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NUR77-dependent PPARγ2 deregulation in fasted white adipose tissue

Doctoral Thesis

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Abstract

Background

Nur77 has already been shown to be induced in white adipose tissue (WAT) upon β-adrenergic receptor (β-AR) stimulation. Its similar upregulation in muscle and liver is known to have metabolic implications. Nevertheless, the impact of the nuclear receptor on WAT's energy expenditure remains obscure.

Objectives

The objective of this thesis was to investigate the role of *Nur77* expression induction upon β-AR stimulation in WAT.

Results

Gene expression profiling revealed an increased level of *Nur77* mRNA in WAT from fasted mice and in β-AR stimulated adipocytes in culture. Microarray-based gene expression analyses were applied and evaluated in order to identify *Nur77*-dependent regulation of metabolism-associated factors during fasting. Among altered cellular processes the PPAR signalling pathway was found. Analysis of mRNA levels of *in vivo* and *in vitro* models indicated reverse expression patterns of *Nur77* and *Pparγ2*. Additionally, PPARγ2 target genes were deregulated in WAT of *Nur77-/-* mice. Chip-qRT-PCR and luciferase assays proved that *Nur77* binds the *Pparγ2* promoter and represses its activity. Finally, metabolite assays on mice sera and conditioned cell culture medium showed that under the evaluated conditions *Nur77* does not influence the levels of lipid metabolites. However, it impacts the glucose metabolism rate.

Conclusion

The presented data implicates that NUR77 is a significant gene-regulatory factor in metabolically demanding conditions in WAT.

Key words

Adipocyte, nuclear receptor, promoter, Chip-qRT-PCR, microarray.

Zusammenfassung

Hintergrund

Es wurde bereits gezeigt, dass die Expression von *Nur77* in weißem Fettgewebe (WAT) ansteigt, wenn der β-adrenerge Rezeptor (β-AR) stimulierert wird. Weiters ist bekannt, dass die ähnliche Hochregulation von *Nur77* in Muskel und Leber metabolische Auswirkungen hat. Die Rolle des nuklearen Rezeptors im WAT Energiehaushalt sind jedoch noch unbekannt.

Ziele

Das Ziel dieser Arbeit war die Untersuchung der Auswirkungen der *Nur77* Hohregulation in WAT nach β-AR Stimulation.

Ergebnisse

Genexpressionanalysen zeigten ein erhöhtes Niveau von *Nur77* mRNA sowohl in WAT von gefasteten Mäusen als auch in β-AR stimulierten Adipozyten in Kultur. Microarray basierte Genexpressionsanalysen wurden angewandt und evaluiert, um NUR77-abhängige Regulation von Stoffwechsel-assoziierte Faktoren während des Fastens zu identifizieren. Der PPAR Signalweg ist dabei einer von vielen veränderten zellulären Prozessen. Die Analyse der mRNA-Expression von *in vitro* und *in vivo* Modellen zeigte gegensätzliche Gen-expressionsmuster von *Nur77* und *Pparγ2*. Zudem wurden PPARγ2 Zielgene im WAT *Nur77*-/ - Mäusen dereguliert. Chip qRT-PCR and Luciferase Assays bestätigen dass NUR77 an den *Pparγ2* Promotor bindet und dessen Aktivität unterdrückt. Schließlich haben Untersuchungen an Metaboliten aus Mäuse Serum und Zellkulturmedium gezeigt, dass NUR77 unter den betrachteten Bedingungen keinen Einfluss auf das Niveau der Lipid-Metaboliten hat. Sehr wohl kommt es aber zu einem erhöhten Glukose Umsatz.

Abschluss

Die vorgestellten Daten zeigen die hohe Gen-regulatorische Bedeutung von NUR77 unter metabolisch herausfordernd Umständen in WAT.

Schlagworte

Adipozyt, Kernrezeptor, Promotor, Chip-qRT-PCR, microarray.

Table of content

1 Introduction

1.1 NUR77 and NR4A family

NUR77, also known as TR3 or NGFI-B, is a nuclear receptor (Chang et al., 1989; Hazel et al., 1988; Milbrandt, 1988) meaning it possesses transcription factor properties and upon binding to DNA regulates gene expression. Currently, an endogenous ligand of NUR77 is not known, thus, the protein was designated as orphan. The protein has a predicted molecular mass of 64kD, and contains a characteristic nuclear receptor modular structure consisting of regions assigned as A-E: an N-terminal with ligand-independent activation function-1 (AF-1) transactivation domain (region A/B), a DNA-binding domain (DBD) with two conserved zinc fingers (region C), a variable linker (region D), a ligand-binding domain (LBD), also responsible for dimerisation, (region E) and a C-terminus (region F) (Chang et al., 1989; Hazel et al., 1988; Wansa et al., 2002). NUR77 belongs to the NR4A nuclear receptor family together with neuron-derived orphan receptor 1 (NOR1), and nuclear receptor related 1(NURR1) (Chang et al., 1989; Hazel et al., 1988; Law et al., 1992; Ohkura et al., 1996; Wansa et al., 2002). NOR1 and NURR1 are said to be paralogous genes of *Nur77* as all three share significant sequence homology (Aranda and Pascual, 2001; Giguere, 1999; Mangelsdorf et al., 1995; Ohkura et al., 1994; Paulsen et al., 1992; Saucedo-Cardenas et al., 1997). The NR4A family members are 91-97% identical at the amino acid level in their DNA binding domains, approximately 60% similar in their putative ligand binding domains, however in the N-terminus they diverge (26-40% identity) (Law et al., 1992; Milbrandt, 1988; Ohkura et al., 1994). Furthermore, the gene structure of the NR4A subfamily members exhibit similarities as all three genes contain six translated exons with similar exon/intron splice sites (Castillo et al., 1997; Milbrandt, 1988; Ohkura et al., 1996). The NR4A receptors can bind to three defined DNA sequences named NGFI-B response element (NBRE), Nur-responsive element (NurRE), and a direct repeat (DR5) with five base pair spacing. Binding specificity depends on dimerisation. As monomers NR4A members demonstrate affinity to NBREs, as homodimers or heterodimes between the family members to NurREs, upon heterodimerizing with RXR NUR77 and NURR1 bind DR5 (Castillo et al., 1997; Perlmann and Jansson, 1995; Zetterstrom et al., 1996). NR4A member's basal expression occurs in some tissues, it can be also rapidly and transiently induced by various stimuli, hence it has potential to function as an early immediate gene. Often the same stimuli are common for two or three members of NR4A inducing them in a similar spatio-temporal manner. Interestingly, several of

the activities are overlapping between the NR4A members and upon silencing of one, the others can compensate for its absence (Cheng et al., 1997; Kanzleiter et al., 2005; Kumar et al., 2008; Pei et al., 2006b). The family members are known to be involved in numerous hormonal, physiological and pathophysiological processes including apoptosis, oncogenesis, steroidogenesis, cardiovascular disease, inflammation and metabolic disease (Arkenbout et al., 2003; Chao et al., 2009; Clark et al., 1996; Fu et al., 2007; Labelle et al., 1995; Mullican et al., 2007; Murphy et al., 2001; Wilson et al., 1993; Woronicz et al., 1994; Zetterstrom et al., 1996; Pires et al., 2007; Woronicz et al., 1994).

1.2 Expression of NUR77

NUR77 has the most widespread tissue distribution of all three NR4A members. It has been found in various tissues including thymus, lung, liver, testis, ovary, adrenal, thyroid, pituitary glands and numerous parts of the nervous system (Davis and Lau, 1994; Nakai et al., 1990; Ohkubo et al., 2002; Song et al., 2001; Stocco et al., 2000; Woronicz et al., 1994). Its expression profiles in the metabolically demanding and energy-dependent tissues, such as skeletal muscle, brain, heart, liver, and white and brown adipose tissue implies a energy metabolism related activity (Myers et al., 2009).
Nur77 expression is induced in response to diverse signals, among them are fatty acids, stress, prostaglandins, growth factors, calcium, inflammatory cytokines, peptide hormones, phorbol esters, and neurotransmitters, magnetic fields, mechanical agitation, and membrane depolarization (Bandoh et al., 1997; Hazel et al., 1988; Hazel et al., 1991; Honkaniemi et al., 1994; Honkaniemi et al., 2000; Kagaya et al., 2005; Miyakoshi et al., 1998; Roche et al., 1999; Tetradis et al., 2001; Williams and Lau, 1993; Woronicz et al., 1995; Youn and Liu, 2000; Borghaei et al., 1998; Fahrner et al., 1990; Katagiri et al., 1997; Yeo et al., 2005). Moreover, numerous publications suggest β-adrenergic (β-AR) signalling as a *Nur77* expression regulator in multiple tissues and under distinct physiological conditions. The β-AR signalling is a key modulator of metabolism, particularly in the metabolically active peripheral tissues. I has been proven to affect glucose homeostasis and oxidative metabolism in skeletal muscle, lipolysis in white adipose tissue (WAT), energy expenditure in brown adipose tissue (BAT), gluconeogenesis and lipogenesis in liver and glycogen utilisation in the heart (Bachman et al., 2002; Chao et al., 2008; Fortier et al., 2004; Kumar et al., 2008; McConville et al., 2003; McConville et al., 2007; Pei et al., 2006b; Pols et al., 2008; Robidoux et al., 2006; Yamamoto et al., 2007). The β-adrenergic receptor knock out mice are unusually susceptible to diet-induced obesity what emphasizes the importance of this signalling in the energy balance (Bachman et al., 2002).

Nur77 expression has been reported to be induced also by several of the β-AR-mediated processes. It has been detected in BAT and WAT in response to cold, in skeletal muscle in response to exercises and an excessive caloric intake (Kanzleiter et al., 2005; Kawasaki et al., 2009; Mahoney et al., 2005; Maxwell et al., 2005). Transient *Nur77* activation takes place upon β-AR agonist treatment in several tissues including the heart, liver, muscle, white adipose tissue, brown adipose tissue and the brain (Kanzleiter et al., 2005; Kawasaki et al., 2009; Mahoney et al., 2005; Maxwell et al., 2005). In the skeletal muscle β-AR stimulation induces expression of all three NR4A members *in vitro* and *in vivo.* It has capacity to elevate *Nur77* level 10 to 100-fold in both slow-twitching, oxidative and fast-twitching, glycolytic muscles (Chao et al., 2007; Kanzleiter et al., 2005; Lim et al., 1995; Maxwell et al., 2005; Myers et al., 2009; Pearen et al., 2006; Pearen et al., 2008). Upon muscle denervation or β-AR antagonist application the expression is significantly decreased demonstrating that the sympathetic innervation is necessary for the *Nur77* induction (Maxwell et al., 2005; Pearen et al., 2006). NUR77 has been reported as a cAMP downstream mediator upon exercise in the muscles as well as after fasting and a glucagon treatment in the liver (Kanzleiter et al., 2009; Pei et al., 2006b). Given that the β-AR signalling, similarly to other *Nur77* activators including IBMX, TNF and LPS, leads through cAMP, the pathway arises as an important *Nur77* regulator (Chao et al., 2008; Pei et al., 2005; Haverstick and Gray, 1992; Pei et al., 2006b; Zhang et al., 2002).

1.3 Regulation of NUR77 activity

NUR77 is said to work in a ligand-independent manner as it stays constitutively active. Nevertheless Yhan and colleagues described cytosporone B, an octaketide isolated form *Dothiorella sp*, HTF3, an endophylic fungus, as a NUR77-activating compound. Its capacity to bind the NUR77 LBD has been confirmed despite a tightly packed bulky hydrophobic group shown by crystallography in that domain (Wang et al., 2003; Zhan et al., 2008). Cytosporone B has been proven to regulate expression of NUR77 target genes connected with gluconeogenesis, it increases the blood glucose level and additionally, induces apoptosis in cancer cells (Wang et al., 2003; Zhan et al., 2008). Furthermore, a number of compounds based on 1,2-di(3'-indolyl)-1-(p-substituted phenyl)methanes was found to demonstrate a NUR77 agonist properties. Though the molecules activity was proven to depend on the *Nur77* LBD it is not clear whether they bind to it directly. Crucially, none of the agonists occurs endogenously which allows the presumption that the nuclear receptor stays fully functional, independently of a ligand. This raised the question of how its activity is regulated *in vivo*. Endogenous NUR77 is said to

be regulated at the levels of transcription and posttranslational modifications. Both mRNA and protein for all three NR4A members appear to be unstable *in vivo* and their expression is transient following stimulation (Darragh et al., 2005). Thus, the nuclear receptor occurrence in the cell is regulated by an induction or a repression of expression as well as by mRNA and protein degradation regulation. Nevertheless, in certain situations the NUR77 stability can be increased. Its interaction with the protein arginine methyltransferase (PRMT1) leads to stabilisation and delayed degradation (Darragh et al., 2005; Lei et al., 2009). Similarly, the presence of IBMX has been demonstrated to increase the NUR77 level by protection from proteasome degradation (Fumoto et al., 2007).

NUR77 activity is also influenced by posttranslational modifications such as phosphorylation and acetylation. Acetylation by p300 results in an increased NUR77 stability and transcriptional activity whereas HDAC1 activity was associated with its transcriptional repression (Kang et al., 2010). NUR77 can be phosphorylated on multiple sites and the modification can be carried out by several kinases *in vitro* including members of the MAP kinase family, the protein kinase Akt, Jun N-terminal kinase, pp90rsk, ERK2 (Davis and Lau, 1994; Fisher and Blenis, 1996; Hazel et al., 1991; Hirata et al., 1993; Kolluri et al., 2003; Pekarsky et al., 2001; Slagsvold et al., 2002). Akt transfers phosphate group on ser350 in the DNA binding domain of NUR77, what results in its reduced DNA binding affinity and predominant localisation in the cytoplasm (Pekarsky et al., 2001). Possibly the phosphorylation-dependent nucleo-cytoplasimc shuttling of NUR77 is connected with a nuclear localisation signal, found in it the DBD, being inactivated. After the modification the nuclear export signal in the LBD predominates and determines cytoplasmic localisation. The phosphorylation of NUR77 at Ser105 of its N-terminal influences transcription and cofactor recruitment mediated by the AF-1 domain and also regulates the ability of NUR77 to be exported to the cytoplasm (Katagiri et al., 2000).

NUR77 activity can be regulated through interactions with other proteins and nuclear receptors like small heterodimer partner (SHP), which is known to bind and inhibit the function of many nuclear receptors. It has been shown to influence NUR77 transcriptional activity by interacting with its N-terminal, transactivation domain and it counteracts its putative interaction with the coactivators (Yeo et al., 2005).

Another activity-regulating mechanism involves the expression of an N-terminally truncated NUR77. This artificial isoform, named NUR77 dominant negative (NUR77 DN), has abolished ability to transactivate gene expression. However, its DBD remains intact and it competes with the full length counterparts for DNA binding sites (Ohkura et al., 1999).

1.4 Role of NUR77

NUR77 as a nuclear receptor can bind to DNA and regulate its target genes promoter activity. In addition to the direct influence on gene expression, it can also interact with other proteins, change their properties and through that influence cellular processes. It has been reported to heretodimerize with retinoid X receptor (RXR) and chicken ovalbumin upstream-promoter transcription factor (COUP-FT) to regulate their transcriptional activity concerning cell growth and apoptosis. (Wu et al., 1997a; Wu et al., 1997b). It binds to the catalytic domain of protein arginine methyltransferase (PRMT1) and inhibits its methyltransferase activity. That results in modulation of transcription, RNA metabolism, signal transduction, DNA repair and apoptosis (Lei et al., 2009). It has been shown to induce expression of p300 but on the other hand it binds and negatively regulates p300 activity concerning acetylation of histons and transcription factors, resulting in a repression of their transcriptional activity (Kang et al., 2010; Lei et al., 2009).

Since *Nur77* is expressed in such a wide spectrum of tissues and conditions it plays role in various processes. It has been reported to take part in nerve system development, female sexual differentiation, negative selection in T-cells and protection against atherosclerosis (Arkenbout et al., 2002; Calnan et al., 1995; Cheng et al., 1997; Goto et al., 2006; Zhou et al., 1996). It is involved in carcinogenesis, cell death, regulates proliferation, cell cycle progression and differentiation (Au et al., 2008; Kolluri et al., 2003; Mu and Chang, 2003; Wu et al., 2002; Baek et al., 2009; Chao et al., 2008; Fumoto et al., 2007; Maruoka et al., 2010; Matuszyk, 2009; Wu et al., 2002). It plays a key role in expression induction of various genes in the HPA axis related to inflammation and steroidogenesis (Crawford et al., 1995; Fernandez et al., 2000; Stocco et al., 2002). A lot of attention has been paid to clarify NUR77 role as an apoptosis regulator in various cell types. In response to apoptotic stimuli it is translocated from the nucleus to the cytoplasm, where it binds Bcl2 and induces its conversion from a protector to a killer. The process is followed by cytochrome c release from mitochondria and apoptosis. A truncated version of NUR77, lacking the DNA-binding domain, localizes exclusively in the cytoplasm, where it associates with mitochondria and potently induces apoptosis what suggests that the apoptotic effect of NUR77 is a transcriptional regulation independent mechanism (Li et al., 2000; Lin et al., 2004).

The expression in metabolically demanding tissues, in energy expenditure associated conditions, and upon stimuli considered to be metabolism modulator, indicates metabolic function of NUR77. Numerous research groups were trying to discover the contribution of this nuclear receptor to the balance in energy mobilisation. By now it has been proven that NUR77 is a

substantial regulator of glucose and lipid metabolism. Abundant evidence indicates that the receptor influences glucose metabolism in liver and skeletal muscle, mainly in response to fasting. Pei and colleagues reported that *Nur77* expression is induced in liver by the cAMP in vitro and by glucagon and fasting in vivo. In hepatocytes, expression of key gluconeogenic genes is controlled by the cAMP axis. Cyclic AMP serves as a central downstream signalling molecule of hormones, including insulin, glucagon and glucocorticoids (Montminy, 1997; Montminy et al., 2004; Pilkis and Granner, 1992). It has been observed that in liver, upon induction, NUR77 stimulates expression of multiple genes involved in gluconeogenesis (*G6pc, FbBp1, Fbp2, eno3*) and glucose transport out of the cell (*Glut2*). The receptor appeared to be a direct regulator of some of those enzymes including *Eno3, Glut2* and *Fpb2*. Consistently, it increases the hepatic glucose production and a raises the blood glucose level upon overexpression, whereas the presence of NUR77DN leads to glucose level depletion. NUR77 has been observed to cooperate with peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) to promote transcription of certain gluconeogenic genes. In some cases both proteins act in an additive manner in others their target genes are complementary, with certain genes preferentially responsive to PGC-1a and others to NUR77 (Pei et al., 2006a).

The NUR77-dependent aerobic metabolism has also been reported in skeletal muscles, the organ where the role of the nuclear receptor has been the most extensively investigated. It has been demonstrated that silencing or overexpression of *Nur77*, similarly to denervation, results in expression changes of genes connected with glucose metabolism. Hence, the nuclear receptor has been proposed to be the downstream signalling molecule of β-AR. NUR77 modulates expression of genes associated with glucose uptake (*Glut4*), glycolysis (*Pfkm, Pgam2, Bpgm*, hexokinase, phosphofructokinase), glycogenolysis (*Phkg1, Pygm*, glycogenin), glycerophophate shuttle (*Gpd1*) (Chao et al., 2007; Kanzleiter et al., 2009). Consistently, it increases glucose uptake via GLUT4, glucose oxidation and glycogen synthesis in skeletal muscle (Chao et al., 2009; Kanzleiter et al., 2009).

Concerning lipid metabolism, it has been shown to be affected by the receptor in liver and in muscle. Under fasting conditions NUR77 lowers expression of genes connected with lipogenesis, including sterol regulatory element binding protein 1c (*Srebp1c*), known as a master regulator of lipid synthesis, as well as fatty acid synthase (*Fas*) and Glycerol-3-phosphate acyltransferase 1 (*Gpam*). Similarly, the over-expression of *Nur77* in mouse liver resulted in a expression inhibition of *Srebp1c*, and other lipogenic genes, which culminates in reduced hepatic triglycerides level (Pols et al., 2008). The data is consistent with the publication concerning *Nur77-/-* mice on high fat diet (HFD). The animals exhibited hepatic steatosis due to an elevated, compared to the wild type mice, triglicetride and cholesterol accumulation. Coherent, the mice livers

displayed enhanced *Srebp1c*, *Fas* and stearoyl-Coenzyme A desaturase 1 (*Scd1*) expression (Chao et al., 2009).

For the reason that few and contrary results have been published concerning a NUR77 impact on lipid metabolism in muscle, its role remains controversial. First, Maxwell reported that *Nur77* attenuation in the C2C12 muscle cells reduces lipolysis and alerts expression of proteins associated with lipid and energy metabolism. The nuclear receptor has been demonstrated to elevate AMP-activiated protein kinase subunit gamma 3 (*Ampkγ3*), fatty acid translocase (*Cd36*), fatty acid binding protein 3 and 4 (*Fabp3*, *Fabp4*), uncoupling protein 3 (*Ucp3*), adiponectin receptor 2 (*AdipoR2*), caveolin 3 (*Cav3*) and to diminish *Srebp1c* level. The results implicate NUR77 as a lipolysis activator and a lipogenesis inhibitor in skeletal muscle. The mentioned genes expression changes are coherent with the phenotype of β-AR null mice (Maxwell et al., 2005). Additionally, they stay consistent with the Nur77-/- animals metabolic profile as the animals exhibit increased intramuscular lipid content. They were also reported to have increased fatty acid (FA) uptake and decreasd peroxisomal B-oxidatin enzyme (*Ehhadh*) expression (Chao et al., 2009). Contrary, Chao et al. did not observe differences in expression levels of genes associated with lipid metabolism in myocytes overexpressing *Nur77*. They also have proven that *Nur77* expression is preferential in fast twitching, glycolytic, compared to slow twitching, oxidative muscles (Chao et al., 2007). Likewise, Kanzleiter noticed that *Nur77* overexpression does not affect lipid metabolism *in vivo* and *in vitro* (Kanzleiter et al., 2010).

Very little is known about NUR77's role in adipocyte tissue even though it has many times been documented to be expressed there. NR4A family members have been shown to be induced in brown adipose tissue (BAT) and cultured brown adipocytes in response to β-AR and cold which implicates the proteins in the thermogenesis process. Thermogenesis in BAT is mediated by β-AR signalling induced by cold exposure. It elevates cAMP level which results in lipolysis and UCP1 expression and activity increase. The NUR77 dominant negative mutant downregulates UCP1, which implies association of NR4A family with the thermal energy production. For that reason it was surprising that *Nur77*-/- mice exhibit normal thermogenesis regulation. However, it has been suggested to result from NOR-1 expression super-inducion compensating the loss of NUR77 (Kanzleiter et al., 2005; Kumar et al., 2008).

Nur77 is known to be implicated in white adipocytes differentiation (Au et al., 2008; Chao et al., 2008; Fumoto et al., 2007). The induction in WAT upon β-AR stimulation suggests a role in the fasting response, what in case of this tissue might indicate lipolysis regulation. Nevertheless NUR77's function in WAT remains obscure. So far it has only been shown to upregulate UCP1 expression (Kumar et al., 2008). There are data indicating NOR1 as insulin

sensitizing protein, increasing phosphorylation of insulin receptor substrate and Akt as well as augmenting glucose transport by increasing GLUT4 recruitment to the plasma membrane. Given that NUR77 and NOR1 activity is said to be overlapping, and that both of them were shown to be induced by the same stimuli in WAT it is very likely that NUR77 plays a role in insulin sensitivity in WAT (Fu et al., 2007).

Of interests, Nur77 is also associated with metabolic diseases. It affects glucose tolerance and insulin sensitivity since *Nur77*-/- mice on HFD display decreased insulin sensitivity and insulin receptor phosphorylation in muscle (Chao et al., 2009). *Nur77* has been shown to be reduced in skeletal muscle and adipose tissue in rodent models of insulin resistance in skeletal muscle. Its expression in human skeletal muscle was negatively correlated with body-fat content and positively with insulin sensitivity. It was also downregulated muscle of in obese compared to lean men (Fu et al., 2007; Kanzleiter et al., 2010). However, there have been contradictory publications concerning putative negative or positive impact of the nuclear receptor on metabolic diseases. That is due to the fact that its expression is elevated in hepatocytes in models of type 1 and 2 diabetes, and it contributes to elevated gluconeogenesis resulting in hyperglycemia. Nevertheless, in the same time it downregulates lipogenesis in the liver what is supposed to mitigate diabetic, hyperinsulinemic conditions (Pei et al., 2006a; Pols et al., 2008). According to Pei et al. NUR77 induces pro-inflammatory cytokines in macrophages. Contrary, Bonta et al. and Hu et al. claim it reduces human macrophage lipid loading and inflammatory responses and protects against neointima formation (Bonta et al., 2006; Pei et al., 2006a; Pires et al., 2007).

In summary, NUR77 participation in the response to energy demanding conditions seems to contribute to the balance of metabolic fuel utilization and thus energy expenditure.

2 Objectives

The main objective of the thesis was to investigate role the of *Nur77* expression induction in white adipose tissues (WAT) upon a β-adrenergic stimulation (β-AR). In order to fulfil that objective it was necessary:

- To analyse *Nur77* expression profile in mature adipocytes in culture upon β-AR stimulation;
- To evaluate *Nur77* expression profile in WAT from fasted mice;
- To compare results of both, *in vivo* and *in vitro* approaches and elucidate its relevance for studying *Nur77* induction during β-AR stimulation;
- To perform gene expression profiling of mature adipocytes in culture upon β-AR stimulation and WAT from fasted mice;
- To identify NUR77 target genes in WAT upon β-AR stimulation;
- To elucidate which cellular pathways are affected during fasting and β-AR stimulation;
- To establish which cellular pathways are affected by NUR77 during fasting and β-AR stimulation;
- To study the role of NUR77 in metabolism regulation of WAT upon β-AR stimulation. To investigate that issue it was important to:
    - To question if Nur77 target genes are associated with metabolism;
    - To determine in which metabolic processes NUR77 target genes are involved;
    - To examine if predicted Nur77 influence on metabolism can be confirmed for *in vivo* and *in vitro* models;
- To analyse obtained data and propose a model of NUR77's influence on metabolism of WAT upon β-AR stimulation.

3 Results

3.1 Fasting and β-AR stimulation enhance *Nur77* expression

It has in recent years been established that β-AR stimulation induces *Nur77* expression as has already been described in muscle (Chao et al., 2007; Kanzleiter et al., 2009; Maxwell et al., 2005) and adipose tissue (Kanzleiter et al., 2005; Kumar et al., 2008). Likewise, in the data presented here treatment of mature 3T3-L1 adipocytes with a β-AR agonist, isoprenaline, resulted in a 9 fold increase in *Nur77* mRNA level. Its expression rise was rapid; it peeked at 2 hours and returned to the basic level by 12 hours (Figure 1). The expression changes were observed on a RNA and protein level (Figure 1 a, c). Additionally, *Nur77* induction was examined in WAT of wt mice 24h fasted and fasted followed by 8h refeeding. The *Nur77* mRNA level was 4 times higher in the fasted than refed WAT sample (Figure 1b).

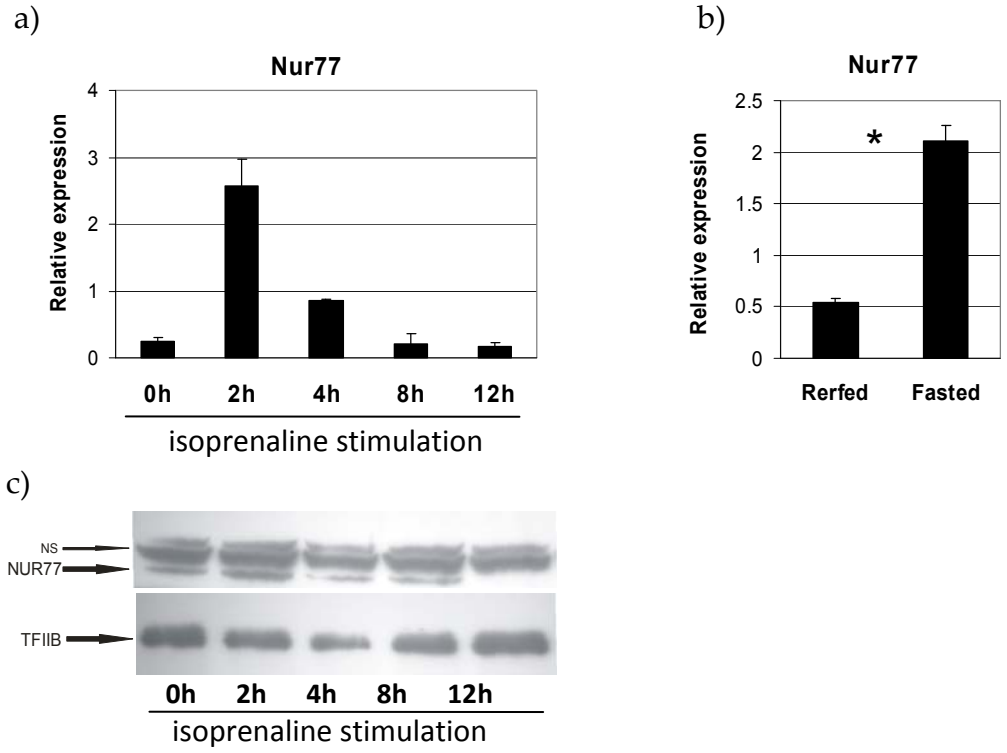


Figure 1 *Nur77* expression indiction upon fasting and a β-AR stimulation. a, c) Mature 3T3-L1 adipocytes were treated with 1μM isoprenaline and harvested at depicted time points. Gene expression was assayed on a mRNA level using RT-PCR (a) and protein with western blot (c); b) White adipose tissue (WAT) was harvested from wild type (wt) mice fasted 24h (fasted) and fasted fallowed by 8h refeeding (refed). Extracted RNA was used as a cDNA template and submitted to a gene expression quantification with RT-PCR. n=3; $p \leq 0.05$ *.

3.2 Fasting and a β-adrenergic stimulation alert expression of genes associated with metabolism in white adipose tissue

With the aim of identifying expression changes characteristic to fasting, a gene expression profiling for WAT from fasted and refeed wt mice was performed. The WAT RNA of three animals was pooled, transcribed to labelled cDNA and hybridised to cDNA microarrays. This experiment allowed the obtainment of background data helpful to indicate a further direction of the investigation. Microarray readout was submitted to normalisation, an extraction of genes demonstrating significant expression changes and a PathwayExplorer analysis. The PathwayExplorer was used to perform the obtained gene list filtration against regulatory, metabolic and cellular pathways from KEGG, BioCarta and GenMAPP. The evaluation allowed to further indicate which processes are affected by fasting, to list them according a number of regulated genes (Passed UniqID) and to quantify the data set significance (% Passed UniqID). A number of genes associated with metabolic pathways was found to be regulated (Table 1). Surprisingly the highest positions in the ranking were occupied by glucose utilisation associated processes i.e. insulin signalling, pyruvate metabolism, glycolysis/gluconeogenesis, and the TCA cycle. Nevertheless, also glycerolipid, glycerophospholipid, ketone bodies and fatty acid associated pathways could be found listed. The data emphasizes a tight regulation between glucose and lipid metabolism occurring in the tissue considered to depend largely on lipid-derived energy.

Expression of some of the metabolically relevant genes indicated by the microarray-based expression analysis were further analysed with RT-PCR (Figure 2). Among them were forkhead transcription factor 1 (*Foxo1*), peroxisome proliferator activated receptor gamma 2 (*Pparγ*2), fatty acid synthase (*Fas*), glucose transporter 4 (*Glut4*). FOXO1 is a transcription factor downstream of an insulin signalling pathway influencing glucose and lipid metabolism and it is known to modulate a the *PPARγ* activity. Fasting conditions influenced the glucose transport and metabolism by inducing changes in the mRNA amount of insulin-regulated glucose transporter (*Glut4*) and *Pck1*. PCK1 constitute a main regulatory point between glycolysis and gluconeogenesis and its enhanced amount is connected with stimulation of a glucose synthesis. There were no changes in the *Glut1* mRNA amount detected, however, its expression level is known to be low in that tissue. A reduction of *Fas* and *Pparγ*2 expression implies an inhibition of lipid synthesis. Of notice, *Glut4* and *Fas* have earlier been reported to be NUR77 target genes (Chao et al., 2007; Kanzleiter et al., 2010; Pols et al., 2008). In summary, the results indicate a repression of lipogenesis and a modulation of glucose metabolism.

Id	Section	Subsection	Pathway	Pathway UniqID	Passed UniqID	% Passed UniqID
mmu03320	KEGG Pathways	4.8 Endocrine System	PPAR signaling pathway	79	6	7.59
mmu04910	KEGG Pathways	4.8 Endocrine System	Insulin signaling pathway	135	6	4.44
mmu_hackl_0001	Pathways	Adipogenesis	Adipogenesis Regulatory Network	45	6	13.33
mmu04010	KEGG Pathways	3.2 Signal Transduction	MAPK signaling pathway	334	4	1.2
mmu00620	KEGG Pathways	1.1 Carbohydrate Metabolism	Pyruvate metabolism	40	4	10.0
mmu00010	KEGG Pathways	1.1 Carbohydrate Metabolism	Glycolysis / Gluconeogenesis	57	3	5.26
mmu00020	KEGG Pathways	1.1 Carbohydrate Metabolism	Citrate cycle (TCA cycle)	27	3	11.11
mmu_hackl_0001	KEGG Pathways	3.3 Ligand-Receptor Interaction	Adipogenesis Regulatory Network	23	3	13.04
mmu04020	KEGG Pathways	3.2 Signal Transduction	Calcium signaling pathway	199	2	1.01
mmu00561	KEGG Pathways	1.3 Lipid Metabolism	Glycerolipid metabolism	113	2	1.77
mmu00564	KEGG Pathways	1.3 Lipid Metabolism	Glycerophospholipid metabolism	79	2	2.53
m_ox40Pathway	BioCarta Pathways	Cell Signalling	Nuclear Receptors in Lipid Metabolism and Toxicity	32	2	6.25
m_metPathway	BioCarta Pathways	Cell Signalling	mTOR Signaling Pathway	19	2	10.53
m_PpargPathway	BioCarta Pathways	Expression	Role of PPAR-gamma Coactivators in Obesity and Thermogenesis	9	2	22.22
mmu00072	KEGG Pathways	1.3 Lipid Metabolism	Synthesis and degradation of ketone bodies	8	2	25.0
mmu00071	KEGG Pathways	1.3 Lipid Metabolism	Fatty acid metabolism	93	1	1.08

Table 1 Ranking of metabolic pathways associated with differentially expressed genes in fasted WAT. WAT was extracted from 24h fasted and fasted fallowed by 8h refeeding wild type (wt) mice. Its RNA was utilised as a template for synthesis of labelled cDNA which was further hybridised on a high density cDNA microarray chip. A gene list obtained within data normalisation and differentially expressed genes filtration was submitted to analysis with in house developed software, PathwayExplorer. Pathway UniqID represents a number of genes comprised by the pathway; Passed UniqID indicates a number of genes from the analysed list found to be associates with the pathway; % Passes UniqID demonstrates a number of passed genes in percentage of total number of genes affiliated with the pathway.

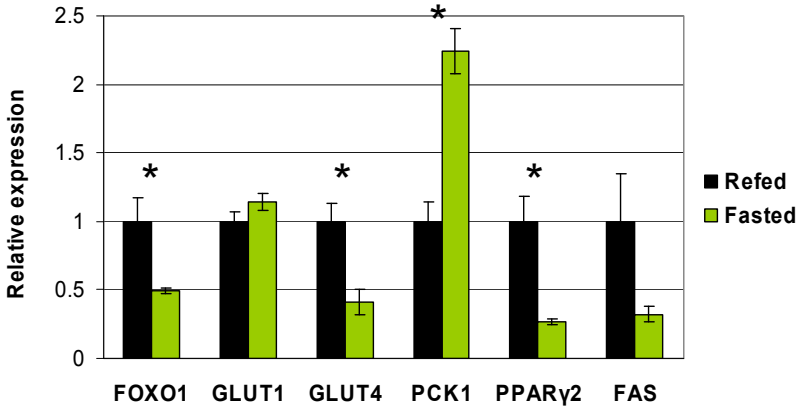


Figure 2 Expression profiles of some of metabolically relevant genes in fasted and refed mice WAT. WAT was harvested from wt mice 24h fasted (fasted) and fasted fallowed by 8h refeeding (refed). Gene expression was quantified with RT-PCR. n=3; p≤0.05*.

The β-adrenergic signalling pathway is induced in a number of tissues upon various stimuli, also in fasting in WAT where it results in stimulation of lipolysis. Therefore, a β-AR ligand, isoprenaline, was used to simulate fasting conditions for adipocytes cells in culture. The experiment allowed elucidating whether the gene expression changes in the *in vitro* β-AR stimulation are consistent with those observed *in vivo* in fasting. For that purpose 3T3-L1 mature adipocytes were incubated with medium containing 1μM isoprenaline and harvested at various time points. Changes in mRNA levels were analysed for some of the selected factors: *Foxo1*, glucose transporters, *Pck1*, *Pparγ*, *Fas* (Figure 3). Shown gene expression changes stay consistent with the results observed in the mouse study, indicating that the *in vitro* approach constitutes a valuable model supplementing animal experiments for investigating fasting. Nevertheless, in case of the cell culture the reaction to β-AR stimulation was very rapid, the gene expression changes could be observed as fast as 30 minutes after the induction and most of the changes were abrogated by 24 hours (Figure 3 b).

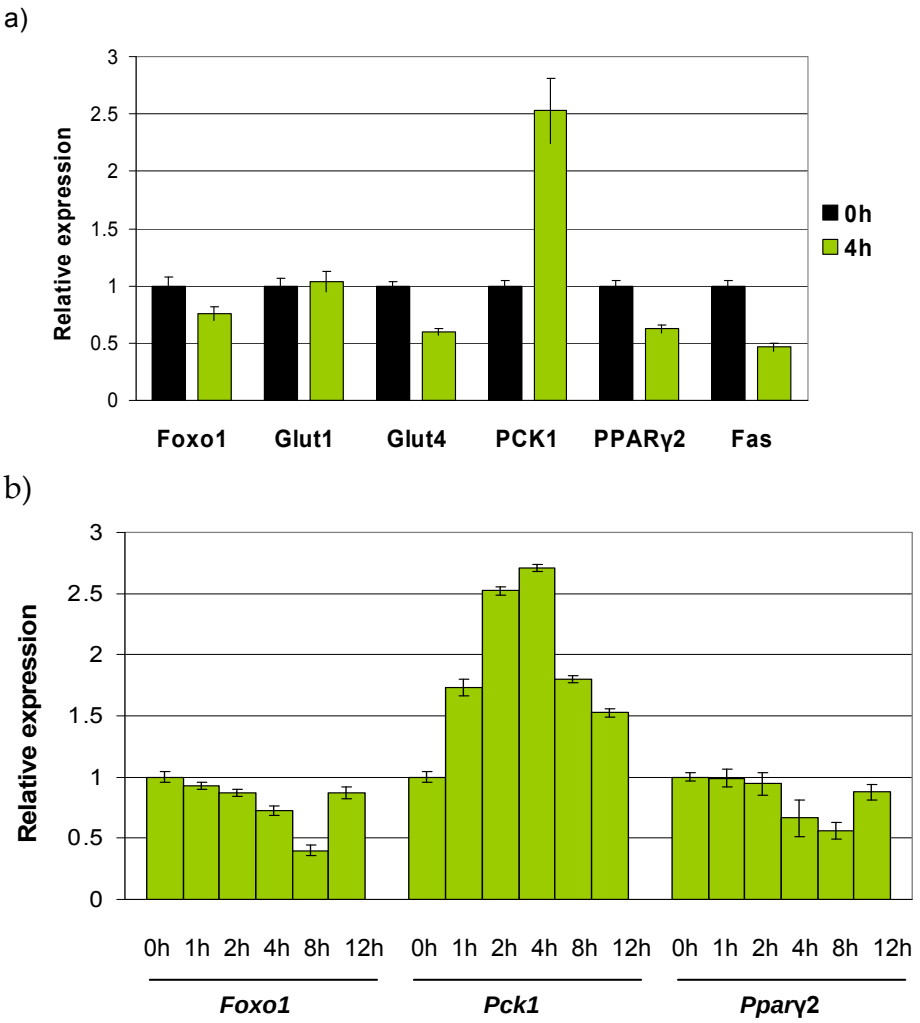


Figure 3 Expression profiles of some of metabolically relevant genes in β-AR stimulated mature 3T3-L1 adipocytes. Mature 3T3-L1 adipocytes were treated with 1μM isoprenaline and harvested at depicted time points. The mRNA level was quantified with RT-PCR. The graphs compare gene expression at 0h and 4h after induction (a) and its changes within series of time points (b).

3.3 Fasting and *Nur77* expression changes influence PPAR signalling pathway

Series of microarray analyses were performed in order to identify *Nur77*-dependent changes in metabolism-associated gene expression during fasting. One of the experiments compared WAT from mice fasted 24h or fasted and then refed. For a second microarray WAT of over night fasted Nur knockout *versus* similarly fasted wt mice used. The obtained results analysis represent the average of four replicates and revealed 156 transcripts significantly regulated ($p<0.05$; Supplementary table 1). A third experiment involved mature 3T3-L1 adipocytes harbouring TET-off regulated *Nur77* overexpression. The TET-off system allowed to overexpress *Nur77* in a

regulated manner by addition or withdrawal of doxycycline from culture medium. It utilisation was unavoidable as a high *Nur77* level is known to inhibit adipogenesis and due to the experimental design the mature adipocytes were indispensable. The TET-off activation resulted in 2-5 fold overexpression and was consistent on RNA and protein level (Supplementary figure 1). Complementary DNA generated from total RNA from the *Nur77* overexpressing (without doxycycline) cells was hybridised on a microarray cDNA chip against not overexpressing (with doxycycline), both stimulated with 1μM isoprenaline. The *Nur77* expression profile served as a control for evaluation of successful TET-off system activation (Supplementary figure 2). Due to the fact that the experiment was performed with only one replicate the obtained data were treated as indicative. Results of each of the three assays indicated alteration of numerous genes affiliated with metabolism. Ranking of differentially expressed genes, for each of the experiments, displayed a high score for a the PPAR signalling pathway in PathwayExplorer analysis (Table 2). The fact that is has also been observed in *Nur77* overexpressing cells and *Nur77-/-* mice suggests *Nur77* as a *Ppar* expression regulator.

A bibliography-based analysis of associations and interactions between the differentially expressed genes revealed that PPARγ is a central component in a network between the detected factors (Figure 4). Hence, PPARγ emerged as a key regulator-protein through which NUR77 impacts fasted WAT metabolism.

PPAR signaling pathway				
Microarray experiment	Pathway UniqID	Passed UniqID	% Passed UniqID	Position in pathways ranking
WAT of fasted *vs* refed mice	79	6	7.59	2
WAT of fasted *Nur77-/- vs* fasted wt mice	79	5	6.33	3
3T3-L1 mature adipocytes with *vs* without *Nur77* overexpression, 1μM isoprenaline	79	17	21,52	8

Table 2 PPAR signalling pathway genes expression is alerted in fasting and upon *Nur77* expression changes. A PathwayExplorer analysis was performed for a list of differentially expressed genes extracted from the results of microarray assays. The experiments utilised WAT of 24h fasted versus 8h refed wild type mice, WAT of *Nur77-/-* fasted versus wild type fasted mice and mature 3T3 –L1 adipocytes stimulated with 1μM isoprenaline overexpressing *Nur77* versus wt cells.

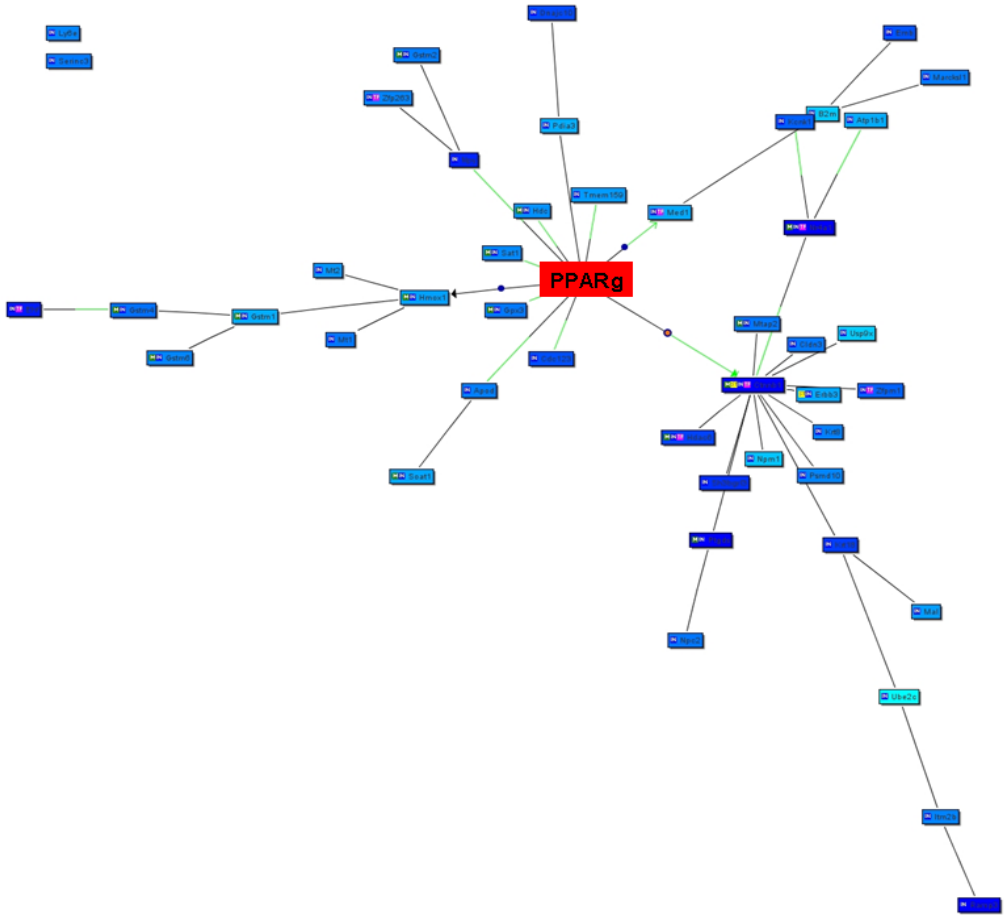


Figure 4 Bibliography-based analysis of differentially expressed genes in WAT of *Nur77*-/- fasted mice. Bibliography-based analysis of associations and interactions between the differentially expressed genes in fasted *Nur77*-/- mice compared to fasted wild type siblings (n=4; Genomatix Bibliosphere). The analysis was performed for results of microarray differential expression profiling.

3.4 *Nur77* expression antagonises *Ppar*γ2 expression

Observed NUR77-dependent expression changes among the PPARγ associated genes suggested that the nuclear receptors might influence each others synthesis. In order to validate that hypothesis expression profiles comparison of both nuclear receptors in various experimental designs was assayed. For the reason that PPARγ2 is the only member of the NR1C family which is known to be abundantly expressed in WAT its mRNA level was examined. The outcome indicated that in WAT of fasted mice, when *Nur77* was significantly overexpressed, the *Ppar*γ2 level was diminished. A reverse pattern was observed upon refeeding (Figure 5 a). Consistently, in the knockout mice, where *Nur77* is absent, the *Ppar*γ2 amount was not reduced (Figure 5b). Additionally, both nuclear receptors were proportionally reverse

regulated in wt and *Nur77* overexpressing cells in culture (Figure 5 c). As it has been shown that *Nur77* expression is strongly elevated upon isoprenaline treatment, *Pparγ2* level was analysed in similarly treated samples. Its mRNA amount appeared to be diminished as fast as 2 hours after the stimulation, at the mRNA and protein levels (Figure 5 d, e). The results indicate that the *Nur77* induction contribute to the *PPARγ2* level depletion.

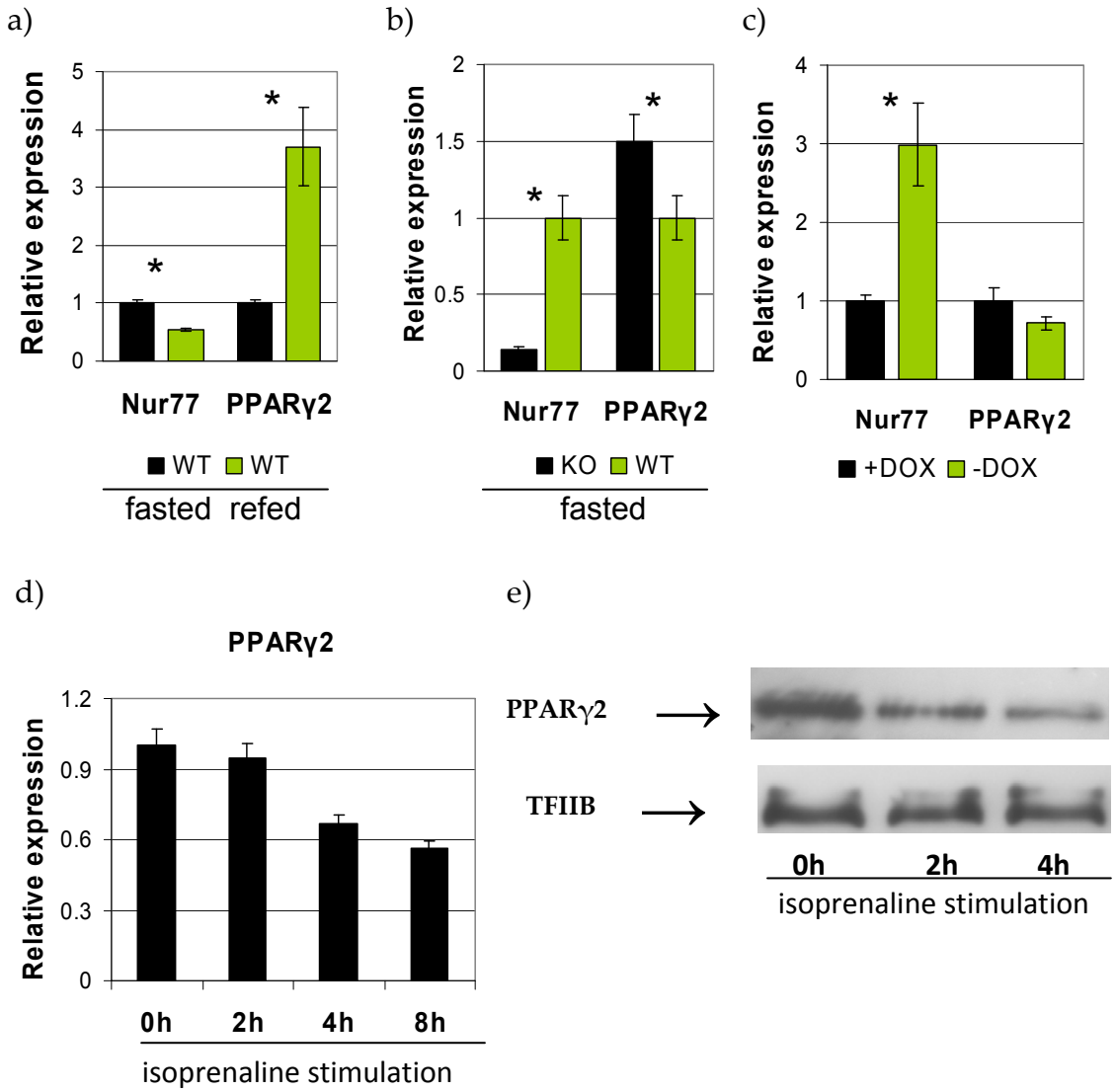


Figure 5 Contrary regulation of *Nur77* and *Pparγ2* expression. RT-PCR gene expression measurements were performed for WAT harvested from wt mice fasted over night (fasted) and fasted fallowed by 8h refeeding (refed) (n=3, a), WAT of 24h fasted *Nur77* -/- (KO) and wt mice (n=3, b), 3T3-L1 mature adipocytes overexpressing *Nur77* (-DOX) and control cells (+DOX, c). RT-PCR and western blot detecting a *Pparγ2* level was carried out for mature 3T3-L1 adipocytes incubated in medium containing 1μM isoprenaline (d, e). n≥3 for a and c, n=10 for b; p≤0.05 *.

Nur-/- mice are said not to have an obvious phenotype. Meaning that the animals are born at an expected Mendelian rate, develop without differences in size, growth or behaviour compared to the wt mice (Lee et al., 1995).

Nevertheless, a recent publication showed that the mice challenged with high fat diet exhibit certain characteristic features indicating that the *Nur77*-/- phenotype is pronounced in physiologically demanding conditions (Chao et al., 2009). In order to obtain a more pronounced picture of the nuclear receptor impact on the metabolism, *Nur77*-/- and wt mice were put on HFD for 6 weeks. During the last three weeks of the diet they were injected with a *Nur77* agonist, cytosporone B, or DMSO as a control (Zhan et al., 2008). No difference in a weight gain, liver and WAT mass between the animal groups was observed (Supplementary figure 4 a, c, d). The mice were fasted over night. Likewise, the fasting body weight changes were comparable between the knock out and the wt animals (Supplementary figure 4 b). Similarly to the previous experiments, in the WAT of the mice *Nur77* expression was induced upon fasting. Nevertheless, its level was significantly lower when compared to the WAT of fasted mice on chow diet (Supplementary figure 3). There was no significant difference in the nuclear receptor expression between the mice injected with cytosporone B and DMSO (Figure 6 a). Importantly, in the same samples *Ppar*γ2 expression was not affected in the wt compared to *Nur77*-/- mice suggesting that for obese animals fasting does not induce *Nur77* to the level sufficient enough to influence *Ppar*γ2 (Figure 6 b). Nevertheless there was a reduction of *Ppar*γ2 mRNA amount observed in the WAT of the mice injected with cytosporone B compared to the control mice. Surprisingly, there was also a difference between the knockout mice injected with the ligand and the control. Those results indicate that the cytosporone B is not a unique *Nur77* ligand but it affects the activity of other factors, which are able to influence *Ppar*γ2 expression.

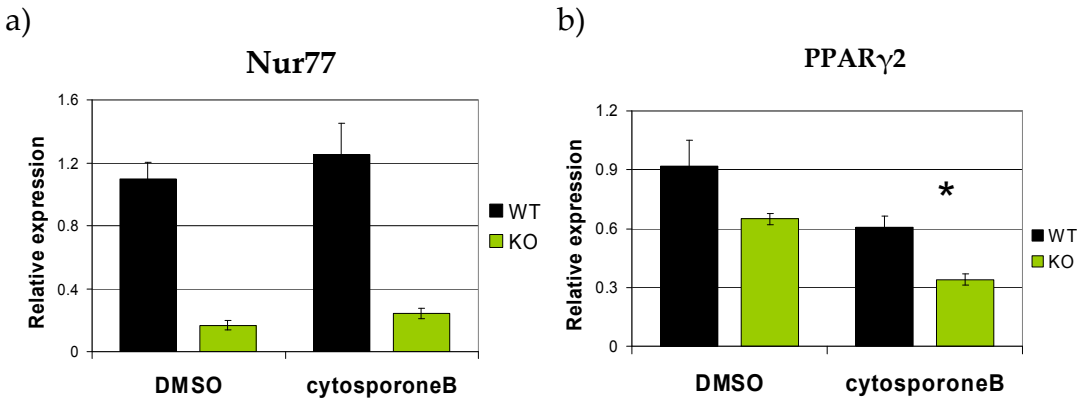
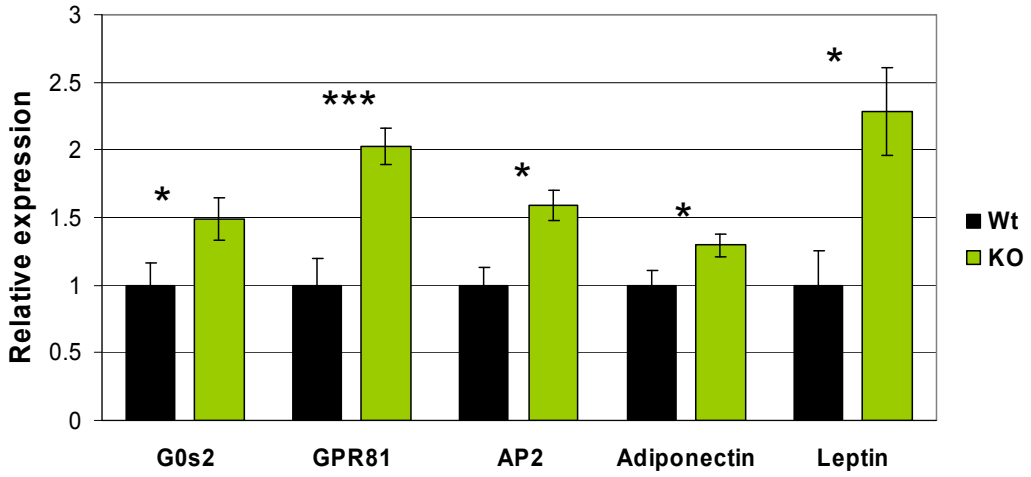


Figure 6 *Nur77* and *Ppar*γ2 expression in WAT of HFD mice. *Nur77*-/- and wt mice were fed HFD for 6 weeks, injected with cytosporone B or a control solution for three weeks. The animals were scarified after overnight fasting. RT-PCR gene expression analysis was performed for cDNA of harvested WAT using *Nur77* (a) and *Ppar*γ2 (b) specific primers. n≥5, p≤0.05 *.

3.5 *Nur77* influences PPARγ2 target genes expression

The observed correlation between the mRNA levels of the two nuclear receptors, prompted the investigation into whether expression of PPARγ2 targeted genes might also be NUR77-dependent. All of the tested genes showed an increased mRNA level in knockout compared to wt animals WAT (Figure 7 a). Among examined genes there were *Ap2*, adiponectin and leptin, which are important lipid metabolism regulators. G0s2 has been proven to bind and inhibit ATGL activity and thus decreasing lipolysis level. GRP81 has recently been identified as a lactate sensor. Upon the ligand binding it blocks β-AR signalling, inhibiting lipolysis. Thus, the *G0s2* and *Gpr81* expression reduction found here, could contribute to an increase in the induced lipolysis. Furthermore, in TET-off Nur77 3T3-L1 cells *Gpr81* and *Ap2* expression was lowered upon the *Nur77* overexpression.

a)



b)

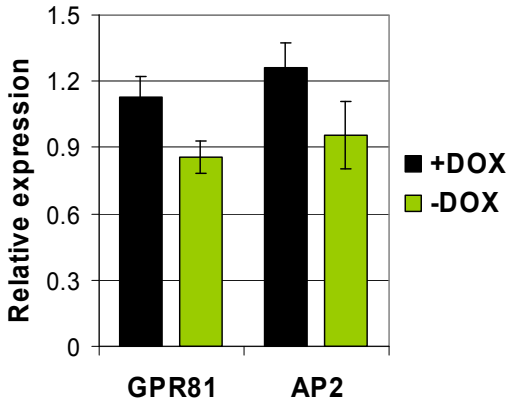


Figure 7 *Nur77* influcences PPARγ target genes expression. RNA extracted from WAT of fasted wild type and *Nur77-/-* mice (a) as well as *Nur77* overexpressing and wt 3T3-L1 mature adipocytes (b) was submitted to reverse transcription. Gene expression was assayed by RT-PCR; n=7, p≤0.05 *, p≤0.00* * *.

3.6 *Nur77* is recruited to the *Pparγ2* promoter and represses its activity

Pparγ2 promoter analysis for potential Nur77 binding sites with a Nur77 binding response element (NBRE) position weight matrix (PWM) revealed five NBRE like sequences: at -600 bp, -906 bp, -1688 bp, -2181 bp, -3490 bp from the transcription start site (TSS, Figure 8). Interestingly, the NBRE at -600 bp was found to be conserved across species, including humans. In an effort to examine if NUR77 is recruited to the *Pparγ2* promoter Chromatin Immunoprecipitation with RT-PCR read-out (ChIP-qRT-PCR) was assayed. The experiment was performed using 3T3-L1 cells stably overexpressing *Nur77*. The predicted NBREs at -600 bp from TSS showed a significant signal enrichment compared to a non-NBRE *Pai-1* sequence and emerged as being a NUR77 affected site. Similarly, binding at -900 bp could be detected. Nevertheless, the signal appeared weaker when compared to the -600 site. No significant enhanced signal was detected at the other predicted sequences. The experiment proved that NUR77 is recruited to the *Pparγ2* promoter.

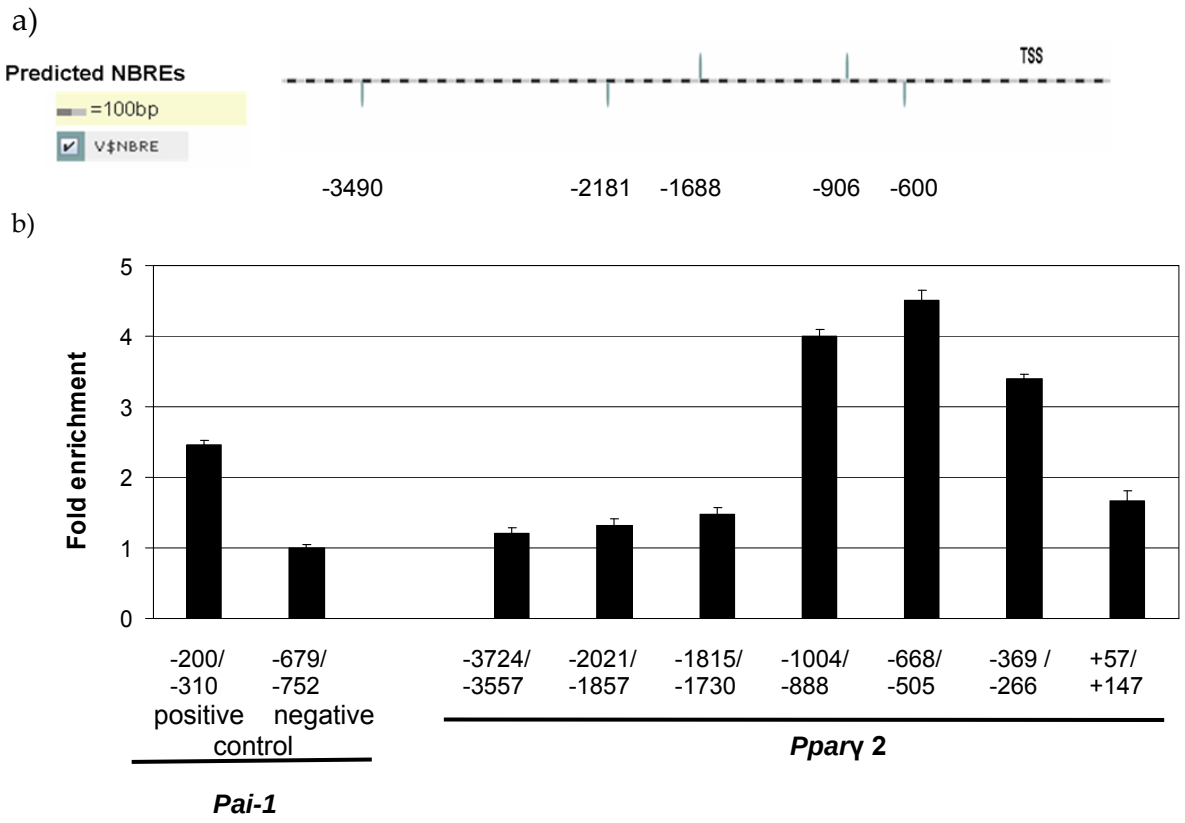


Figure 8 NUR77 is recruited to the *Pparγ2* promoter. a) Position of five predicted *Nur77* binding response elements on the *Pparγ2* promoter; b) results of ChIP-qRT-PCR on nuclear extract from 3T3-L1 cells, stably expressing *Nur77*, with an antibody against *Nur77*. A fold enrichment was calculated relative to a non-NBRE site of *Pai-1* promoter. The numbers indicate detectors position. Detector for known NBRE in *Pai-1* promoter served as a positive control. All amplicons were normalized to the S18 gene with total input as a reference; n=6.

In order to investigate whether the NUR77 recruitment affects the PPARγ2 promoter activity, luciferase reporter gene assays were performed. For the experiment NIH-3T3 preadipocytes transfected with luciferase expression vectors containing four different length *Pparγ*2 promoter fragments were used (Figure 9 a). Most of the examined fragments comprise sequence known to be CEBPα biding site (Clarke et al., 1997). For that reason a *Cebpα* expressing plasmid served as a positive control in the relevant assays. Its addition resulted in an elevated luciferase activity ratio validating the functionality of the experiment. Titration with a *Nur77* expressing plasmid in the presence the reporter construct (pGL4.21) containing the -1078bp to +62bp fragment of the *Pparγ*2 promoter led to a reduction of luciferase activity showing that NUR77 attenuates the activity of the promotor (Figure 9 b). Nevertheless, a fragment containing only the -906bp NBRE did not show any signal (Figure 9 c). Other vectors containing three (Figure 9 d) and all four (Figure 9 e) of the predicted binding sites also showed an induction by *Cebpα* but a milder decrease in luciferase signal upon a *Nur77* titration. The results show that, the inhibitory activity of *Nur77* did not increase with a growing promoter length.

Subsequently, cytosporone B was applied to examine whether *Nur77* activity changes would influence the activity of the promoter. NIH-3T3 cells incubated with an increasing concentration of cytosporone B exhibited a gradual decreased luciferase signal (Figure 9). Thereby suggesting *Nur77* mediated cytosporone B inhibition of the promoter activity.

To further explore the observed occurrence NIH-3T3 cells were transfected with a vector containing the *Nur77* dominant negative (*Nur77*DN) version of the receptor. In contrary to the full length receptor, the truncated version of *Nur77* does not exhibit repressive activity on the *Pparγ*2 promoter.

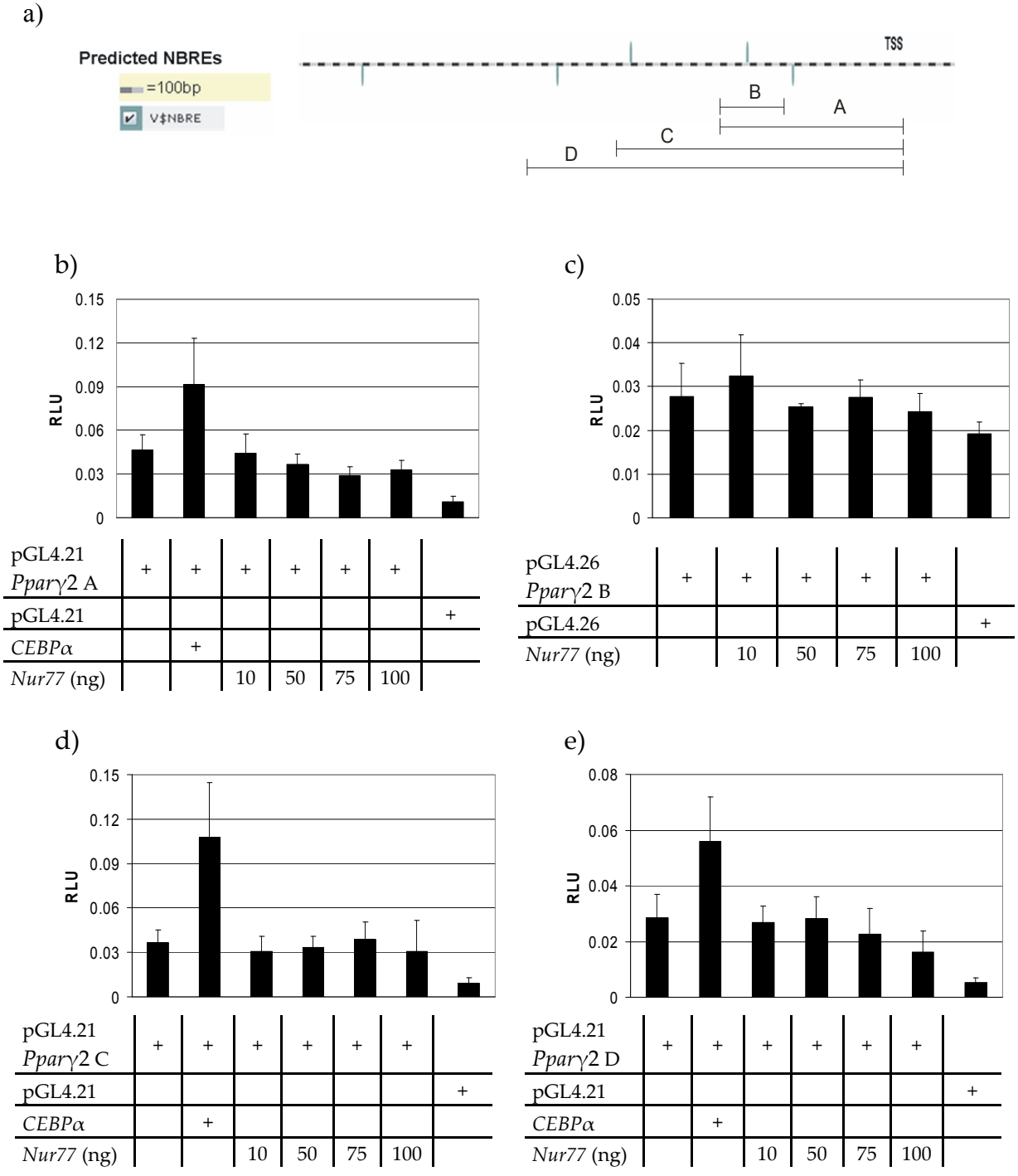


Figure 9 *Nur77* represses the *Pparγ2* promoter activity. a) The graph presents examined fragments of *Pparγ2* promoter. The luciferase assay was performed for *Pparγ2* promoter fragements containing predicted NBREs: -1078/+62 (b), -1078/-768 (c), -1860/+62 (d), -2327/+62 (e). *Cebpα* was employed as a positive control. A *Nur77* expressing plasmid amount was titrated as indicated in the table below the pictures. Renilla expression vector was used as a transfection efficiency control. n≥3.

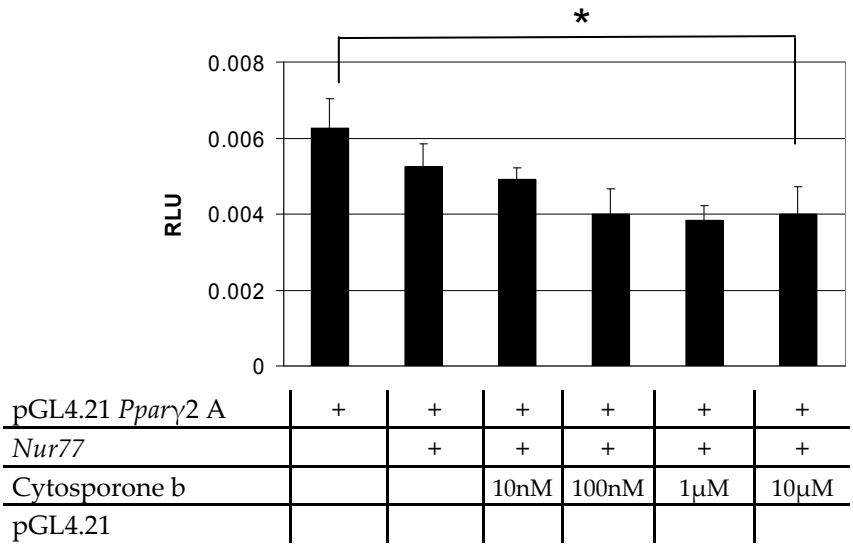


Figure 10 Cytosporone B reduces activity of the *Pparγ*2 promoter. The luciferase assay was carried out by a transfection of -1078/+62 *Pparγ*2 promoter fragment (A) in the presence of 75 ng of a *Nur77* expressing plasmid. Medium applied after the transfection contained depicted cytosporone B concentrations. Renilla expression vector was employed as a transfection efficiency control. n=3; p≤0.05*.

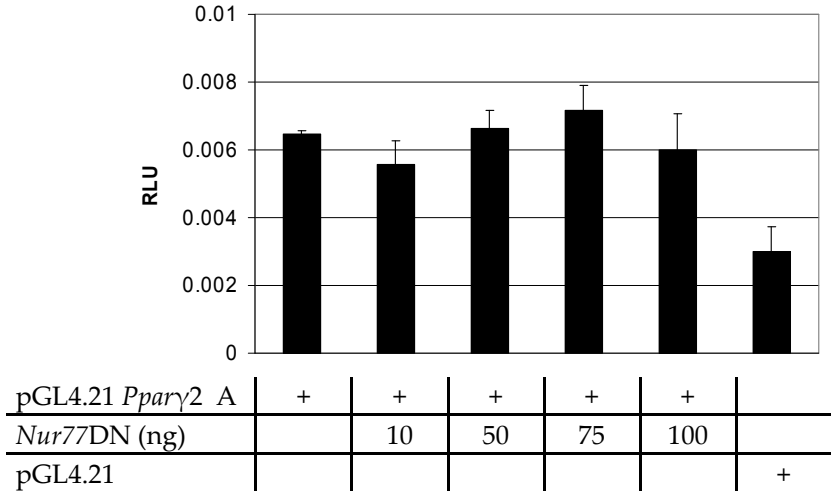


Figure 11 *Nur77*DN does not repress *PPAR* promoter activity. Luciferase assay was accomplished cotransfecting -1078/+62 *PPARγ*2 promoter fragment (A) in pGL4.21 vector with depicted amounts of *Nur77*DN expressing plasmid. Renilla expression vector was used as a transfection efficiency control.

For the reason that a *Cebpα* binding site lies at -340 bp fromTSS on the *Pparγ*2 promoter, in the proximity to the discovered NBRE a possibility that the two nuclear receptors influence each others binding to the promoter was examined (Clarke et al., 1997). To elucidate that issue luciferase/renilla ratio of -1078/+62 promoter fragment incubated with *Cebpα* was compared with the same sequence simultaneously transfected with *Cebpα* and *Nur77* (Figure 12). Consistently *Cebpα* enhanced the promoter activation but there was no impact of the *Nur77* presence detected.

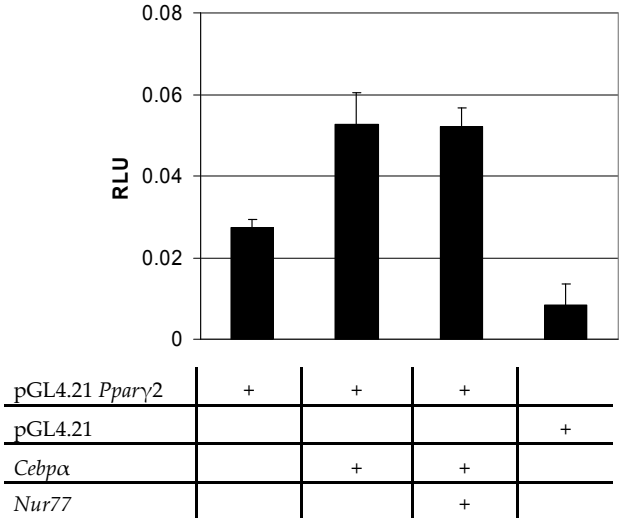


Figure 12 *Nur77* competes with *Cebpα* for biding site on *Pparγ2* promoter. NIH-3T3 fibroblasts were transfected with luciferase reporter vector containing -1078/+62 *PPARγ2* promoter fragment and *Cebpα* or *Cebpα* with 75ng *Nur77* expression vectors. Renilla expression vector was used as a transfection efficiency control.

3.7 Metabolic implications of NUR77 in WAT

To determine whether NUR77 influences WAT lipid metabolism series of metabolite detection assays were employed. For the in vitro experiment 3T3-L1s cell with silenced *Nur77* expression (Si) and control cells (Si-C) as well as the 3T3-L1 cells with inducible *Nur77* overexpression and a proper control were utilised. Mature adipocytes were incubated with isoprenaline to induce β-AR mediated lipolysis. Cell culture medium was collected at five time points and assayed for glycerol concentration measurement. There was no difference detected between 3T3-L1 adipocytes with silenced *Nur77* expression and the control cells through all the time points (Figure 13a). The subsequent comparison of a glycerol level in medium of the cells overexpressing *Nur77* and the control cells also did not demonstrate a significant variance between the samples (Figure 13b).

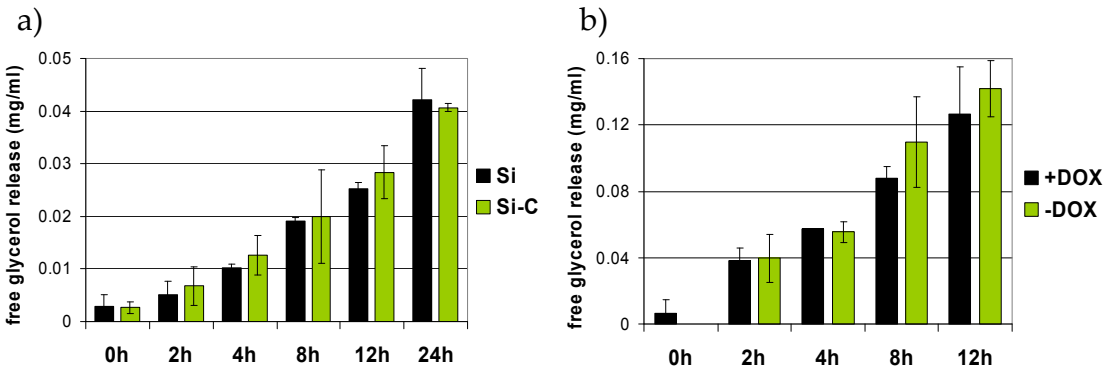


Figure 13 Silencing and overexpression of *Nur77* does not influence a free glycerol release level. 3T3-L1 mature adipocytes stably transfected with *Nur77* silencing (Si) and control construct (Si-C, a) as well as cells overexpressing *Nur77* (-DOX) and its control (+DOX; b) were induced with 1μM isoprenaline in low glucose, phenol red free medium. The medium was harvested at depicted time points and assayed for a free glycerol level.

Triglyceride (TG) and free fatty acid (FFA) levels were also measured in mouse serum. Upon fasting there was, as expected, due to activated lipolysis, an increased TG and FFA level detected (Figure 14 a, b). Nevertheless, there was no significant difference between the wild type and *Nur77*-/- mice in the serum metabolites concentrations (Figure 14 a, b). Also between the fasted wt and the *Nur77*-/- mice treated with cytosporone B and DMSO the TG and FFA levels were comparable (Figure 14 c, d). Interestingly, there was a difference between samples treated with DMSO and cytosporone B. The presented results show that in the analysed metabolic situations *Nur77* does not influence the FFA and TG serum level.

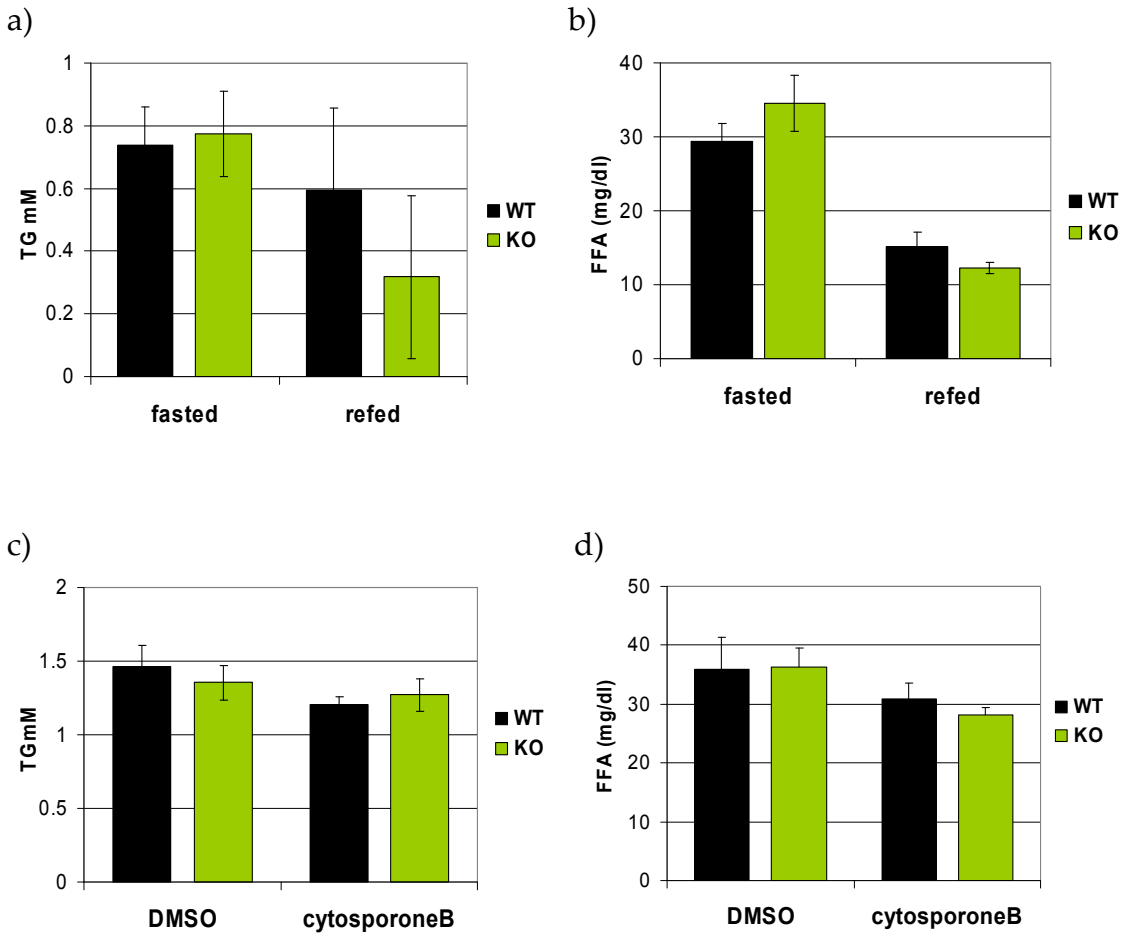


Figure 14 *Nur77* knockout or activity stimulation does not influence triglycerides (TG) and free fatty acid (FFA) serum levels. a) *Nur77*-/- (ko) and wild type (wt) mice were taken the food away in the evening and scarified the next day (fasted) or after 8 h refeeding (refed). fasted n≥6, refed n=3; b) HFD fed *Nur77*-/- and wt mice injected with cytosporone B or DMSO as a control were fasted overnight and sacrifed. Serum was harvested and submitted TG and FFA concentration measturment. *Nur77*-/- n≥4, wt n≥6.

Even though *Nur77*-dependent changes in the lipid metabolites concentration were not noticed there were other indications suggesting that the nuclear receptor influences energy homeostasis. Mature adipocyte cultures were observed to exhibit variable medium colour depending on the *Nur77* expression. The variability resulted from differences in pH which were mediated to the medium colour by the pH indicator, phenol red, added to the solution. Medium of activated TET-off *Nur77* cells turns yellow much faster than medium of the control cells indicating lower pH (Figure 15a). Consistently, in the case of cells stably transfected with *Nur77*DN, medium stayed redder (Figure 15b). The noticed discrepancy in pH suggests that *Nur77* increased metabolic rate. Metabolites concentration measurement in

the medium showed that the cells overexpressing *Nur77* produce more lactate, probably resulting from a more intensive glucose metabolism (Figure 16).

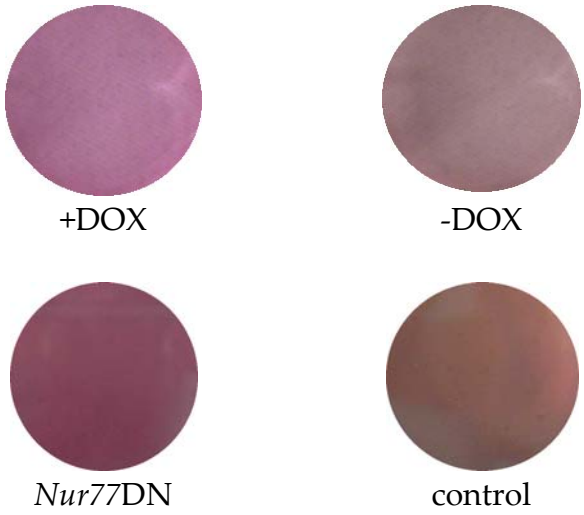


Figure 15 Cell culture medium colour changes upon overexpression of *Nur77* or *Nur77*DN. Mature adipocytes overexpressing *Nur77* (+DOX) and control cells (-DOX) as well as expressing *Nur77*DN and its control cells were maintained in high glucose, phenol red supplemented DMEM medium.

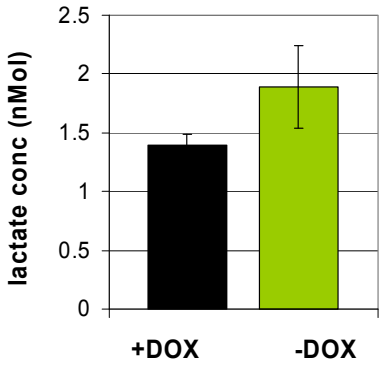


Figure 16 Lactate concentration in cell culture medium of *Nur77* overexpressing and control cells. Mature adipocytes overexpressing *Nur77* (+DOX) and control cells (-DOX) were maintained in high glucose DMEM medium. Medium was harvested and submitted lactate concentration measurement.

4 Discussion

White adipose tissue regulates whole body energy homeostasis by responding to nutrient intake, storing the energy excess and releasing it in demanding conditions like fasting or exercise. Additionally, it serves as an endocrine organ secreting adipokines, which have impact on multiple tissues. Therefore it is not surprising that WAT is tightly associated with the regulator PPARγ, known to influence metabolism and insulin response in an entire organism. Adipocytes apparently, constitute the main regulatory response target of the nuclear receptor as most of the gene expression changes displayed in *Pparγ*+/- phenotype in mice can be detected in WAT (Anghel et al., 2007). Selective activation of PPARγ in WAT is sufficient to improve insulin sensitivity as well as adipokine, inflammatory and lipid profiles (Sugii et al., 2009). The role of PPARγ in whole body metabolic homeostasis emphasizes its significance and underlines how crucial it is to learn about its activation mechanism in adipose tissue. Herewith presented results, indicate NUR77-dependent regulation of PPARγ2 expression in WAT. The discovery was confirmed by gene expression profiles analysis in several *in vivo* and *in vitro* models as well as by Chip-qRT-PCR and luciferase assays. Additionally, the shown data suggests a possible mechanism of NUR77 influence on fasted WAT metabolism.

Here demonstrated results show that *Nur77* expression is induced in WAT upon β-AR agonist treatment and in WAT of fasted mice. It has already been reported that *Nur77* level increases after β-AR stimulation in muscle and adipose tissue (Chao et al., 2007; Kanzleiter et al., 2005; Kanzleiter et al., 2009; Kumar et al., 2008; Maxwell et al., 2005). However, the upregulation *in vivo* upon fasting in WAT has never been presented before.

In order to gain an overview of global transcriptional changes in WAT during fasting gene expression profiling of WAT from fasted and refed wild type mice as well as β-AR stimulated adipocytes in culture was performed. The resulting list of regulated genes includes genes associated with glucose and lipid metabolism. The observed profiles of *Glut4*, *Pck1*, *Fas* and *Pparγ2* suggested inhibition of lipogenesis as well as modulation of glucose metabolism. In both, *in vivo* and *in vitro* models, the *Pparγ*2 level was downregulated in response to fasting and β-AR stimulation. PPARγ2 is a known lipogenesis stimulator however, its role concerning lipolysis remains controversial. This is due to the fact that the PPARγ agonists, rosiglitazone and pioglitazone, induce multiocularisation of adipocytes and increased expression of lipases ATGL, HSL and MGL. On the other hand, the ligands diminish the number of β-adrenergic receptors and increases expression of lipid droplet coating proteins like perilipin, S3-12, and Cidea, thereby limiting

the lipid droplet surface accessible to lipases (Bakopanos and Silva, 2000; Dalen et al., 2004; Kim et al., 2007; Koh et al., 2009; Puri et al., 2008; Viswakarma et al., 2007). Additionally, PPARγ2 has recently been reported to stimulate expression of GPR81 and G0s2, which are known lipolysis inhibitors (Jeninga et al., 2009; Zandbergen et al., 2005; Zandbergen et al., 2005). Expression profiles presented here demonstrate that *Pparγ2* is downregulated during fasting, implying that it is unrequited to perform lipolysis.

Importantly, the measured *Pparγ2* mRNA levels in the applied *in vivo* and *in vitro* models were inverse to the measured *Nur77* expression levels. Further investigation showed that in WAT of fasted *Nur77*-/- mice the mRNA levels of PPARγ2 target genes: *Ap2*, adiponectin, leptin, *Gpr81,* and *G0s2,* was elevated compared to fasted wild type siblings. Similarly, in mature 3T3-L1 adipocytes *Gpr81* and *Ap2* expression was diminished upon *Nur77* overexpression. This regulation may have metabolic implications as AP2, adiponectin and leptin are important lipid turnover modulators. Interestingly, published results from Chao et al. show that in mature adipocytes in culture, maintained in high glucose medium, no influence of *Nur77* overexpression on *Ap2* level was observed, indicating that the NUR77-dependent *Ap2* regulation is dependent on physiological context (Chao et al., 2008).

GPR81 has recently been identified as a lactate sensor, which upon activation blocks β-AR signalling, inhibiting lipolysis (Jeninga et al., 2009). G0s2 has been proven to bind and deplete ATGL activity (Zandbergen et al., 2005). Thus, here presented, NUR77-dependent *G0s2* and *Gpr81* expression modulation could contribute to increase in the induced lipolysis level.

Despite the observed NUR77-dependent regulation of metabolism associated genes, in the study presented here, no changes in lipid metabolism were detected upon *Nur77* silencing or overexpression. There were no differences in the FFA, TG and free glycerol concentration in conditioned media from the cell culture experiments nor in blood sera from the *Nur77*-/- and wild-type mice. Notably, this is consistent with reported data from Chao et al. showing no difference in glycerol release between isoprenaline stimulated wt and *Nur77*DN expressing mature adipocytes in culture (Chao et al., 2008). It is important to mention that a NUR77-mediated impact on lipid catabolism has never been reported in adipose tissue. What is known is that its expression rises in WAT in situations where also lipolysis is induced. It has only once been demonstrated that *Nur77* silencing decreases glycerol release but that experiments was performed using C2C12 muscle cells (Maxwell et al., 2005). The possible explanation is that NUR77 stimulates lipolysis as well as induces increased metabolite turnover. The hypothesis correlates well with an experiment proving that the *Nur77* overexpressing cells produce more lactate than the wt cells, probably resulting from more intensive glycolysis. That effect was confirmed by a publication describing increased acidification of cell culture medium of *Nur77* overexpressing C2C12 cells (Chao et al., 2009).

Hence, the glycerol released during lipolysis could be directed to glycolysis and metabolised. A previous publication concerning *Nur77-/-* mice indicates that the animals have downregulated expression of peroxisomal oxidation enzymes (Chao et al., 2009). The NUR77-regulated oxidation could therefore, serve as a way to remove remaining FA. Additionally, the same report indicates that *Nur77-/-* mice exhibit decreased energy expenditure which further confirms the presented hypothesis. According to the mentioned results, a model of Nur77's influence in fasted WAT can be proposed (Figure 17). Nur77 stimulates lipolysis, by regulation of, among others, G0s2 and GPR81. It affects degradation of lipid metabolites by increasing the FFA oxidation and directing glycerol to the glycolysis pathway. Additionally, it upregulates the glycolytic rate which results in an elevated lactate release. Furthermore, NUR77 has been described to stimulate gluconeogenesis in liver which results in increased glycerol and lactate clearance (Pei et al., 2006b). The only controversy in the presented model is the fact that lactate may stimulate GPR81 activity leading to β-AR signalling inhibition. Nevertheless, NUR77 is known to downregulate also GPR81 expression.

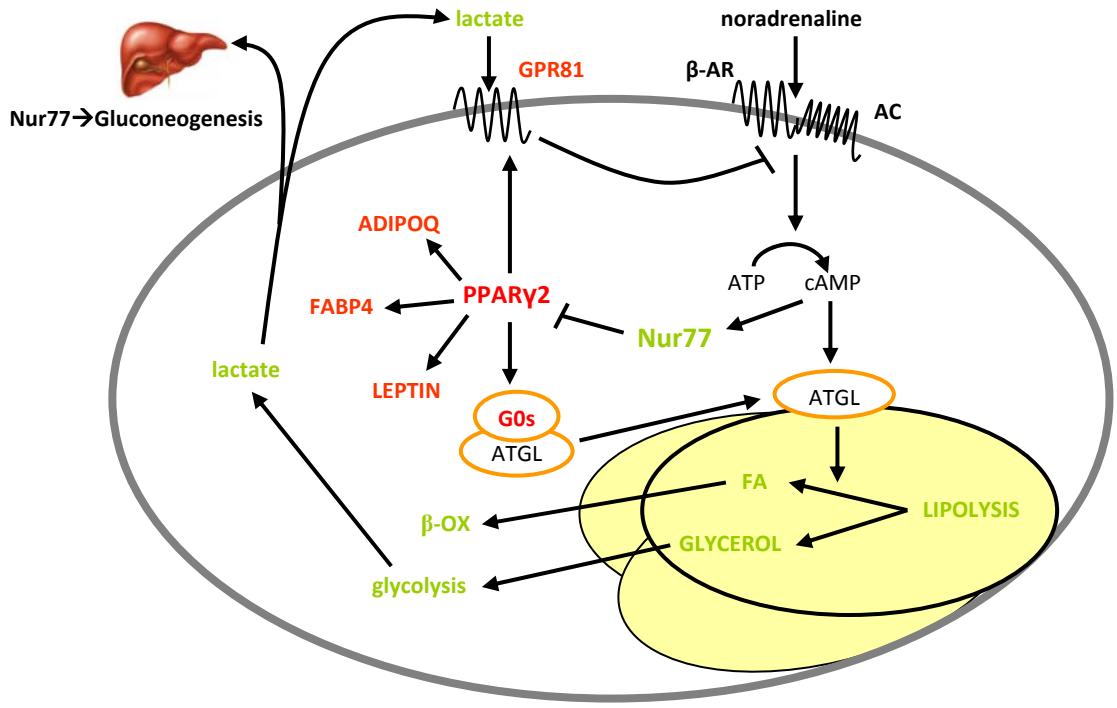


Figure 17 Proposed mechanism of *Nur77* influence on lipid metabolism in WAT. Green-upregulared, red-downregulated

On the other hand, most of the published research results concerning role of *Nur77* in the liver and the muscle metabolism associate Nur77 with carbohydrate turnover. Some of the reported studies on Nur77 in metabolism fail to find a correlation to lipid metabolism and conclude that glucose metabolism remains the main target of Nur77-dependent regulation (Chao et al., 2007; Chao et al., 2007; Kanzleiter et al., 2010; Pei et al., 2006b). Presented here results support the hypothesis indicating that NUR77 modulates glucose metabolism.

The most important finding of the presented work is NUR77-dependent PPARγ2 deregulation. The results indicate that an increase in *Nur77* expression resulted in a *Pparγ2* mRNA level reduction. Consistently, in WAT from fasted *Nur77-/-* mice, where NUR77 is absent *Pparγ2* expression is not reduced. This regulatory relationship is further supported by the observation that in WAT from fasted HFD-fed mice, where the fasting-induced increase in *Nur77* expression is diminished, the *Pparγ2* expression level is not significantly different in the *Nur77-/-* compared to wt mice. This result is consistent with the here presented results showing that *Nur77* induction upon fasting in HDF mice is lower than in chow fed mice as well as with the published fact that *Nur77* expression is reduced in obese and insulin resistant individuals (Fu et al., 2007; Kanzleiter et al., 2009; Kanzleiter et al., 2010). Chao et al. described study, performed in mature adipocytes in culture in fed state, showing that there is no correlation between mRNA levels of the two nuclear receptors (Chao et al., 2008). This result, again, suggests that the regulation is strongly dependent on metabolic situation. In several other reports a reverse expression pattern of the two receptors has been presented. The analysis of circadian clock-linked expression of nuclear receptors showed that in WAT *Nur77* expression peaks at 4 am and is downregulated afterwards. That is followed by a *Pparγ* mRNA level increase which reaches its highest level at 4 pm. Similarly, in the liver *Nur77* peaks at 4 am and *Pparγ* at 8 am(Yang et al., 2006). Likewise, upon dietary restriction in muscle, liver and brain the pattern of occurrence of *Nur77* and *Pparγ* is reverse (Oita et al., 2009). The mentioned publications confirm antagonistic expression profiles for the two nuclear receptors and indicate that this takes place in many tissues, what implies that the controlling pattern discovered in WAT may be applicable to other organs. Additionally, many indirect opposite regulatory mechanisms and processes have been associated with these factors. NUR77 has been described to inhibit PPARγ/RXR complex mediated induction of *Ucp1* in BAT (Kanzleiter et al., 2005). Additionally, these receptors are known to play opposite roles in regulation of metabolism. NUR77 inhibits lipogenesis, stimulates lipolysis and gluconeogenesis. PPARγ2 on the, contrary, upregulates lipid and decreases glucose synthesis.

Here presented results show that there was no difference between the wild type and Nur77-/- mice on HFD with respect to whole body, liver and

WAT mass. Likewise, the body weight changes upon fasting were comparable in the knockout and the wild type animals. Chao and colleagues noticed that mice kept on HFD from birth exhibited body mass deviation between *Nur77-/-* and wild type group. The animals display increased lipid and cholesterol accumulation in the liver which affects weight of the organ (Chao et al., 2009). The discrepancy, concerning mice whole body and liver mass, between the study by Chao et al. and the results shown here probably result from differences in the experimental design. The mice applied here were, already adult, when they were put on HFD for 6 weeks. In the study by Chao et al. the mice were fed HFD for 3 months starting from birth.

Surprisingly, twice within this study Nur77-independent activity of cytosporone B was observed. First, it resulted in reduction of *Pparγ2* expression in WAT of *Nur77-/-* and wt mice. The ligand also influenced the FA and TG concentrations in mice sera. It thus appears that cytosporone B is not a unique NUR77 ligand but also influences metabolism by NUR77-independent action. The authors of the cytosporone B discovery reported that the compound also influences the activity of NOR1 but with very low efficiency (Zhan et al., 2008). Importantly, the results imply that also other members of the NR4A family may influence the expression of *Pparγ2*.

To sum up, the presented data show that *Nur77* modulates the expression of *Pparγ2* and its target genes in WAT. Results of gene expression profiling of WAT from *Nur77-/-* mice and 3T3-L1 TET-off *Nur77* adipocytes show NUR77-dependent regulation of genes involved in metabolic pathways, suggesting that NUR77 influences metabolism modulation during fasting. Nevertheless, the functionality of the discovered regulation, at metabolite level, remains to be revealed.

5 Materials and methods

5.1 Animal models

Nur77 knockout mice were kindly provided by M. Klingenberg. All animals were maintained on a regular light-dark cycle (10 h light, 14 h dark) and kept on a standard laboratory chow diet with free access to water. Mice tail tips samples were used for genotyping with the following primers: GTACTCCCAGGAAGTGACTG, CGGAATAGCTCTCCCCCTCC, CTCGTGCTTTACGGTATCGC. Tissue samples were harvested from 2h or 8h fed and over night or 24h fasted animals. For an obesity conditions experiment mice were fed with a high fat diet for 6 weeks. Within last 3 weeks of the diet the animals were regularly, twice a week, intraperitonealy injected with a cytosporone B solution. Both wild-type and *Nur77*-/- mice received 50mg/g body of the ligand dissolved in DMSO. The control mice obtained a DMSO injection. Before scarifying mice were submitted to an over night fasting.

5.2 Cell culture

3T3-L1 adipocytes were maintained in high glucose (4,5 g/L) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 33,7 μM pantothenic acid, 32μM biotin, 2 mM L-glutamin, 100 U/ml penicillin, 100 U/ml streptomycin (all from Invitrogen/Gibco) and normocin (InvoGen) under a humidified atmosphere of 5% CO_2 at 37°C. TET-off *Nur77* cells were additionally, constantly supplemented with 100 ng/ml doxycycline (Sigma). Preadipocytes were grown to 100% confluence and after two days differentiation was initiated. Adipogenesis was induced *via* an incubation with medium containing a standard DMI mix (1 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine (IBMX) and 1 μg/ml insulin; all from Sigma) along with 1 μM rosiglitazone (Alexis). After two days, the medium was changed leaving insulin and rosiglitazone in. Subsequently, the standard proliferation medium was used every other day. The cells were cultivated till 10-14 day after the differentiation induction. A day before harvesting, adipocytes were washed twice with PBS fallowed by a repeated medium change in an approximately 4 hours intervals, in order to remove the doxycycline. The control cells were subjected to the same procedure but obtaining doxycycline. The adipocytes were incubated over night in low glucose (1 g/L), phenol red free DMEM (Invitrogen/Gibco) with 3% BSA

(PPA) or low FFA BSA (Sigma). Subsequently, 1 μM isoprenaline (Sigma) was added to the medium. Samples were harvested 0h, 2h, 4h, 8h, and 12 h after for a RNA, protein and medium examination.
NIH 3T3-L1 and HEK 293FT cell were maintained in high glucose (4,5 g/L) DMEM supplemented with 2 mM L-glutamin, 100 U/ml penicillin, 100 U/ml streptomycin (all from Invitrogen/Gibco) and normocin (InvoGen) at 5% CO_2 and 37°C. NIH 3T3-L1 cells were cultured in 10% BCS (Sigma) and HEK 293FT in 10% FBS.

5.3 TET-off system

The TET-off system was purchased from Clontech. *Nur77* was coned, using EcoRI and BamHI, restriction sites, into pTRE-Tight plasmid with the Tet-responsive P_{tight} promoter. The successful cloning was confirmed by sequencing. pTRE-Tight-*Nur77* and TET-off plasmid together with a packaging mix were transfected in to HEK 293T cells using Lentiphos ™ HT (Clontech) according manufacturer's instructions. The cell culture medium with the viral particles was collected 48 hours later and used for a 3T3-L1 adipocytes transduction. Obtained TET-off Nur77 3T3-L1 cells were subjected to a puromycin (Sigma) and geneticin (Invitrogen/Gibco) selection. The *Nur77* expression was controlled by an addition or a removal of doxycycline (100 ng/ml) from the culture medium.

5.4 RNA isolation

Cells were collected, and mice tissues homogenized, in Trizol (Invitrogen). Total RNA was isolated according to the manufacturer's instructions. The quality of the RNA was examined using Agilent 2100 Bioanalyzer RNA assays (Agilent Technologies, Palo Alto, CA, USA).

5.5 RT-PCR

Quantitative real-time reverse transcriptase PCR (RT-PCR) was performed to a analyze target cDNA level. Superscript II and random primers were used for the reverse transcription step. The qPCR reaction was performed using 4,5ng cDNA, 200nM primer pairs and SybrGreen master mix (Invitrogen) in 18μl reaction volume on an ABI Prism 7000 sequence detection system (Applied Biosystems). PCR was carried according the program: 2 min at 50°C; 10 min at 95°C and then 40 cycles of 15 sec at 95°C and 1 min at 60°C followed by a melting curve analysis. Ubiquitously-expressed transcript (Uxt) was applied as a housekeeping gene for normalization. Data normalisation

(AnalyzeMiner) and analysis was performed using a qPCR data base https://rtpcr.genome.tugraz.at/rtpcr/ (Pabinger et al., 2009).

5.6 Microarrays

cDNA samples were labelled with cyanine dyes (Amersham), using an indirect labeling procedure, to be visualized on a microarray chip. In order to do that 10-20 μg of total RNA was reverse transcribed using SuperScript II, random hexamer primers and amino-allyl-dUTP in a nucleotide mix. Residual RNA was hydrolyzed and the cDNA was purified with QIAquick PCR purification kit (Qiagen). Cy3 or Cy5 ester was coupled to the integrated amino-allyl molecules and the remaining dye was washed away. Complementary DNA was dried in a vacuum centrifuge and restored in a hybridization buffer (50% formamide, 5x SSC, 0.1% SDS). The samples assigned to be co-hybridized on one slide were pooled and cot1-DNA and polyA-DNA (both Invitrogen) was added to reduce a non-specific sequences binding. Microarrays were incubated for 45 minutes at 42°C in a pre-hybridizing buffer (5x SSC, 10% SDS and 1% BSA) to block non-specific signals. cDNA samples were applied on the array surface and placed in Corning chambers. Hybridization was performed by incubation at 42°C over night. All hybridizations were performed with a reversed dye assignment (dyeswap). Afterwards the slides were washed with buffers of an increasing stringency and scanned at two wave lengths (635 nm and 532 nm) at 10μm resolution in an Axon GenePix 4000B scanner (Axon Instruments). The obtained TIFF images were subjected to filtering for low intensity, inhomogeneity and satured spots using GenePix Pro 4.1 (Axon Instruments). The results were normalized with the CARMAWeb (Rainer et al., 2006) using a global median sub grid normalization. The data was submitted modified T-test (Limma-package) Benjamini-Hochberg correction for small datasets (FDR<0.05). The obtained result files were subjected to cluster analyses with the Genesis software using SOM (Sturn et al., 2002). Pathway analyses were performed with the in house developed Pathway Explorer software (Mlecnik et al., 2005). The bibliography based analysis was carried out using Genomatix genome analyzer.

5.7 Chip-qRT-PCR

A *Pparγ*2 promoter scan, performed using Genomatix MatInspector with Nur77 binding response element (NBRE) position weight matrix (PWM), revealed five putative Nur77 binding sites. Chromatin Immunoprecipitation with RT-PCR read-out (ChIP-qRT-PCR) was carried out on nuclear extract

from 3T3-L1 cells, stably expressing Nur77, with an antibody against Nur77. Fold enrichment was calculated relative to a non-NBRE site of the *Pai-1* promoter. Detector for known NBRE in the *Pai-1* promoter served as a positive control. All amplicons were normalized to the S18 gene with total input as a reference.

5.8 Luciferase reporter assays

Pparγ promoter fragments were cloned in luciferase reporter vectors: pGl4.26 containing minimal promoter and pGl4.21 without minimal promoter (both from Promega). The successful cloning was confirmed by sequencing. NIH-3T3 cells were seeded 10000 cells/weel in 96-well plates. The next day transfection was carried out using MetafectenePro (Biontex) in ratio of 3:1 (µl MFP: µg DNA) fallowing the provider's instructions. One-hundred ng of luciferase reporter vectors and 25ng of each pCMX-*Pparγ*2, pCMX-Rxr*α* and pMSCV-*Cebpα* as well as a titration of pBABE-*Nur77* (10ng, 50ng, 75ng, 100ng) was used. Addition of empty pCMX, pMSCV and pBABE vectors was calculated to balance a promoter load and pBLUESCRIPT as fill DNA. As a control for varying transfection efficiencies Renilla reporter vector pGl4.75 (Promega) was cotransfected in all the experiments in a ratio of 1:100 to luciferase reporter vectors. After 48 hours the cells were lysed and assayed fallowing the Dual-luciferase assay system (Promega) protocol. Luminescence measurements were accomplished using an Orion II luminometer (Berthold). Relative Light Unit (RLU) pictures a ratio of firefly to renilla values. Cotransfected pCMX-*Pparγ*2 and pCMX-*Rxrα* expression vectors were kindly provided by M. Schupp.

5.9 Western Blot

Proteins were harvested by scraping cell in lysis buffer (50mM Tris-HCl, 10% Glycerol, 2,5% SDS, 10mM beta-Glycerophosphate, 10mM NaF, 100 mM Na-Orthovanadate) with 7 mM PMSF (Sigma) and PIC (Roche) after rinsing with ice-cold TBS. The collected solutions were incubated at 96°C for 5 minutes and Benzonase (Merck) treated for one hour at room temperature. The protein concentration was measured with the BCA protein assay kit (Pierce) according to the provider's protocol. The proteins (20-50 µg) were separated by electrophoresis on a 4-12% Bis-Tris Gel (NuPAGE, Invitrogen) and transferred onto nitrocellulose membranes (Pall) afterwards. The blots were blocked in 3% skim milk (Fluka) PBST (0,05% tween, AppliChem) and incubated with the antibody against NUR77 (M-210x, 1:1500, Santa Cruz), TFIIB (c18, 1:500, Santa Cruz), PPARγ2 (A3409A, 1:2000, abcam) over night at 4°C. For chemiluminescent detection horseradish peroxidase-conjugated anti-

rabbit and anti-mouse (both Dako) secondary antibodies and an ECL substrate (Pierce) were employed.

5.10 Metabolites assays

Glycerol assay was performed using Free Glycerol Reagent with glycerol standard solution (both from Sigma) according to the manufacturer's protocol. Fallowing the provider's description Triglycerides reagent (Thermo scientific) and NEFA C (Wako) were applied to determine trigliceride and free fatty acid concentration. Lactate concentration was determined using Lactate assay kit (BioVision) consistently with the producer's instructions.

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7 Publications and conference proceedings

Publications:

Comparative transcriptomics of human multipotent stem cells during adipogenesis and osteoblastogenesis. Scheideler M, Elabd C, Zaragosi LE, Chiellini C, Hackl H, Sanchez-Cabo F, Yadav S, Duszka K, Friedl G, Papak C, Prokesch A, Windhager R, Ailhaud G, Dani C, Amri EZ, Trajanoski. BMC Genomics. 2008 Jul 17;9:340.

Reconstruction of gene association network reveals a transmembrane protein targeted by *PPARγ* and required for adipogenesis. Bogner-Strauss J, Prokesch A, Sanchez-Cabo F, Rieder D, Duszka K, Krogsdam A, Hackl H, Di Camillo B, Lass A, Pinent M, Toffolo G, Cobelli C, Zechner R, Trajanoski Z. Cell Mol Life Sci. 2010 Jun 15.

Nur77-dependent Regulation of Genes associated with metabolically important Pathways in fasted white adipose Tissue. Duszka K, Krogsdam A, Bogner-Strauss J, Neuhold C, Prokesh A, Trajanoski Z. In preparation

Conference proceedings:

Nur77-dependent deregulation of *PPARγ*2 in fasted white adipose tissue. Duszka K, Krogsdam AM, Bogner-Strauss JG, Prokesch A, Trajanoski Z. Nuclear Receptors & Disease, CSHL, NY, USA, August 31-September 4 2010.

Nur77-dependent Regulation of Genes associated with metabolically important Pathways in fasted white adipose Tissue. Duszka K, Krogsdam A, Bogner-Strauss J, Trajanoski Z. 3rd International Symposium on Lipid and Membrane Biology, March 18-20, 2010, Graz, Austria

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9 Supplementary tables

EST	Ref Seq	Name	p value	BH	mean	median
AA016913		Unknown	0.041344	0.999816	-0.77019	-0.91801
AA407434		Unknown	0.02985	0.999816	1.024706	1.053519
AA407614		Unknown	0.001075	0.315854	-0.98321	-1.2279
AA407681		, Moderately similar to S12207 hypotheti	0.009028	0.999816	0.114959	0.091518
AA408153	XM_921983	PREDICTED: expressedequence AI464131, transcript variant 4 (AI464131)	0.02561	0.999816	0.23324	0.185576
AA409038	NM_133716	stromalembrane-associated protein 1-like (Smap1l)	0.038744	0.999816	0.736235	0.740786
AA409100		putative senescence-associated protein {	0.021226	0.999816	1.412344	1.438801
AI834768	NM_026950	OCIA domainontaining 2 (Ociad2)	0.015202	0.999816	0.956085	0.991133
AI835003	XM_918022	PREDICTED:imilar to Aspartate aminotransferase, mitochondrial precursor (Transaminase A) (Glutamate oxaloacetate transaminase 2) (LOC640847)	8.84E-05	0.053339	-1.61563	-1.70786
AI835784	NM_030750	sphingosine-1-phosphate phosphatase 1 (Sgpp1)	0.044792	0.999816	-0.783	-0.82222
AI836521		ABC transporter [Mus musculus]; ATP-bind	0.029121	0.999816	-0.89719	-1.01374
AI837042	NM_007470	apolipoprotein D (Apod)	0.002927	0.684471	-0.61138	-0.92792
AI837521	XM_918037	PREDICTED:nc-13 homolog C (C. elegans) (Unc13c)	0.005638	0.999816	1.124091	1.20218
AI837598		gag {provirus} [Mus musculus, MrV, Evi-2	1.44E-05	0.021211	-1.27642	-1.47044
AI837639		Unknown	0.031055	0.999816	-0.6919	-0.84191
AI837944		Unknown	0.044976	0.999816	1.187853	1.354585
AI838060	NM_024181	DnaJ (Hsp40) homolog,ubfamily C, member 10 (Dnajc10)	0.005114	0.999816	-1.10634	-1.34819
AI838490	NM_007467	amyloid beta (A4) precursor-like protein 1 (Aplp1)	0.033118	0.999816	0.167083	0.167083
AI838612	NM_008183	glutathione S-transferase,u 2 (Gstm2)	0.016669	0.999816	-0.84799	-0.91609
AI839328		Unknown	2.13E-05	0.026121	-1.6914	-1.98065
AI839411	NM_133949	prostate tumor over expressed gene 1 (Ptov1)	0.044883	0.999816	-0.73815	-0.75779

AI839572	NM_007952	protein disulfide isomerase associated 3 (Pdia3)	0.014813	0.999816	-0.56145	-0.56806
AI839842		mel	0.02202	0.999816	-0.40209	-0.48869
AI839913	NM_010762	myelin andymphocyte protein, T-cell differentiation protein (Mal)	0.044653	0.999816	-0.71617	-0.7273
AI840035		GP|12847111|dbj|BAB27441. putative {Mus	0.000186	0.092793	-1.26359	-1.42327
AI840560		Unknown	0.004208	0.876789	-1.01776	-0.98634
AI840671	NM_008963	prostaglandin D2ynthase (brain) (Ptgds)	4.72E-09	2.70E-05	-2.14202	-2.36295
AI840674	NM_025535	SAR1 gene homolog B (S.erevisiae) (Sar1b)	0.037252	0.999816	-0.39288	-0.41339
AI841111	NM_008529	lymphocyte antigen 6omplex, locus E (Ly6e)	0.03589	0.999816	-0.88757	-0.87093
AI841201	NM_178714	leucine rich repeat and fibronectin type III domainontaining 5 (Lrfn5)	0.016625	0.999816	-1.12559	-1.31849
AI841291	NM_009121	spermidine/spermine N1-acetyl transferase 1 (Sat1)	0.006403	0.999816	-0.83874	-0.85016
AI841308	NM_009721	ATPase, Na+/K+ transporting, beta 1 polypeptide (Atp1b1)	0.036789	0.999816	-0.45606	-0.58194
AI841346	NM_010358	glutathione S-transferase,u 1 (Gstm1)	0.025825	0.999816	-0.6408	-0.65909
AI841410		homologue to GP|12835837|dbj|BAB23383.	0.000635	0.228996	-1.03796	-1.09036
AI841412		Unknown	0.000132	0.07546	-1.17687	-1.26297
AI841771	NM_026859	MAF1 homolog (S.erevisiae) (Maf1)	0.007446	0.999816	-0.95761	-0.95767
AI841954	NM_173756	RIKENDNA 5830457H20 gene (5830457H20Rik)	7.40E-06	0.016949	-1.35697	-1.55872
AI841956		Unknown	1.48E-05	0.021211	-1.33148	-1.50773
AI842171	NM_010442	heme oxygenase (decycling) 1 (Hmox1)	0.017842	0.999816	-0.60784	-0.67536
AI842431	NM_001015046	GTPase activating RANGAP domain-like 4 (Garnl4)	0.036844	0.999816	-0.65862	-0.72877
AI842576	NM_008430	potassiumhannel, subfamily K, member 1 (Kcnk1)	0.000762	0.249647	-1.21222	-1.23578
AI843200	NM_007805	cytochrome b-561 (Cyb561)	0.010971	0.999816	-0.99035	-0.90518
AI844373		Unknown	0.001866	0.477742	-1.03868	-1.17708
AI844374		homologue to SP|Q93009|UBP7_HUMAN Ubiqu	0.03964	0.999816	-0.55972	-0.64405
AI844386		Unknown	0.036172	0.999816	-0.69199	-0.66804
AI844417		GP|13905234|gb|AAH06914.1 Similar to hy	0.026655	0.999816	-0.48283	-0.45298

AI844663	NM_145586	RIKENDNA 8430420C20 gene (8430420C20Rik)	0.037467	0.999816	-0.6788	-0.71078
AI844691	NM_012032	serine incorporator 3 (Serinc3)	0.047419	0.999816	-0.68477	-0.74515
AI844743		Unknown	0.02881	0.999816	-0.5131	-0.71893
AI844743		Unknown	0.047046	0.999816	-0.50146	-0.68092
AI845449	NM_145377 .2	Mus musculus tripartite motif-containing 41 (Trim41), mRNA	0.007904	0.999816	1.181904	1.492734
AI847977	NM_016883	proteosome (prosome,acropain) 26S subunit, non-ATPase, 10 (Psmd10)	0.025523	0.999816	-0.86145	-0.87266
AI848382	NM_146144	ubiquitinpecific peptdiase 1 (Usp1)	0.029973	0.999816	-0.94884	-0.99283
AI848386	NM_023456	neuropeptide Y (Npy)	0.000152	0.082871	-1.70961	-1.62987
AI849250	NM_013477	ATPase, H+ transporting,ysosomal V0 subunit D1 (Atp6v0d1)	0.039644	0.999816	-0.68116	-0.68026
AI849257	NM_007614	catenin (cadherin associated protein), beta 1 (Ctnnb1)	3.22E-09	2.70E-05	-2.1705	-2.29389
AI849649	NM_008161	glutathione peroxidase 3 (Gpx3)	0.003342	0.750845	-0.92413	-0.95605
AI849758	XM_901540	PREDICTED:icrotubule-associated protein 2, transcript variant 12 (Mtap2)	0.002373	0.56642	-0.98001	-0.98938
AI849795	NM_016773	nucleobindin 2 (Nucb2)	0.000174	0.090853	-1.26819	-1.40918
AI850208	XM_922867	PREDICTED: glutathione S-transferase,u 7, transcript variant 12 (Gstm7)	0.008812	0.999816	-0.8337	-0.95629
AI850209	NM_009569	zinc finger protein,ultitype 1 (Zfpm1)	0.0115	0.999816	-1.07878	-1.14981
AI850291	NM_008410	integralembrane protein 2B (Itm2b)	0.017913	0.999816	-0.78067	-0.89167
AI850306	NM_019511	receptor (calcitonin) activityodifying protein 3 (Ramp3)	8.89E-07	0.003395	-1.90048	-1.94248
AI850439	NM_148924	zinc finger protein 263 (Zfp263)	0.010808	0.999816	-0.80452	-0.88593
AI850656	NM_010413	histone deacetylase 6 (Hdac6)	0.003042	0.697188	-1.28658	-1.39194
AI850990	NM_009846	CD24a antigen (Cd24a)	0.000763	0.249647	-0.83443	-0.9315
AI851647	NM_080559	SH3 domain binding glutamic acid-rich protein-like 3 (Sh3bgrl3)	0.000846	0.267756	-1.33322	-1.53714
AI851928	NM_009902	claudin 3 (Cldn3)	0.03628	0.999816	-0.84281	-0.97829
AI851985		22 kDa neuronal tissue-enriched acidic p	0.009072	0.999816	-2.26739	-2.26739
AI852028	NM_009481	ubiquitinpecific peptidase 9, X chromosome (Usp9x)	0.0315	0.999816	-0.33019	-0.33082
AI852075	NM_153166	copine V (Cpne5)	0.016874	0.999816	-1.18451	-1.38238

AI852220	NM_172430	RIKENDNA 4930544G21 gene (4930544G21Rik)	0.020438	0.999816	-0.91604	-1.10741
AI853333	NM_172787	l(3)mbt-like 3 (Drosophila) (L3mbtl3)	0.024529	0.999816	-0.83778	-0.94461
AI853887	NM_019789	Kvhannel interacting protein 3, calsenilin (Kcnip3)	0.004461	0.89677	-0.74696	-0.79387
AI854274	NM_025824	basiceucine zipper and W2 domains 1 (Bzw1)	2.84E-05	0.029552	-1.60011	-1.92341
AI854302	NM_178811	tetratricopeptide repeat domain 15 (Ttc15)	0.006542	0.999816	-1.03122	-1.23813
AI854317	NM_178619	RIKENDNA 1810026J23 gene (1810026J23Rik)	0.045247	0.999816	-0.81943	-0.90532
AU015471	NM_025952	RIKENDNA 2610529C04 gene (2610529C04Rik)	0.024178	0.999816	-0.52732	-0.80125
AU018794		, Weakly similar to POL3_MOUSE Retroviru	0.020389	0.999816	-0.47524	-0.74428
AU019064	NM_007914	ets homologous factor (Ehf)	0.000334	0.152995	-1.57637	-1.68843
AU019213	NM_028060	solutearrier family 35, member F2 (Slc35f2)	1.10E-05	0.020945	-1.88925	-1.99815
AU019655	NM_023409	Niemann Pick type C2 (Npc2)	0.017467	0.999816	-0.89643	-1.0003
AU020016		Mus musculus 12 days embryo spinal gangl	0.046874	0.999816	-0.76783	-0.87657
AU020382		hypothetical protein 1 (rRNA external tr	0.012319	0.999816	1.113699	1.240375
AU020467	NM_172943 .4	Mus musculus alkB, alkylation repair homolog 5 (E. coli) (Alkbh5),	0.005447	0.999816	0.941474	0.949405
AU020776		, Weakly similar to RIKEN cDNA 5730493B1	0.025435	0.999816	0.096994	0.087759
AU022607		Unknown	0.015868	0.999816	-0.28077	-0.59899
AU022808		Unknown	0.00614	0.999816	-0.39215	-0.69293
AU023036		Unknown	0.005289	0.999816	-0.44868	-0.70741
AU023454		Unknown	0.001876	0.477742	-0.58215	-0.89896
AU040207		Unknown	0.000646	0.228996	-1.24623	-1.44281
AU040460	NM_028930	transmembranehannel-like gene family 5 (Tmc5)	6.79E-05	0.045744	-1.05095	-1.24294
AU040533	NM_178055	DnaJ (Hsp40) homolog,ubfamily B, member 10 (Dnajb10), transcript variant 2	0.031568	0.999816	-0.57982	-0.66188
AU040774		Unknown	0.001214	0.347858	-0.95694	-1.12323
AU042518	NM_008230	histidine decarboxylase (Hdc)	0.019302	0.999816	-0.41322	-0.79136
AU043432		Unknown	0.026306	0.999816	0.402335	0.384568
AU045081		, Moderately similar to RIKEN cDNA 57304	0.038792	0.999816	0.14539	0.14539
AU045358		Unknown	0.001715	0.456973	-0.96633	-1.00112
AU045358		Unknown	0.008514	0.999816	-0.84221	-0.83297

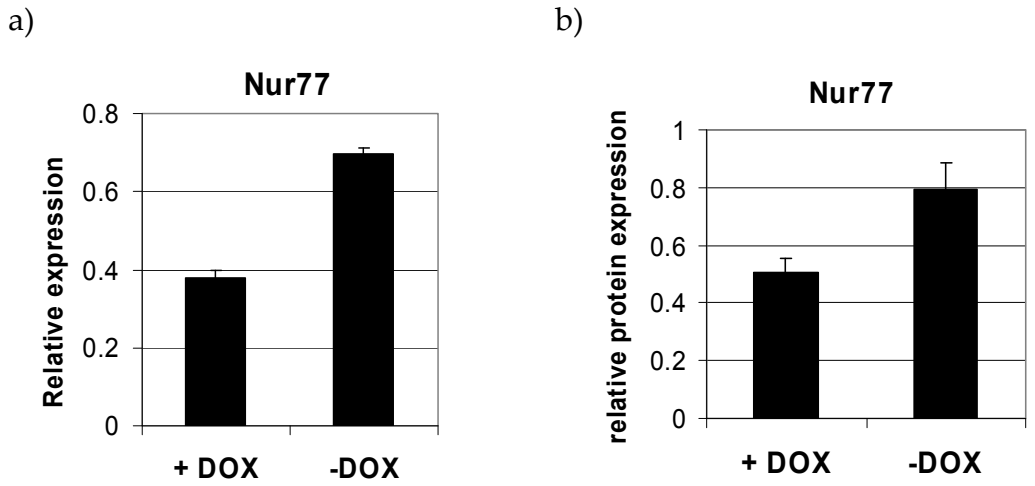
AW496303	NM_026524	Mid1 interacting protein 1 (gastrulationpecific G12-like (zebrafish)) (Mid1ip1)	0.019711	0.999816	-0.82577	-0.77841
AW536176	NM_026785	ubiquitin-conjugating enzyme E2C (Ube2c)	0.011351	0.999816	0.040936	0.040936
AW536222	NM_010330	embigin (Emb)	0.000471	0.192681	-1.09238	-1.08893
AW536719		RIKEN cDNA 7030407O06 gene	0.043342	0.999816	0.189569	0.197449
AW536807	NM_010807	MARCKS-like 1 (Marcksl1)	0.02006	0.999816	-0.78637	-0.94693
AW536834	NM_175541	melanoma associated antigen (mutated) 1-like 1 (Mum1l1)	5.89E-05	0.045005	-1.26982	-1.3403
AW537584	XM_894929	PREDICTED:imilar to keratin complex 2, basic, gene 8 (LOC628679)	0.02688	0.999816	-0.6613	-0.84939
AW537892		Unknown	0.000659	0.228996	-1.07998	-1.04681
AW538107	NM_010664	keratinomplex 1, acidic, gene 18 (Krt1-18)	0.00042	0.178376	-1.31809	-1.4356
AW539788	NM_008630	metallothionein 2 (Mt2)	0.0108	0.999816	-0.49817	-0.73784
AW541488	NM_009735	beta-2icroglobulin (B2m)	0.018191	0.999816	-0.36001	-0.35135
AW542449	NM_031170	keratinomplex 2, basic, gene 8 (Krt2-8)	0.035119	0.999816	-0.91494	-0.91494
AW543947	NM_030108	transmembrane protein 33 (Tmem33), transcript variant 2	0.013471	0.999816	-0.81144	-0.85309
AW544317	NM_011968	proteasome (prosome,acropain) subunit, alpha type 6 (Psma6)	0.03782	0.999816	-0.52355	-0.50333
AW544499	NM_009190	vacuolar proteinorting 4b (yeast) (Vps4b)	0.00229	0.558354	-1.39723	-1.45604
AW544601	NM_023054	charged amino acid richeucine zipper 1 (Crlz1)	0.036022	0.999816	0.172011	0.187207
AW544811	NM_013602	metallothionein 1 (Mt1)	0.000611	0.228996	-0.74789	-0.98616
AW545135	NM_133837	RIKENDNA G431001I09 gene (G431001I09Rik)	0.000929	0.280018	-1.2152	-1.29821
AW547143	NM_026078	phosphatidylinositol glycan,lassC (Pigc), transcript variant 1	0.037598	0.999816	-0.51274	-0.57253
AW549913		Mus musculus activated spleen cDNA, RIKE	0.017959	0.999816	0.010127	0.060164
AW553142		Unknown	0.001658	0.452244	-0.90336	-1.0242
AW553151		KIAA1513 protein {Homo sapiens}	0.008232	0.999816	-0.36451	-0.672
AW553155	NM_021296	GrpE-like 2,itochondrial (Grpel2), nuclear gene encoding mitochondrial protein	0.011024	0.999816	-0.38258	-0.67904
AW553157		Mus musculus 7 days embryo whole body cD	0.046491	0.999816	-0.414	-0.72727
AW553181		axonal-associated cell adhesion molecule	0.019848	0.999816	-0.39659	-0.75776
AW553207	NM_010097	SPARC-like 1 (mast9,	0.026592	0.999816	-0.33701	-0.62092

		hevin) (Sparcl1)				
AW553526	NM_008722	nucleophosmin 1 (Npm1)	0.022656	0.999816	-0.31	-0.394
AW554397	NM_009230	sterol O-acyltransferase 1 (Soat1)	0.033911	0.999816	-0.59637	-0.62587
AW555156	NM_026764	glutathione S-transferase,u 4 (Gstm4)	0.027639	0.999816	-0.93668	-1.03701
AW555539	NM_001081094.1	Zinc finger, HIT type 6 (Znhit6)	4.98E-05	0.040796	-1.18259	-1.33985
AW556216		data source:SPTR source key:Q9NSY6 evi	0.000214	0.102192	-1.3876	-1.67491
AW556218		RIKEN cDNA 2900076A13 gene	0.044405	0.999816	0.089475	0.118207
AW558377		Unknown	0.0198	0.999816	0.065141	0.065141
AW646959		Unknown	0.040496	0.999816	0.672614	0.09149
AW647169		Unknown	6.78E-05	0.045744	-1.28353	-1.54687
AW647181		spermidine/spermine N1-acetyltransferase	0.000356	0.156771	-0.96701	-0.9083
AW647524		24p3 protein;lipocalin;ORF	0.037188	0.999816	-0.27885	-0.51724
AW741846	NM_013634	peroxisome proliferator activated receptor binding protein (Pparbp), transcript variant 1	0.040483	0.999816	-0.60552	-0.60552
BE334798	NM_027852	retinoic acid receptor responder (tazarotene induced) 2 (Rarres2)	0.006442	0.999816	-0.8654	-0.93109
C76071	NM_019990	START domainontaining 10 (Stard10)	0.002283	0.558354	-0.7808	-0.71951
C76256		Receptor protein-tyrosine kinase erbB-3	0.024451	0.999816	-0.64735	-0.61255
C77432	NM_024460	RIKENDNA 2310016E02 gene (2310016E02Rik)	0.036038	0.999816	-0.37381	-0.34995
C78661		RIKEN cDNA 1200016E24 gene	0.003585	0.775092	-0.52029	-0.88861
C79532	NM_145529	cleavagetimulation factor, 3' pre-RNA, subunit 3 (Cstf3), transcript variant 1	0.028248	0.999816	-0.35389	-0.61499
C79711		Unknown	0.00401	0.851013	-0.46132	-0.75023
C79743		, Weakly similar to CAMP [Mus musculus]	0.049084	0.999816	0.061287	0.076108
C79821	NM_146149	cDNAequence BC026682 (BC026682)	0.050131	0.999816	0.123743	0.13855
C80205		Unknown	0.010747	0.999816	0.259449	0.311349
C85666		Unknown	0.017986	0.999816	-0.26899	-0.21558
C85725	NM_029013	RIKENDNA 4933428M03 gene (4933428M03Rik)	0.034552	0.999816	-0.41697	-0.63876
C86601		Unknown	0.027631	0.999816	-0.2584	-0.21946
C86610	NM_028058	FUN14 domainontaining 1 (Fundc1)	0.033476	0.999816	-0.43025	-0.77379
C86940		Unknown	0.007916	0.999816	-0.33619	-0.61914
C87015		Unknown	0.005496	0.999816	-0.40211	-0.67316
C87085		RIKEN cDNA A130040M12 gene	0.034668	0.999816	-1.00771	-1.00771

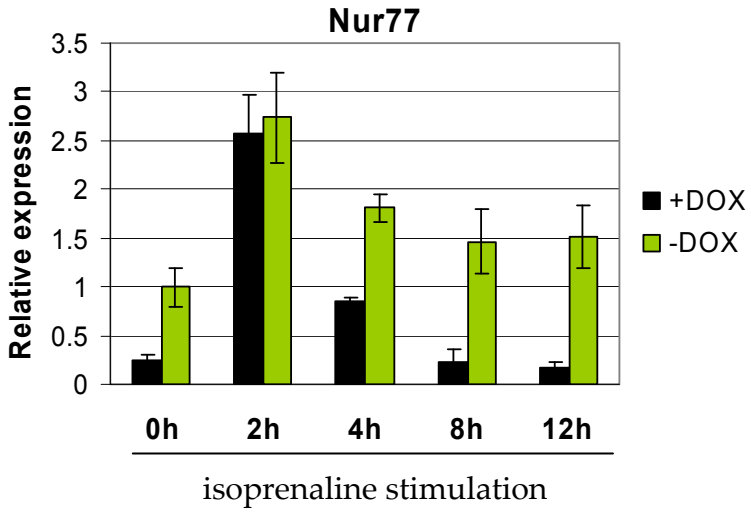
C87415	NM_009197	solutearrier family 16 (monocarboxylic acid transporters), member 2 (Slc16a2)	0.019735	0.999816	-0.79073	-0.83249
C87418	NM_194058	NACHT, LRR and PYDontaining protein 9b (Nalp9b)	0.03229	0.999816	-0.52	-0.82934

Supplementary table 1 List of differentially regulated genes in WAT of fasted Nur7-/- and wt mice. RNA of fasted *Nur77-/-* and wt mice WAT was extracted and used for cDNA synthesis. With the obtained material gene profiling was performed using high density microarray cDNA chips. The microarray read-outs were submitted normalisation using CARMAWeb with Benjamini-Hochberg (BH) correction (FDR<0.05) and extraction of significantly regulated genes ($p<0.05$). The table presents mean and median of four replicates.

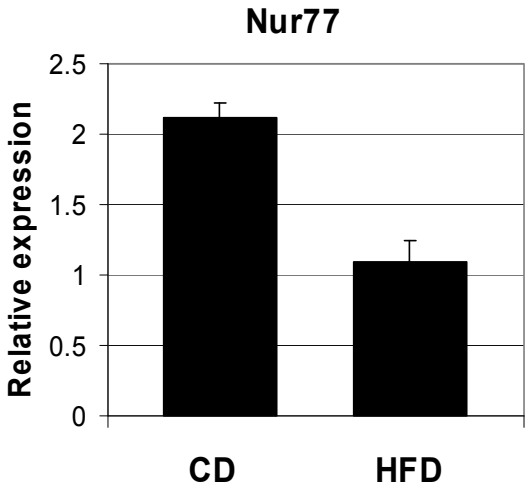
10 Supplementary figures



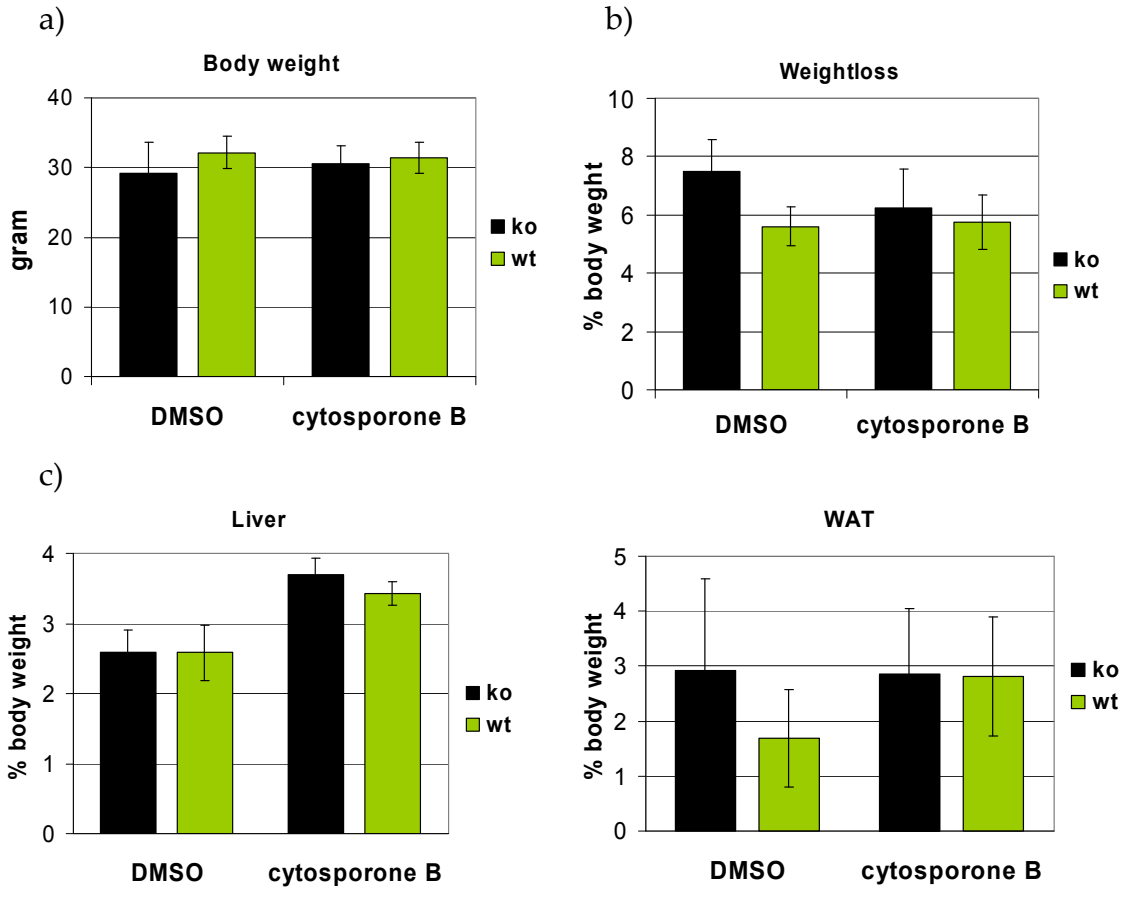
Supplementary figure 1 Nur77 expression level is conserved on RNA and protein level. *Nur77* mRNA level was measured with qPCR and protein with western blot for 3T3-L1 adipocytes overexpressing (-DOX), what allowed to overexpress *Nur7,* and control cells (+DOX). The obtained western blot picture was scanned and band intensity was measured with Gbox using GeneSnap and GeneTools software (SynGene).



Supplementary figure 2 *Nur77* expression profile after β-AR stimulation in wt and *Nur77* overexpressing cells. 3T3-L1 mature adipocytes transfected with TET-off *Nur77* expression system were incubated in medium with (+DOX) or without (-DOX) doxycycilne what allowed to overexpress *Nur77*. The cells were induced with 1μM isoprenaline and harvested at depicted time points. *Nur77* mRNA level was quantified with RT-PCR.



Supplementary figure 3 Comparison of *Nur77* expression induction in response to fasting in WAT of mice on chow and high fat diet. WAT was harvested from mice kept on chow diet or HFD for 6 weeks after overnight fasting. RNA level was quantified with RT-PCR.



Supplementary figure 4 *Nur77* knockout and wild type mice on HFD weight measurements. The animals were fed HFD for 6 weeks and during last 3 weeks they were injected with cytosporone B or a control solution (DMSO). The animals were taken away the food and scarified after an overnight fasting; a) a mice weight after 6 weeks of HFD; b) the weight change after overnight fasting c) liver weight d) WAT weight.