM 10 (Philips).

## *IN VITRO* STUDIES

**General notes about cell culture**

Cells grew at temperature 37°C in incubators with 10% $CO_2$ and 21% $O_2$. For all cultures temperature of media was strictly 37°C. Media was changed each second day in all cell types. For each type of cells batch of serum has been carefully tested and its percentage in media was determined to get the best growth and differentiation. Also enzymes used for tissue dissociation were tested for the adequate concentration in advance.
For passing cells were washed with PBS and incubated with 0.05% trypsin-EDTA (Lonza) solution for 5 minutes at 37°C. Trypsin was neutralized with serum containing medium.
If cells had to be propagated they were not allowed to overgrow 90% of confluence.

**Primary mouse brown adipocytes isolation and culture**

Primary mouse brown adipocytes were isolated from intrascapular fat pads of two days old newborns. With this aim fat pads were  digested with isolation buffer (3.0 mg/ml of collagenase type IV, 123 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 5 mM glucose, 100 mM HEPES, 4% BSA) diluted

with PBS in ratio 1:1 for 40 min at 37°C with vigorous shaking by hands every 10 minutes. The time of digestion was estimated empirically. Too long digestion will result in damage of cells; instead too short digestion will be not sufficient for adipocytes isolation. In both cases cells will not undergo insulin induced differentiation. After digestion cells were spinned down (5 minutes at 200 $g$), pellet was resuspended in growth media (DMEM-HG (Cambrex) supplemented with 20% FBS (Invitrogen) and 20 mM HEPES) and filtered through 70 μm cell strainer. Cells were plated in the volume of 2.5 ml of media per one well of 12-well dish. Amount of plated cells was calculated on the basis that cells isolated from 0.5 newborn were plated in one well. Next day cells were washed twice with prewarmed DMEM. Upon 90% of confluence, cells were splitted on the basis of 50000 cells/cm$^2$ in DMEM-HG supplemented with 10% FBS (differentiation media). The day after, differentiation was induced with supplementation of media with 100 nM of insulin. From this time point we did not use vacuum aspiration in order not to detach differentiating adipocytes, which become weakly attached to plastic. Lipid accumulation was evaluated at day six after induction.

In parallel experiments differentiation was induced with differentiation media supplemented with 40 ng/ml of insulin; with 250 μM Lipoic acid; with 1mM NAc; with 0.1 μM Antimycin A; with 1 μM dexamethasone and 1 mM IBMX.


**Primary mouse white adipocytes isolation and culture**


**Primary mouse white adipocytes** were isolated from abdominal white fat pads of one- month-old mice. Fat pieces were minced and digested with the same isolation buffer under the same conditions as for brown adipocytes. Cells were spinned down for 5 minutes at 200 $g$, pellet was resuspended and filtered through 100 μm cell strainer and plated in growth media (DMEM-HG supplemented with 20% FBS and 20 mM HEPES). At day 2 upon confluence cells were stimulated with 1 μg/ml of insulin for differentiation. The level of differentiation was evaluated after 10 days after the induction.


**Primary mouse fetal hepatocytes isolation and culture**


For fetal hepatocytes isolation livers were isolated from mouse embryos at day 13.5, minced and digested for 20 min with isolation buffer. The composition of 5x isolation buffer is 0.0238 M CaCl$_2$, 0.344 M NaCl, 0.034 M KCl, 0.5 M HEPES, 0.33 M NaOH, 0.25% collagenase type IV. This solution one day before use was diluted till working concentration and pH was adjusted till 7.6 at 37°C and stored at +4°C. The solution was prewarmed at 37°C at least for 15 min before use. After

digestion two rounds of centrifugation for 5 minutes at 400 $g$ were performed. At the end pellet was resuspended in M199 media supplemented with 10% FBS, 10 mM HEPES, 10 nM cortisol, and 2 µg/ml of insulin and plated on collagen type I treated dishes and allowed to reach confluence after what further experiments were performed.

**Primary mouse myocytes isolation and culture**

Muscles from limbs of newborns at day 2 were collected, cleaned carefully from the skin, minced and digested with the buffer on the base of dispase/collagenase type IV solution for 40 minutes at 37°C, filtered through 70µm cell strainer, spinned down for 5 minutes at 350 $g$. Pellet was resuspended in F10-based primary myoblast growth medium (Hams F-10 (Invitrogen) supplemented with 20% of FBS, 2.5 ng/ml of bFGF) and plated on calf skin collagen coated dishes. For enrichment of cultures with myoblasts method of twice 15 minutes preplating when passing cells was used. After fibroblasts were no longer visible in culture the medium has been changed for 1:1 F-10/DMEM-based primary myoblasts growth medium.  Cultures were splitted every 5 days. For differentiation fusion media (DMEM plus 5% of horse serum) has been used.

**Primary mouse fibroblasts isolation and culture**

Carcasses of eviscerated living embryos at day 13.5 were minced and digested with small volume of 0.05% trypsin for 10 minutes at 37°C. Suspension obtained after digestion was resuspended in DMEM-HG supplemented with 10% FBS and plated.

**Determination of glucose consumption**

Brown adipocytes, fibroblasts and hepatocytes were plated on 12 well dishes in usuall for each cell type growth media. Media were then switched to equivalent low glucose (1 g/L) media supplemented with 0.5% serum (with or without 100 ng/ml insulin). Glucose concentration in the medium was determined after 24 hours with Ascensia Elite XL (Bayer).

For glucose determination in myoblasts, cells were plated on 24-well dish (30000 cells per well), kept 4 days in fusion medium and then 24 hours in serum free medium supplemented with 4.5 mM/L glucose and 1% BSA. Cells were then washed with a buffer containing 20 mM HEPES, pH 7.4, 120 mM NaCl, 2.5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1% BSA and incubated in the same buffer with or without insulin for 30 min at 37°C. After washing with the same buffer cells were treated

with a DOG solution (1 μCi/mL) for 15 min, then washed with cold PBS and fixed with 0.1 N of NaOH. Radioactivity was counted with a liquid scintillation analyzer and normalized for protein content.


## Fatty acids intake and oxidation rate

Brown adipocytes at passage 1 were grown till confluence without stimulation with insulin. Cultures were washed with 0.5% BSA solution in PBS, incubated at 37°C for 90 min with 1 mCi/ml [$^{14}$C]oleate (Amersham-GE Healthcare) in DMEM supplemented with 10% FBS and 0.5% BSA, washed twice with 0.5% BSA solution in PBS and lysed in 200 ml of 0.5 N NaOH. After neutralization, radioactivity was counted with a liquid scintillation analyzer and normalized for protein content.

Fatty acids β-oxidation rate was assessed by the release of $^{14}CO_2$ after [$^{14}$C] oleate uptake. To assay oxidation products, semi-dry filter paper saturated with 2 M NaOH was placed over 24-well incubation dishes and tightly covered with a foam pad and the plate cover. $^{14}CO_2$ produced by cells was driven from the media to the filter-paper trap by adding 100 μl of 70% (v:v) perchloric acid to each well. After 60 minutes in a shaking bath at 37°C, the filter paper discs corresponding to each well were dried, cut and washed in 2.0 ml of distilled water. $^{14}CO_2$ was quantified in 1.0-ml aliquots of the water wash in a liquid scintillation analyzer and normalized for protein content.


## Retroviral infection of adipocytes

Packaging cell line Phoenix were maintained till 70-80% of confluence when they were transfected. Before transfection media in Phoenix cells has been changed for a fresh growth media which was normally used by culture subjected to infection (10 ml per 10 cm dish). For transfection 20 μg of empty pBABE vector or vectors expressing WT p66[Shc] or the p66Shc*q* or p66Shc*a* mutants were diluted in 0.5 ml of 0.2 M CaCl$_2$ water solution. While bubbling 0.5 ml of 2x HBSS solution, prepared DNA solution was added dropwise. Thus we obtained small aggregates of Ca$_3$(PO$_4$)$_2$ containing DNA. This aggregates-containing solution has been added to Phoenix cell. 5 minutes before this step chloroquine was added dropwise to Phoenix cells to obtain final 25 nM concentration to inhibit lysosomal DNases by neutralizing vesicle pH, because DNA delivered by Ca$_3$(PO$_4$)$_2$ transfection is thought to transit through lysosomes. In 8 hours upon transfection media has been changed for a fresh, because chloroquine is toxic for cells. In 48 hours upon transfection media containing virus has been collected, filtered through 0.45 μm filter to remove cell debris and

cells. It was used for infection of adipocytes at passage 0 on their day 2 of culture. Infection included two cycles of 5 hours of adipocyte incubation with virus containing media. After selection with 2 μg/ml puromycin cells were splitted for differentiation. Equal levels of expression of p66$^{Shc}$ and its mutants upon infection in adipocytes have been demonstrated with western blotting.

**Oil red O staining**

Cells were washed with PBS and fixed in a 10% formalin PBS solution, washed once with water and once with 60% isopropanol. After air drying, Oil Red O solution was added (6 parts of 0.6% Oil Red O in isopropanol plus 4 parts of 1% sucrose water solution) for 15 min. After staining, cells were washed extensively with 60% isopropanol and then with water and used for observation. In some experiments, Oil Red O was washed out with 100% isopropanol and OD was read at $\lambda = 490$.

**FOXO translocation determination**

Primary brown adipocytes at passage 1 (not stimulated before with insulin) were seeded on glass slides and serum starved for 15 hours in serum free media supplemented with 0.5% BSA and $10^{-8}$ M sodium selenite. Afterwards cells were stimulated with 1μg/ml of insulin for 90 min. After that cells were washed with PBS and fixed in formalin solution in PBS during 10 min with following washing with PBS. Cells were permibilazied for 10 min in 0.1% Tryton-X 0.2 % BSA solution, blocked with 2% BSA, incubated for 1 hour with primary antibodies in 2% BSA solution in PBS, washed 3 times for 5 min with PBS, incubated with secondary antibodies conjugated with Cy5 in 2% BSA solution in PBS and washed with PBS again 3 times for 5 minutes. For nuclear staining incubation with 200 ng/ml DAPI solution in PBS was performed. Glass slides were air dried, mounted with moviol and observed under Olympus BX 61 microscope.

**Akt and MAPK activation**

Adipocytes at passage 1 were serum starved for 15 hours in serum free media supplemented with 0.5% BSA and $10^{-8}$ M sodium selenite. Then cells were stimulated (t = 0 minutes) with 1μg/ml of insulin for 5 minutes and at time point t = 5 minutes were washed and media was replaced with equivalent without insulin. Cells were collected at time points 5, 15 and 45 minutes after stimulation.

**ROS level determination**

Levels of intracellular $H_2O_2$ were determined using 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). It is cell-permeant indicators for reactive oxygen species and is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. The higher intracellular amount of ROS is, (namely amount of hydrogen peroxide), the higher oxidation of the dye happens and the higher fluorescent signal from the probe can be detected (170). WT and p66$^{Shc-/-}$ preadipocytes stained with 10μM of DCFDA (45 minutes at 37°C) were detached from plates using 0.05% trypsin (5 minutes at 37°C). This protocol allows complete cell recovery (> 95%), as evaluated by microscopic examination of plates after trypsinization. The cell suspension obtained was then analyzed by FACS (FACSCalibur, Becton Dickinson). The histograms obtained were analyzed using a specifically designed computer program (Cell Quest, Becton Dickinson). A trypan blue exclusion test (performed in parallel experiments) showed > 95% viability of trypsinized DFCDA-stained and -not stained cells. During FACS analysis we included control of unstained cells.

**Mitochondria isolation and studies**

All steps were performed at 4°C. Brown adipose tissues were minced and homogenized in MTC buffer (0.25M sucrose, 10mM Tris-MOPS buffer, pH 7.2 at 4°C, 1mM EGTA), filtered through gauze. Obtained suspension was centrifuged for 10 minutes at 8500 *g*, pellet was resuspended in MTC buffer and centrifuged again for 10 minutes at 800 *g*. Supernatant has been collected and recentrifuged for 10 minutes at 8500 *g*. Pellet consisiting of mitochondria, was resuspended on a basis of each mitochondrial preparation contained 0.5 mg of protein /ml mitochondrial suspension in (for state 3 determinations): 100 mM KCl, 50 mM MOPS, 10 mM $K_2PO_4$, 10 mM $MgCl_2$, 1mM EGTA, and 0.2% BSA when indicated, pH 7.2. Respiration was determined using a Clark's type oxygen electrode in the presence of different energetic substrates (5 mM succinate, 1 mM Pyruvate, 1 mM malate, 10 mM glycerol-3-phosphate) and modulators (0.1 μM DNP, 5 μM FCCP, 0.4 mM ADP, 0.4 mM GDP, 5 mM palmitate), which were added to glycerol-3-phosphate energized mitochondria.

For the investigation mitochondrial properties in brown fat, the tissue was minced and approximately 20 mg of minced tissue were investigated directly with Clark's electrode.

**Mitochondrial transmembrane potential studies**

Mitochondrial transmembrane potential was measured with tetramethylrhodamine methyl ester (TMRM), which is a fluorescent probe. This dye represents itself lipophilic cations accumulated by mitochondria in proportion to $\Delta\psi$ (171). Upon accumulation, it exhibits a red shift in both absorption and fluorescence emission spectra. TMRM was dissolved in methanol and used directly. The methanol concentrations in all incubations of mitochondria was kept to < 0.5% (v/v). Fluorescent measurements of mitochondria were made using a PERKIN ELMER LS 35 Fluorescent spectrofotometr.

**STUDIES OF PROTEINS, DNA, RNA**

**Cells lysis and protein detection**

Cell lysis was performed at 4°C in a buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 500 μM sodium orthovanadate, supplemented with a protease inhibitors cocktail from Calbiochem. Cells were lysed for 10 minutes, vortexed, spinned down at 130000 $g$ for 15 minutes. Supernatant was collected and protein concentration determination was performed with Bradford reagent. For calibration curve plottering BSA solution in the same lysis buffer was used. Proteins were separated by 8 or 12 % PAAG. After SDS-PAGE, proteins were transferred to PVDF membrane. Mebranes have been blocked in 5% BSA in 0.3% Tween 20 TBS solution for one hour, incubated with primary antibodies in the same solution during time which was required for each antibody by manufacters, washed three times during 10 minutes with 0.3% Tween 20 solution in TBS, incubated with secondary horse-radish peroxidase conjugated antibodies and washed in the way described above. Chemiluminescence was detected with ECL Western blotting detection reagent from Amersham.

**Immunoprecipitation**

Immunoprecipetations were performed on a platform rocker at 4º C. Equal amounts of proteins were incubated with anti-Shc antibodies and then with 50 μl of protein A-agarose, followed by three washes with lysis buffer. Beads were resuspended in 2x Laemmli loading buffer and proteins were separated by 8% SDS-PAGE. After SDS-PAGE, proteins were transferred to PVDF membrane.

Membranes were blocked in 5% BSA in 0.1% Tween 20 TBS solution for one hour, incubated with primary antibodies in the same solution during time which was required for each antibody by manufacters, washed three times during 10 minutes with 0.1% Tween 20 solution in TBS, incubated with secondary horse-radish peroxidase conjugated antibodies and washed in the way described above. Chemiluminescence was detected with ECL Western blotting detection reagent from Amersham.

**Total RNA extraction**

Total RNA was extracted using RNeasy isolation kit (Qiagen). In order to avoid genomic DNA contamination that could produce aspecific amplification products, before reverse-transcription, isolated total RNA was treated with RNase free DNase set (Qiagen).

**cDNA synthesis and QPCR**

RNAs of each sample were reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). For each sample two identical reactions were set up. With this aim 1 μg of RNA was incubated in reaction mixture containing random primers and dNTP for 5 minutes at 65°C in 12 μl reaction volume. The mixture was cooled down on ice, briefly centrifuged and combined with salt-buffer, DTT, RNase OUT (Invitrogen) solution on the base that final composition of the mixture was 50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, dNTP 0.5 mM each, RNase OUT 2 units/μl, 3 ng/μl random primers in final 20 μl reaction volume. After incubation at 25°C for 2 minutes, 1 μl of SuperScript II RT has been added (with final activity 10 units/μl) to the mixture. Reaction mixture was incubated at 25° for 10 minutes, followed by incubation at 42°C for 50 minutes and inactivated by heating at 70°C for 15 minutes. Obtained cDNA was used for determination of relative levels of specific mRNA with a 5′ nuclease assay (TaqMan) chemistry system. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a control gene for normalization. Applied Biosystems Assay-on-Demand products, consisting of pre-designed TaqMan probes, were used. Each sample was run in triplicate. All PCRs were performed with an ABI 7900HT sequence detection system.

**Gene expression analysis**

Primary brown adipocytes were grown till confluence and incubated with or without 100 nM insulin for 4 hours. Cells then were lysed in a guanidine-isothiocyanate containing buffer and total RNA was extracted using RNeasy isolation kit (Qiagen). For each experiment, total RNA was isolated and pooled from triplicate culture dishes and used for microarrays Affymetrix GeneChip hybridization. Biotin-labeled cRNA targets were obtained from 30 μg of total RNA derived from samples as described above. cDNA synthesis was performed with Gibco SuperScript Custom cDNA Synthesis Kit and biotin-labelled antisense RNA was transcribed using the *in vitro* MEGAscript High Yield Transcription Kit (Ambion Inc., Austin, Texas, USA) and included Bio-11-UTP and Bio-11-CTP (PerkinElmer Life Sciences) in the reaction. GeneChip hybridization, washing, staining, and scanning were performed according to Affymetrix (Santa Clara, California, USA) protocols. A copy of the Mouse Genome 430 2.0 GeneChip Array was hybridized with each target. Absolute and comparative analyses were performed with Affymetrix GeneChip Operating Software (GCOSv1.4) software, scaling all images to a TGT value of 500. Results were further exported to GeneSpring GX software version 7.3 (Agilent Technology). This work has been performed with the Gene Expression Analysis Service of the Campus.

**RNA interference**

For RNA interference, 100000 cells were seeded into one well of 6 well-plate. 3 μl of Oligofectamine and 12 μl of Opti-MEM (both from Invitrogen) were mixed (mix A). For mix B p66[Shc] specific (5'- GTACAACCCACTTCGGAATG (Nemoto, 06); and scrambled nucleotide sequence as negative control siRNA (Dharmacon) (final concentration 100 nM) were mixed with Opti-MEM in final volume 185 μl. After 15 minutes of incubation both mixes were pulled together and left for additional 15 minutes. After this 200 μl of solution obtained plus 800 μl of Opti-MEM were used to cover well with cells, previously washed with Opti-MEM. After 4 hours incubation 500 μl of growth media were added, containing triple concentration of serum. Control of protein knockdown was performed after 24 hours after the beginning of interference.

**STATISTICAL ANALYSIS**

All data are expressed as mean +/- S.D. and analyzed by Student's t-test. Differences between means were assessed by one-way analysis of variance. The minimum level of significance was set at $p < 0.05$.

# RESULTS

# P66[SHC] PREDISPOSES TO METABOLIC SYNDROME

## P66[SHC] REGULATES FAT TISSUE DEVELOPMENT AND PREDISPOSES TO OBESITY

It was previously noticed that p66[Shc-/-] mice had slightly decreased body weight. We analyzed the dynamics of body weight in WT and p66[Shc-/-] mice throughout their lives. We noticed that animals of both genotypes had similar body weight from the birth and till around two months of age, at which point the difference in 5-10% appeared. With age the difference became more profound. In fact, we noticed that p66[Shc-/-] animals stopped the accumulation of body weight at around 6-7 months, whereas WT animals continued to gain it (Fig. 6).
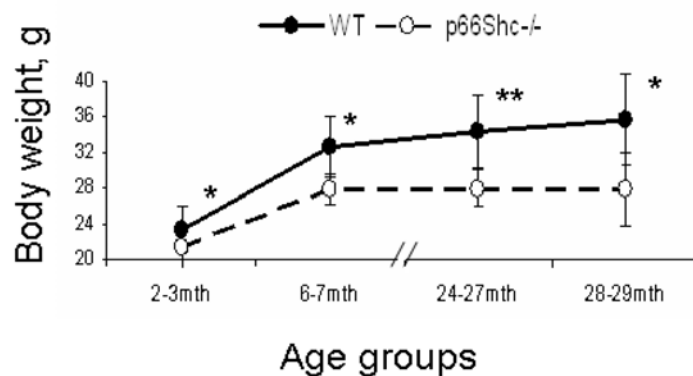


**Figure 6. Dynamics of body weight in p66[Shc-/-] and WT mice.** P66[Shc-/-] mice gain less body weight during their lives. N = 15 per group. * $p < 0.05$, ** $p < 0.001$.

Body weight is the result of a balance between processes of energy intake and dissipation. Main factors which regulate energy intake are food intake and calorie absorption in intestines. Locomotory activity and the level of metabolism in tissues determine energy dissipation. We found that calorie intake was the same in WT and p66[Shc-/-] mice (Fig. 7). We investigated intestinal calorie absorption by measuring the residual caloric content of faeces by calorimetric bomb. It was similar between animals of two genotypes (Fig. 8). Locomotory activity in WT and p66[Shc-/-] mice was also the same (Berry A. *et al*, manuscript in preparation).

Total body weight is influenced by the weight of single organs, mainly of fat. We measured the weight of fat deposits in p66[Shc-/-] mice. We isolated inguinal, abdominal and intrascapular fat pads from WT and p66[Shc-/-] animals and compared their weights. We discovered that difference in body weight between WT and p66[Shc-/-] animals was accompanied with much more pronounced difference in weight of fat deposits. In fact, abdominal, inguinal and inter-scapular fat pads were all reduced in p66[Shc-/-] mice (Fig. 9). Differences were more marked in the abdominal district (up to 60% reduction in the p66[Shc-/-] mice) (Fig. 9). Mice both of 129Sv and C57Bl6 strains showed the same tendency in fat weights. The differences were more profound in male.
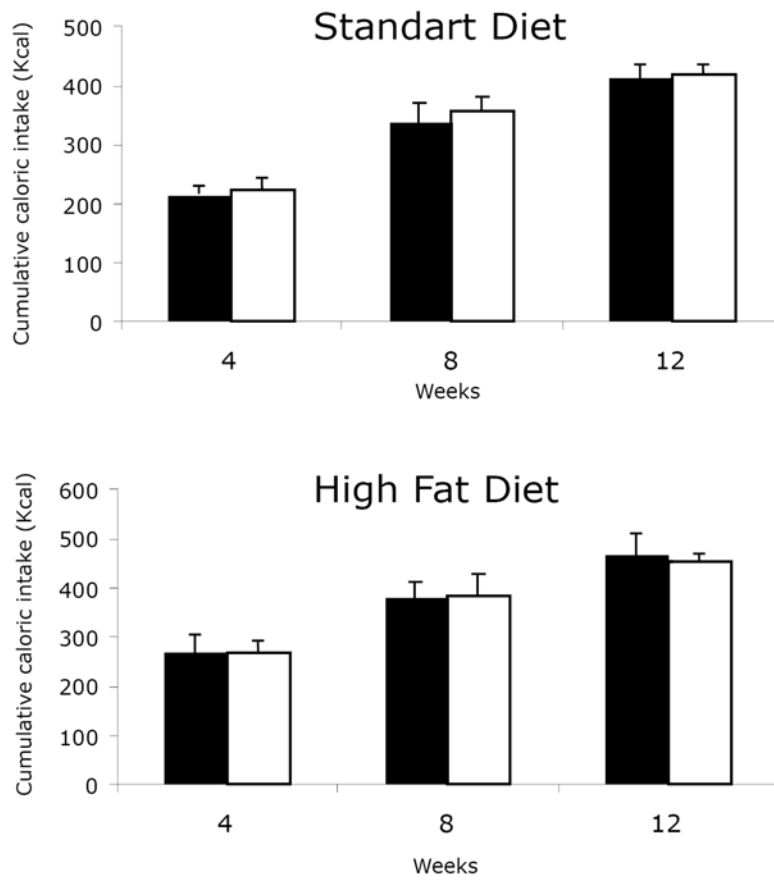
**Figure 7. Calorie intake in WT and p66<sup>Shc-/-</sup> mice.** P66[Shc-/-] mice consume the same amount of calories both on standrad and high fat diet in comparison with WT controls. N = 10 per genotype.
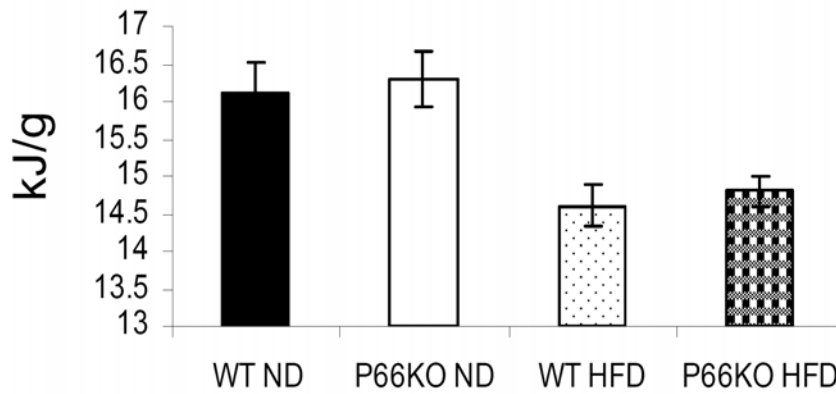


**Figure 8. Energy content in feces of WT and p66<sup>Shc-/-</sup> mice**. Energy content in feces of WT and p66[Shc-/-] mice is equal both on normal (ND) and high fat (HFD) diet. N = 10 per genotype.
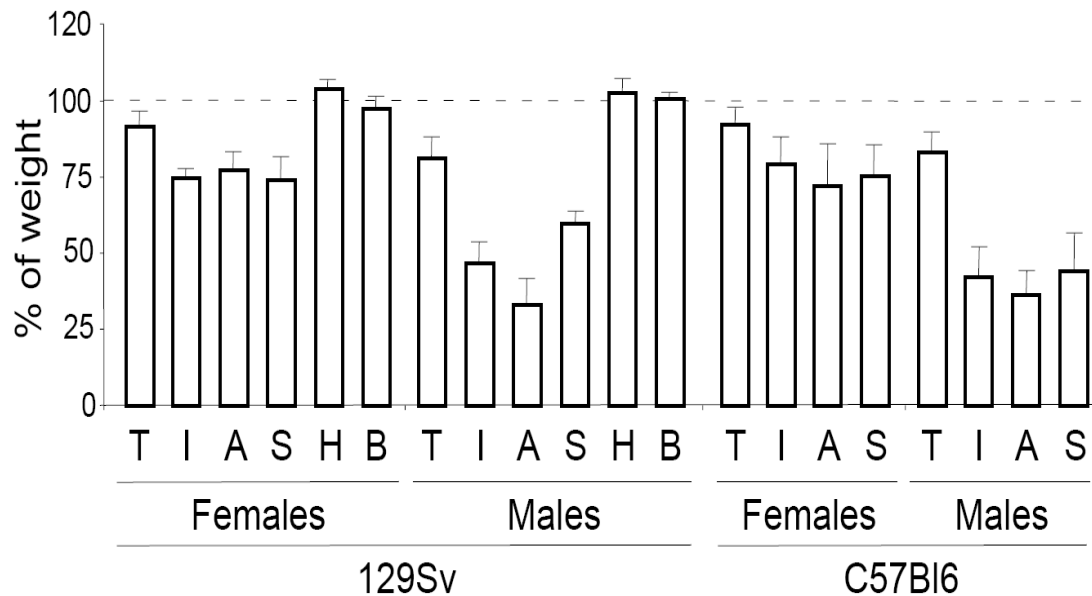
**Figure 9. P66<sup>Shc-/-</sup> regulates weight of fat depots *in vivo*.** Total body fat (T), fat pad (inguinal, I; abdominal, A; intrascapular, S), heart (H) and brain (B) weights in 129Sv or C57Bl6 p66$^{Shc-/-}$ mice on standard diet expressed as percentage of (the weight of) matched WT (n = 20 per group). The difference in weights was significant in 129Sv background, $p < 0.001$ and in C57Bl/6 male, $p < 0.05$.

We checked if there was the difference in the weight of other organs. We studied the weight of brain and heart, however did not find significant difference in the weight of these organs between WT and p66$^{Shc-/-}$ animals (Fig. 9).

All together these findings indicate that p66$^{Shc}$ regulates the development of fat tissue *in vivo*.

Diet induced obesity is the most frequent reason of obesity in human modern society. We asked if a deletion of p66$^{Shc}$ could protect from diet induced obesity. With this aim p66$^{Shc-/-}$ and WT mice were subjected to 12 weeks treatment with high fat diet (HFD) with increased calorie content and 60% of calories coming from fat. Upon treatment we studied the body weight gain and the weight of fat depots. We discovered that loss of p66$^{Shc}$ had protective effect against diet induced obesity. Indeed, as shown in Fig. 10, WT male gained significantly more body weight than the p66$^{Shc-/-}$ mice (Fig. 10) and accumulated more fat (Fig. 11). The difference in body size was obvious even upon visual examination (Fig. 12). Indeed, gross appearance at sacrifice after 12 weeks of HFD revealed markedly reduced fat mass in the p66$^{Shc-/-}$ animals, as compared to WT controls (Fig. 12). Notably, the treatment of mice with antioxidant NAc also resulted in the protection from body weight gain upon high fat diet treatment (Fig. 13). Thus, we hypothesized, that p66$^{Shc}$ is involved in the regulation of fat tissue development and diet induced obesity through oxidative stress dependent mechanisms.
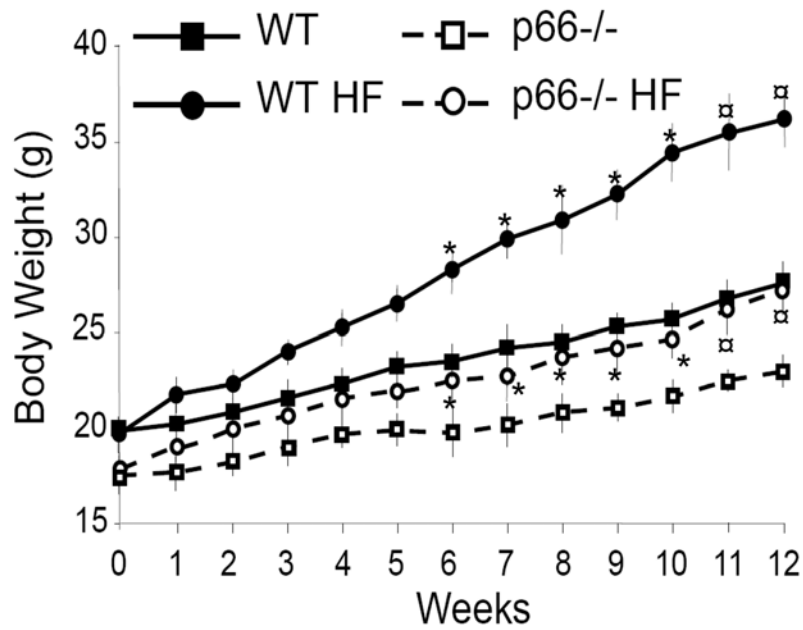
**Figure 10. P66^Shc regulates dynamic of body weight upon HFD.** Body weight curves of WT and p66$^{Shc-/-}$ mice fed standard or high fat (HF) diets. N = 10 per group. *  $p < 0.05$, ¤ $< 0.001$
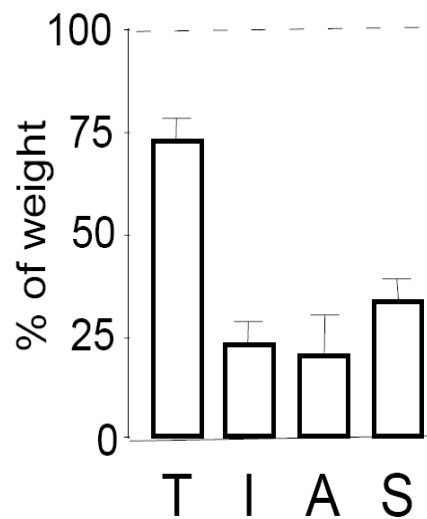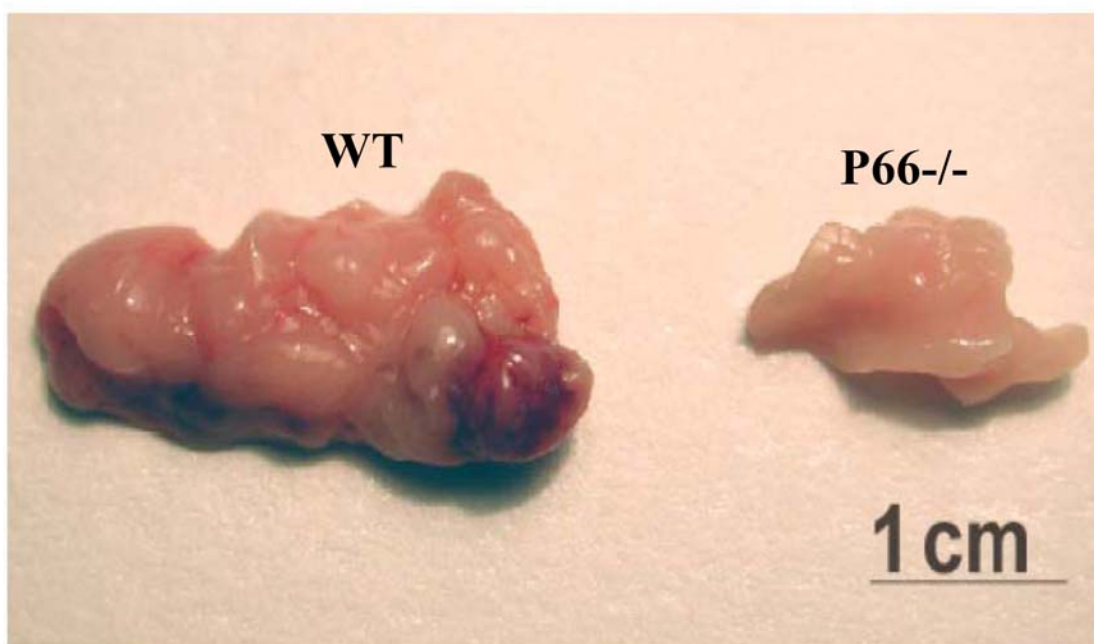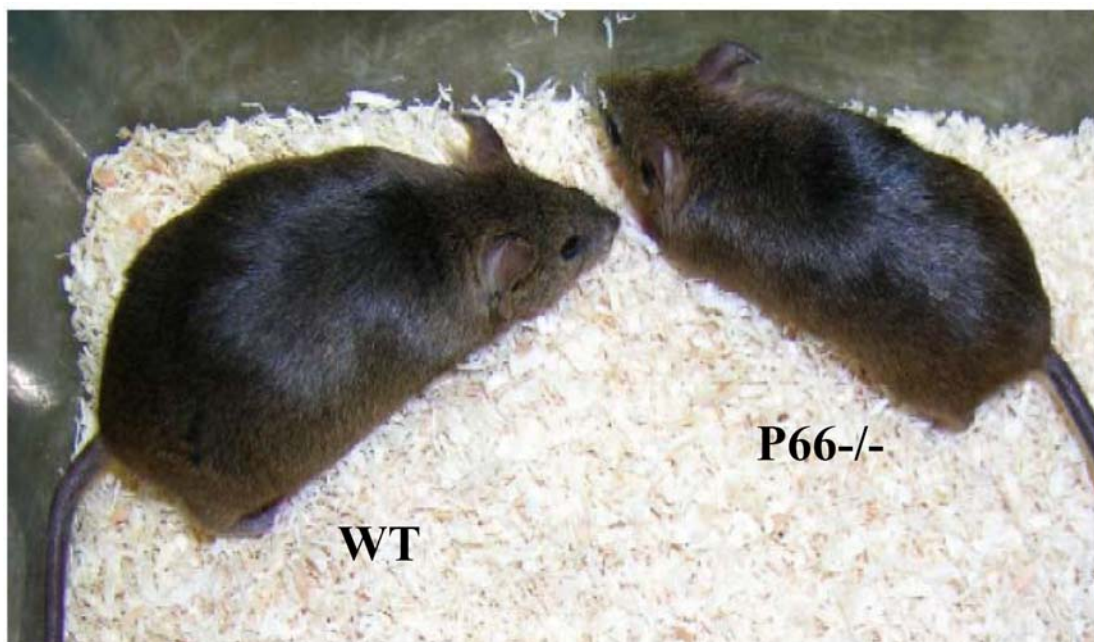


**Figure 11. P66^Shc regulates fat accumulation upon HFD.** Total body fat (T) and fat pads (inguinal, I; abdominal, A; intrascapular, S) weights upon HF diet in p66$^{Shc-/-}$ mice, expressed as percentage of (the weight of) matched WT (n = 10 per group). The difference in weights was always statistically significant, $p < 0.05$.

**Figure 12. P66<sup>Shc-/-</sup> mice are protected from diet-induced obesity.** Comparative view of representative p66$^{Shc-/-}$ and WT mice and inguinal fat pads upon HFD treatment.
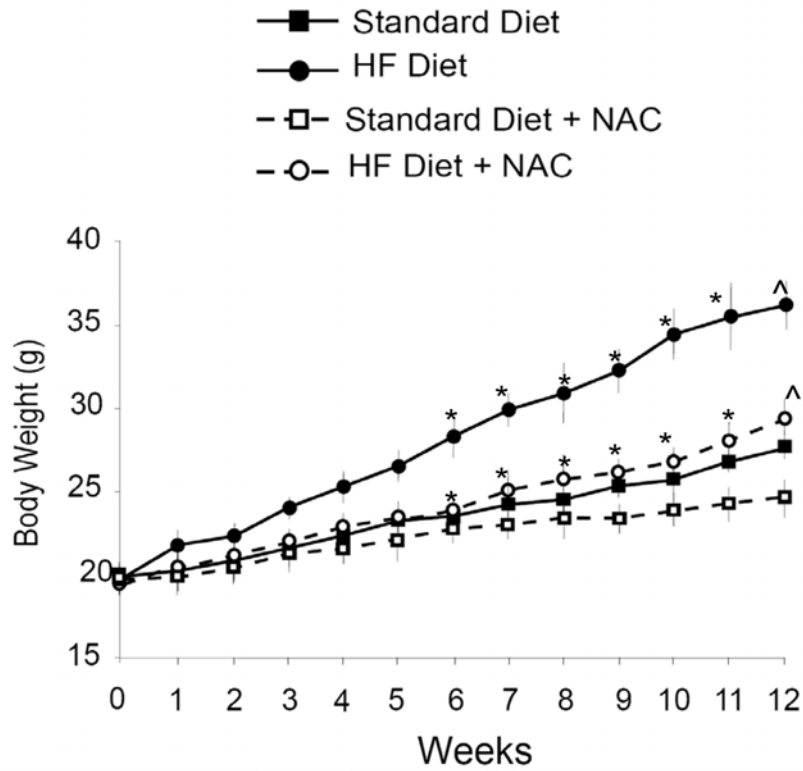
**Figure 13. N-acetylcisteine decreases weight gain on HFD treatment.** Body weight curves of WT mice fed standard or high fat (HF) diets with or without 40 mM NAc in the drinking water. N = 12 mice per group. * $p < 0.05$, ^ $p < 0.001$

Further, we performed examination of histological sections of brown fat and perigonadal white fat depots in adult mice. We discovered that defect in the development of fat tissue in p66^Shc-/- mice was associated with abnormalities in its architecture. In fact, adipocytes from p66^Shc-/- mice showed higher heterogeneity in size (Fig. 14).
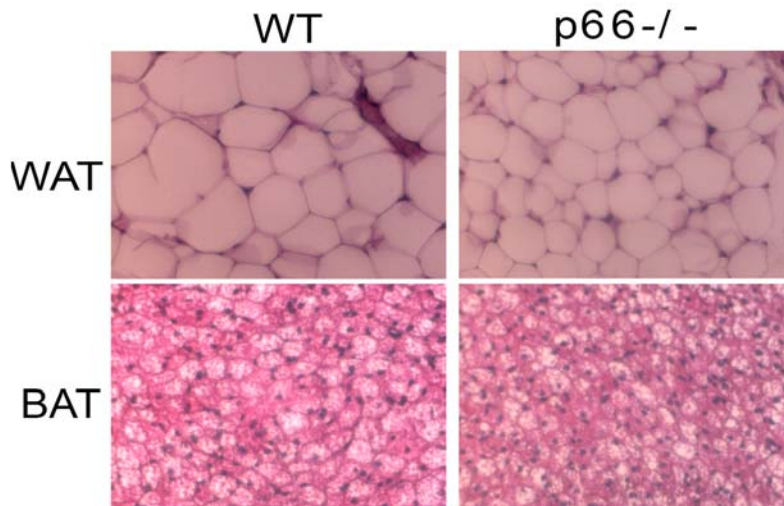


**Figure 14. P66^Shc regulates cell size in adipocytes.** Figure represents hematoxylin/eosin-staining of histological sections of abdominal white depot (WAT) and brown fat tissue (BAT) from 8-months-old p66^Shc-/- and WT mice.
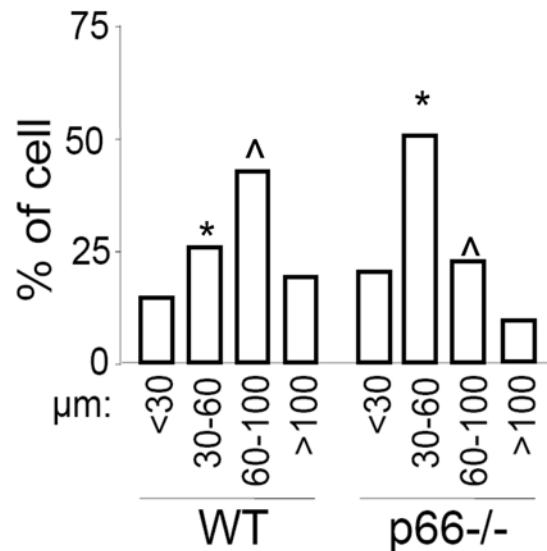
**Figure 15. P66<sup>Shc</sup> regulates size of adipocytes.** Distribution of adipocytes of abdominal fat depot according to cell size upon analysis of histological sections. Data represent the result of the analysis of 10 different mice per group. *, ^ $p < 0.05$.

Analysis of the distribution of adipocytes according to size revealed that the majority of cells were shifted from the size box 60-100 µm in WT into 30-60 µm in p66$^{Shc-/-}$ animals (Fig. 15).

It is known, that higher heterogeneity of adipocytes size can be associated with deregulated pattern of expression of key adipocyte proteins, as it was shown on example of FIRKO mice (127). Thus, loss of p66$^{Shc}$ might be connected with alteration of properties and function of fat. We came to the same conclusion after electron microscopy analysis of ultra-structure of adipocytes in the same samples. As shown in Fig. 16, electron microscopy sections revealed drastic alteration in the fat cell structure upon deletion of p66$^{Shc}$.
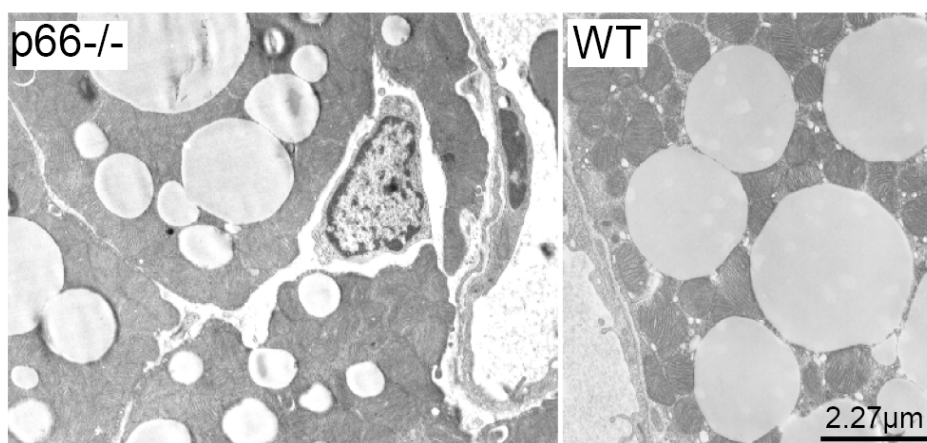


**Figure 16. Electron microscopy of WT and p66$^{Shc-/-}$ brown adipose tissue.** Brown adipocytes in p66$^{Shc-/-}$ mice have smaller lipid drops and are more tightly packed with mitochondria.

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Indeed, p66^Shc-/- brown adipocytes showed smaller sized lipid drops as compared to wild type. The increased area of lipid-drops free cytoplasm in the p66^Shc-/- adipocytes was filled with tightly packed mitochondria, suggesting that the relative number of mitochondria per surface unit is increased in these cells. This brings to the suggestion that brown adipocytes from p66^Shc-/- animals might be more metabolically active.

## P66^SHC IMPAIRS INSULIN SENSITIVITY AND GLUCOSE TOLERANCE

Fat tissue is a critical determinant of systemic insulin sensitivity (see Preface). It is known, that insulin resistance is one of the most frequent and essential components of MS. It is often associated with obesity, and it is a crucial determinant of other risk factors seen in MS (172,173). Thus we investigated if p66^Shc is involved in the regulation of insulin resistance and glucose intolerance.

Total body insulin resistance is associated with decreased insulin stimulated glucose consumption in tissues. Progression of insulin resistance is accompanied with an increase in insulin and glucose blood levels. Abnormalities in glucose/insulin metabolism at earlier stages can be detected with insulin and glucose tolerance tests. In these tests injection of high doses of insulin or glucose stimulates peripheral tissues to consume the glucose from the blood. The dynamics of blood glucose level lowering upon such challenges reflects the state of insulin responsive tissues and secretion of insulin by pancreas.

We measured the levels of circulating fasting insulin and glucose. Insulin level was slightly reduced in p66^Shc-/- mice (Table 1).

| | WT SD | WT HFD | p66-/- SD | p66-/- HFD |
|---|---|---|---|---|
| Free Fatty Acid (meq/L) | $0.57 \pm 0.12$ | $1.24 \pm 0.32$ | $0.45 \pm 0.11$ | $1.11 \pm 0.28$ |
| Triglycerides (mg/dL) | $68 \pm 9$ | $143 \pm 30$ | $61 \pm 6$ | $118 \pm 35$ |
| Cholesterol (mg/dL) | $53 \pm 6$ | $112 \pm 23$ | $55 \pm 8$ | $103 \pm 17$ |
| Insulin (ng/dL) | $1.34^{*} \pm 0.22$ | $1.47^{\wedge} \pm 0.18$ | $0.93^{*} \pm 0.15$ | $0.90^{\wedge} \pm 0.12$ |
| Glucose (mg/dL) | $96 \pm 5.4$ | $103 \pm 7.9$ | $89 \pm 4.5$ | $90 \pm 4.3$ |

**Table 1. Lipid profile and blood levels of insulin and glucose in WT and p66^Shc-/- mice.** Plasma levels of free fatty acids, triglycerides, cholesterol, insulin and glucose in WT and p66^Shc-/- mice fed standard (SD) and high fat (HF) diet. N = 20 mice per group. *, ^ $p < 0.05$.

Glycaemia was not significantly different between animals of two genotypes (Table 1). Preliminary data suggest that p66[Shc-/-] animals have higher glucose tolerance and insulin sensitivity.

We further confirmed our data with insulin tolerance test. Results revealed that both young and old p66[Shc-/-] mice had increased insulin sensitivity. Indeed, young p66[Shc-/-] mice were around 50% more effective in the consumption of glucose upon introduction of exogenous insulin at the end time point of experiment than their WT littermates. Actually, this experiment resulted in hypoglycemic coma almost for 50% of transgenic animals; meanwhile only around 7% of WT littermates experienced it. Insulin sensitivity impaired during age both in WT and p66[Shc-/-] animals and increased insulin dose was required to lower blood glucose level. However, old p66[Shc-/-] mice were still more insulin sensitive than WT matched animals and faster utilized blood glucose upon endogenous injection of insulin (Fig. 17).
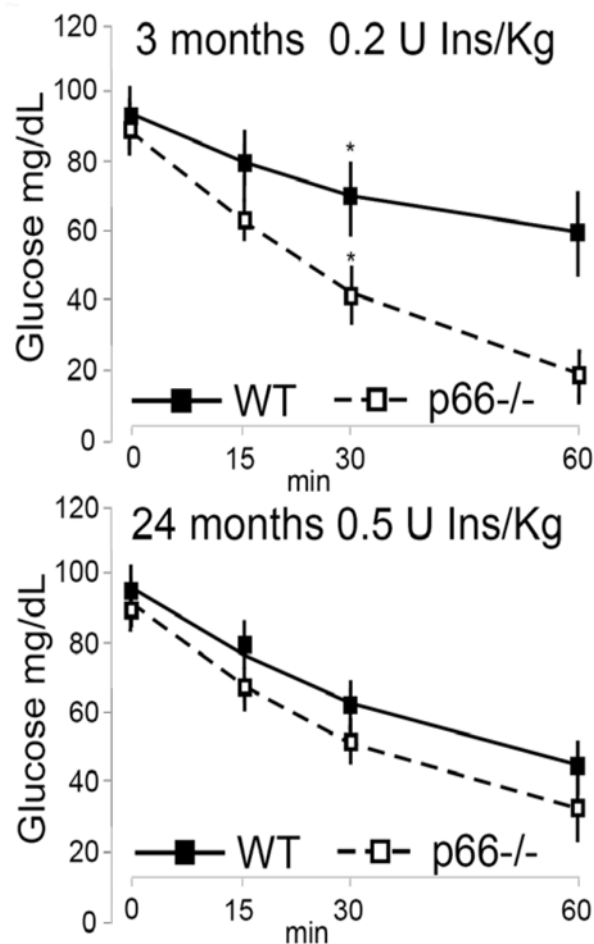


**Figure 17. Increased insulin sensitivity in p66[Shc-/-] mice.** Insulin tolerance tests in WT and p66[Shc-/-] mice. Figure depicts lowering of blood glucose upon injection of insulin in 3-months (0.2 U/kg of body weight) and 24-months old (0.5 U/kg of body weight) p66[Shc-/-] and WT mice (n = 10), $* p < 0.05$.

We performed glucose tolerance test in p66$^{Shc-/-}$ animals. Its result showed that utilization of blood glucose under endogenous insulin production was much more effective in p66$^{Shc-/-}$ mice both in old and young ages (Fig. 18). Maximum blood level of glucose in p66$^{Shc-/-}$ animals was raised only up to around 65% of that of WT in this test.
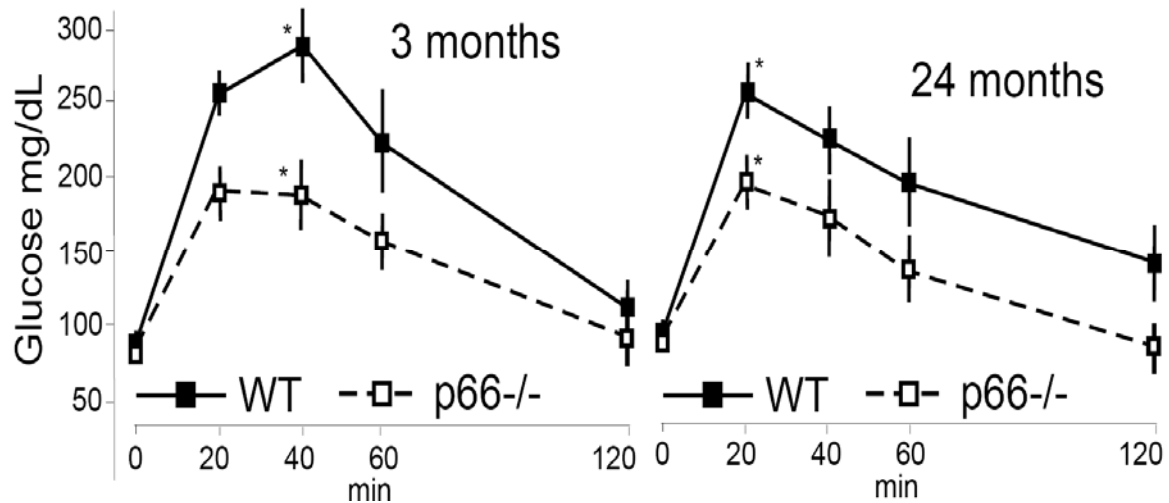


**Figure 18. Increased glucose tolerance in p66$^{Shc-/-}$ mice.** Glucose tolerance tests in WT and p66$^{Shc-/-}$ mice. Both 3- and 24- months old p66$^{Shc-/-}$ male utilize glucose faster than WT controls upon injection of 2g/kg of body weight D-glucose ($n = 10$), * $p < 0.01$.

Thus, we received multiple confirmations of significantly increased insulin sensitivity and glucose tolerance in p66$^{Shc-/-}$ mice.

# P66$^{SHC}$ IS REGULATED BY DIET

Fat development is subjected to a complex genetic regulation. Genes, which are regulated by diet themselves, could represent master elements in this system.

We asked if there was a link between food intake and p66$^{Shc}$ regulation.

We investigated therefore the expression of p66$^{Shc}$ protein by western blot in the white adipose tissue extracted from mice fed different diets. Groups of mice were treated with high fat diet for 10 days or were subjected to 48 hours of starvation. After treatment, the expression of p66$^{Shc}$ protein in perigonadal depot was checked. We noticed that the expression of p66$^{Shc}$ varies a lot between different individuals (Fig. 19). Notably, there were no differences in the individual expression of p66$^{Shc}$ in lungs and livers upon diet treatment (Fig. 20).
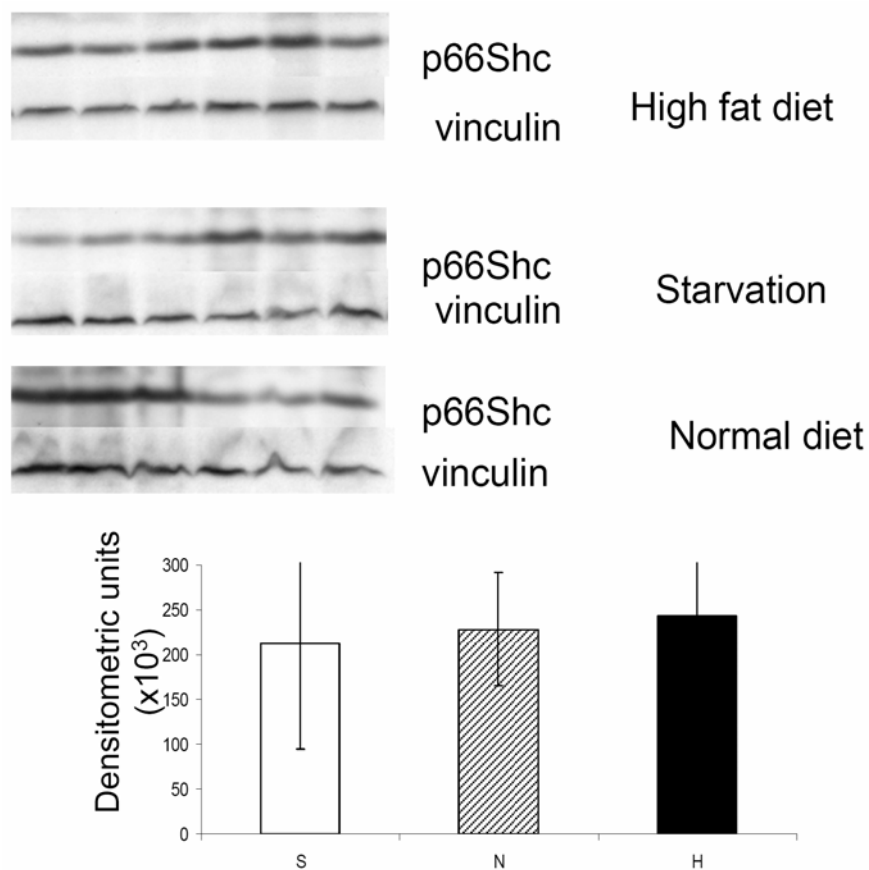
**Figure 19. P66<sup>Shc</sup> expression in white fat upon diet treatment.** Upper panel. P66$^{Shc}$ expression is heterogenious in perigonadal fat depot from mice treated with high fat diet for 10 days or upon 48 hours of starvation as evaluated by western blotting. Lower panel. Densitometric analysis of p66$^{Shc}$ expression from the blots shown in upper panel (S, upon starvation, N, upon normal diet, H, upon high fat diet treatment).
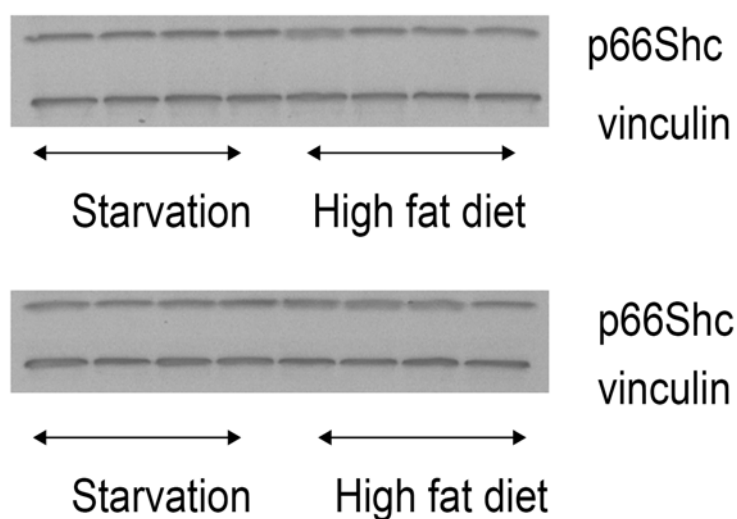


**Figure 20. Expression of p66<sup>Shc</sup> in livers (upper panel) and lungs (lower panel) upon treatment with starvation and high fat diet.** P66$^{Shc}$ is equally expressed in livers and lungs from mice treated with high fat diet for 10 days or upon 48 hours of starvation as evaluated by western blotting.

To avoid the influence of individual genetic backgrounds, we compared the level of p66$^{Shc}$ expression before and after treatment with diet in the same individuals. With this aim a part of perigonadal fat depot was extracted before and remaining symmetrical part of the same depot was removed after diet treatment. Strikingly, we found that the level of p66$^{Shc}$ was dependent on the diet applied. The most obvious effect was observed upon starvation which decreased p66$^{Shc}$ expression. Upon treatment with high fat diet there was a trend of increase in protein expression (Fig. 21).
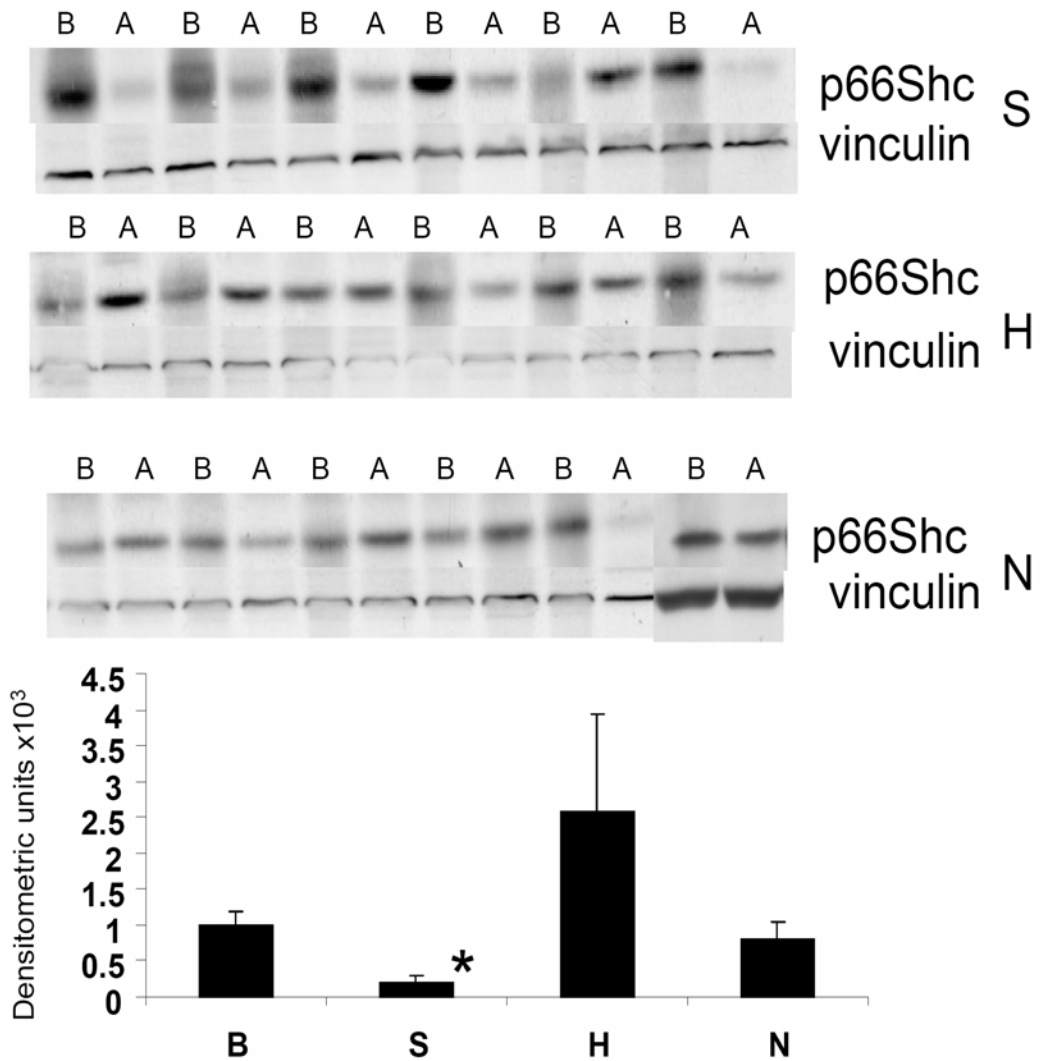


**Figure 21. Diet regulates expression of p66$^{Shc}$ in fat.** Upper panel. P66$^{Shc}$ expression in perigonadal fat of mice before (B) and after (A) treatment with 10 days of high fat diet (H), 48 hours of starvation (S) and normal diet (N) as evaluated by western blotting. Lower panel. Densitometric analysis of bands of p66$^{Shc}$ from the same experiment. Difference between S and B, S and H, S and N is statistically significant, $p < 0.05$

We investigated if the regulation of p66$^{Shc}$ expression happens at the level of transcription or involves post-transcriptional regulation. We checked the level of mRNA of p66$^{Shc}$ upon different diet treatments. Strikingly, the level of p66$^{Shc}$ mRNA expression was significantly increased upon starvation, suggesting that the real level of p66$^{Shc}$ protein in the cell is a result of post-transcriptional regulation (Fig. 22).
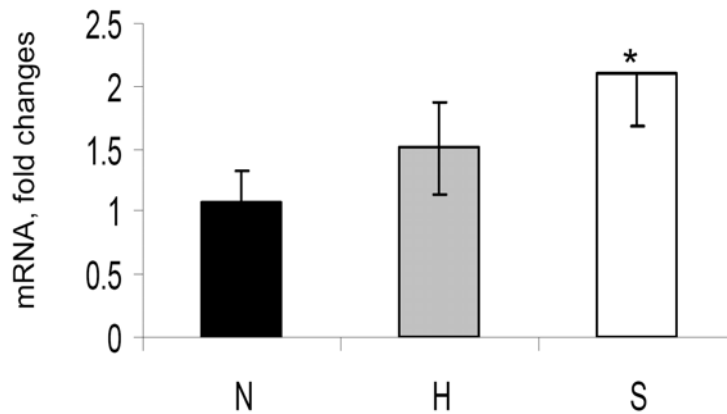


**Figure 22. Starvation increases p66$^{Shc}$ mRNA level.** Expression of p66$^{Shc}$ mRNA in fat samples upon treatment with high fat diet (H), normal diet (N) and starvation (S) expressed in fold changes to the level in samples before treatment. The difference is significant between S and N, S and H, $p < 0,001$.

Thus, p66$^{Shc}$ is a novel diet regulated protein.

Our ongoing experiments have the aim to investigate if p66$^{Shc}$ is modified by diet also in humans. With this aim we study possible correlation between the body mass index and p66$^{Shc}$ expression in human fat samples obtained upon liposuction or during the surgical interventions.

## SYSTEMIC CONSEQUENCES OF ALTERED FAT TISSUE DEVELOPMENT IN P66$^{SHC-/-}$ MICE

We checked systemic consequences of impaired fat tissue development in p66$^{Shc-/-}$ mice. Since fat tissue is crucially involved in the overall body metabolism we investigated energy metabolism in p66$^{Shc-/-}$ mice. Under basal conditions, body temperature of p66$^{Shc-/-}$ mice was slightly higher than in WT (Fig. 23). Then we exposed WT and p66$^{Shc-/-}$ mice to cold (5°C) for 6 hours. The maximal loss of body heat after cold exposure in the range of 3°C occurred in the WT mice within 4 hours. P66$^{Shc-/-}$ mice, instead, dropped 6°C of their temperature within 3 hours (Fig. 24). However, both WT and p66$^{Shc-/-}$ mice returned to their basal body temperature after 6 hours, suggesting that thermogenesis is not impaired in p66$^{Shc-/-}$ mice (Fig. 24).
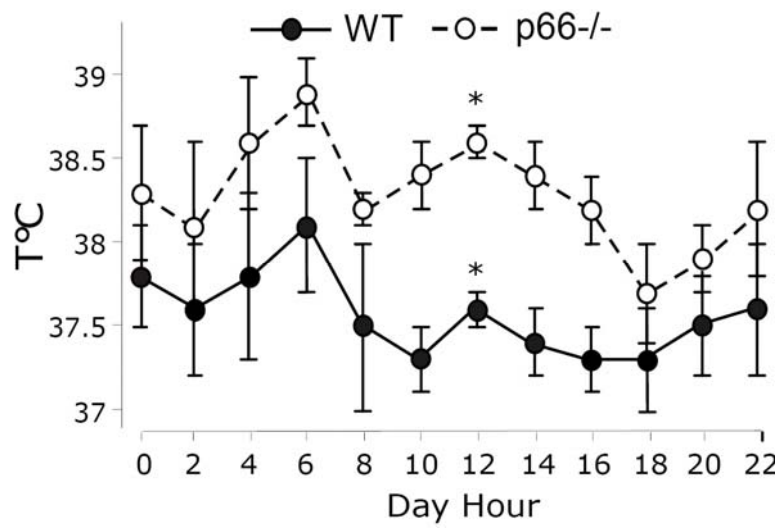
**Figure 23. P66<sup>Shc</sup> and body temperature dynamics.** Daily body temperature values of WT and p66$^{Shc-/-}$ mice. N = 12 per group. *p < 0.01.
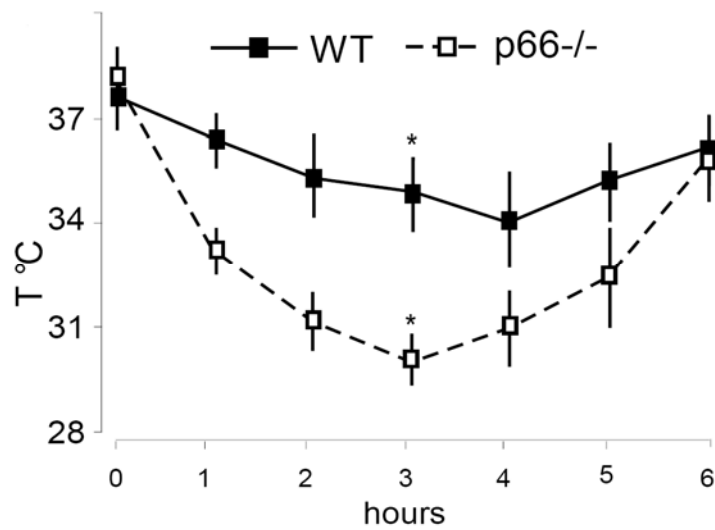


**Figure 24. P66<sup>Shc</sup> is involved in thermoregulation.** Body temperature values of WT and p66$^{Shc-/-}$ mice during cold exposure. N =12 per group. *p < 0.01.

Thus, abnormal adaptation to cold in p66$^{Shc-/-}$ mice is the consequence of accelerated heat loss, which probably happens due to the reduced thermal insulation effect of reduced white fat depots. We checked as well expression of UCP1 upon cold exposure. Notably, levels of UCP1 in p66$^{Shc-/-}$ mice kept at 22°C were as high as those of WT mice after cold exposure (Fig. 51). It suggests that brown adipose tissue of p66$^{Shc-/-}$ mice was more activated for the heat generation initially.

Next we checked systemic respiration in p66$^{Shc-/-}$ mice. With this aim we measured total oxygen consumption. It was slightly, yet significantly increased in p66$^{Shc-/-}$ males, as compared to matched controls (differences did not reach statistical significance in female) (Fig. 25).
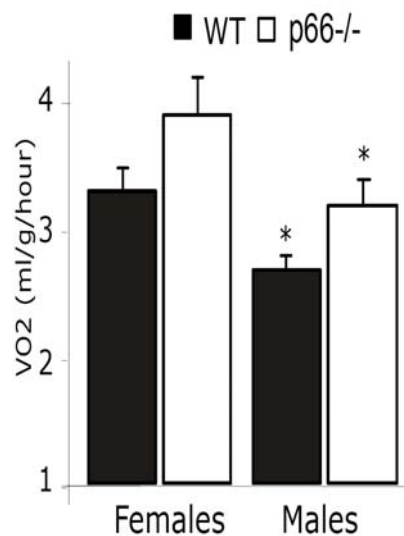


**Figure 25. P66$^{Shc}$ down-regulates systemic respiration.** O$_2$ consumption over 24 hours in WT and p66$^{Shc-/-}$ mice (n = 8 per group), * p < 0.01.
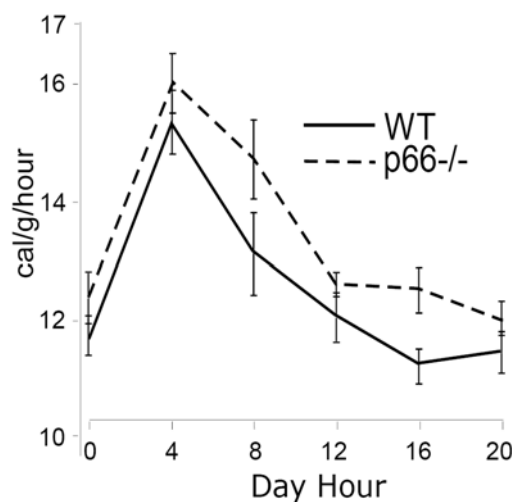


**Figure 26. P66$^{Shc}$ down regulates daily energy expenditure.** Daily energy expenditure of male WT and p66$^{Shc-/-}$ mice (n = 8 per group), * p < 0.01.

Likewise, total energy expenditure, measured in a 24-hours interval, was moderately higher in p66$^{Shc-/-}$ males (Fig. 26).

These data suggest that in p66$^{Shc-/-}$ animals energy consumed is less stored in form of TAG in adipose tissue, but is more dissipated.

Besides storage of TAG and thermoregulation, adipose tissue functions as an active endocrine organ that secretes adipokines with profound effect on organism. Their secretion influences metabolic function of other organs such as liver and muscles (174). Deregulation of adipokines is an important mechanism by which adipose tissue contributes to systemic insulin resistance and metabolic diseases (174). It is known that secretion of adipokines is deregulated in obesity and lipodistrophia. We checked if p66$^{Shc}$ was important for the secretion of adipokines, crucially involved in the onset of systemic insulin resistance. We found that the level of circulating leptin was significantly lower in p66$^{Shc-/-}$ mice. This difference was increased even more upon high fat diet treatment (Fig. 27).
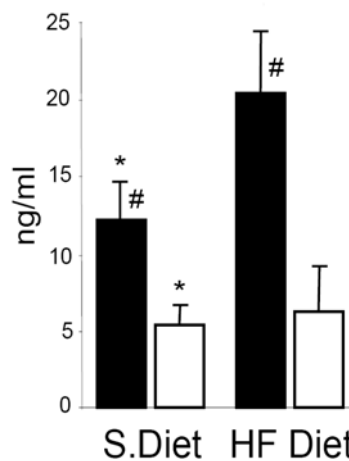


**Figure 27. P66$^{Shc}$ regulates leptin secretion.** Leptin concentrations in the serum of WT and p66$^{Shc-/-}$ 8-months-old male mice fed standard or HF diets. N = 10. * $p < 0.01$; # $p < 0.05$.

High-fat diet treated WT mice had also higher level of TNFα in agreement with the fact of higher amount of fat in the organism (Fig. 28).

Lipid blood profile was however similar in p66$^{Shc-/-}$ and WT animals (Table 1).

Thus, alteration of fat tissue development upon p66$^{Shc}$ loss has as a consequence altered endocrine properties of adipose tissue, altered systemic thermoregulation and energy metabolism.
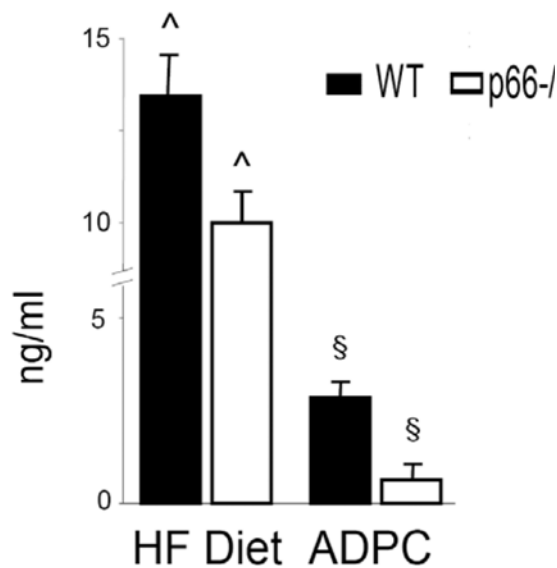
**Figure 28. P66^Shc regulates TNFα secretion.** TNFα concentrations in the serum of WT and p66^Shc-/- 8-months-old male mice fed HF diet (n = 10), and in the supernatants from WT and p66^Shc-/- adipocytes cultures (ADPC), ^ $p < 0.01$; § $p < 0.05$.

# MECHANISM OF P66^SHC REGULATION OF FAT TISSUE DEVELOPMENT

Since obesity is major component and etiological factor of MS, we restricted our research on the role of the p66^Shc in the predisposition to MS to the studies on p66^Shc involvement in the regulation of fat tissue development.

## P66^SHC REGULATES INSULIN SIGNALING IN ADIPOCYTES
### P66^Shc regulates insulin-induced lipid accumulation *in vitro*

Our next step was the identification of the intracellular mechanisms regulated by p66^Shc in adipocytes. With this aim we established *in vitro* model representing fat tissue development. We established protocols for the isolation of primary brown and white adipocytes and their propagation and differentiation in culture. For the differentiation of adipocytes we used insulin. Insulin treatment resulted in a drastic change of shape of cells into spherical one, accumulation of lipid drops and high increase in cellular metabolic activity (see below and data not shown). For the detection of TAG accumulation, we used staining of adipocytes with Oil Red O dye, which shows high degree specificity for lipids and is routinely used for their staining. Cultures of both brown (Fig. 29) and white adipocytes (Fig. 30) expressed p66^Shc at equal levels during differentiation. We checked if the loss of p66^Shc in adipocytes was associated with decrease in ROS level like it was demonstrated for other cells (155,156). We studied ROS level in cell cultures upon their treatment with fluorescence

dye 2', 7' –dichlorofluorescin diacetate (DCFDA), a molecular probe that becomes fluorescent upon oxidation by $H_2O_2$ (170). FACS analysis of cells stained with DCFDA showed significantly decreased fluorescence in p66$^{Shc-/-}$ adipocytes suggesting that concentration of intracellular $H_2O_2$ is decreased in p66$^{Shc-/-}$ cells. In fact, steady-state levels of ROS in p66$^{Shc-/-}$ adipocytes were reduced ~35%, as compared to control WT cells (Fig. 31).
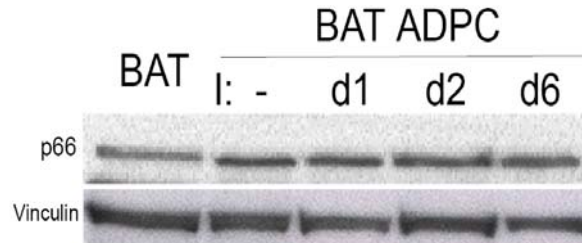


**Figure 29. P66$^{Shc}$ protein expression in brown adipocytes (BAT ADPC) and in brown adipose tissue (BAT).** P66$^{Shc}$ is equally expressed during differentiation of brown adipocytes as evaluated by western blotting. I: insulin treatment; d: days of treatment.



**Figure 30. P66$^{Shc}$ protein expression in white adipocytes (WAT ADPC) and white adipose tissue (WAT).** P66$^{Shc}$ is equally expressed during differentiation of brown adipocytes as evaluated by western blotting. I: insulin treatment; d: days of treatment.
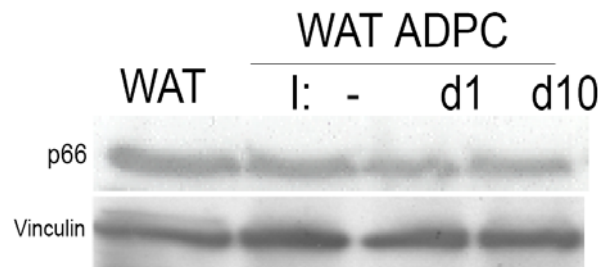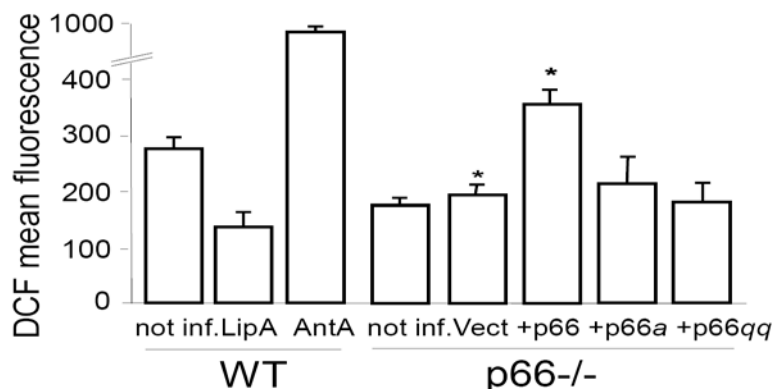


**Figure 31. ROS level in brown WT and p66$^{Shc-/-}$ adipocytes.** FACS analysis of the mean DCF fluorescence of DCFDA-stained WT brown adipocytes, under basal conditions (not inf.) or after lipoic acid (LipA) or antimycin A (AntA) treatment, and p66$^{Shc-/-}$ brown adipocytes under basal conditions (not inf.) or after infection with empty retroviral vector (Vect), or retroviral vectors expressing p66$^{Shc}$, p66Shc*a* or p66Shc*qq*.
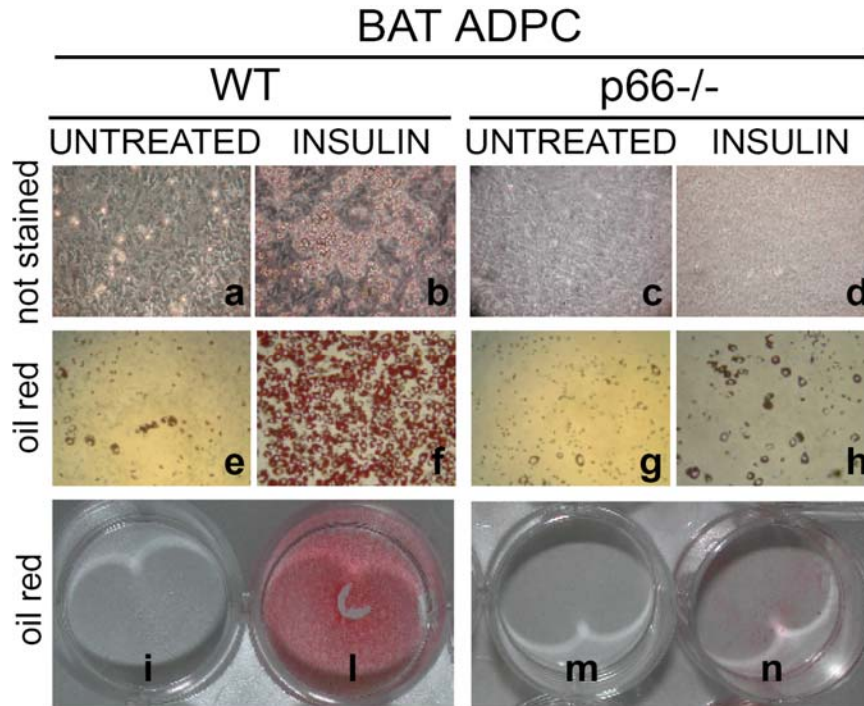
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**Figure 32. P66$^{Shc}$ regulates lipid accumulation in brown adipocytes (BAT ADPC).** White light microscopy pictures (a-h) and plate micrographs (i-n) of unstained (a-d) and Oil red stained (e-h, l-n) WT and p66$^{Shc-/-}$ brown adipocytes. Cells were left untreated (a, e, c, g, i, m) or treated with insulin for 6 days (b, f, d, h, l and n).

It is known, that increase in fat depots happens mainly due to increased TAG accumulation in adipocytes. Since p66$^{Shc}$ promotes the processes of fat development *in vivo*, we checked how p66$^{Shc}$ regulates processes of lipid accumulation *in vitro*. We compared the level of TAG accumulation in cultures of WT and p66$^{Shc-/-}$ adipocytes upon the treatment with insulin.
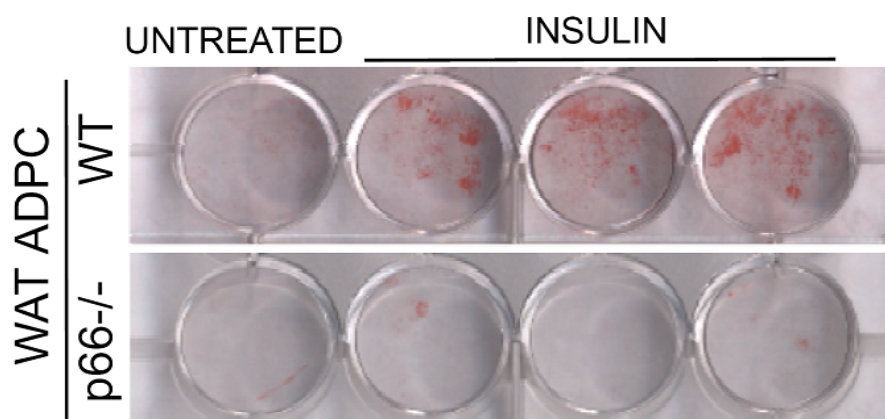


**Figure 33. P66$^{Shc}$ regulates lipid accumulation in white adipocytes (WAT ADPC).** Micrographs of plates of Oil red-stained of WT and p66$^{Shc-/-}$ white adipocytes, left untreated or treated with insulin for 10 days.

We found that treatment of WT brown (Fig. 32) and white (Fig. 33) adipocytes with 40 ng/ml or 1 µg/ml of insulin correspondingly, induced TAG accumulation in virtually all cells to day 6 and 10 after treatment, respectively (Fig. 32, 33), as it was estimated with staining with Oil Red O. Strikingly, p66[Shc-/-] brown and white adipocytes accumulated less lipids after the same insulin treatment (Fig. 32, 33).
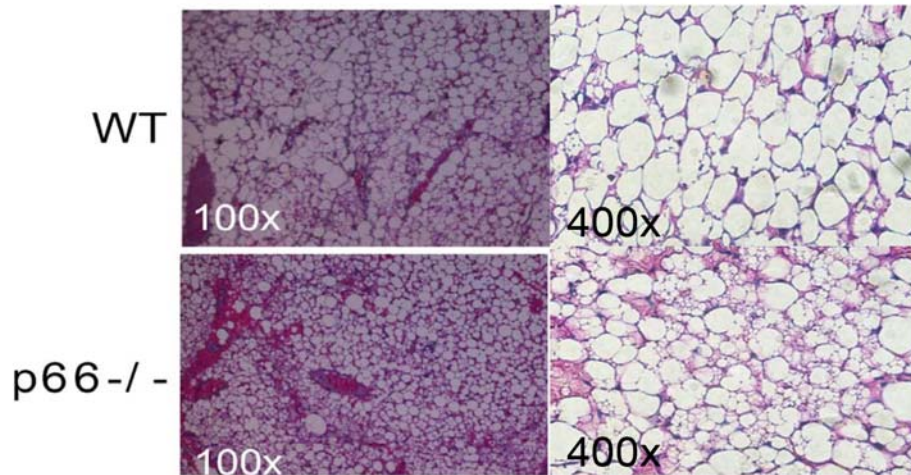


**Figure 34. Histology of WT and p66[Shc-/-] fat upon transplantation.** Representative hematoxylin/eosin-staining of sections of fat mass developed from WT and p66[Shc-/-] transplanted brown adipocytes into athymic nude mice. Higher heterogeneity is seen in fat mass obtained upon injection of p66[Shc-/-] adipocytes.

**P66[Shc] regulates fat tissue development through alteration of intrinsic properties of adipocytes**

We demonstrated that p66[Shc] participates in the fat tissue development *in vivo* and regulates lipid accumulation in adipocytes *in vitro*. However, this phenomenon *in vivo* may be due to a complex systemic alteration (for instance, hormonal) in p66[Shc-/-] mice or may be connected with alteration of inherent processes of adipocytes. To finally prove that p66[Shc] influences intrinsic properties of adipocytes we isolated and propagated both p66[Shc-/-] and WT adipocytes. These cells were transplanted into athymic nude mice and were allowed to grow for one month. In this experiment surrounding conditions of transplanted adipocytes are equal and all possible differences in their development could be explained only due to the intrinsic differences of injected cells. Results of experiment showed that adipocytes isolated from WT and p66[Shc-/-] animals developed differently upon transplantation. Whereas WT adipocytes grew into homogenous fat mass with a dramatic increase in size, p66[Shc-/-] adipocyte transplants formed smaller fat mass with heterogenous histological structure (Fig. 34 and data not shown). This data evidences that p66[Shc] regulates some inherent properties of adipocytes.

**P66<sup>Shc</sup> regulates insulin-induced lipid accumulation *in vitro* in ROS dependent manner**

It is known, that intracellular ROS are critically involved in the ability of insulin to induce lipogenesis (140). In fact, treatment of WT brown adipocytes with antioxidants such as lipoic acid (Fig. 35) or N-acetylcysteine (not shown) decreased the amount of ROS in WT adipocytes to the level seen in p66$^{Shc-/-}$ adipocytes and inhibited the lipid accumulation (Fig. 35). On the contrary, treatment with antimycin A, an inhibitor of respiration that increases mitochondrial ROS generation, increased level of ROS (Fig. 31) and slightly increased the level of lipid accumulation in adipocytes cultures (Fig. 35). Thus, we suggested that ROS generated by p66$^{Shc}$ are important for normal lipid accumulation in adipocytes. To verify this hypothesis we reproduced into p66$^{Shc-/-}$ cells wild type form of p66$^{Shc}$ or their mutant forms p66Shca and p66Shcqq, which are unable to be translocated to mitochondria  and oxidize cytochrome *c* respectively (159,175). <u>The net consequence of both these mutations is the loss of p66$^{Shc}$ ability to generate mitochondrial H$_2$O$_2$.</u>
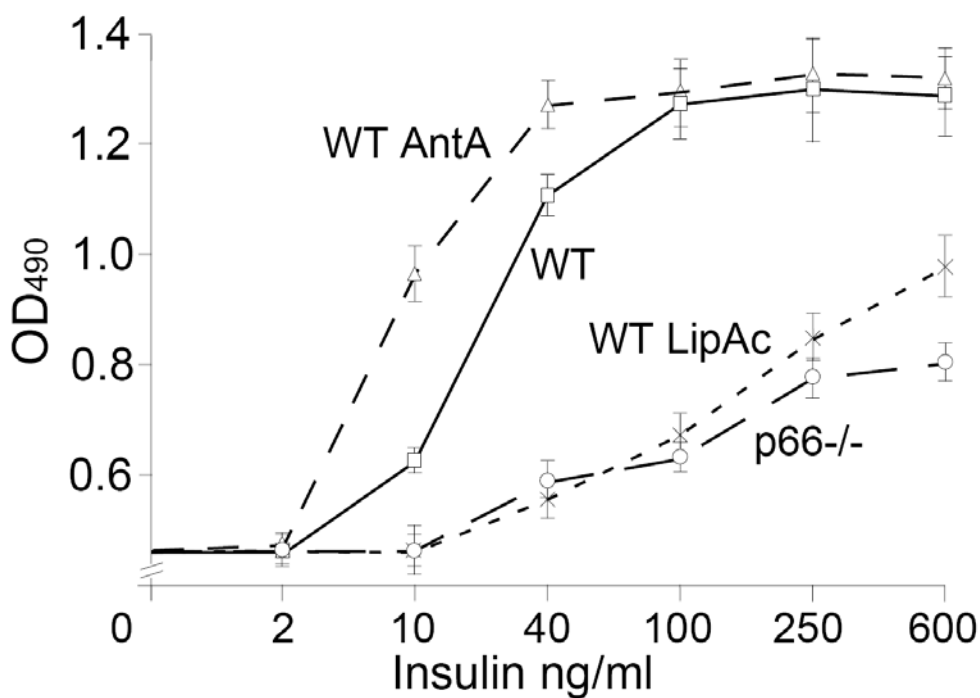


**Figure 35. P66<sup>Shc</sup> and ROS treatment regulate lipid accumulation in brown adipocytes.** OD values of Oil red extracts from stained WT, antimycin A (AntA)- or lipoic acid (LipAc)- treated WT and p66$^{Shc-/-}$ brown adipocytes (average of 8 independent experiments for each condition) at day 6 upon induction of differentiation with insulin.

Reintroduction by retroviral infection of mutants of p66[Shc] did not restore the normal level of ROS in adipocytes (Fig. 31) and failed to rescue insulin-induced TAG accumulation (Fig. 36). We discovered, that only re-expression of wild type form of p66[Shc] was able to rescue phenomenon (Fig. 36) and was associated with increase in ROS level (Fig. 31).
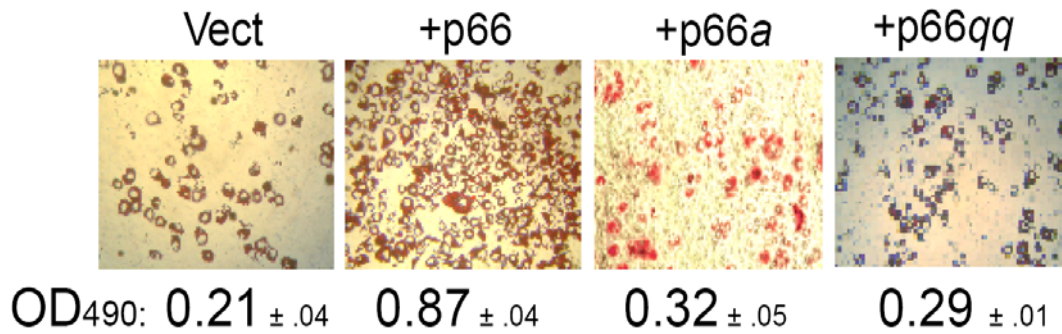


**Figure 36. P66[Shc] regulates lipid accumulation in ROS dependent manner in brown adipocytes.** White light microscopy pictures of Oil red-stained p66[Shc-/-] brown adipocytes infected with empty (Vect), p66[Shc], p66Shc*a* or p66Shc*qq* retroviruses and treated with insulin for 6 days, and their corresponding optical density (OD; 490nm) values.
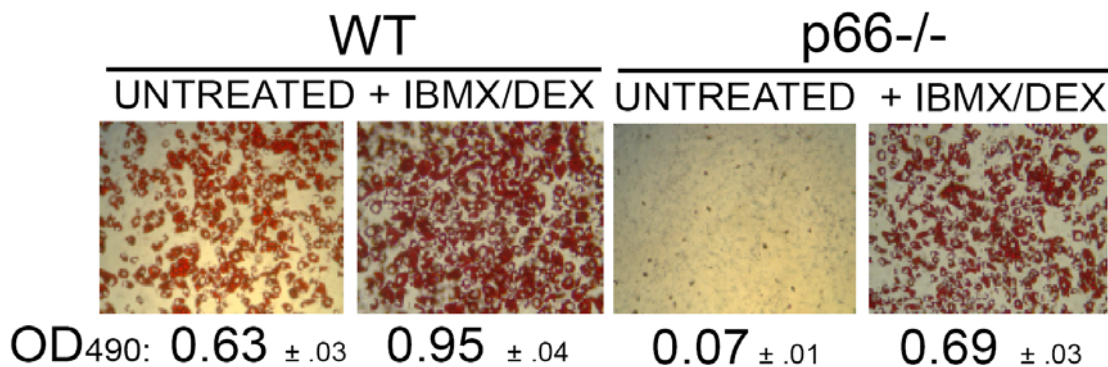


**Figure 37. P66[Shc] does not influence IBMX/DEX induced differentiation in brown adipocytes.** White light microscopy pictures of Oil red-stained brown adipocytes from WT and p66[Shc-/-] mice, untreated or after treatment with IBMX-dexametasone (IBMX-DEX), and their corresponding OD values (this experiment is representative of three that gave comparable results).

These data demonstrate that p66[Shc] regulates insulin induced accumulation of lipids in adipocytes in ROS dependent manner. Notably, adipocyte differentiation by other stimuli (IBMX and dexamethasone) (Fig. 37) and insulin-stimulated glucose uptake (Fig. 38) proceeded normally in p66[Shc-/-] adipocytes. From these data we conclude that biological contribution of p66[Shc] in adipocytes is restricted to the regulation of specific branches of insulin signaling, in particularity to insulin induced lipid accumulation.

**Figure 38. P66$^{Shc}$ does not affect insulin induced glucose consumption.** Glucose consumption prior or after insulin treatment (24 hours), as indicated, of undifferentiated brown adipocytes and primary myoblasts, hepatocytes and fibroblasts (MEFs) prepared from WT and p66$^{Shc-/-}$ mice. Results are expressed as percentage of glucose consumption in p66$^{Shc-/-}$ versus WT cells.
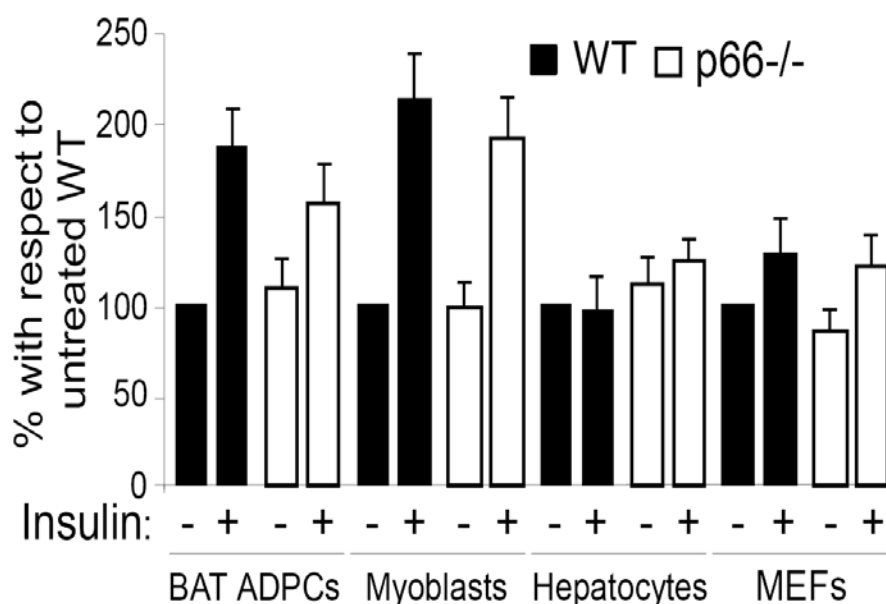
## Insulin – p66$^{Shc}$ interactions

## Insulin activates the redox activity of p66$^{Shc}$

P66$^{Shc}$ contains sites for the activation by tyrosine and serine kinases. It was shown that insulin treatment results in the preferential serine activation of p66$^{Shc}$ (176).

Here we demonstrated that insulin induced serine phosphorylation of p66$^{Shc}$ also in adipocytes. In fact, analysis of anti-p66$^{Shc}$ immunoprecipitates using antibodies against phosphorylated Ser36 showed rapid and transient phosphorylation of p66$^{Shc}$ after insulin treatment in WT brown adipocytes (Fig. 40).

It is known, that insulin treatment induces wave in ROS concentration in some cell types (135). In fact, we discovered that insulin treatment induced 2-3 fold increase in ROS level in WT brown adipocytes. However, the ability of insulin to induce ROS was abrogated in p66$^{Shc-/-}$ cells (Fig. 41). Only reintroduction of wild type form of p66$^{Shc}$, but not of the phosphorylation-defective p66$^{Shc}$ Ser36Ala (p66Shca) mutant, into p66$^{Shc-/-}$ adipocytes restored insulin-induced ROS up-regulation (Fig. 41). Thus, serine activation of p66$^{Shc}$ is indispensible for insulin induced ROS generation in adipocytes.
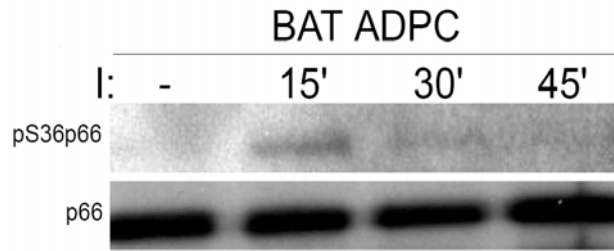
**Figure 40. Insulin induces serine phosphorylation of p66$^{Shc}$ in brown adipocytes (BAT ADPC).** P66$^{Shc}$ Ser36 phosphorylation before and after insulin (I) treatment (at the indicated time points) of brown adipocytes. Anti-Shc immunoprecipitates were analysed by western blotting using antibodies against phosphorylated (upper panel) or total (lower panel) p66$^{Shc}$.
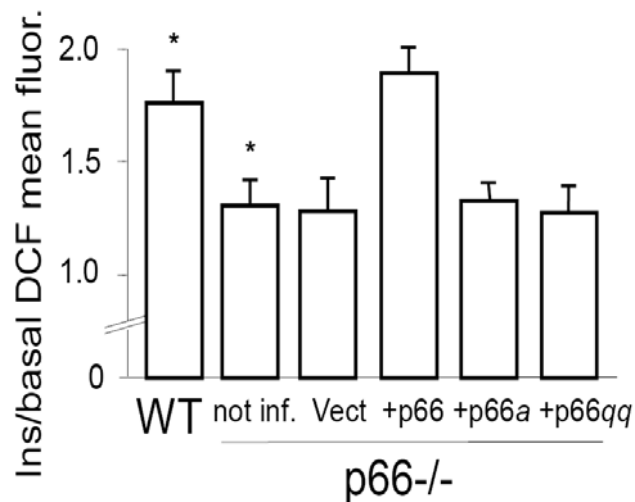


**Figure 41. Insulin regulates redox activities of p66$^{Shc}$.** FACS analysis of the mean DCF fluorescence of DCFDA-stained WT and p66$^{Shc-/-}$ brown adipocytes not infected (not inf.) or after infection with empty retroviral vector (Vect), or retroviral vectors expressing p66$^{Shc}$, p66Shc*a* or p66Shc*qq* upon insulin stimulation. Expressed as fold increase in comparison to insulin not stimulated condition.

To demonstrate that insulin induced up-regulation of ROS through redox activity of p66$^{Shc}$, we used also p66Shcqq mutant. Expression of the p66Shcqq mutant in p66$^{Shc-/-}$ adipocytes failed to mediate ROS up-regulation upon insulin stimulation (Fig. 41). Together, these findings demonstrate that insulin activates the redox activity of p66$^{Shc}$.

# P66[Shc] modulates insulin signal transduction pathway

We then studied the role of p66[Shc] on insulin sensitivity of adipocytes. We performed dose dependent studies of lipid accumulation in adipocytes upon insulin stimulation.

We found that the absence of p66[Shc] expression reduced insulin sensitivity. In fact, the half-maximal differentiating doses of insulin were around 20 and 600 ng/ml in WT and p66[Shc-/-] adipocytes, respectively (Fig. 35).

It is known that cellular oxidant activated by insulin have different effects on downstream pathways: they activate phosphatidylinositol 3-kinase, while they have no effect on Ras (136).

To characterize which branch of downstream insulin signaling is effected by p66[Shc], we first analyzed the intracellular levels of phosphorylated Akt and MAPK, which were activated by PI3K and Ras, respectively. In WT adipocytes insulin increased phosphorylation of Akt and Erk-1 (Fig. 42, 43), with peaks at 5'-15' after treatment.
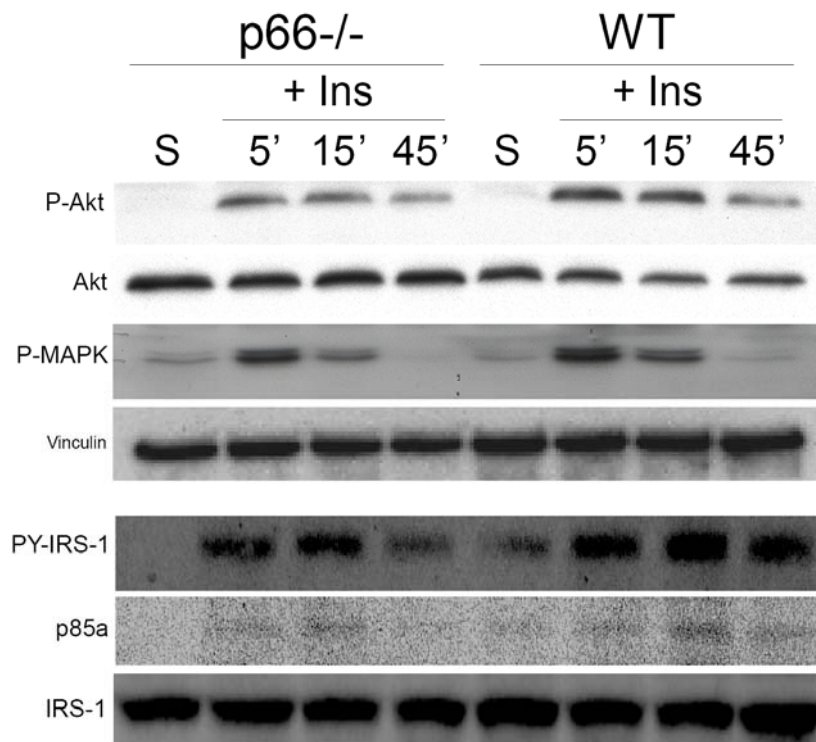


**Figure 42. Influence of p66[Shc] on insulin signal transduction in brown adipocytes.** WT and p66[Shc-/-] brown adipocytes, were not (S) or treated with insulin and analyzed at the indicated time points after treatment. Top four panels: western blot of total (Akt) and phosphorylated (P-Akt) Akt, phosphorylated MAPK (P-MAPK) and vinculin. Bottom three panels: western blot of anti-IRS1 immuniprecipitates using antibodies against IRS-1, pY-IRS-1 and p85a.
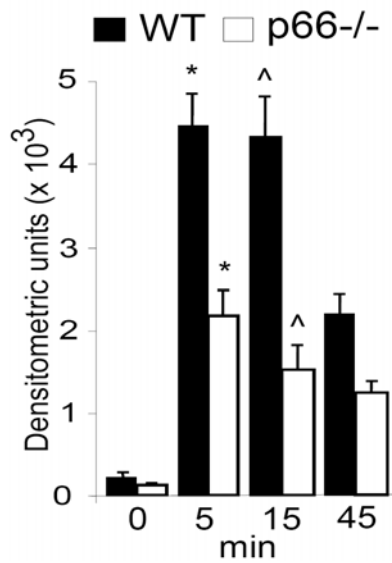
**Figure 43. P66^Shc regulates insulin induced Akt phosphorylation in brown adipocytes**. Densitometric analysis of P-Akt signals in WT and p66$^{Shc-/-}$ brown adipocytes upon insulin treatment. The phopsho-Akt signal was normalized against total-Akt signal (average values from three independent experiments: * and ^ $p < 0.01$).
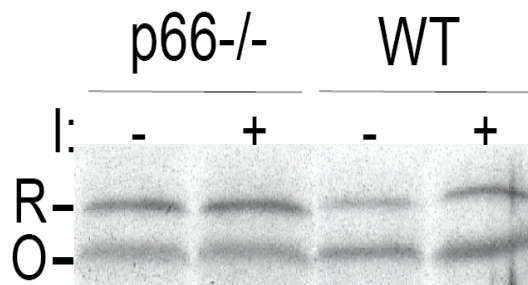


**Figure 44. P66^Shc regulates PTEN oxidation.** Western blot analysis using non reducing PAGE of reduced (R) vs. oxidized (O) forms of PTEN in brown adipocytes, prior (-) and after (+) insulin treatment. D, E and F are representative of 4 experiments which gave comparable results.

In p66$^{Shc-/-}$ adipocytes, the extent of insulin-induced Akt phosphorylation was markedly reduced, at the same time as MAPK phosphorylation proceeded normally (Fig. 42, 43). These findings indicate that loss of p66$^{Shc}$ specifically affects the PI3K/Akt part of the insulin signaling pathway.

Next we checked at which level the regulation of Akt activation by p66$^{Shc}$ happens. IRS1, a direct target of activated insulin receptor, was equally phosphorylated and expressed in WT and p66$^{Shc-/-}$ adipocytes (Fig. 42). It is known that one common way of the regulation of signal transduction in cell by oxidants is the regulation of activity of PTPs through their reversible oxidation. We found

that in p66$^{Shc-/-}$ adipocytes the level of oxidation of PTEN was altered. PTEN is a protein-tyrosine phosphatase, which dephosphorylates and inactivates PIP3, a lipid second messenger produced by PI3K (177) and thus modulates propagation of insulin signaling. PTEN was shown to be inactivated by endogenous ROS through the formation of a disulfide bond between the active site Cys124 and Cys71. This oxidative dependent alteration in structure of PTEN results in the shift of its electrophoretic mobility (178). Thus, reduced and oxidized forms of PTEN migrate differently in non-reducing PAGE. Our studies showed that in p66$^{Shc-/-}$ adipocytes the amount of reduced PTEN, (the slowly migrating form) was moderately, yet consistently, increased, both in basal conditions and after insulin treatment (Fig. 44).

We then looked at Akt downstream effectors of insulin signaling transduction pathway. It is known that Akt is direct and crucial regulator of FOXO1. FOXO1 plays a critical role in coupling of insulin signaling to adipocyte differentiation (126). Akt phosphorylates FOXO1, and this event leads to sequestering of FOXO1 in the cytoplasm and inhibition of transcription of FOXO1-target genes (126). We checked if the subcellular distribution of FOXO1 is normal in p66$^{Shc-/-}$ brown adipocytes. Immunofluorescence analysis of WT adipocytes showed massive relocalization of FOXO1 in the cytoplasm upon insulin treatment. In fact, nuclear localization was noted in 80% of investigated cells in WT adipocytes under basal conditions. Upon treatment with insulin nuclear presence of FOXO1 was observed only in 5% of cells. In opposition, insulin almost had no effect on relocalization of FOXO1 in p66$^{Shc-/-}$ cultures (80% of cells with nuclear localization of FOXO1 were detected under basal conditions and 75% upon insulin stimulation) (Fig. 45). It appears, therefore, that p66$^{Shc}$ expression is indispensable for the activation of the insulin-Akt-FOXO1 pathway. Notably, the effect of p66$^{Shc}$ on FOXO1 appeared more pronounced than its effect on Akt activation, suggesting that p66$^{Shc}$ regulates insulin signaling at multiple levels.

We checked if p66$^{Shc}$ regulated FOXO1 relocalization in ROS dependent way. With this aim we re-introduced wild type and mutant forms of p66$^{Shc}$ into p66$^{Shc-/-}$ adipocytes. Only re-expression of wild type of p66$^{Shc}$ restored normal response of FOXO1 to insulin treatment, while the p66Shca or p66Shcqq mutants had no effects (Fig. 45).


**P66$^{Shc}$ regulates insulin induced gene expression in adipocytes**

To obtain large-scale picture of the impact of p66$^{Shc}$ on insulin signaling in adipocytes, we performed global gene-expression analysis of WT and p66$^{Shc-/-}$ brown adipocytes treated, or not, with insulin (4 hours). Biotinylated cRNA targets were synthesized from RNA pools of three
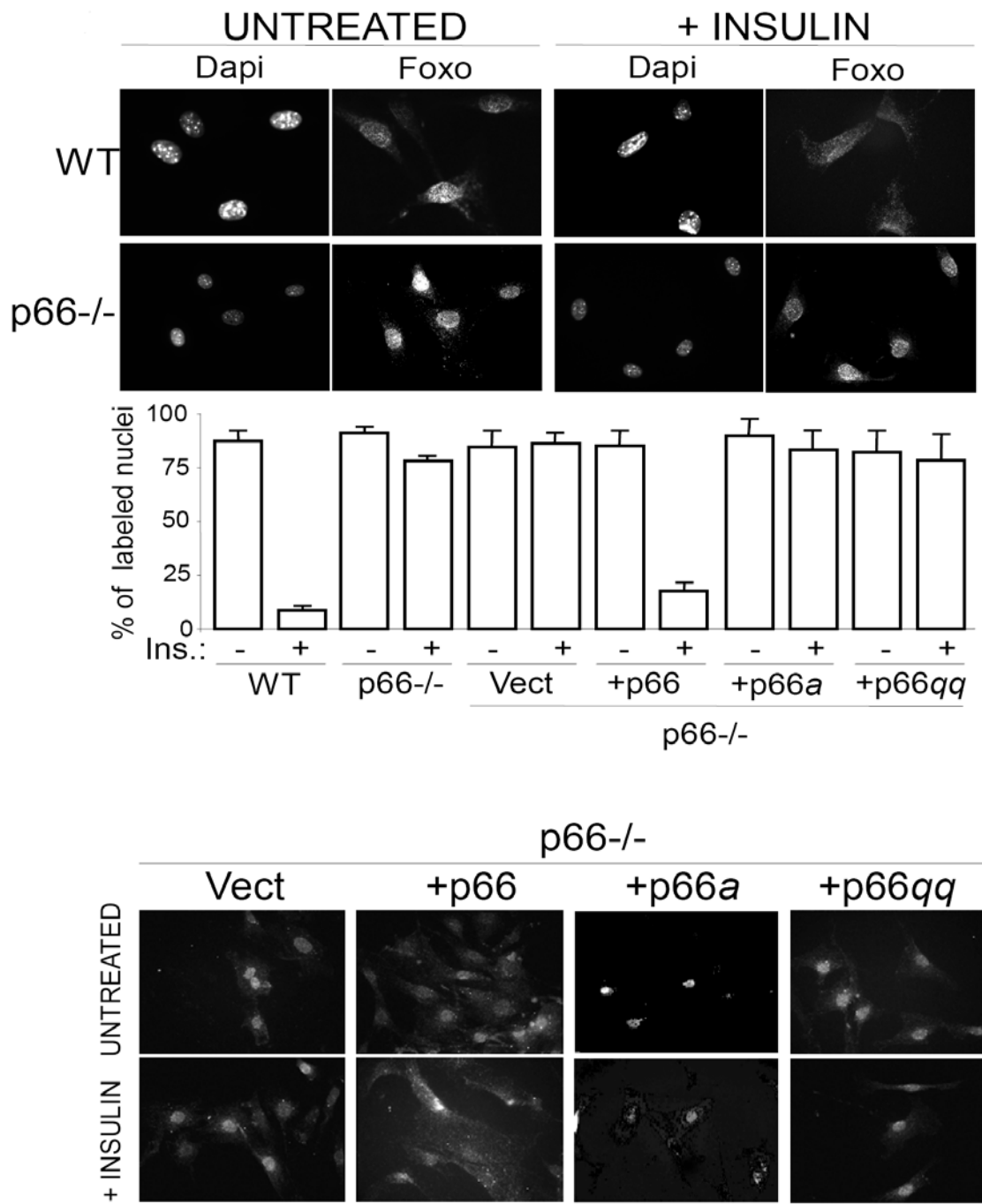
**Figure 45. P66^Shc regulates insulin-induced FOXO1 cellular distribution.** Dapi staining of nuclei and immunofluorescence analysis of FOXO1 localization in untreated and insulin treated brown adipocytes (upper panel; results from three experiments). Percentage of cells with FOXO1 nuclear localization is shown in the middle graph. Nuclear localization of FOXO1 in p66^Shc-/- brown adipocytes infected with empty (Vect), p66^Shc or p66Shc*qq* retroviral vectors is shown in the lower panels.

independent experiments and hybridized to Affymetrix oligonucleotide chips. Analysis of microarray data identified two overlapping lists of 598 and 610 genes regulated by insulin in WT and p66[Shc-/-] adipocytes, respectively (Fig. 46).

| | | a | Chst2 | Cnot2 | Copg2as2 | Cxcr4 |
|---|---|---|---|---|---|---|
| P66-dependent insulin targets | | Foxo | Hbs1l | Homer1 | Hrmt1l6 | Lztfl1 |
| | | Myod1 | Ncor1 | Nptx1 | Parp6 | Pkd1l3 |
| | | Prkx | Pvr | Scin | Slc16a11 | Tnni3k |
| P66-independent insulin targets | | Bsf3 | Egr2 | **Egr3** | Ereg | Errfi1 |
| | | Fosl1 | Gm337 | Hbegf | Lipl2 | Maff |
| | | Obox1 | Pla2g1b | **Ptgs2** | Rad51ap1 | Rbbp8 |
| | | Rgs16 | Sema7a | **Pai-1** | Tm7sf3 | Zfp606 |
| P66-suppressed insulin regulation | | Asb12 | Cml2 | Creb1 | Eya3 | Flot2 |
| | | Hecw1 | Lpp | Mapkap1 | Nef3 | Nktr |
| | | Pabpc1 | Pdcd10 | Pde10a | Pkd2l1 | Prss2 |
| | | Rnf103 | Rttn | Stx8 | Syk | Tnip2 |

**Figure 46. P66[Shc] regulates insulin induced gene expression in adipocytes.** Venn diagrams representing overlapping gene regulations in WT and p66[Shc-/-] brow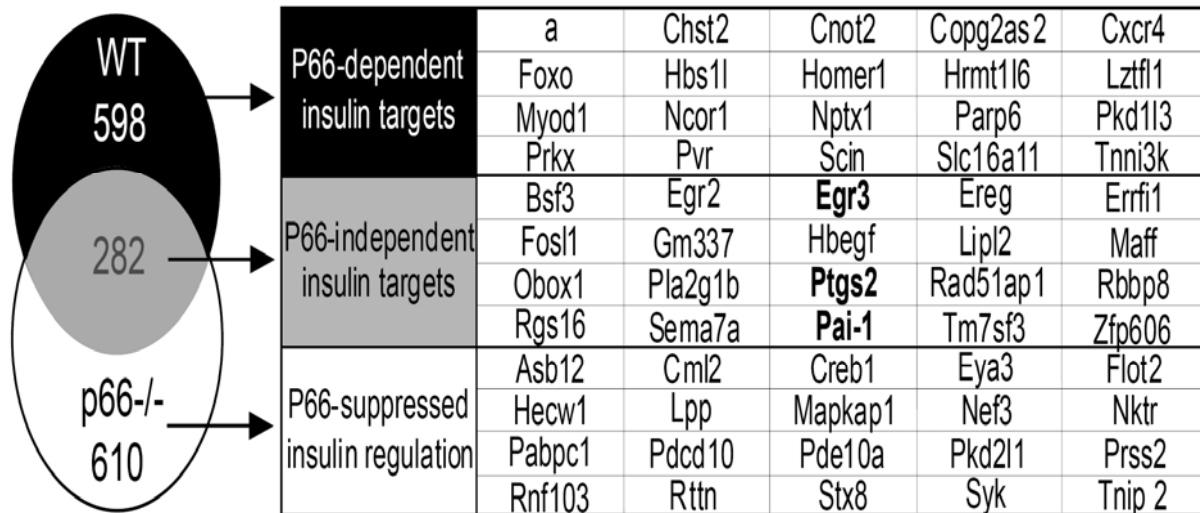n adipocytes treated with insulin. The table reports the top 20 insulin-regulated genes in each group. In bold are indicated the genes whose analysis is showed in the following figures.

Results of microarray experiments were validated by quantitative PCR (QPCR) of 30 genes which revealed full concordance (not shown). Cross comparisons of WT and p66[Shc-/-] gene-sets distinguished three groups of insulin targets: i) 282 genes were regulated by insulin in both WT and p66[Shc-/-] adipocytes (p66[Shc]- independent insulin targets; ii) 316 genes were regulated by insulin in WT, but not p66[Shc-/-] adipocytes (p66[Shc]- dependent insulin targets) and iii) 328 genes regulated by insulin in p66[Shc-/-], but not WT adipocytes (p66[Shc]- repressed insulin targets) (Fig. 46). Thus, results of microarray showed that p66[Shc] regulated a vast number of genes in response to insulin expression. Notably, the insulin dependent gene-regulations in p66[Shc-/-] adipocytes also included genes whose activity is linked to lipogenesis (Egr1, Egr3, Ptgs2, Pai-1, Fabp3, UCP1, CD36, App; Fig. 47) (179-184). QPCR validation of these targets in p66[Shc-/-] adipocytes, or in WT adipocytes after depletion of p66[Shc] expression by siRNA, is shown in Fig. 47, 48. Together, these data indicate that the effect of p66[Shc] on insulin-dependent gene expression is restricted to specific transcriptional targets, which might explain why p66[Shc] selectivity affects only some particular branches of insulin effect on the cell (in particularity, lipogenesis) and does not affect others (glucose consumption).
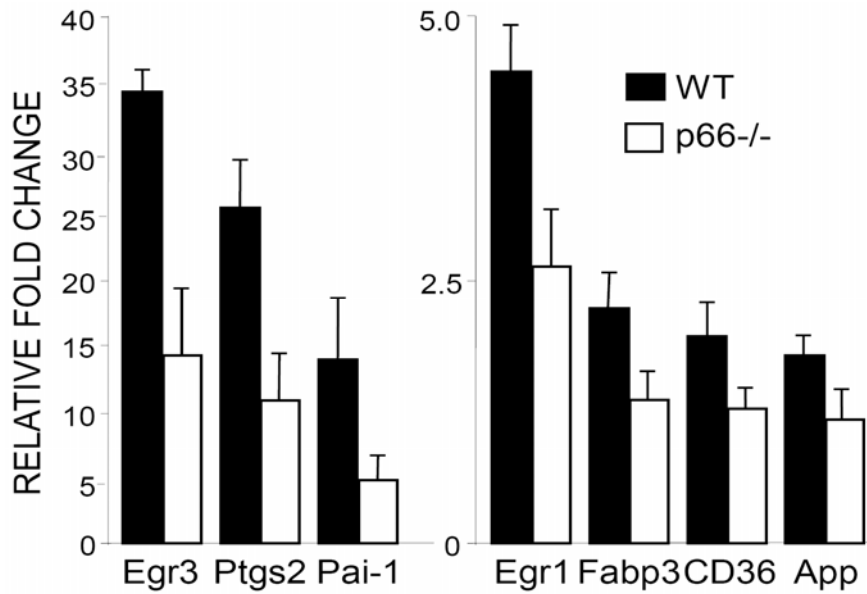
**Figure 47. QPCR validation of microarray data**. Quantitative RT-PCR analysis of transcripts from the indicated genes in WT and p66[Shc-/-] brown adipocytes upon insulin stimulation. Results are shown as ratio of values from insulin treated vs. untreated samples.
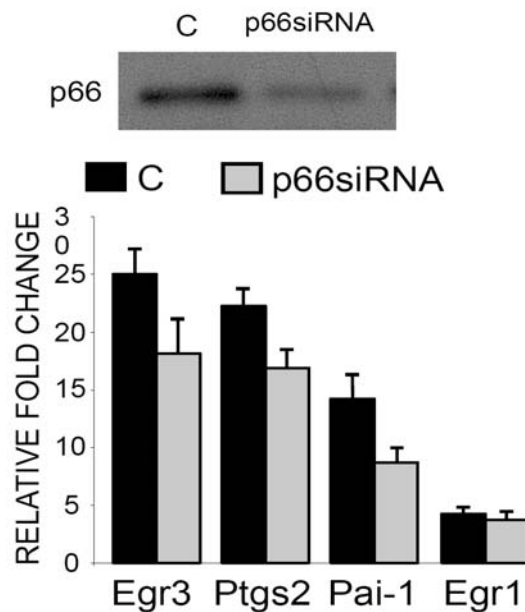


**Figure 48. Validation of microarray results upon down regulation of p66[Shc] in brown adipocytes**. QPCR analysis of transcripts from the indicated genes in WT brown adipocytes treated (P66Shc-siRNA), or not (C), with siRNA oligos for p66[Shc] upon insulin stimulation (resulting p66[Shc] levels are shown in the upper panel).

## P66[Shc] regulates energy balance of brown adipocytes

## P66[Shc] inhibits lipolysis

In adipocytes, insulin stimulated TAG accumulation is determined by increased uptake of glucose or FA and / or inhibition of FA oxidation. Glucose consumption was the same in p66[Shc-/-] and WT

adipocytes (see above). Thus we determined FA uptake and oxidation in adipocytes. We pre-incubated cells with $^{14}$C-oleate and measured incorporation of $^{14}$C and its release for evaluation of processes of fatty acid uptake and breakdown (β-oxidation), respectively. We found that the rate of intake of $^{14}$C oleate was similar in WT and p66$^{Shc-/-}$ adipocytes under basal conditions and upon insulin treatment (Fig. 49). On the contrary, the release of $^{14}CO_2$ was increased in p66$^{Shc-/-}$ adipocytes, both at the steady state and after insulin treatment (Fig. 50).

These results indicate that p66$^{Shc}$ promotes insulin induced TAG accumulation through the inhibition of lipolysis and FA oxidation.
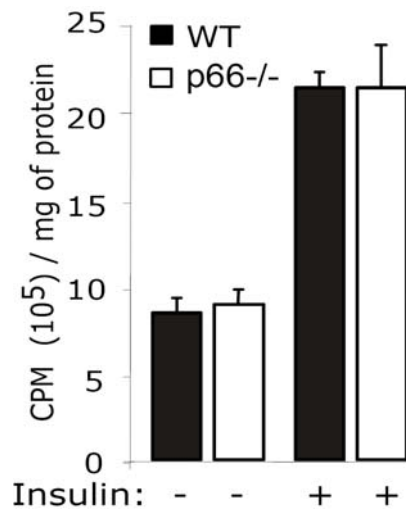


**Figure 49. Fatty acid uptake is equal in WT and p66$^{Shc-/-}$ brown adipocytes.** Cells were labeled with $^{14}$C-oleate, left untreated or treated with insulin, as indicated. Fatty acid uptake was measured as $^{14}$C incorporation.
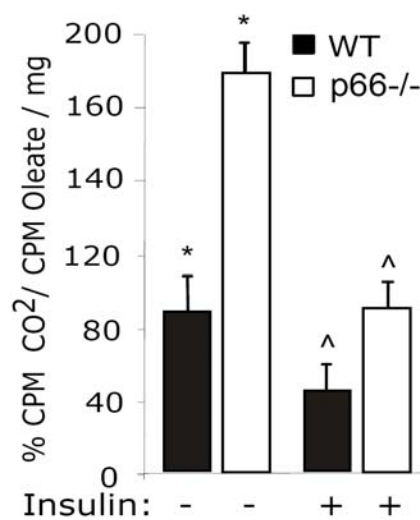


**Figure 50. Fatty acid oxidation is increased in p66$^{Shc-/-}$ brown adipocytes.** Cells were labeled with $^{14}$C-oleate, left untreated or treated with insulin, as indicated. Fatty acid uptake and release were measured as $^{14}CO_2$ release.

# P66<sup>Shc</sup> improves mitochondrial coupling in adipocytes

Presented findings suggest that in p66<sup>Shc-/-</sup> adipocytes part of lipids is oxidized instead of to be stored. Thus, the energy released during this oxidation must be connected with conversion into another form in p66<sup>Shc-/-</sup> mice. One possible way is its dissipation in the form of heat during thermogenesis. Indeed, deletion of p66<sup>Shc</sup> resulted in increased expression of UCP1, regulator of thermogenesis. QPCR confirmed that UCP1 is up-regulated in p66<sup>Shc-/-</sup> adipocytes both under basal conditions and after insulin treatment (Fig. 51).
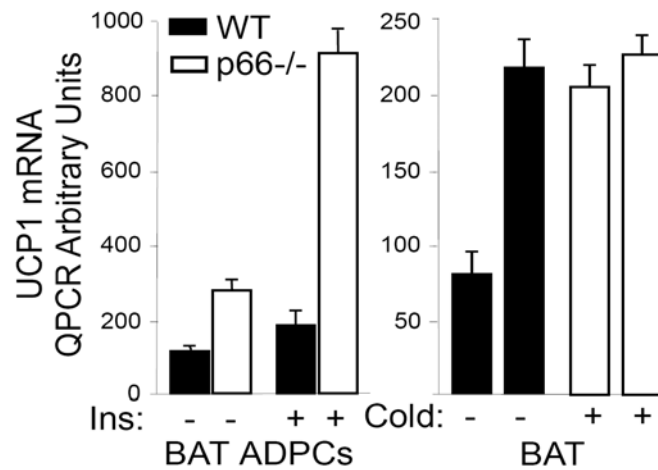


**Figure 51. P66<sup>Shc</sup> regulates expression of UCP1 mRNA.** Quantitative RT-PCR analysis of UCP1 gene expression in WT and p66<sup>Shc-/-</sup> brown adipocytes(BAT ADPC) (untreated or after insulin treatment; left panel) and brown adipose tissue (BAT), basal and upon cold exposure (right panel).
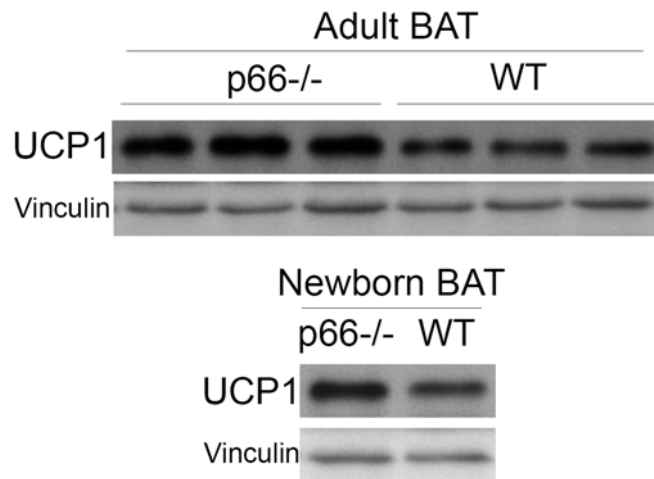


**Figure 52. P66<sup>Shc</sup> regulates UCP1 expression.** Western blot analysis of UCP1 protein expression in brown adipose tissue (BAT) from adult and newborn WT and p66<sup>Shc-/-</sup> mice.

We checked the amount of UCP1 protein by western blotting in brown adipose tissue of newborn and adult p66$^{Shc-/-}$ mice. In all cases results showed increased levels of UCP1 protein expression, as compared to WT controls (Fig. 52). These data indicate that p66$^{Shc}$ inhibits basal and insulin-induced UCP1 expression in adipocytes. We checked if p66$^{Shc}$ deletion was indeed associated with energy dissipation during thermogenesis. With this aim we studied uncoupling status of p66$^{Shc-/-}$ brown adipocytes mitochondria since thermogenesis is directly associated with uncoupling of proton flux through inner membrane of mitochondria from ATP synthesis.

*Studies in isolated mitochondria from brown fat tissue*

Polarographic analysis of isolated energized mitochondria (using either pyruvate (Pyr.) and malate (Mal.) or glycerol-3-phosphate (Glyceriol3P) and in the presence of fatty acid-free albumin (to adsorb free fatty acids) revealed similar levels of oxygen consumption in the WT and p66$^{Shc-/-}$ brown adipose tissues mitochondria (Table 2). It means that their mitochondria were coupled to the same degree under condition that UCP1 was not stimulated with free fatty acids. Instead, in the absence of fatty acid-free albumin, when FA stimulate leakage of protons through UCP1, p66$^{Shc-/-}$ mitochondria showed higher levels of oxygen consumption (Table 2), reflecting the fact that part of energy was lost through the uncoupled proton leakage in p66$^{Shc-/-}$ mitochondria. Consistently, addition of palmitate (which stimulates proton leakage through available UCP1) increased respiration more potently in the p66$^{Shc-/-}$ mitochondria (Table 2), suggesting that p66$^{Shc-/-}$ mitochondria had more uncouplers which allowed protons back flux to matrix. Treatment with the inhibitor of uncoupling GDP blocks the leakage of protons through UCP1. Under this condition, WT and p66$^{Shc-/-}$ mitochondria reduced respiration to the same level suggesting that now they were equally coupled (Table 2). Treatment with the protonophore FCCP (which allows leakage of protons through membrane) increased respiration of WT, but not p66$^{Shc-/-}$, energized mitochondria, meaning that protons in p66$^{Shc-/-}$ mitochondria had initially higher possibility to come back to matrix. All these findings suggest that the difference in oxygen consumption between WT and p66$^{Shc-/-}$ mitochondria is attributable to increased uncoupling of mitochondria.

*Studies in minced brown adipose tissue*

**Respiration studies**

We also studied energy status of brown adipose tissue*.* As seen for the isolated BAT mitochondria, minced brown adipose tissue from p66$^{Shc-/-}$ mice upon addition of succinate showed increased respiration rate in comparison with WT, indicating that part of energy was lost due to uncoupled transport of protons through the membrane (Fig. 53). Treatment with FCCP further enhanced the

respiration only in WT, suggesting that mitochondria in p66[Shc-/-] cells are already in uncoupled state (Fig. 53). Notably, also basal respiration (sustained by endogenous substrates) was higher in p66[Shc-/-] brown adipose tissue, suggesting that uncoupled passage of protons was unblocked in p66[Shc-/-] cells in *ex-vivo*, probably due to free fatty acids availability (Fig. 53).

| | Isolated MTC nmol/ml/mg of protein | | | |
|---|---|---|---|---|
| | + fat free BSA | | w/o fat free BSA | |
| | WT | p66-/- | WT | p66-/- |
| Basal | 11.76 ± 2.43 | 9.14 ± 3.55 | 6.95 ± 1.86 | 6.14 ± 0.40 |
| Pyr./Mal. | 36.12 ± 5.24 | 35.10 ± 6.78 | **52.68 ± 6.13°** | **64.24 ± 5.37°** |
| Glycerol3P | 50.61 ± 5.94 | 46.82 ± 5.30 | **70.32 ± 4.51^** | **96.15 ± 7.16^** |
| GDP | 35.23 ± 6.36 | 36.51 ± 6.27 | 39.71 ± 5.18 | 42.07 ± 4.23 |
| FCCP | 102.34 ± 8.12 | 113.45 ± 9.26 | 108.22 ± 9.41 | 104.26 ± 8.51 |
| Palmitate | **79.24 ± 8.19*** | **102.11 ± 9.03*** | | |
| ADP | 94.56 ± 8.22 | 110.07 ± 7.48 | | |

**Table 2. Polarographic analysis of isolated mitochondria (MTC) from WT and p66[Shc-/-] brown adipose tissue.** Level of respiration is shown in isolated mitochondria from brown adipose tissue of WT and p66[Shc-/-] mice in the presence or absence of bovine serum albumin (BSA). The average values and standard deviations of three independent experiments are indicated. *, and " $p < 0.01$, ° $p < 0.05$
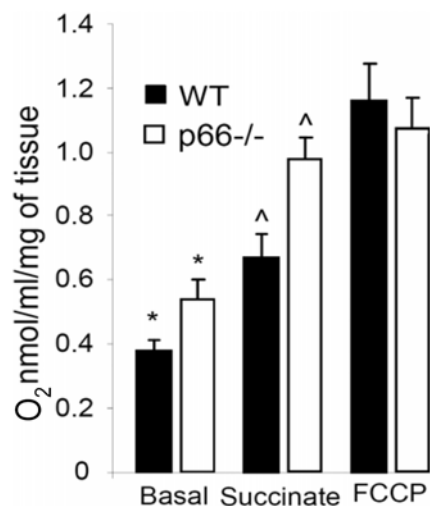


**Figure 53. Polarographic analysis of WT and p66[Shc-/-] minced brown adipose tissue.** Results of polarographic analysis of WT and p66[Shc-/-] mitochondria under basal condition and stimulation with succinate and FCCP. * and ^ $p < 0.01$.

**Membrane potential studies**

To characterize directly coupling efficiency, we measured the proton-motive force of WT and p66[Shc-/-] brown adipose tissue mitochondria using the potentiometric die tetramethylrhodaminemethylester (TMRM). Results showed a slight, yet statistically significant, reduction of the membrane potential of p66[Shc-/-] energized mitochondria, suggesting that proton gradient is lost through the uncoupling processes (Fig. 54). Treatment with GDP abolished this difference and increased it to the same level in WT and p66[Shc-/-] brown adipose tissue mitochondria, further supporting our conclusion that p66[Shc-/-] mitochondria were uncoupled. Together, these findings suggest that the increased oxygen consumption of p66[Shc-/-] mitochondria is attributable to increased uncoupled state. High levels of UCP1 might be responsible for this phenomenon.



**Figure 54. Membrane potential studies in WT and p66[Shc-/-] minced brown adipose tissue.** The graph reports membrane potential under basal conditions and upon GDP stimulation. Presented results are from three independent experiments. * $p < 0.01$.

# DISCUSSION

# P66<sup>SHC</sup> IS GENETIC DETERMINAT OF METABOLIC SYNDROME
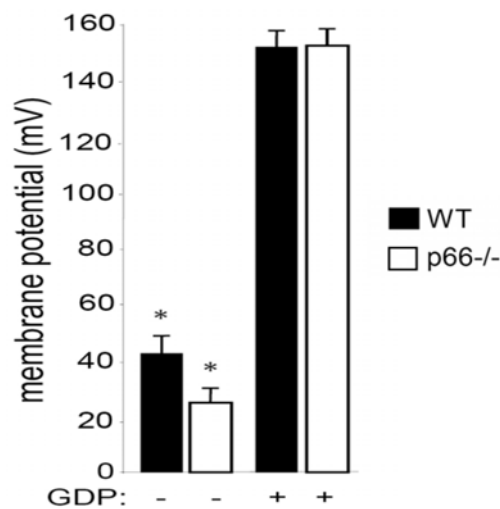
P66$^{Shc}$ is known to promote aging and degenerative diseases (165). Apparently p66$^{Shc}$ loss is associated only with positive effect and increase in lifespan in mice. However, the physiological role of p66$^{Shc}$ remains not clear. The beneficial effects of p66$^{Shc}$ deletion were attributed mainly to the decreased oxidative stress. Indeed, p66$^{Shc}$ generates $H_2O_2$ upon the oxidation of cytochrome $c$ within mitochondria (159).

Here we propose that generation of $H_2O_2$ through p66$^{Shc}$ is part of a multi-steps signaling process that participates in the transduction of energetic intake towards storage (Fig. 55). Firstly, food intake induces expression of p66$^{Shc}$. We show that the level of p66$^{Shc}$ in fat is dependent on weather the animal was fed or not. Secondly, food intake is associated with insulin secretion (185). Insulin stimulates serine phosphorylation of p66$^{Shc}$, thus regulating its translocation into the mitochondria (175). As a consequence, p66$^{Shc}$ accumulates in the mitochondria upon food intake. Thirdly, food intake provides mitochondria with substrates and maintains them in energized state. Energized state of mitochondria is important requirement for oxidation of cytochrome $c$ by p66$^{Shc}$ with a consequent $H_2O_2$ generation (159).  So, increase in $H_2O_2$ production by p66$^{Shc}$ (due to increased protein expression, increased translocation into the mitochondria and increased availability of substrate for mitochondrial respiratory chain) is a final result of food intake.

In adipocytes, $H_2O_2$ produced by p66$^{Shc}$ upon food intake enhances insulin signaling. Indeed, we demonstrated that p66$^{Shc}$ increased insulin induced phosphorylation of Akt. Also, p66$^{Shc}$ is crucial for relocalization of anti-adipogenic factor FOXO1 from the nucleus into the cytoplasm. Furthermore, p66$^{Shc}$ is involved in the regulation of PTPs which regulate insulin pathway: PTEN, the main PTP of insulin signaling (177), is less oxidized and consequently less inactivated in p66$^{Shc-/-}$ than in WT adipocytes.

P66$^{Shc}$ has also profound effect on insulin induced global gene expression in adipocytes (160). Among p66$^{Shc}$ regulated genes is UCP1, a key factor of non shivering thermogenesis. UCP1 provides dissipation of electrochemical gradient across the inner membrane of mitochondria uncoupling it from ATP synthesis (119). P66$^{Shc}$ suppresses expression of UCP1 both on mRNA and protein levels both in newborn and adult mice. This phenomenon is responsible for increased mitochondrial coupling and for more efficient utilisation of energy. In fact, respiration in brown adipocyte mitochondria, energy expenditure and basal body temperature of p66$^{Shc-/-}$ animals are increased. Increased mitochondrial uncoupling together with increased β-oxidation might explain inhibition of TAG accumulation in p66$^{Shc-/-}$ adipocytes. Thus, p66$^{Shc}$ increases storage of energy. Upon the same amount of energy consumed, WT adipocytes will store in the form of TAGs that part

of energy which will be dissipated in p66$^{Shc-/-}$ adipocytes through the uncoupling of mitochondria. Decreased lipid accumulation inside of adipocytes is associated with decreased development of fat deposits in p66$^{Shc-/-}$ animals.

The features of p66$^{Shc-/-}$ mice (decreased fat weight, increased heterogeneity of adipocytes in size, altered insulin sensitivity and life span) resemble those seen in FIRKO mice, in which insulin signaling is completely abrogated specifically in fat tissue (127). In our study, some effects of p66$^{Shc}$ on insulin signaling were also restricted to fat. P66$^{Shc}$ did not regulate insulin induced FOXO1 translocation in hepatocytes and fibroblasts (data not shown). Likewise, food intake regulated expression of p66$^{Shc}$ exclusively in adipose tissue, suggesting a specific role of p66$^{Shc}$ in the fat.

Accumulation of nutrients in the form of fat is very important for the survival in wild condition where food availability is always limited. Mechanisms involved in fat tissue development regulate fitness of animals from the evolutionary point of view. Therefore p66$^{Shc}$ might have evolved to serve a specific role in the fat.

Thus, regulation of insulin induced lipid accumulation and fat tissue development by p66$^{Shc}$ is for a first time identified role of p66$^{Shc}$ in organism.

However, the same process involving p66$^{Shc}$ become deleterious upon excess food intake. In this case processes of fat accumulation are viciously increased and stimulate the development of pathological conditions such as obesity and MS.

Indeed, p66$^{Shc-/-}$ mice are less susceptible to obesity. Obesity is considered as the main underlying factor of MS and is *sine qua non* for the diagnosis of MS according to the last united definition by IDF (9). However, manifestation of MS requires the presence of some other metabolic abnormalities such as insulin resistance and glucose intolerance, dyslipidemia, increased blood pressure, prothrombotic and proinflammatory state. P66$^{Shc}$ contributes also to some of these components of MS in mice.

We found that deletion of p66$^{Shc}$ was responsible for the increased insulin sensitivity and glucose intolerance in p66$^{Shc-/-}$ animals. We suggest that deregulated adipokine (TNFα, leptin, PAI1) secretion in p66$^{Shc-/-}$ animals might contribute to this phenomenon. Other risk factors composing MS are prothrombotic and proinflammatory states. One of the markers of an inflammatory state is TNFα blood circulating level. It was decreased in p66$^{Shc-/-}$ animals upon treatment with high fat diet. Prothrombotic state is characterized by increased circulating level of PAI-1. According to microarray data its expression is also decreased in p66$^{Shc-/-}$ adipocytes. In summary, deletion of p66$^{Shc}$ protects from obesity, insulin resistance and glucose intolerance, and decreases inflammatory and prothrombotic states. Additionally, from previously published data it is known that p66$^{Shc-/-}$ mice

are protected from dysfunction of cardiovascular system such as atherosclerosis and hindlimb ischemia (157,161).

Taking together all this findings, we propose p66$^{Shc}$ as genetic determinant of MS.

## COMPLEX GENETIC-ENVIRONMENTAL INTERACTIONS ARE CENTRAL IN METABOLIC SYNDROME

Genetic regulation of MS and alleles associated with the higher risk of MS were demonstrated (see Preface). However, extremely high prevalence of MS in humans in our time is originally driven by diet and affects various populations in spite of differences in genetic background. This consideration suggests that MS arises from the complex interactions between environment and genes involved in the regulation of energy metabolism.

Genes which are subjected themselves to diet induced modifications might represent starting points through which diet exerts its influence on the organism. Thus they can be key elements in the mechanism of MS onset upon diet. Not many genes regulated by diet have been identified so far. One example is the family of sirtuin proteins, involved in the regulation of several intracellular processes. It was shown that sirtuins are regulated by food availability. Notably, Sirt1 was identified as a fundamental determinant of the metabolic disorders resulting from high fat diet treatment (113).

We demonstrated that p66$^{Shc}$ is a mediator of the effect of food intake in mammals. Its ability to produce ROS links p66$^{Shc}$ to many metabolic reactions. Finally, p66$^{Shc}$ is a clear diet regulated protein. So p66$^{Shc}$ is central to MS etiology and it could be considered as genetic determinant of MS.

Thus, main conclusions from my work are:

1.  We identified p66$^{Shc}$ as a novel diet regulated protein involved in environmental-genetic interactions.

2.  We identified the physiological role of p66$^{Shc}$ which is the regulation of fat tissue development through the regulation of insulin signaling and thermogenesis in adipocytes.

3.  We identified p66$^{Shc}$ as a genetic determinant of MS. Thus, drug repression of p66$^{Shc}$ and its targets and regulators hopefully would be beneficial for therapeutic treatment of MS.

4.  We propose p66$^{Shc-/-}$ mice as animal model resistant to MS useful for the study of MS etiology and treatment.
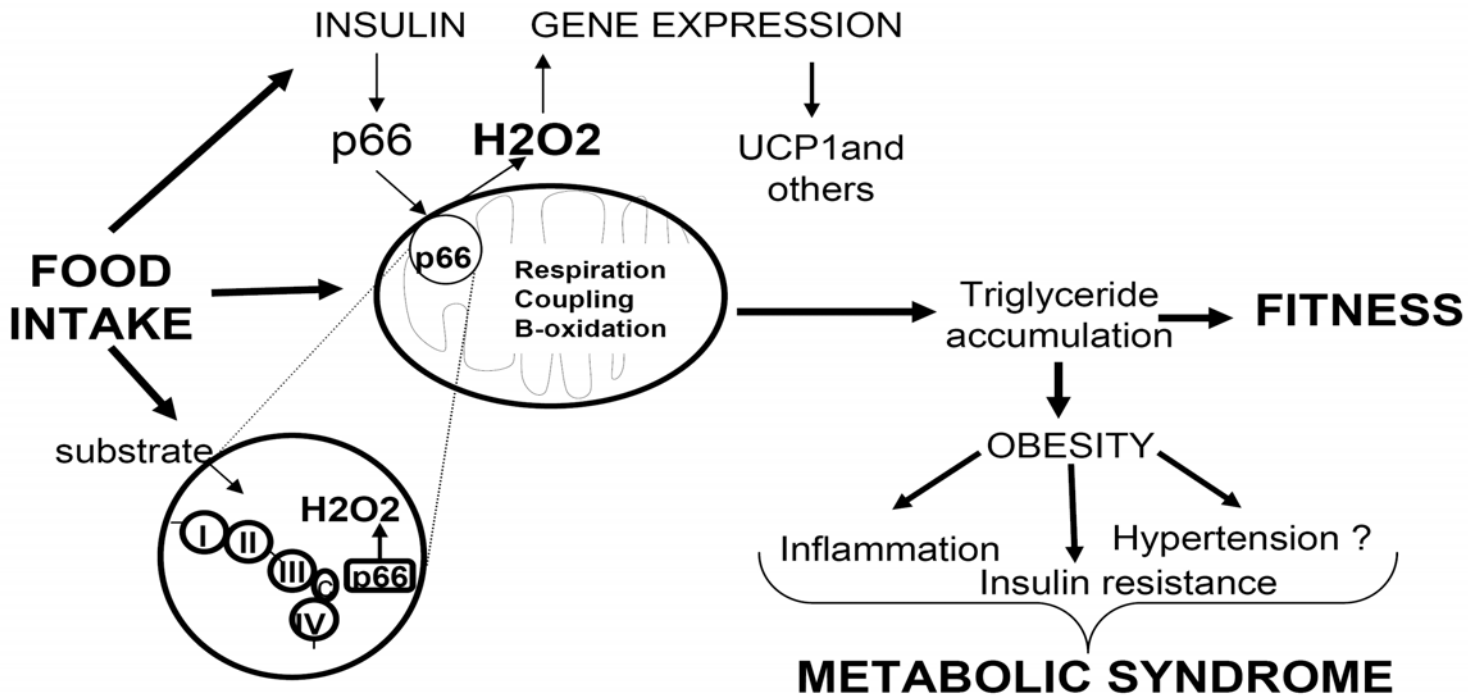
**Figure 55. P66<sup>Shc</sup> regulation of fat functions by food intake**. Food intake stimulates insulin secretion. Insulin induces serine phosphorylation of p66$^{Shc}$, the fact which is important for mitochondrial translocation of the protein. Food intake also increases p66$^{Shc}$ expression. As a consequence, the mitochondrial amount of p66$^{Shc}$ increases. In the mitochondria, food intake provides ETC with substrates thus activating it. Energized ETC is a necessary condition for production of $H_2O_2$ through the oxidation of cytochrome $c$ by p66$^{Shc}$. Thus, food intake stimulates multiple events which all lead to increased generation of mitochondrial $H_2O_2$ by p66$^{Shc}$. P66$^{Shc}$ generated $H_2O_2$ in turn modulates intracellular insulin signaling. P66$^{Shc}$ also globally changes the pattern of gene expression in response to insulin. One major outcome of this regulation is inhibition of UCP1 expression leading to increased mitochondrial coupling and reduced β-oxidation. The final outcome of p66$^{Shc}$ –mediated oxidative signal is the direction of fat metabolism towards adipogenesis. In this way p66$^{Shc}$ increases fat deposits. Under wild conditions it causes higher fitness of animals from the evolutionary point of view. However, excess calorie intake promotes obesity. Obesity promotes a complex pattern of disorders linked to MS. Thus p66$^{Shc}$ is involved in predisposition to inflammatory state, prothrombotic state, insulin resistance and glucose intolerance, which all together resemble features of MS.

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