Modulation of the adipogenic master regulator PPARγ

Ismayil Taşdelen

Tasdelen, Ismayil Taşdelen
Modulation of the adipogenic master regulator PPARγ

ISBN: 978-90-8891-825-4

The printing of this thesis was financially supported by the UMC Utrecht

Cover design: Ismayil Taşdelen

Printed & Lay Out by: Ismayil Tasdelen & Proefschriftmaken.nl || Uitgeverij BOXPress

Published by:Uitgeverij BOXPress, 's-Hertogenbosch

Modulation of the adipogenic master regulator PPARγ

Modulatie van de adipogene meester regulator PPARγ
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 25 maart 2014 des middags te 12.45 uur

door

Ismayil Taşdelen

geboren op 25 oktober 1984 te Amsterdam

Promotor: Prof. dr. R. Berger

Co-promotor: Dr. E. Kalkhoven

Table of contents

Chapter 1

General Introduction

Obesity

Obesity is characterized by an increase in adipose tissue (AT) due to increased cell proliferation (hyperplasia) and increased cell size (hypertrophy) [1]. This metabolic disorder has been acknowledged by the WHO as a major public health problem since 1997 [2]. Since then, the obesity epidemic has been increasing steadily and as of 2008 more than 1.4 billion adults have been estimated to be overweight (http://www.who.int/topics/obesity/en/). Obesity has been linked to various diseases amongst which diabetes type 2 and cardiovascular diseases [3,4,5]. Due to its major implications on public health more extensive research has been performed in recent years to unravel the processes of weight gain and thus AT itself.

Adipose Tissue

For a long time, AT was seen as connective tissue with droplets of fat and would seemingly have a role in the prevention of heat loss and mechanical support for certain parts of the body [6]. Later studies showed that that AT had a more complex identity and versatile role in metabolic homeostasis. AT is dispersed over the entire body in different depots. The physiological roles of subcutaneous (sc) and visceral AT depots are best characterized. For example, fat stored in the visceral depots are known to be more harmful than storage under the skin [7]. It was already shown in 1956 that especially abdominal obesity led to higher mortality rates. [8]. Experiments in which sc AT were transplanted into abdominal cavity of mice led to a more beneficial metabolic profile than transplantation of visceral transplants to the subcutaneous region [9]. Strikingly, there even appears to be differences between different types of sc AT. Sc AT in the abdomen is associated with deleterious metabolic consequences in contrast to gluteal sc fat tissue [10].

Beside different depots, there are two different types of AT; brown and white AT. White adipose tissue (WAT) is composed of multiple cell types such as macrophages, fibroblasts, endothelial cells and white (pre)adipocytes [11,12]. WAT is involved in triacylglycerol (TAG) synthesis/storage and lipolysis, and is

highly regulated by hormones and other factors such as nutritional status and exercise. In the 'fed' state, excess fuel substrates are stored as TAG in WAT, which can subsequently be converted and released as free fatty acids (FFA) in times of energy demand. The classical view of WAT as being solely responsible for energy storage is starting to disappear. Studies have illustrated that the WAT is an endocrine and paracrine organ which can secrete hormone-like proteins, called adipokines, that are capable of influencing inflammation, blood pressure, angiogenesis, insulin sensitivity and the general lipid and glucose metabolism[12,13]. For example, WAT is among others capable of indirectly influencing the nervous system by releasing the adipokine leptin which promotes energy expenditure and has a repressive effect on food intake [14].

Whereas white adipocytes are able to store energy, the mitochondria-rich brown adipocytes predominantly dissipate energy in the form of heat via the respiratory chain [15]. In general, brown adipose tissue is predominantly present in infants and decreases gradually with aging [16]. However, exposure to cold induces expansion of BAT and activates it peaking in the supraclavicular region in healthy young men [17,18]. Recent investigations have revealed a novel type of adipocyte, the so-called beige adipocyte[19]. These adipocytes, like their white counterparts, have very little basal expression of UCP1 which is a hallmark protein of brown adipocytes. However, in contrast to white adipocytes, beige adipocytes can respond to cAMP stimulation leading to increased UCP1 and respiration rates. PRDM16, a protein that is mainly expressed in brown adipocytes, appears to play a crucial role in the transcriptional control of brown fat cell differentiation by activation of PGC-1α and PGC-1β[20]. Interestingly, overexpression of PRDM16 in white fat progenitors shifted the differentiation towards the brown fat phenotype. Moreover, a PGC-1α dependent myokine called irisin, is capable of stimulating brown-fat-like development of white fat and thermogenesis [21]. Mice studies have shown that even moderately increased levels of circulating irisin increases energy expenditure, reduces body weight and improves diet-induced insulin resistance. Subsequently, the beneficial effects of irisin in humans remain to be determined but are promising indeed.

Adipogenesis

In literature, adipogenesis is defined as the conversion of pre-adipocytes into full mature adipocytes and is a complex process in which proteins at different cellular levels are involved [22]. In 1999, it had been estimated that expression levels of approximately 300 proteins are altered during this morphogenic change, but since then more proteins have been found to play a role [23]. Since the process takes relatively long, some proteins have a role in the early differentiation whereas others have functions later on. In general differentiation comes in three waves; it first starts with the activation of very early pro-adipogenic transcription factors such as C/EBPβ, C/EBPδ, KLF5, CREB, SREB-1c and Krox20 (Figure 1) [24]. For C/EBPβ and C/EBPδ it was shown that already 4 hours after the induction of differentiation they had associated with genomic regions, called hotspots where master adipogenic regulator PPARγ would bind to 6 days later, revealing cross-talk between early and late transcription factors [25]. Moreover, C/EBPβ is binding to some of these hotspots even before differentiation is started. A plausible explanation for this interesting finding is that this way other early TFs such as GR and Stat5a will be able to bind more efficiently to the hotspots after adipogenic initiation. The PPARγ promotor region itself is also remodeled several hours after the start of differentiation and c-Fos and C/EBPβ seem to play a role in this process [26]. Eventually the early transcription factors will lead to the expression of the two most important proteins in adipogenesis, C/EBPα and PPARγ and these can then induce the genes that are necessary for proper differentiation. C/EBPα seems to be crucial for adipogenesis since inducible C/EBPα knockout mice display a significant decrease of WAT [27]. Global genome-wide occupation of C/EBPα and PPARγ has been extensively studied and there was a significant overlap [28]. C/EBPα increases PPARγ expression and vice versa. To go into more mechanistic detail, PPARγ is required to release the HDAC1 from the C/EBPα promotor so that C/EBPα can induce its expression [29]. In addition, C/EBPα cannot rescue adipogenesis in PPARγ knockout MEFs whereas the opposite is possible [30]. C/EBPα does seem to have a role in insulin sensitivity of adipocytes, since PPARγ could not rescue that feature in C/EBPα deficient cells [31,32]. However, in general PPARγ is the transcription factor that regulates most other lipid and glucose homeostasis genes which are required for lipid storage; hence earning the title of master regulator [33].

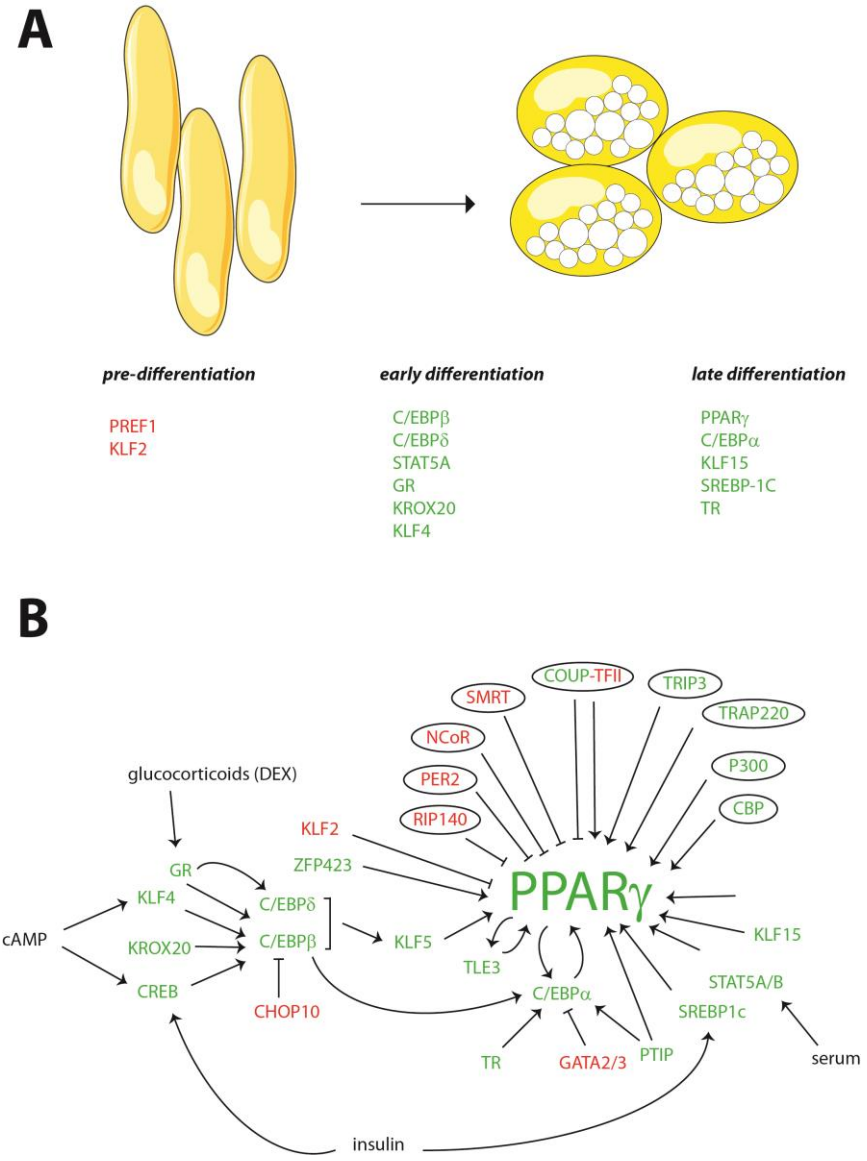


Figure 1 Selected regulators of adipogenesis. A. selected regulators of in vitro differentiation. Different factors which play a role prior to and during differentiation are depicted. Red factors are anti-adipogenic whereas green are pro-adipogenic. B. Selected signaling pathways that play a role in adipogenesis. Factors that are circled are direct co-regulators of PPARγ.

Both brown and white pre-adipocytes require the nuclear peroxisome proliferator-activated receptor γ (PPARγ) to be able to differentiate into adipocytes [22]. This essential role was established in an elegant assay in which forced expression of PPARγ in fibroblasts appeared to be sufficient to promote adipogenesis [34]. Moreover, PPARγ regulates many adipocyte genes that are necessary for lipid and glucose metabolism (Table 1). In vivo models show that PPARγ$^{-/-}$ mice are severely lipodystrophic, while PPARγ$^{+/-}$ mice have reduced amounts of adipose tissue [35,36,37,38]. PPARγ is also crucial for the maintenance of adipose tissue, since conditional knock-out of the *PPARg* gene resulted in reduced in vivo survival of mature adipocytes [39].To conclude, human Familial partial lipodystrophy subtype 3 (FPLD3, MIM604357) patients, harbouring heterozygous mutations in the PPARG gene, are characterized by aberrant fat distribution and metabolic disturbances, including insulin resistance and dyslipidemia [40]. Hence, the importance of PPARγ in adipose biology was firmly established.

PPAR structure and function

PPARγ is a member of the nuclear receptor PPAR family that consists of PPARα, PPARβ and PPARγ. These ligand inducible transcription factors were identified in rodents three decades ago and were named as such because of their ability to proliferate peroxisomes [41]. All PPARs consist of a ligand-independent activation domain (AF-1) at the N-terminus, adjacent to a centrally located DNA binding domain which consists of two zinc fingers (figure 2).

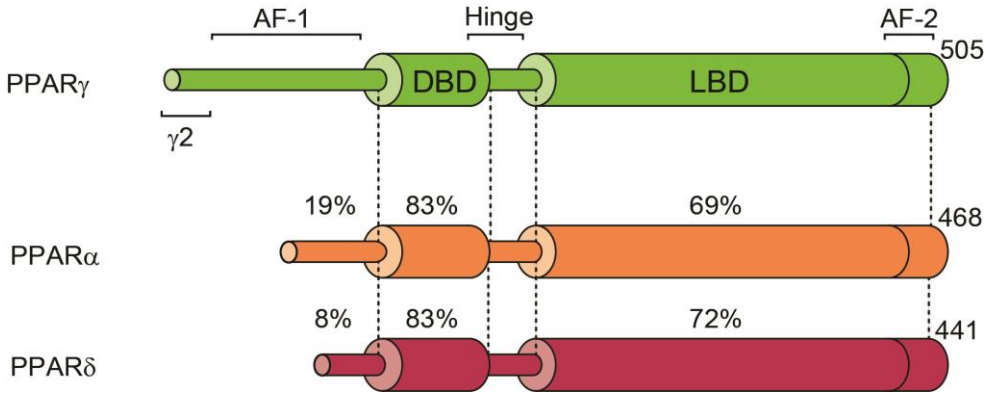


Figure 2 Comparison between different PPAR members. Indicated are the DNA binding domain (DBD) and ligand binding domain (LBD) and the two activation functions (AF-1 and AF-2). Homology is indicated in percentages.

Importantly, PPARs do not bind DNA as monomers, but rather as a dimer with RXRα. This complex binds to the so called PPAR responsive elements (PPRE) which in the case of PPARγ:RXR is called DR-1 and is a double hexamer sequence of AGGTCA separated by one nucleotide [42]. The inability of PPARs to bind to DNA as a monomer seems to be caused by the AF-1 domain, since deletion of this domain in the case of PPARα did allow the nuclear receptor to bind to DNA without RXR[43]. Next to the DBD-domain is the so-called hinge region which is spatially highly flexible and connects the DBD to the C-terminal, ligand dependent AF-2 domain. The AF-2 domain, which also embodies the ligand binding domain (LBD), is necessary for ligand binding, heterodimerisation with RXR and is the interaction site for most co-regulators through its LxxLL motif.

Recently, the crystal structure of PPARγ:RXR has been found [44] and strikingly the AF-1 domain displayed high flexibility even though it is to bind to other proteins for its role in gene regulation. Moreover, the PPARγ LBD is involved in binding to DNA by cooperating with both PPARγ DBD and RXR DBD, which explains why a mutation in that LBD results in overall decreased DNA binding capabilities of the complex[44].

Despite the PPARs displaying a high degree of homology in their amino acid sequence for the LBD and DBD, the AF-1 and hinge region are less well conserved. Therefore, the gene regulating profiles of these nuclear receptors are supposedly mainly dictated by their AF-1 domains [45]. Combining this fact with the different

cellular context in which the PPARs are exerting their physiological role, may explain the differences in target gene profiles. PPARα transcripts for example have been found in the liver, heart, kidney, skeletal muscle and the large intestine [43]. PPARα, responding to fibrates and unsaturated fatty acids, seems to be involved in fatty acid oxidation and has important roles in fasting since PPARα knock-out mice fail to cope with long term food deprivation [41,46]. PPARβ/ PPARδ have a relative high expression in the brain, AT and skin but are ubiquitously expressed in general [47]. Similar to PPARα, activation of PPARβ/δ in AT will lead to upregulation of genes that are required for fatty acid oxidation and energy dissipation [48]. Moreover, overexpression of PPARδ in muscle leads to increased production of type I muscle fibers and also induces genes implicated in oxidative metabolism [49,50]. Consequently, endurance training increases the amount of PPARδ protein in the muscle. Though PPARδ and PPARα seem to have similar roles, there are also differences. PPARδ can act as a plasma free fatty acid sensor in liver by positively regulating levels of lpin2 and st3gal5, whereas PPARα cannot [51].

PPARγ has two isoforms, PPARγ1 and PPARγ2, the latter having an extra 30 (or 28 in humans) amino acids at the N-terminus [52,53]. Whereas the PPARγ1 transcript is ubiquitously expressed, PPARγ2 seems to be exclusively present in AT. In adipocytes PPARγ2 seems to be the more potent activator of adipogenesis. Experiments have shown that ectopic expression of PPARγ1 in PPARγ knockout MEFs is capable of inducing adipogenesis, but in comparison PPARγ2 specific knockout animals had less AT and were also more insulin resistant [54,55]. As stated above, PPARγ is essential for adipogenesis and has a broad role in adipocyte biology. This is traced back to its role in the upregulation of a subset of genes that have roles in adipogenesis and adipocyte biology.

Target genes of PPARγ

Similar to other nuclear receptors, PPARγ is also capable of cell-specific gene regulation. For example, PPARγ is able to induce genes that are involved in growth regulatory pathways, epithelial cell maturation and immune modulation in colorectal cancer cells [56]. In adipocytes however, PPARγ has a more important role and is therefore called the master regulator. Moreover, PPARγ and C/EBPα

positively promote gene expression of themselves and of each other [57,58]. This will result in a strong positive forward loop in which PPARγ will directly induce expression of genes such as fabp4, Lpl, plin2, cd36, pepck, cidec, and others that have direct roles in lipid storage and accumulation and glucose metabolism (see table 1). To elaborate on this in more detail, Fabp4 for example is a lipid transporter, whereas perilipin is a lipid droplet binding protein and cd36 a fatty acid transporter. On the other hand, some genes are not directly involved in adipogenesis, but rather have a role in adipocyte biology. These genes are for example adiponectin and chemerin, which have effects on glucose and lipid homeostasis. Interestingly, some of the PPARγ target genes can sometimes also be capable of interacting with themselves after being expressed. An example of this mechanism involves G0S2 and ATGL. Both are PPARγ target genes, but in addition, G0S2 also binds to ATGL blocking the latter from exerting its TAG hydrolase activities [59,60,61].

PPARγ can both repress and activate target genes dependent on the presence of a ligand; in the case of a ligand it will usually activate genes. This is the case for the VLDL receptor for example, of which the expression is increased in 3T3-L1 adipocytes upon addition of rosiglitazone [62]. This is also the case with another target gene, glycerol kinase (gyk) of which expression is only elevated upon incubation with an exogenous ligand [63]. The mechanism behind this effect is that co-repressor complexes are recruited to unliganded PPARγ at the gyk promotor blocking gene transcription until liganded PPARγ will recruit co-activators. This is in contrast with the fabp4 promotor which is constititously associated with co-activators and consequently results in gene transcription without an exogenous ligand. These findings tell us that PPARγ function can be modulated in different ways and that these can interact with each other. Some of these include regulation of PPARγ protein levels by transcription factors, regulation of PPARγ activity by co-regulators, post-translational modifications (PTMs) and ligands.

Table 1: Selected target genes of PPARγ and their function.

Type	Gene	Function	Reference
Adipokine	ADIPOQ	Insulin sensitizing hormone	(Iwaki, et al. 2003)
Adipokine	RARRES2	Also known as chemerin, promotes MSC adipogenesis	(Muruganandan, Parlee et al. 2011)
Fatty acid release	LPL	Releases fatty acids from lipoprotein-bound triglycerides in the extracellular space	(Schoonjans, Peinado-Onsurbe et al. 1996)
Fatty acid release	ATGL	Hydrolysis of triglycerides	(Kershaw, Schupp et al. 2007)
Glucose transporter	GLUT4	Import of glucose	(Wu, Xie et al. 1998)
Lipid droplet formation	FABP4	Intracellular fatty acid transport	(Tontonoz, Hu et al. 1994)
Lipid droplet formation	CIDEC	Lipid droplet-associated protein, suppressing lipolysis, accumulation of triglycerides	(Kim, Cho et al. 2008)
Lipid droplet formation	PLIN1	Coats the lipid droplet protecting it from hormone sensitive lipase for lipolysis	(Arimura, Horiba et al. 2004)
Lipid droplet formation	S3-12	Lipid droplet associated protein	(Dalen, Schoonjans et al. 2004)
Lipid synthesis	ACSL1	Conversion (esterification) of free long-chain fatty acids into fatty acyl-CoA esters	(Schoonjans, Watanabe et al. 1995)
Lipid synthesis	ME1	Generation of NADPH for FFA biosynthesis by oxidative decarboxylation of malate to pyruvate	(Castelein, Gulick et al. 1994)
Lipid synthesis	PEPCK	Production of glycerol for storage of fatty acids as TGA	(Devine, Eubank et al. 1999)

Lipid synthesis	GYK	Produces glycerol-3-phosphate, the backbone for esterification of FFAs in the production of TGA	(Guan, Ishizuka et al. 2005)
Lipid uptake	FATP	Import of long-chain fatty acids	(Frohnert, Hui et al. 1999)
Lipid uptake	CD36	Import of long-chain fatty acids	(Motojima, Passilly et al. 1998)
Lipid uptake	ACBP	Intracellular lipid-binding protein that selectively binds medium and long chain acyl-CoA esters	(Helledie, Grontved et al. 2002)
Lipid uptake	AQP7	Import of glycerol for the formation of TAG	(Kishida K 2000)
Lipid uptake	VLDLR	VLDLR binds apolipoprotein E-triglyceride-rich (apoE-TG rich) lipoproteins and mediates lipid uptake	(Tao, Aakula et al. 2010)
Other	CAP	Protein that plays a role in insulin sensitization	(Ribon, et al. 1998)
Other	GPR81	Lactate receptor with anti-lipolytic properties	(Jeninga, Bugge et al. 2009)
Other	C/EBPα	TF that cooperates with PPARg for promoting adipogenesis through gene regulation	(Nielsen, Pedersen et al. 2008)
Other	GOS2	Unclear; however GOS2 most likely plays a role in growth arrest in mitotic clonal expansion	(Zandbergen, Mandard et al. 2005)
Other	NR1D1	Unclear, but plays a role in enhancing the adipogenic properties of PPARg	(Fontaine, Dubois et al. 2003)
Other	P18	Unclear; however p18 most likely plays a role in terminal differentiation	(Morrison and Farmer 1999)
Other	P21	Unclear; however p21 most likely plays a role in growth arrest	(Morrison and Farmer 1999)

PPARγ ligands

Endogenous ligands of PPARγ are supposedly prostaglandin J2 derivatives (15-deoxy-$\delta^{12,14}$-PGJ2), nitro-oleic acidin, hepoxilins and some polyunsaturated fatty acids [64,65,66]. Synthetic ligands also exist, and members of the class of thiazolidinediones have been used in the clinic to elevate insulin sensitivity in patients with type 2 diabetes [67]. The use of ligands is one way to affect PPARγ, but since it is expressed in multiple tissues such as colon, spleen, skeletal muscle , liver and heart, side effects could occur and a different strategy would be necessary [68]. Rosiglitazone for example, a potent ligand for PPARγ is associated with increased risk myocardial infarction and death from cardiovascular causes [69].

In general, ligand binding causes a structural conformational change of PPARγ prompting release of co-repressors such as NCoR and SMRT and giving rise to binding sites for co-activators such as CBP/p300 and SRC/p160 which further activate gene transcription. Interestingly, at the present time it is also known that ligands modulate PPARγ activity by PTMs such as phosphorylation, ubiquitilation and sumoylation [70]. Hence, these factors are somehow connected to each other which will be discussed further on.

Post-translational modifications of PPARγ

Recent work has shown that PPARγ activity can be modulated by post translational modifications such as phosphorylation, ubiquitilation and sumoylation (Figure 3). Serine 112 phosphorylation by ERK 1/2 or p38/JNK after stimulation by growth factors, exposure to prostaglandin PHF2a or stress, decreases PPARγ activity [71,72,73,74,75,76]. Conversely, cdk7 or cdk9 mediated phosphorylation of S112 leads to an increase in activity [77,78]. Seemingly, the cellular context determines whether the effect is inhibition or activation. Interestingly, Shao et al. have shown that S112 phosphorylation does lower ligand binding affinity of PPARγ [79]. Recently another phosphorylation site for PPARγ has been found. The serine at position 273, targeted by cdk5 seems to have a repressive effect only on specific genes such as adipsin and adiponectin but not for the general adipogenic capacity pleading for gene subset specific regulation [70]. The effect of cdk5 phosphorylation was abolished upon use of PPARγ ligands

such as rosiglitazone and MRL24, which seems to overrule the subtle regulation of PPARγ. In line with the hypothesis, a novel partial agonist of PPARγ called GQ-16 was able to block cdk5 S273 phosphorylation and thereby improve insulin sensitivity in diabetic mice [80]. This finding also indicates that it is possible for ligands to subtly finetune PPARγ through its PTM status.

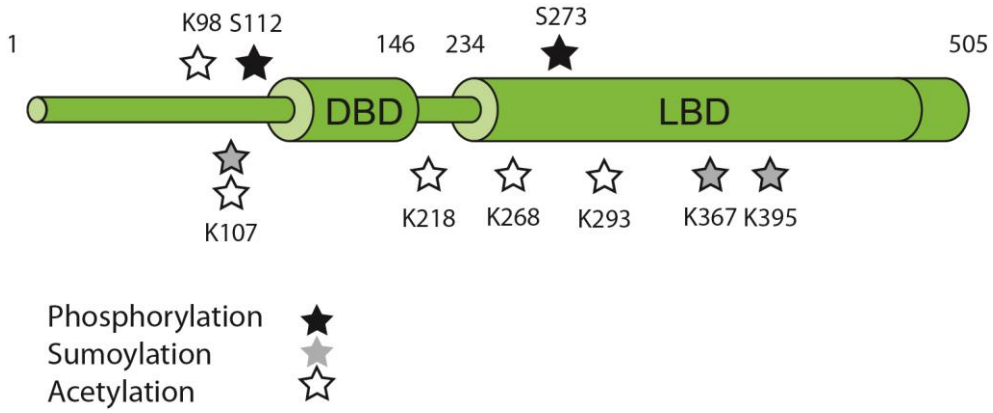


Figure 3 Schematic representation of the PTMs for PPARγ2. Black stars indicate phosphorylation, grey stars sumoylation and white stars acetylation.

The use of thiazolidinediones on the other hand can cause PPARγ to be degraded much faster by enhanced ubiquitination at the AF2 domain [81]. This paradox of simultaneous activation but degradation may be puzzling but is re-enforced by the physiological finding that heterozygous PPARγ knockout mice had elevated insulin sensitivity when compared to their wild type littermates [82]. The ubiquitin ligase Siah2 has recently been found to interact with PPARγ but the mechanism by which it controls PPARγ and its role in adipogenesis remains poorly understood [83].

Another post-translational modification is sumoylation and for PPARγ this can occur at position K107 and leads to lowered activity (Table 2) [84]. This observation was corroborated by the fact that overexpression of a de-sumoylating protein SENP2 increased expression of PPARγ target genes after de-sumoylating PPARγ in C2C12 myotubes [85]. Sumoylation of PPARγ at K365 by Pias1 in the context of inflammation is to transrepress iNos expression by preventing clearance of co-repressor complexes [86]. Recently, this mechanism could also be attributed to a novel sumo site; in vascular cells PPARγ is sumoylated by Ubc9 at position K367 which blocks SMRT release and thus leading to transrepression of

MMP-9, a protein that is involved in the development of atherosclerosis [87]. No effect for transrepression has been found when PPARγ was sumoylated at K77, even though PPARγ target gene expression was diminished [88]. In the previous cases however, the link between PTMs and co-regulators of PPARγ should be evident.

Table 2: PTMs of PPARγ in various cell lines and effects

	Position	Cell	Factor	Notes
Acetylation	K98	293T	CBP?*	
	K107	293T	CBP?*	
	K218	293T	CBP?*	
	K268	293T, 3T3L1	CBP?*	Deacetylated by Sirt1, which increases both brown and white adipogenic genes
	K293	293T, 3T3L1	CBP?*	Deacetylated by Sirt1, which increases both brown and white adipogenic genes
Sumoylation	K107**	Adipocytes	FGF21	PPARγ transcriptional activity lowered, but transrepression feat remains
	K367	Vascular cells	Ubc9	inhibition through blockade of SMRT release from PPARγ
	K395**	Macrophages	Pias1	PPARγ transrepresses expression of inflammatory genes by inhibiting co-repressor release
Phosphorylation	S112	NIH-3T3, CHO.T	ERK1/2, JNK	PPARγ transcriptional activity lowered
	S112	3T3-L1, Adipocytes, SF9	cdk7, cdk9	PPARγ transcriptional activity increased
	S273	3T3-L1	cdk5	PPARγ transcriptional activity lowered for subset of genes

* CBP was co-transfected in order to recover the acetylatedpeptides. This suggested that CBP was involved in the acetylation of the peptides.
**K107 is K77 in PPARg1, K395 is K365 in PPARg1

Very recently, a study by Qiang et al. showed that PPARγ can be acetylated at five lysines, K98, K107, K218, K268 and K293 [89]. Their research has further demonstrated that Sirt1 is able to deacetylate PPARγ at K268 and K293 leading to the recruitment of PRDM16, which will then bind to PPARγ and induce upregulation of BAT genes and repression of WAT genes. This is an example on how co-regulators can affect PPARγ activity.

Co-regulators of PPARγ

Aside from ligands and PTMs, PPARγ activity can also be influenced via its co-regulators. The possible therapeutic advantage here is that PPARγ can be affected

more specifically according to the tissue where the regulator has to be present. Co-regulators can to bind to PPARγ via conserved binding sites, such as the LxxLL motif for co-activators including CBP/p300 and the LXXXIXXXL motif for co-repressors such as NCOR/SMRT [90,91]. In the unliganded state, co-repressors such as the NCoR and SMRT are binding to PPARγ which is now unable to promote gene expression. When a ligand is present this will bind to the AF2 domain, PPARγ changes conformation leading to release of the co-repressor complex and docking sites are exposed for co-activators such as CBP/p300 and SRC/p160. This switch from co-repressor to co-activator is mediated by TBL1 and TBLR1 [92].Another example of a co-activator is TRIP3, which also plays a role in adipogenesis by binding to PPARγ via the hallmark of co-activators for nuclear receptors, the LxxLL motif.

Since PPARγ comes up late in adipogenesis, it is most likely that it can be directed by other proteins that come up earlier. PER2, a circadian protein, can bind to S112 phosphorylated PPARγ subsequently blocking PPARγ from transcriptionally activating its target genes leading to an inhibition in adipogenesis. [93] In this context PER2 determines lipid metabolism by regulating the master regulator. Another example is the zinc finger protein Zfp423. Zfp423 regulates PPARγ expression and both brown and white adipocyte differentiation is impaired in $Zfp423^{-/-}$ mouse embryos, indicating the important role of this protein [94]. With regard to epigenetics there is PTIP, a component of a H3K4 histone methyltransferase complex, which is also involved in regulating the PPARγ and C/EBPα gene and thus adipogenesis itself [95]. Knockdown of PTIP impairs enrichment of H3K4 methylation and RNA polymerase II at the gene promotors of PPARγ and C/EBPα and will also impair differentiation.

In the previous sections it should have become clear that ligands, PTMs and co-regulators can somehow be related to one another with regard to PPARγ activity. For example, Cdk5 mediated phosphorylation of PPARγ at position S273 was reduced in adipocytes devoid of NCOR leading to enhanced PPARγ activity and insulin sensitivity which resembles TZD treatment [96]. To acquire more insight in these layers of complex regulation, high-throughput screens have been developed.

Screening platforms

Screens have been developed for many cellular processes in order to distinguish crucial factors. The platforms involved techniques such as proteomics, RNAi lethality, yeast two-hybrid, RNAi loss of function, and microarrays (Table 3). It is important to differentiate between screens that focus on regulators of (regulators of) PPARγ (target genes) or adipogenesis, since these do not always coincide.

The important role of adipogenesis in physiology led to the development of platforms for high-throughput screens in order to discover novel and essential keyplayers. It has been shown in earlier studies that these types of screens can be very useful in finding important adipogenic regulators. For example, Guo et al. used an RNAi screen to identify MAP4K4 as being a negative regulator of PPARγ levels and thus adipogenesis [97]. With a similar system Sohle et al. identified multiple genes that are involved in adipogenesis and fat storage in primary adipocytes [98]. Moreover, an RNAi screen in drosophila has led to the discovery that Hedgehog is a crucial factor in determining adipose cell fate; interestingly, AT with constant expression of Hedgehog displayed BAT but almost no WAT [99]. RNAi screens are usually set up for a loss of function phenotype which is absence of fat droplets in the case of adipocytes. Van Beekum et al. however showed that RNAi screening by lethality can be a good alternative because differentiation is usually not homogenous and so a cleaner signal with less background is achieved [100].

Table 3 Overview on different types of screens to identify regulators of PPARγ function and/or adipogenesis

Type	Technique	System	Gene	Notes	Reference
Adipogenesis	Microarray	hMSC	multiple	-	(Hung, Chang et al. 2004)
Adipogenesis	Proteomics	hMSC	PDE9	-	(Lee, Lee et al. 2006)
Adipogenesis	RNAi	hMSC	GNAS	-	(Zhao and Ding 2007)
Adipogenesis	RNAi	Drosophila S2 cells	Arf-COP1	Lipid droplet formation	(Guo, Walther et al. 2008)
Adipogenesis	RNAi	HeLa	TMEM97	Cholesterol regulating genes	(Bartz, Kern et al. 2009)
Adipogenesis	RNAi	Primary h.adipocytes	multiple	-	(Sohle, Machuy et al. 2012)
Adipogenesis	RNAi	Adult drosophila	Hedgehog	Brown versus white adipocyte	(Pospisilik, Schramek et al. 2010)
Adipogenesis and PPARγ	Pamchip	3T3-L1	TRIP3	co-activator PPARγ	(Koppen, Houtman et al. 2009)
Adipogenesis and PPARγ	RNAi	3T3-L1	SCD2	induction PPARγ levels	(Christianson, Nicoloro et al. 2008)
Adipogenesis and PPARγ	RNAi	3T3-L1	MAP4K4	represses PPARγ levels	(Tang, Guilherme et al. 2006)
Adipogenesis and PPARγ	RNAi	U2OS	UCHL3	induction PPARγ levels	(van Beekum, Gao et al. 2012)
PPARγ co-regulator	Y2H	in vitro	multiple	-	(Albers, Kranz et al. 2005)
PPARγ co-regulator	Y2H	293T	PDIP1	enhances PPARγ activity	(Tomaru, Satoh et al. 2006)
PPARγ co-regulator	Y2H	293T	FOXO1	inhibits PPARγ activity	(Dowell, Otto et al. 2003)

Another type of screen is the yeast two hybrid (Y2H) setup that has been developed in order to identify protein-protein interactions. PDIP and FOXO1 were discovered as PPARγ regulators this way. A new approach to identify co-regulators of nuclear receptors is the so-called PamChip assay. In technique, peptides of many known co-regulators have been spotted on a chip and are subsequently incubated with recombinant nuclear receptor fused to GST [101]. The binding affinity can be read out by a fluorescent signal which is conjugated to the antibody targeting GST. TRIP3 was identified as a co-regulator of PPARγ using this technique.

Aim and outline of this thesis

Understanding adipocyte biology is essential for the development of therapeutic interventions with regard to obesity and T2D. In this thesis we have mainly focused on the characterization of metabolic master regulator PPARγ and its co-regulators and target genes. In chapter 2 we discuss our findings on PPM1B, a phosphatase of PPARγ in adipocytes. We show here that PPARγ activity and a subset of specific target genes are affected by the presence of PPM1B. Chapter 3 and 4 describe the results on Baf57 and Phf12 which were identified as essential players of adipogenesis using an siRNA-based knockdown screen. After validation, it became clear that Baf57 and Phf12 control adipogenesis by regulation of *PPARG/Pparg* expression. In chapter 5 we focus on Chst11, a novel target gene of PPARγ. Chst11 was discovered using Chip-Seq data and this gene plays a role in intracellular lipid accumulation. Finally, in chapter 6 the findings described in this thesis are discussed, integrated in current literature and future directions are suggested.

References

1. Hausman DB DM, Bartness TJ, Hausman GJ, Martin RJ (2001) The biology of white adipocyte proliferation.
2. James WP (2008) The epidemiology of obesity: the size of the problem. JInternMed 263: 336-352.
3. Colditz GA WW, Rotnitzky A, Manson JE. (1995) Weight gain as a risk factor for clinical diabetes mellitus in women. Ann Intern Med.
4. Stevens J CD, Pankow J, Folsom AR, Duncan BB, Nieto FJ, Jones D, Tyroler HA (2001) Sensitivity and specificity of anthropometrics for the prediction of diabetes in a biracial cohort. Obes Res.
5. Yusuf S HS, Ounpuu S, Bautista L, Franzosi MG, Commerford P, Lang CC, Rumboldt Z, Onen CL, Lisheng L, Tanomsup S, Wangai P Jr, Razak F, Sharma AM, Anand SS; INTERHEART Study Investigators (2005) Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. Lancet.
6. Lafontan M (2012) Historical perspectives in fat cell biology: the fat cell as a model for the investigation of hormonal and metabolic pathways. Am J Physiol Cell Physiol 302: C327-359.
7. Gesta S, Bluher M, Yamamoto Y, Norris AW, Berndt J, et al. (2006) Evidence for a role of developmental genes in the origin of obesity and body fat distribution. Proc Natl Acad Sci U S A 103: 6676-6681.
8. Vague J (1956) The degree of masculine differentiation of obesities: a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease. Am J Clin Nutr 4: 20-34.
9. Tran TT, Yamamoto Y, Gesta S, Kahn CR (2008) Beneficial effects of subcutaneous fat transplantation on metabolism. Cell Metab 7: 410-420.
10. Karastergiou K, Fried SK, Xie H, Lee MJ, Divoux A, et al. (2013) Distinct developmental signatures of human abdominal and gluteal subcutaneous adipose tissue depots. The Journal of clinical endocrinology and metabolism 98: 362-371.
11. Otto TC LM (2005) Adipose development: from stem cell to adipocyte. Crit Rev Biochem Mol Biol.
12. Hajer GR vHT, Visseren FL (2008) Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J.
13. Lau DC DB, Yan H, Szmitko PE, Verma S (2005) Adipokines: molecular links between obesity and atheroslcerosis. Am J Physiol Heart Circ Physiol.
14. Rosen ED, Spiegelman BM (2006) Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444: 847-853.
15. Lowell BB FJ (1997) Brown adipose tissue, beta 3-adrenergic receptors, and obesity. Annu Rev Med.
16. Lean ME (1989) Brown adipose tissue in humans. Proc Nutr Soc 48: 243-256.
17. Klingenspor M (2003) Cold-induced recruitment of brown adipose tissue thermogenesis. Exp Physiol 88: 141-148.

18. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, et al. (2009) Cold-activated brown adipose tissue in healthy men. N Engl J Med 360: 1500-1508.
19. Wu J BP, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G, Huang K, Tu H, van Marken Lichtenbelt WD, Hoeks J, Enerbäck S, Schrauwen P, Spiegelman BM (2012) Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell.
20. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, et al. (2007) Transcriptional control of brown fat determination by PRDM16. Cell Metab 6: 38-54.
21. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, et al. (2012) A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 481: 463-468.
22. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM (2000) Transcriptional regulation of adipogenesis. Genes Dev 14: 1293-1307.
23. Morrison RF, Farmer SR (1999) Insights into the transcriptional control of adipocyte differentiation. J Cell Biochem Suppl 32-33: 59-67.
24. Siersbaek R, Nielsen R, Mandrup S (2012) Transcriptional networks and chromatin remodeling controlling adipogenesis. Trends Endocrinol Metab 23: 56-64.
25. Siersbaek R, Nielsen R, John S, Sung MH, Baek S, et al. (2011) Extensive chromatin remodelling and establishment of transcription factor 'hotspots' during early adipogenesis. EMBO J 30: 1459-1472.
26. Xiao H, Leblanc SE, Wu Q, Konda S, Salma N, et al. (2011) Chromatin accessibility and transcription factor binding at the PPARgamma2 promoter during adipogenesis is protein kinase A-dependent. J Cell Physiol 226: 86-93.
27. Yang J, Croniger CM, Lekstrom-Himes J, Zhang P, Fenyus M, et al. (2005) Metabolic response of mice to a postnatal ablation of CCAAT/enhancer-binding protein alpha. J Biol Chem 280: 38689-38699.
28. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, et al. (2008) Genome-wide profiling of PPAR{gamma}:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev 22: 2953-2967.
29. Zuo Y, Qiang L, Farmer SR (2006) Activation of CCAAT/enhancer-binding protein (C/EBP) alpha expression by C/EBP beta during adipogenesis requires a peroxisome proliferator-activated receptor-gamma-associated repression of HDAC1 at the C/ebp alpha gene promoter. J Biol Chem 281: 7960-7967.
30. Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, et al. (2002) C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 16: 22-26.
31. Wu Z, Rosen ED, Brun R, Hauser S, Adelmant G, et al. (1999) Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. Mol Cell 3: 151-158.
32. El-Jack AK, Hamm JK, Pilch PF, Farmer SR (1999) Reconstitution of insulin-sensitive glucose transport in fibroblasts requires expression of both PPARgamma and C/EBPalpha. J Biol Chem 274: 7946-7951.
33. Spiegelman B, Brun R, Tontonoz P, Hu E, Altiok S (1995) Ppar-Gamma - A Master Regulator of Adipogenesis. MolBiolCell 6: 1343-1343.

34. Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79: 1147-1156.
35. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, et al. (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. MolCell 4: 585-595.
36. Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, et al. (1999) PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. MolCell 4: 597-609.
37. Nadra K, Quignodon L, Sardella C, Joye E, Mucciolo A, et al. (2010) PPARgamma in placental angiogenesis. Endocrinology 151: 4969-4981.
38. Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, et al. (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. MolCell 4: 611-617.
39. Imai T, Takakuwa R, Marchand S, Dentz E, Bornert JM, et al. (2004) Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. ProcNatlAcadSciUSA 101: 4543-4547.
40. Hegele RA (2005) Lessons from human mutations in PPARgamma. IntJObes(Lond) 29 Suppl 1:S31-5.: S31-S35.
41. Issemann I, Green S (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347: 645-650.
42. Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, et al. (2008) PPAR{gamma} and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes Dev 22: 2941-2952.
43. Desvergne B, Wahli W (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev 20: 649-688.
44. Chandra V, Huang P, Hamuro Y, Raghuram S, Wang Y, et al. (2008) Structure of the intact PPAR-gamma-RXR- nuclear receptor complex on DNA. Nature 456: 350-356.
45. Hummasti S, Tontonoz P (2006) The peroxisome proliferator-activated receptor N-terminal domain controls isotype-selective gene expression and adipogenesis. MolEndocrinol 20: 1261-1275.
46. Grimaldi PA (2007) Peroxisome proliferator-activated receptors as sensors of fatty acids and derivatives. Cell Mol Life Sci 64: 2459-2464.
47. Peters JM, Lee SS, Li W, Ward JM, Gavrilova O, et al. (2000) Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). Mol Cell Biol 20: 5119-5128.
48. Wang YX, Lee CH, Tiep S, Yu RT, Ham J, et al. (2003) Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. Cell 113: 159-170.
49. Luquet S, Lopez-Soriano J, Holst D, Fredenrich A, Melki J, et al. (2003) Peroxisome proliferator-activated receptor delta controls muscle development and oxydative capability. FASEB J 17: 2299-+.
50. Wang YX, Zhang CL, Yu RT, Cho HK, Nelson MC, et al. (2004) Regulation of muscle fiber type and running endurance by PPAR delta. PLoSBiol 2: 1532-1539.
51. Sanderson LM, Degenhardt T, Koppen A, Kalkhoven E, Desvergne B, et al. (2009) Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not

PPARalpha serves as a plasma free fatty acid sensor in liver. MolCell Biol 29: 6257-6267.
52. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM (1994) mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 8: 1224-1234.
53. Farmer SR (2006) Transcriptional control of adipocyte formation. Cell Metab 4: 263-273.
54. Mueller E, Drori S, Aiyer A, Yie J, Sarraf P, et al. (2002) Genetic analysis of adipogenesis through peroxisome proliferator-activated receptor gamma isoforms. JBiolChem 277: 41925-41930.
55. Zhang JF, Fu MG, Cui TX, Chen X, Xu KF, et al. (2004) Selective disruption of PPAR gamma 2 impairs the development of adipose tissue and insulin sensitivity. ProcNatlAcadSciUSA 101: 10703-10708.
56. Gupta RA, Brockman JA, Sarraf P, Willson TM, DuBois RN (2001) Target genes of peroxisome proliferator-activated receptor gamma in colorectal cancer cells. J Biol Chem 276: 29681-29687.
57. Tontonoz P, Spiegelman BM (2008) Fat and beyond: the diverse biology of PPARgamma. AnnuRevBiochem 77: 289-312.
58. Steger DJ, Grant GR, Schupp M, Tomaru T, Lefterova MI, et al. (2010) Propagation of adipogenic signals through an epigenomic transition state. Genes Dev 24: 1035-1044.
59. Yang X, Lu X, Lombes M, Rha GB, Chi YI, et al. (2010) The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. Cell Metab 11: 194-205.
60. Kershaw EE, Schupp M, Guan HP, Gardner NP, Lazar MA, et al. (2007) PPARgamma regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. AmJPhysiol EndocrinolMetab 293: E1736-E1745.
61. Zandbergen F, Mandard S, Escher P, Tan NS, Patsouris D, et al. (2005) The G0/G1 switch gene 2 is a novel PPAR target gene. Biochem J 392: 313-324.
62. Takazawa T, Yamauchi T, Tsuchida A, Takata M, Hada Y, et al. (2009) Peroxisome proliferator-activated receptor gamma agonist rosiglitazone increases expression of very low density lipoprotein receptor gene in adipocytes. J Biol Chem 284: 30049-30057.
63. Guan HP, Ishizuka T, Chui PC, Lehrke M, Lazar MA (2005) Corepressors selectively control the transcriptional activity of PPARgamma in adipocytes. Genes Dev 19: 453-461.
64. Baker PR, Lin Y, Schopfer FJ, Woodcock SR, Groeger AL, et al. (2005) Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. JBiolChem 280: 42464-42475.
65. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, et al. (1995) 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell 83: 803-812.
66. Hallenborg P, Jorgensen C, Petersen RK, Feddersen S, Araujo P, et al. (2010) Epidermis-type lipoxygenase 3 regulates adipocyte differentiation and peroxisome proliferator-activated receptor gamma activity. MolCell Biol 30: 4077-4091.

67. Yki-Jarvinen H (2004) Thiazolidinediones. NEnglJMed 351: 1106-1118.
68. Tham M RS, Gan HT, Ramachandran A, Poonepalli A, Yu YH, Ahmed S (2010) CSPG is a secreted factor that stimulates neural stem cell survival possibly by enhanced EGFR signaling. PLoS One.
69. Nissen SE, Wolski K (2007) Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. NEnglJMed 356: 2457-2471.
70. Choi JH BA, Kamenecka TM, Busby SA, Chalmers MJ, Kumar N, Kuruvilla DS, Shin Y, He Y, Bruning JB, Marciano DP, Cameron MD, Laznik D, Jurczak MJ, Schürer SC, Vidović D, Shulman GI, Spiegelman BM, Griffin PR (2011) Antidiabetic actions of a non-agonist PPARγ ligand blocking Cdk5-mediated phosphorylation. Nature 466: 451-456.
71. Hu E, Kim JB, Sarraf P, Spiegelman BM (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. Science 274: 2100-2103.
72. Tang XQ, Guilherme A, Chakladar A, Powelka AM, Konda S, et al. (2006) An RNA interference-based screen identifies MAP4K4/NIK as a negative regulator of PPAR gamma, adipogenesis, and insulin-responsive hexose transport. ProcNatlAcadSciUSA 103: 2087-2092.
73. Camp HS, Tafuri SR (1997) Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase. JBiolChem 272: 10811-10816.
74. Reginato MJ, Krakow SL, Bailey ST, Lazar MA (1998) Prostaglandins promote and block adipogenesis through opposing effects on peroxisome proliferator-activated receptor gamma. JBiolChem 273: 1855-1858.
75. Zhang B, Berger J, Zhou GC, Elbrecht A, Biswas S, et al. (1996) Insulin- and mitogen-activated protein kinase-mediated phosphorylation and activation of peroxisome proliferator-activated receptor gamma. JBiolChem 271: 31771-31774.
76. Adams M, Reginato MJ, Shao D, Lazar MA, Chatterjee VK (1997) Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. JBiolChem 272: 5128-5132.
77. Compe E, Drane P, Laurent C, Diderich K, Braun C, et al. (2005) Dysregulation of the peroxisome proliferator-activated receptor target genes by XPD mutations. MolCell Biol 25: 6065-6076.
78. Iankova I, Petersen RK, Annicotte JS, Chavey C, Hansen JB, et al. (2006) Peroxisome proliferator-activated receptor gamma recruits the positive transcription elongation factor b complex to activate transcription and promote adipogenesis. MolEndocrinol 20: 1494-1505.
79. Shao D, Rangwala SM, Bailey ST, Krakow SL, Reginato MJ, et al. (1998) Interdomain communication regulating ligand binding by PPAR-gamma. Nature 396: 377-380.
80. Amato AA, Rajagopalan S, Lin JZ, Carvalho BM, Figueira AC, et al. (2012) GQ-16, a novel peroxisome proliferator-activated receptor gamma (PPARgamma) ligand, promotes insulin sensitization without weight gain. J Biol Chem 287: 28169-28179.

81. Hauser S, Adelmant G, Sarraf P, Wright HM, Mueller E, et al. (2000) Degradation of the peroxisome proliferator-activated receptor gamma is linked to ligand-dependent activation. JBiolChem 275: 18527-18533.
82. Rolls A SR, London A, Segev Y, Jacob-Hirsch J, Amariglio N, Rechavi G, Schwartz M (2008) Two faces of chondroitin sulfate proteoglycan in spinal cord repair: a role in microglia/macrophage activation. PLoS Med.
83. Kilroy G, Kirk-Ballard H, Carter LE, Floyd ZE (2012) The ubiquitin ligase Siah2 regulates PPARgamma activity in adipocytes. Endocrinology 153: 1206-1218.
84. Yamashita D, Yamaguchi T, Shimizu M, Nakata N, Hirose F, et al. (2004) The transactivating function of peroxisome proliferator-activated receptor gamma is negatively regulated by SUMO conjugation in the amino-terminal domain. Genes Cells 9: 1017-1029.
85. Cortes M BA, Schwartz NB (2009) Sulfation of chondroitin sulfate proteoglycans is necessary for proper Indian hedgehog signaling in the developing growth plate. Development.
86. Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, et al. (2005) A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. Nature 437: 759-763.
87. Bloch M, Prock A, Paonessa F, Benz V, Bahr IN, et al. (2012) High-mobility group A1 protein: a new coregulator of peroxisome proliferator-activated receptor-gamma-mediated transrepression in the vasculature. Circ Res 110: 394-405.
88. Zelcer N, Tontonoz P (2005) SUMOylation and PPARgamma: wrestling with inflammatory signaling. Cell Metab 2: 273-275.
89. Qiang L, Wang L, Kon N, Zhao W, Lee S, et al. (2012) Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Ppargamma. Cell 150: 620-632.
90. Plevin MJ, Mills MM, Ikura M (2005) The LxxLL motif: a multifunctional binding sequence in transcriptional regulation. Trends BiochemSci 30: 66-69.
91. Koppen A, Kalkhoven E (2010) Brown vs white adipocytes: The PPARgamma coregulator story. FEBS Lett 584: 3250-3259.
92. Sugii S, Evans RM (2011) Epigenetic codes of PPARgamma in metabolic disease. FEBS letters 585: 2121-2128.
93. Lefterova MI SD, Zhuo D, Qatanani M, Mullican SE, Tuteja G, Manduchi E, Grant GR, Lazar MA (2010) Cell-specific determinants of peroxisome proliferator-activated receptor gamma function in adipocytes and macrophages. Mol Cell Biol.
94. Gupta RK, Arany Z, Seale P, Mepani RJ, Ye L, et al. (2010) Transcriptional control of preadipocyte determination by Zfp423. Nature 464: 619-623.
95. Cho YW, Hong S, Jin Q, Wang L, Lee JE, et al. (2009) Histone methylation regulator PTIP is required for PPARgamma and C/EBPalpha expression and adipogenesis. Cell Metab 10: 27-39.
96. Hamza MS PS, Vega VB, Thomsen JS, Kandhadayar GS, Ng PW, Chiu KP, Pettersson S, Wei CL, Ruan Y, Liu ET. (2009) De-novo identification of PPARgamma/RXR binding sites and direct targets during adipogenesis. PLoS One.

97. Guo Y, Walther TC, Rao M, Stuurman N, Goshima G, et al. (2008) Functional genomic screen reveals genes involved in lipid-droplet formation and utilization. Nature 453: 657-661.
98. Sohle J, Machuy N, Smailbegovic E, Holtzmann U, Gronniger E, et al. (2012) Identification of new genes involved in human adipogenesis and fat storage. PLoS One 7: e31193.
99. Pospisilik JA, Schramek D, Schnidar H, Cronin SJ, Nehme NT, et al. (2010) Drosophila genome-wide obesity screen reveals hedgehog as a determinant of brown versus white adipose cell fate. Cell 140: 148-160.
100. van Beekum O, Gao Y, Berger R, Koppen A, Kalkhoven E (2012) A novel RNAi lethality rescue screen to identify regulators of adipogenesis. PLoS One 7: e37680.
101. Koppen A, Houtman R, Pijnenburg D, Jeninga EH, Ruijtenbeek R, et al. (2009) Nuclear receptor-coregulator interaction profiling identifies TRIP3 as a novel PPARgamma cofactor. MolCell Proteomics 8: 2212-2226.
102. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, et al. (2003) Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 52: 1655-1663.
103. Muruganandan S, Parlee SD, Rourke JL, Ernst MC, Goralski KB, et al. (2011) Chemerin, a novel peroxisome proliferator-activated receptor gamma (PPARgamma) target gene that promotes mesenchymal stem cell adipogenesis. J Biol Chem 286: 23982-23995.
104. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, et al. (1996) PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO J 15: 5336-5348.
105. Wu Z, Xie Y, Morrison RF, Bucher NL, Farmer SR (1998) PPARgamma induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPalpha during the conversion of 3T3 fibroblasts into adipocytes. J Clin Invest 101: 22-32.
106. Kim YJ, Cho SY, Yun CH, Moon YS, Lee TR, et al. (2008) Transcriptional activation of Cidec by PPARgamma2 in adipocyte. BiochemBiophysResCommun 377: 297-302.
107. Arimura N, Horiba T, Imagawa M, Shimizu M, Sato R (2004) The peroxisome proliferator-activated receptor gamma regulates expression of the perilipin gene in adipocytes. J Biol Chem 279: 10070-10076.
108. Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, et al. (2004) Adipose tissue expression of the lipid droplet-associating proteins S3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-gamma. Diabetes 53: 1243-1252.
109. Schoonjans K, Watanabe M, Suzuki H, Mahfoudi A, Krey G, et al. (1995) Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. J Biol Chem 270: 19269-19276.
110. Castelein H, Gulick T, Declercq PE, Mannaerts GP, Moore DD, et al. (1994) The peroxisome proliferator activated receptor regulates malic enzyme gene expression. J Biol Chem 269: 26754-26758.

111. Devine JH, Eubank DW, Clouthier DE, Tontonoz P, Spiegelman BM, et al. (1999) Adipose expression of the phosphoenolpyruvate carboxykinase promoter requires peroxisome proliferator-activated receptor gamma and 9-cis-retinoic acid receptor binding to an adipocyte-specific enhancer in vivo. J Biol Chem 274: 13604-13612.
112. Frohnert BI, Hui TY, Bernlohr DA (1999) Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. J Biol Chem 274: 3970-3977.
113. Motojima K, Passilly P, Peters JM, Gonzalez FJ, Latruffe N (1998) Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor alpha and gamma activators in a tissue- and inducer-specific manner. J Biol Chem 273: 16710-16714.
114. Helledie T, Grontved L, Jensen SS, Kiilerich P, Rietveld L, et al. (2002) The gene encoding the Acyl-CoA-binding protein is activated by peroxisome proliferator-activated receptor gamma through an intronic response element functionally conserved between humans and rodents. J Biol Chem 277: 26821-26830.
115. Kishida K KH, Funahashi T, Shimomura I, Kihara S, Ouchi N, Nishida M, Nishizawa H, Matsuda M, Takahashi M, Hotta K, Nakamura T, Yamashita S, Tochino Y, Matsuzawa Y (2000) Aquaporin adipose, a putative glycerol channel in adipocytes. JBC.
116. Tao H, Aakula S, Abumrad NN, Hajri T (2010) Peroxisome proliferator-activated receptor-gamma regulates the expression and function of very-low-density lipoprotein receptor. Am J Physiol Endocrinol Metab 298: E68-79.
117. Ribon V, Johnson JH, Camp HS, Saltiel AR (1998) Thiazolidinediones and insulin resistance: peroxisome proliferatoractivated receptor gamma activation stimulates expression of the CAP gene. Proc Natl Acad Sci U S A 95: 14751-14756.
118. Jeninga EH, Bugge A, Nielsen R, Kersten S, Hamers N, et al. (2009) Peroxisome proliferator-activated receptor gamma regulates expression of the anti-lipolytic G-protein-coupled receptor 81 (GPR81/Gpr81). JBiolChem 284: 26385-26393.
119. Fontaine C, Dubois G, Duguay Y, Helledie T, Vu-Dac N, et al. (2003) The orphan nuclear receptor Rev-Erbalpha is a peroxisome proliferator-activated receptor (PPAR) gamma target gene and promotes PPARgamma-induced adipocyte differentiation. J Biol Chem 278: 37672-37680.
120. Morrison RF, Farmer SR (1999) Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J Biol Chem 274: 17088-17097.
121. Hung SC, Chang CF, Ma HL, Chen TH, Low-Tone Ho L (2004) Gene expression profiles of early adipogenesis in human mesenchymal stem cells. Gene 340: 141-150.
122. Lee HK, Lee BH, Park SA, Kim CW (2006) The proteomic analysis of an adipocyte differentiated from human mesenchymal stem cells using two-dimensional gel electrophoresis. Proteomics 6: 1223-1229.
123. Zhao Y, Ding S (2007) A high-throughput siRNA library screen identifies osteogenic suppressors in human mesenchymal stem cells. Proc Natl Acad Sci U S A 104: 9673-9678.

124. Bartz F, Kern L, Erz D, Zhu M, Gilbert D, et al. (2009) Identification of cholesterol-regulating genes by targeted RNAi screening. Cell Metab 10: 63-75.
125. Christianson JL, Nicoloro S, Straubhaar J, Czech MP (2008) Stearoyl-CoA desaturase 2 is required for peroxisome proliferator-activated receptor gamma expression and adipogenesis in cultured 3T3-L1 cells. JBiolChem 283: 2906-2916.
126. Albers M, Kranz H, Kober I, Kaiser C, Klink M, et al. (2005) Automated yeast two-hybrid screening for nuclear receptor-interacting proteins. MolCell Proteomics 4: 205-213.
127. Tomaru T, Satoh T, Yoshino S, Ishizuka T, Hashimoto K, et al. (2006) Isolation and characterization of a transcriptional cofactor and its novel isoform that bind the deoxyribonucleic acid-binding domain of peroxisome proliferator-activated receptor-gamma. Endocrinology 147: 377-388.
128. Dowell P, Otto TC, Adi S, Lane MD (2003) Convergence of peroxisome proliferator-activated receptor gamma and Foxo1 signaling pathways. JBiolChem 278: 45485-45491.

Chapter 2

The serine/threonine phosphatase PPM1B (PP2Cβ) selectively modulates PPARγ activity

Ismayil Tasdelen[1#], Olivier van Beekum[1,4#], Olena Gorbenko[2*], Veerle Fleskens[1,4], Niels J.F. van den Broek[1,4], Arjen Koppen[1,4], Nicole Hamers[1,4], Ruud Berger[1,4] , Paul J. Coffer[2,3], Arjan B. Brenkman[1,4] and Eric Kalkhoven[1,4§]

From the [1]Departments of Metabolic Diseases and [2] Cell biology, University Medical Centre Utrecht, Utrecht, The Netherlands, [3]Center for Molecular & Cellular Intervention, Wilhelmina Children's Hospital, University Medical Center Utrecht, The Netherlands, and [4]Netherlands Metabolomics Centre, The Netherlands.

Biochem J. 2013 Apr 1;451(1):45-53

Abstract

Reversible phosphorylation is a widespread molecular mechanism to regulate the function of cellular proteins, including transcription factors. Phosphorylation of the nuclear receptor Peroxisome Proliferator Activated Receptor γ (PPARγ) on two conserved serine residue (S112 and S273) results in altered transcriptional activity of this transcription factor. So far, only a very limited number of cellular enzymatic activities has been described which can dephosphorylate nuclear receptors. Here we used immunoprecipitation assays coupled to tandem mass spectrometry analysis to identify novel PPARγ regulating proteins. We identified the serine/threonine phosphatase PPM1B (also named PP2Cβ) as a novel PPARγ interacting protein. Endogenous PPM1B protein is localized in the nucleus of mature 3T3-L1 adipocytes where it can bind to PPARγ. Furthermore we show that PPM1B can directly dephosphorylate PPARγ, both in intact cells and *in vitro*. In addition PPM1B increases PPARγ-mediated transcription via dephosphorylation of serine 112. Finally, we show that knockdown of PPM1B in 3T3-L1 adipocytes blunts the expression of some PPARγ target genes while leaving others unaltered. These findings qualify the phosphatase PPM1B as a novel selective modulator of PPARγ activity.

Introduction

The transcription factor Peroxisome Proliferator-Activated Receptor gamma (PPARγ) is a ligand-activated transcription factor of the nuclear receptor superfamily that regulates genes involved in differentiation, metabolism and immunity [1]. Like other nuclear receptors, PPARγ consists of distinct functional domains including an N-terminal transactivation domain (AF-1), a highly conserved DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) that contains a ligand-dependent transactivation function (AF-2). The LBD of PPARγ can accommodate a wide variety of ligands like prostaglandins, eicosanoids and fatty acids. Also thiazolidinediones, a class of anti-diabetic drugs including rosiglitazone, function as ligands for PPARγ. Ligand binding stabilizes the active conformation of the PPARγ LBD, resulting in the release of corepressor proteins (e.g. NCoR) and the recruitment of coactivator proteins. Ligand binding thereby serves as a "molecular switch" between activation and repression functions of the receptor. It has however become increasingly clear that post-translational modifications (PTM) represent an additional important molecular mechanism to regulate the activity of nuclear receptors, including PPARγ [2,3]. The first PTM described for PPARγ was phosphorylation of serine residue 112 (serine 82 in PPARγ1) by various kinases like ERK1, p38, JNK, cdk7 and cdk9 (reviewed in [2,3]). Serine 112 phosphorylation can impair PPARγ transcriptional activity, and several, not mutually exclusive, mechanisms have been reported: inhibition of ligand binding [4], stimulation of a repressive PTM named SUMOylation [5,6] or reduced DNA binding due to interaction with the PER2 protein [7]. It should be noted that S112 phosphorylation can also result in increased PPARγ activity [8,9], but the molecular mechanism(s) underlying this differential output are currently unclear. Very recently, a second phosphorylation site, serine 273, was identified [10]. Phosphorylation of this residue by cdk5, a kinase which is activated in obesity, results in reduced expression of specific PPARγ target genes that have anti-obesity effects [10]. Interestingly, treatment with PPARγ agonists leads to decreased phosphorylation at this site, an effect which may be mediated by the corepressor protein NCoR [10,11,12].

Protein phosphorylation events are generally reversible and many nuclear receptors are subject to phosphorylation [13]. However, only a very limited number of cellular enzymatic activities have been described so far which can dephosphorylate nuclear receptors, including the phosphatase PP5 for PPARγ and

the glucocorticoid receptor (GR) ([14,15]; see also Discussion). Recent studies using high-throughput siRNA screening have highlighted the importance of phosphatases in many different cellular processes, including cell survival, apoptosis and cell-cycle progression (e.g.[16]). Protein phosphatases are defined by structurally distinct gene families which can be divided into two major classes: the protein serine/threonine phosphatase family (PP) and the protein tyrosine phosphatase family (PTP), including both tyrosine-specific and dual-specificity phosphatases [16]. Protein serine/threonine phosphatases are further classified into two subfamilies, PPP and PPM, based on substrate specificity, divalent cation dependency and sensitivity to specific inhibitors. The PPP family includes PP1, PP2A and PP2B whereas the PPM family consists of the PP2C isozymes and pyruvate dehydrogenese phosphatase. The PPM family of protein phosphatases differ from other phosphatases since they (i) depend on Mg^{2+} or Mn^{2+} ions for their catalytic activity, (ii) function as monomers, and (iii) are insensitive to the phosphatase inhibitor okadaic acid. PPM family members have been reported to function in the regulation of different cellular processes including apoptosis, cell cycling and differentiation [17,18]. To conform to the nomenclature for the human Mg^{2+} dependent phosphatases, PP2Cα and PP2Cβ will be further denoted as PPM1A and PPM1B, respectively. Here we used immunoprecipitation assays coupled to mass spectrometry analysis to identify novel PPARγ associated proteins. We describe the serine/threonine phosphatase PPM1B as a novel PPARγ interacting protein, which can selectively modulate PPARγ-mediated transcription.

Experimental procedures

Materials-Rosiglitazone was purchased from Alexis. Fugene®6 transfection reagent and protease inhibitor tablets (11697498001) were purchased from Roche Applied Biosciences. PEI (#23966) was purchased from Polysciences Inc. The following antibodies were used: anti-PPARγ (sc-7273) Santa Cruz Biotechnologies; anti-PPM1B (A300-887A), Bethyl laboratories; anti-FLAG M2 HRP (A8592), anti-HA (H9658) and anti-Tropomyosin (T2780), Sigma-Aldrich; anti-rabbit-HRP (111035144) and anti-mouse-HRP (115035146), Jackson Immunoresearch Laboratories Inc.; anti-PPARγ phosphoserine 112, Euromedex and anti-PPARγ phosphoserine 112 (ab60953), Abcam. Anti-FLAG M2 agarose beads (A02220), Oil-Red-O (O-0625) and purified PPM1B (P-1743) were purchased from Sigma-Aldrich, Lipofectamine 2000 was purchased from Invitrogen.

Plasmids-All recombinant DNA work was performed according to standard procedures [19]. pCMV Renilla was purchased from Promega. All mutations were generated by Quickchange mutagenesis (Stratagene) and verified by sequencing. pSport PPM1B isoform 1 and PPM1A expression vector was puchased from RZPD (clone: IRAT p970B0984D, clone: IRAT p970A1077D). The pCDNA-Gal4DBD-PPARγAF1 (amino acids 1-129) was generated by cloning a PCR fragment into the pCDNA3-Gal4DBD [20]. The reporter plasmid containing the aquaporin promoter was a kind gift of Dr. N. Maeda [21]. All other plasmids have been described before [22,23].

Cell culture, transient transfections and reporter assays-The human osteosarcoma cell line U2OS and the human embryonic kidney 293T cell line (HEK293T) were maintained in DMEM Glutamax (Dulbecco) containing 10% foetal calf serum (Gibco Life Technologies), 100 μg of penicillin/ml and 100 μg streptomycin/ml (Gibco Life Technologies). The murine 3T3-L1 cells were cultured in the same media but now with 10% bovine serum (Gibco Life Technologies), 100 μg of penicillin/ml and 100 μg streptomycin/ml (Gibco Life Technologies). For differentiation, 3T3-L1 cells were grown to confluency and after 2 days incubated with culture medium containing dexamethasone (250 nM), 3-isobutyl-1-methylxanthine (500 uM) and insulin (170 nM) for 2 days. On day 3, medium was changed for culture medium supplemented with insulin (170 nM) and left for 2-5

days. Subsequently, cells were stained with Oil-red-O, or lysed and subjected to Western blot analysis as described before [22,23].
Reporter assays were performed in 24-well plates with 1 µg 3xPPRE-tk-Luc reporter construct or AQP7-Luc reporter construct, 2 ng PPAR expression construct, 100 ng PPM1B expression construct (or empty vector) and 2 ng pCMV-Renilla (Promega) as described before [22]. For immunoprecipitation experiments, U2OS cells or 293T cells were grown in 15 cm dishes and transiently transfected with PPARγ2 or PPM1B expression vectors (10 µg) using either Fugene®6 or PEI transfection reagent, as described before [23].

Tandem Mass Spectrometry-Overexpressed FLAG-PPARγ2 was isolated from 20 dishes (15 cm) of 293T cells. Proteolytic digestion of proteins and LC-tandem MS were performed exactly as described [23,24], except that proteins were not only subjected to trypsin, but also chymotrypsin and Arg-C digestion. Moreover, raw data files were processed by Mascot Distiller (version 2.4.2.0, Matrix Science, London, UK) and searched against the Swiss-Prot database (release 2012_07) using Mascot Search Engine (version 2.3.01).

Western blot analysis-For detection of phosphorylated PPARγ in intact cells, FLAG-PPARγ2 was overexpressed in 293T cells. The next day, cells were incubated with or without rosiglitazone (2 µM) for 24 hours prior to lysis in RIPA buffer (200Mm Tris-HCl pH 8.0, 1% Triton X-100, 1% NaDOC, 0.1% SDS, 10mM EDTA, 150mM NaCl, containing protease inhibitors). After immunoprecipitation with anti-FLAG M2 Sepaharose beads for at least 4 hours at 4°C, samples were analysed by Western blotting. Blots were probed with various primary antibodies and immunoreactive complexes were visualised by enhanced chemiluminescence as described [23].

In vitro dephosphorylation assay-FLAG tagged PPARγ2 was immuno-purified from transiently transfected HEK 293T cells. Purified PPM1B was incubated in phosphatase buffer (20mM Tris pH 7.4, 150mM NaCl, 5mM $MgCl_2$, 1mM DTT, 1mg/ml BSA, 0.1% Tween and complete protease inhibitors) together with FLAG PPARγ2 coupled to 25µl FLAG beads for 30 to 45 minutes at 30ºC. Phosphorylated PPARγ2 was detected by Western blot analysis using phospho-serine 112 specific antibody labeling.

Immunofluorescence microscopy and proximity ligation assay (PLA)- Immunofluorescence staining was performed as described [25], using primary antibodies against PPM1B (A300-887A) and PPARγ (sc-7273).
PLA detection was performed using the Duolink II kit (Olink bioscience, Uppsala, Sweden) according to the manufacturer's protocol. In short, differentiated 3T3-L1 cells were enabled to adhere to coverslips and subsequently washed with PBS and fixed with 4% formaldehyde for 20 minutes. Afterwards, the samples were permeabilized with 0.2% triton X-100 for 5 minutes and then incubated for 30 minutes with blocking buffer (10% normal human serum in PBS). After blocking, cells were incubated O/N at 4**°**C with anti- PPM1B antibody (A300-887A) and anti-PPARγ antibody (sc-7273) in blocking buffer containing 10% normal human serum. Cells were washed three times with PBS, followed by PLA according to manufacturer's protocol. Coverslips were mounted and analysed as for immunofluorescence microscopy.

RNA isolation and QPCR - 3T3-L1 fibroblasts were differentiated as described above. Three independent samples of total RNA were extracted at different time points using TRIzol reagent (Invitrogen). cDNA was synthesized using the superscript first strand synthesis system (Invitrogen) according to manufacturer's protocol. Gene expression levels were determined by quantitative real time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to *TFIIb* expression.
The primers used were as follows:
murine TFIIb forward primer,
5'-TCCTCCTCAGACCGCTTTT-3', and reverse primer,
5'-CCTGGTTCATCATCGCTAATC-3'; murine PPARγ forward primer,
5'- CGCTGATGCACTGCCTATGA-3' and reverse primer,
5'-AGAGGTCCACAGAGCTGATTCC-3'; murine PPM1B forward primer,
5'- TCAGAGTTGGATAAGCACTTGG-3', and reverse primer,
5'- CATCACATGGGCAAGATCAG -3'; murine Fapb4 forward primer,
5'-CGCAGACGACAGGAAGGT-3', and reverse primer,
5'-TTCCATCCCACTTCTGCAC-3'; murine Adipoq forward primer,
5'- GGAACTTGTGCAGGTTGGAT-3', and reverse primer,
5'- TCTCCAGGCTCTCCTTTCCT-3'; murine Lpl forward primer,
5'- TTTGTGAAATGCCATGACAAG-3' and reverse primer,
5'- TCAAACACCCAAACAAGGGTA-3'; murine Cd36 forward primer,
5'- TTGTACCTATACTGTGGCTAAATGAGA -3' and reverse primer,

5’- TCTACCATGCCAAGGAGCTT -3’. murine Lipin forward primer,
5’- CGCCAAAGAATAACCTGGAA -3’ and reverse primer,
5’- TGAAGACTCGCTGTGAATGG -3’

siRNA transfection - 3T3-L1 cells were transfected with siRNA oligonucleotides as described before [23] using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s protocol. The siRNA oligonucleotides used were siControl (D-001810-10-20, Dharmacon), siPPARγ (L-040712-00-0010, Dharmacon), siPPM1B #6 (J-040053-06-0005, Dharmacon), siPPM1B #5 (J-040053-05-0005, Dharmacon).

Results

PPARγ interacts with the phosphatase PPM1B (PP2Cβ)

In order to identify novel PPARγ interacting proteins, ectopically expressed FLAG-tagged human PPARγ2 was immunopurified from HEK 293T cells and associated proteins were identified by mass spectrometry analysis [23]. Several peptides corresponding to the serine/threonine phosphatase PPM1B (also named PP2Cβ) were repeatedly identified in independent experiments (Fig. 1A and B and Supplementary Table 1). At least five different splice variants of PPM1B have been reported in humans and four in mice (Fig. 1A and 2A and Supplementary Table 2), all identical in their catalytic domains but differing in the N- and C-terminal domains, which are thought to be involved in localisation and substrate specificity [26,27].

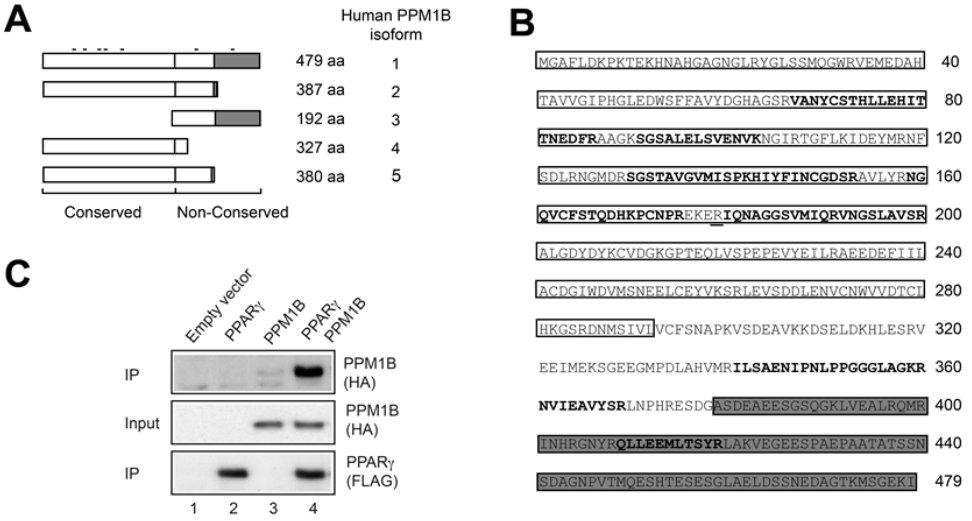


Figure 1 The phosphatase PPM1B is a novel PPARγ interacting protein

A. Schematic representation of human PPM1B isoforms. Indicated are the conserved N-terminus and the non-conserved C-termini, including the isoforms-specific regions (in grey). Also indicated are the peptides identified by mass spectromic analyisis. B**.** Amino acid sequence of human PPM1B isoform 1. Indicated in grey is the catalytic domain conserved between different PPM1B isoforms. The peptides identified by mass spectromic analyisis are shown in bold. The R179 residue that was mutated to inactivate the PPM1B protein is underlined. C. FLAG-PPARγ and HA-PPM1B were overexpressed in HEK 293T cells. Cells were lysed and PPARγ was precipitated using anti-FLAG beads. Immunoreactive proteins were detected on Western blots by anti-HA and anti-FLAG antibodies

Three peptides (e.g. SGSTAVGVMISPK)) corresponded to the N-terminal region not present in isoform 3, while one of the peptides detected (QLLEEMLTSYR) corresponded to a region only present in human PPM1B isoforms 1 and 3. While we cannot formally exclude that PPARγ may interact with isoform 3, PPM1B isoform 1 is more likely to represent a genuine PPARγ interacting protein, as (i) expression of human isoform 3 on the protein level has never been reported, while human isoform 1 is expressed ubiquitously on the mRNA and protein level [28], and (ii) no mRNA corresponding to human isoform 3 has been described in other species (e.g. mouse; Fig. 2A), while isoform 1 is highly conserved (Supplementary Fig. 1). We therefore focused on the interaction between PPARγ and PPM1B isoform 1 in our subsequent experiments. To confirm the interaction between these two proteins by independent means, 293T cells were cotransfected with HA-tagged PPM1B and FLAG-tagged PPARγ, followed by immunoprecipitation. As shown in Fig. 1C, PPM1B co-immunoprecipitated with FLAG-tagged PPARγ. As controls, immunoprecipitations were performed on lysates of cells expressing either PPM1B or PPARγ alone, or neither protein. No co-immunoprecipitations were detected in these lysates (Fig. 1C). Taken together, these findings indicate that PPM1B isoform 1 is a novel PPARγ interacting protein.

Nuclear PPM1B interacts with PPARγ in adipocytes

Given the key role of PPARγ in adipocyte differentiation, function and maintenance [1], we examined the expression and subcellular localization of the PPARγ-interacting phosphatase PPM1B in mouse 3T3-L1 adipocytes. To address which PPM1B isoform(s) are expressed in these cells, expression of the different mouse PPM1B isoforms was analyzed by RT-PCR. While expression of isoforms 2-4 could not be detected, mouse PPM1B isoform1-which encodes a protein highly homologous to the human PPM1B isoform 1 protein (Supplementary Fig. 1) - was clearly expressed in 3T3-L1 adipocytes (Fig. 2A). Next, we examined PPM1B protein expression during differentiation of 3T3-L1 cells into mature adipocytes. Expression levels of PPM1B isoform 1 increased up to day 4, while PPARγ expression was detectable from day 3 onwards (Fig. 2B). As both cytoplasmic and nuclear localizations of PPM1B have been reported [27,29,30,31], we investigated the localization of the endogenous PPM1B protein in differentiated 3T3-L1 cells.

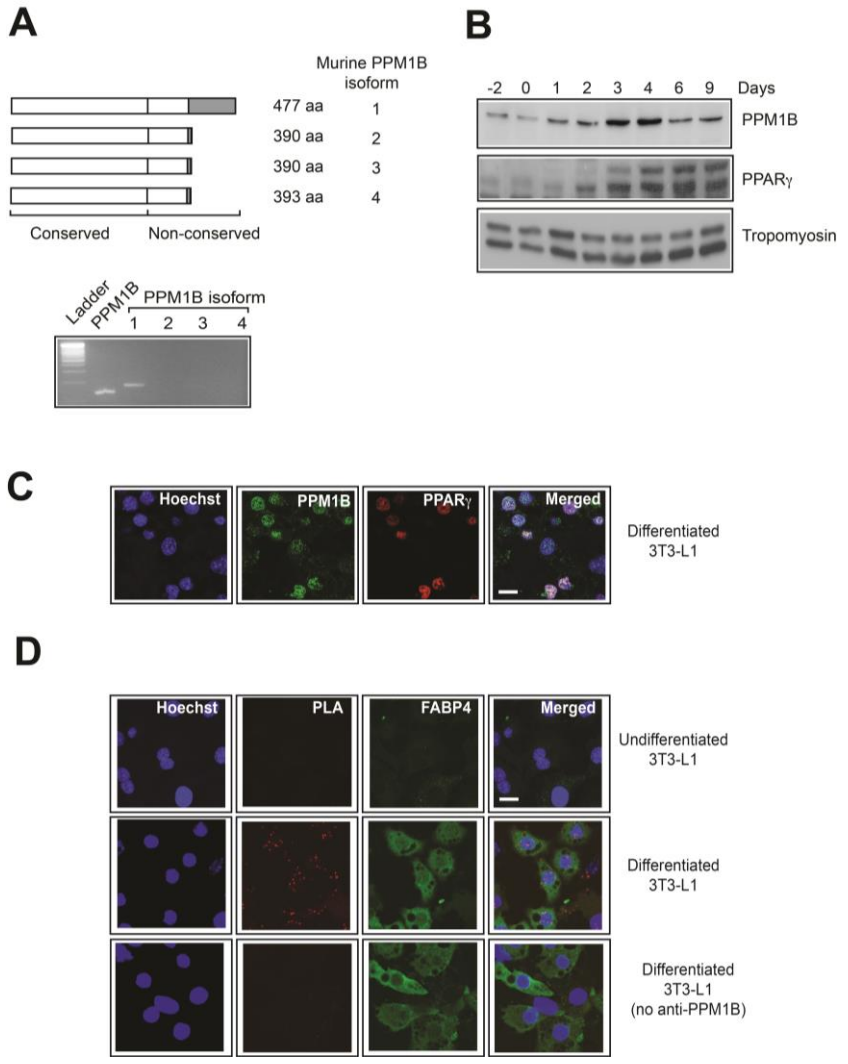


Figure 2 PPARγ and PPM1B interact in 3T3-L1 adipocytes. Expression of mouse PPM1B isoforms in mature 3T3-L1 adipocytes. PPM1B isoforms were detected by RT-PCR, using primer pairs common to all isoforms (PPM1B), or primers that specifically recognize isoform 1-4. B. PPM1B expression is regulated during 3T3L1 differentiation. Mouse 3T3L1 pre-adipocytes were differentiated into mature adipocytes and samples were taken at different time points. Protein expression levels of PPM1B were determined by Western blot analysis. As a control for differentiation PPARγ protein levels were analysed. C. Representative confocal microscopy images of 3T3-L1 adipocytes. Endogenous PPM1B (green) and PPARγ (red) were visualized utilizing specific antibodies, Hoechst was used to visualize the nuclei (blue). D. PPARγ-PPM1B was visualized in 3T3-L1 adipocytes using an *in situ* proximity ligation assay (PLA). Cells were fixed and protein-protein interactions were visualized utilizing anti-PPARγ and anti-PPM1B antibodies as described in the Methods section. Punctate staining (red) indicates a PPARγ-PPM1B interaction as detected by the assay, differentiated cells were identified by staining for FABP4 (green), Hoechst was used to visualize the nuclei (blue).

For this immunofluorescence staining of PPM1B was combined with PPARγ staining to mark differentiating cells. PPM1B and PPARγ both displayed nuclear localization in mature 3T3-L1 adipocytes (Fig. 2c). Finally, we validated the PPM1B-PPARγ interaction by performing an *in situ* proximity ligation assay (PLA) on the endogenous proteins in undifferentiated and differentiated 3T3-L1 adipocytes (see Methods). Since a PLA signal can only be obtained when the proteins of interest are in extremely close proximity, this technique enables the detection of direct protein-protein interactions in cells. Association of PPM1B and PPARγ was observed specifically in differentiated cells, and localized in the nucleus (Fig. 2d). Taken together, these data indicate that PPM1B is expressed in the nuclei of mature 3T3-L1 adipocytes, where it can interact with PPARγ.

PPM1B directly dephosphorylates PPARγ

Given the interaction between the phosphatase PPM1B and PPARγ (Fig. 1 and 2d), and since PPM1B is expressed in mature adipocytes (Fig. 2), we investigated whether PPARγ may be a direct PPM1B dephosphorylation substrate. First, FLAG-PPARγ and HA-PPM1B were transiently co-expressed in HEK 293T cells followed by FLAG immunoprecipitation. To detect PPARγ phosphorylation, a phosphoserine 112-specific antibody was used. Phosphorylated PPARγ was readily detected either in absence or presence of the synthetic PPARγ ligand rosiglitazone (Figure 3A, lane 3 and 4). Coexpression of PPM1B resulted in a significant decrease of PPARγ phosphorylation, both in the absence and presence of ligand (Figure 3A, lane 5 and 6). As a control, a PPM1B catalytic mutant was used in which a conserved arginine residue at position 179 was changed into glycine (R179G; [27]). This mutant displayed reduced dephosphorylation ability towards PPARγ (Figure 3A, lanes 5 and 6). Remarkably, coexpression of either wild type or mutant PPM1B led to increased PPARγ levels (Figure 3A, lanes 5 to 8). In order to determine whether PPM1B can directly dephosphorylate PPARγ *in vitro* assays were performed. FLAG-PPARγ was immunoprecipitated from transiently transfected HEK 293T cells and incubated with purified PPM1B. Phosphospecific antibodies were used to detect PPARγ dephosphorylation and as is shown in figure 3B lane 3 PPM1B functions as a direct PPARγ phosphatase. Taken together, these data indicate that PPM1B functions as a PPARγ phosphatase both in intact cells and *in vitro*.

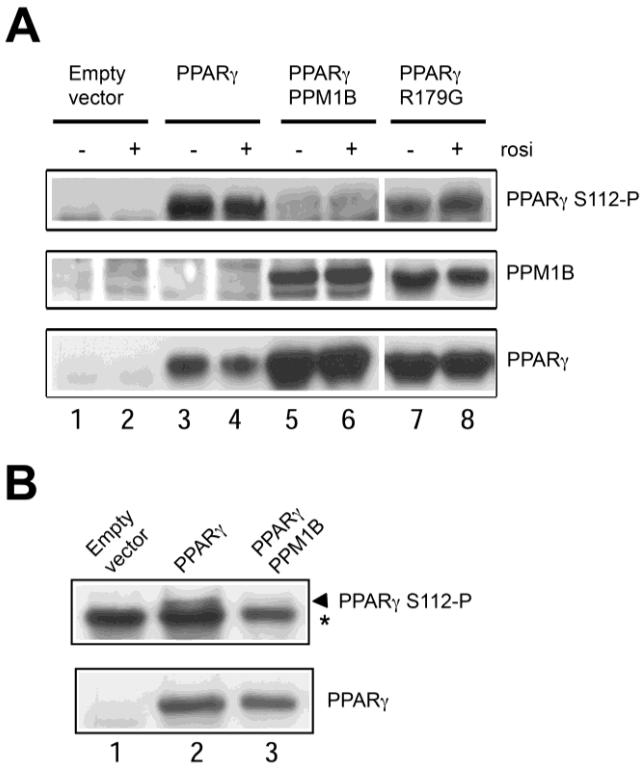


Figure 3 PPM1B functions as a direct phosphatase for PPARγ, dephosphorylating serine 112 within the AF1 region A. In HEK 293T cells PPM1B and FLAG PPARγ were overexpressed in presence or absence of 2μM rosiglitazone. PPARγ was immunoprecipitated in RIPA buffer using FLAG beads. Dephosphorylation of PPARγ S112 was detected with Western blot labeling using phospho S112-specific antibodies. B. *In vitro* dephosphorylation was assesed using PPARγ bound FLAG beads precipitated form transiently transfected HEK 293T cells. Beads were incubated with 1 unit of purified PPM1B and incubated at 30˚C for 45 minutes in phosphobuffer. Phosphorylation was detected by Western blot analysis as described above. An aspecific band is indicated (*).

PPM1B stimulates PPARγ activity

Finally, we examined whether PPM1B affected PPARγ activity. For this the human osteosarcoma cell line U2OS was used, which lacks endogenous PPARγ expression and displays a robust transcriptional response upon introduction of PPARγ (e.g [22,23]). To investigate whether PPM1B predominantly affects the N-terminal AF1 region or the C-terminal AF2 region, the two activation functions were fused individually to a heterologous DNA binding domain (Gal4DBD). Cotransfection of PPM1B isoform 1 clearly enhanced the ligand-independent activity of the Gal4DBD-AF1 protein, while the activity of AF2, either in the presence or absence

of ligand, was unaffected (Figure 4A). These experiments therefore indicate that PPM1B specifically targeted the AF1 region (including S112), and not the AF2 region (including S273).

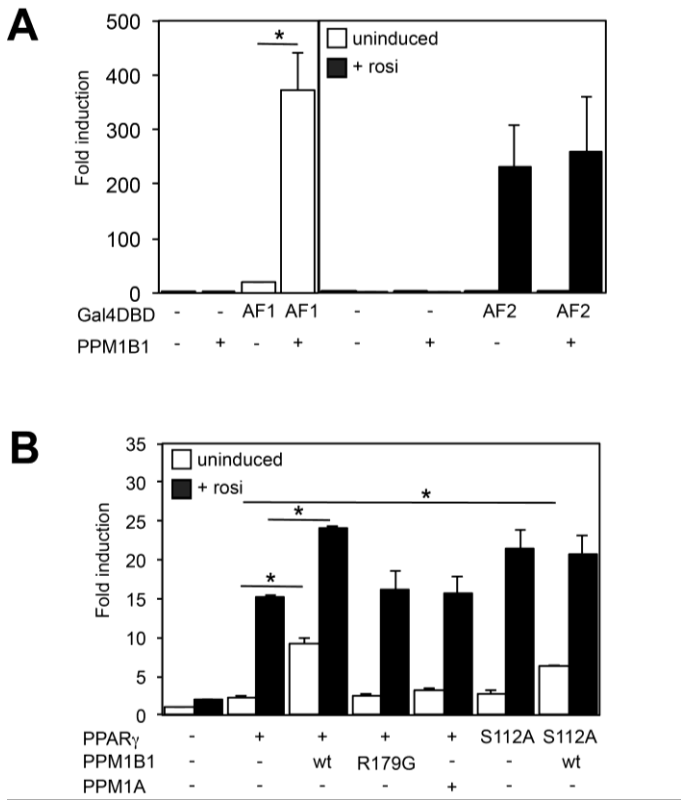


Figure 4 PPM1B is a specific activator of PPARγ

A**.** U2OS cells were transfected with 5xGal4-AdMLTATA-Luc reporter, empty vector or Gal4DBD-PPARγAF1 or -PPARγLBD(AF2), in the absence or presence of PPM1B and incubated with rosiglitazone (1μM) as indicated. Activation of the luciferase reporter is expressed as fold induction over that with empty vector in absence of ligand and normalized for Renilla luciferase activity. Results are averages of three independent experiments performed in duplo ± stand error of the means. Significant differences are indicated by an asterisk ($p<0.05$). B**.** U2OS cells were transfected with expression vectors encoding wt PPARγ, mutant PPARγ (S112A phosphorylation site mutant), wt PPM1B, mutant PPM1B (R179G catalytic inactive mutant), PPM1A and 3xPPRE-tk-Luc reporter. Activation of the luciferase reporter in absence or presence of 1μM rosiglitazone is expressed as fold induction over that with empty vector in absence of ligand and normalized for Renilla luciferase activity. Results are averages of three independent experiments performed in duplo ± stand error of the means. Significant differences are indicated by an asterisk ($p<0.05$).

Next, the effect of PPM1B on the transcriptional activity of the full-length PPARγ protein was assessed. As is shown in Figure 4B, co-transfection of PPARγ together with PPM1B increased PPARγ activity on a synthetic 3xPPRE reporter approximately 2-fold, either in the absence or the presence of the synthetic ligand rosiglitazone. Importantly, the catalytically inactive R179G mutant of PPM1B failed to activate PPARγ. Furthermore, the phosphatase PPM1A, which is 70% identical to PPM1B, did not affect PPARγ mediated reporter activation. Mutating the phosphorylation site at serine serine 112 into alanine resulted in increased activity as described earlier (e.g. [32]). PPM1B was not able to further increase transcriptional activity of this S112A mutant, indicating that PPM1B specifically targets S112 in the AF1 region. PPARγ-mediated activation of a more "natural" promoter (human adipose aquaporin gene; AQP7) was also potentiated by PPM1B, but not by its catalytically inactive R179G mutant (Supplementary Fig. 2). From these findings we conclude that the phosphatase PPM1B can specifically modulate PPARγ transcriptional activity, most likely through dephosphorylation of serine residue 112 in the N-terminal AF1 region.

PP1MB knock down selectively impairs endogenous PPARγ target gene expression

Having established that PPM1B is able to stimulate PPARγ (Fig. 4), we wished to address the role of this phosphatase in regulating endogenous PPARγ target genes. For this siRNA-mediated knock down experiments were performed. Two independent oligonucleotides were used, with oligonucleotide #6 being the most efficient while oligonucleotide #5 only marginally reduced PPM1B expression on the protein and mRNA level (Fig. 5A and C, respectively). Knockdown of PPM1B increased PPARγ phosphorylation at serine 112 (Fig. 5B), in line with a direct role for PPM1B in regulating the phosphorylation status of PPARγ (Fig. 3). When the expression of a number of PPARγ target genes was examined, knock down of PPM1B expression with oligonucleotide #6 was found to blunt the expression of a number of PPARγ target genes including *lpin1, lpl, adipoq, fabp4*, but not *cd36* (Fig. 5C). Introduction of oligonucleotide #5, which reduced PPM1B expression poorly, had no appreciable effect on PPARγ target gene expression (Fig. 5C). In contrast, knock down of PPARγ itself efficiently reduced the expression of all genes examined (Fig. 5C). Importantly, the decrease in PPARγ target gene expression observed upon PPM1B knock down is not caused by downregulation of PPARγ protein or mRNA (Fig. 5A and C, respectively). In agreement with a

selective role for PPM1B in regulating PPARγ activity, knock down of PPM1B did not significantly impair the differentiation of 3T3-L1 cells into adipocytes, as assessed by PPARγ protein expression and Oil-red-O staining (Fig. 5B and D), while knock down of PPARγ efficiently blocked adipogenesis (Fig. 5D). These findings indicate that the phosphatase PPM1B functions a selective modulator of PPARγ activity in adipocytes.

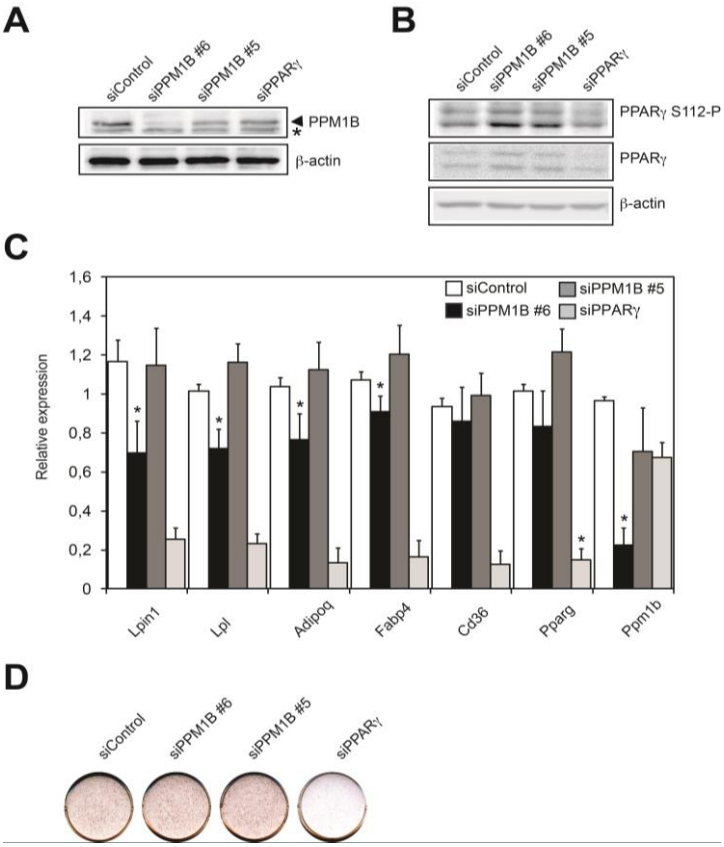


Figure 5 PPM1B knock down selectively impairs PPARγ target gene expression

A, 3T3-L1 cells were transfected with two oligonucleotides targeting PPM1B (#5 and #6), PPARγ and scrambled siRNA 2 days prior to differentiation. Five days after the start of differentiation cells were lyzed and subjected to Western Blot analysis, using antibodies against PPM1B and β-actin. B. Cell lysates prepared as described under A were subjected to Western Blot analysis, using antibodies against S112 phposphorylated PPARγ total PPARγ and β-actin. C**.** PPARγ target gene expression in cells treated as described under A was analyzed by quantitative PCR. As a control expression of *Pparg* and *Ppm1b* was included. Results are depicted relative to the housekeeping gene TFIIb and normalized for the siControl. Error bars represent SEM and significant differences between specific knock downs and siControl are indicated by an asterisk ($p<0.05$). D. 3T3-L1 cells treated as described under A were fixed and stained for triglycerides using Oil-red-O.

Discussion

In the present study we show that the phosphatase PPM1B is a novel PPARγ interacting protein, mediating direct dephosphorylation of serine 112, and thereby stimulating its transcriptional activity. Knockdown of PPM1B reduced the expression of a number of PPARγ target genes (e.g. *Lpin1, Lpl* and *Adipoq*) while leaving others unaltered (e.g. *Cd36*). In agreement with this, PPM1B knock down did not abolish adipogenesis. As such, the phosphatase PPM1B qualifies as a novel selective modulator of PPARγ activity. The finding that phosphorylation of S112 does not dictate but rather fine-tunes the transcriptional and adipogenic activity of PPARγ (e.g. [4,33]) supports this view.

Recently disruption of PPM1B in mice was shown to lead to early pre-implantation lethality [34]. Although PPM1B knock-out ES cells were viable, embryos died between two and eight-cell stage. Also knock-down of PPM1B in wild-type ES cells did not affect proliferation suggesting that PPM1B expression is specifically required during the early stages of embryogenesis and does not affect cell cycle progression [34]. So far, only a limited number of substrates for PPM1B have been identified, including the kinases IKKβ [35] and TAK1 [36], the pro-apoptotic protein Bad [37], the cyclin dependent kinases cdk2 and cdk6 [38], the transcription factor p53 [39] and the cdk9 subunit of the positive transcription elongation factor b (P-TEFb) [40]. Interestingly, Iankova *et al.* showed that cdk9-mediated phosphorylation of PPARγ on S112 results in enhanced transcriptional activity [9]. PPM1B may therefore potentially affect PPARγ activity indirectly, by stimulating cdk9 activity. In general, dephosphorylation of T186 in the T-loop of cdk9 can result in either activation of this enzyme by enabling its release from an inhibitory complex, or subsequent inhibition of its activity as phosphorylated T186 is required for substrate binding [40,41]. As PPM1B can only efficiently dephosphorylate cdk9 in the absence of the inhibitory complex [40], PPM1B-mediated dephosphorylation is most likely to result in reduced cdk9 activity and ultimately reduced gene expression. As we found PPM1B to stimulate rather than inhibit reporter activity, it is most likely that PPM1B regulates PPARγ-mediated transcription directly, and not indirectly via cdk9.

While not identified as a *bona fide* dephosphorylation substrate yet, the transcription factor EKLF/KLF1 was recently reported to interact with PPM1B [42]. Interestingly, both wt and catalytically inactive PPM1B stabilized the EKLF protein,

reminiscent of our findings with PPARγ (Fig. 3A). Future experiments should clarify whether PPM1B can also stabilize other proteins, and unravel the molecular mechanism underlying this phenomenon.

Very recently Hinds *et al.* reported the serine/threonine protein phosphatase 5 (PP5), an enzyme mainly implicated in the response to stress and hormones [43], to interact with and dephosphorylate PPARγ [15]. It should be noted that PP5 also interacts with and dephosphorylates GR [15], which also plays an important role in adipocyte differentiation and function [44]. Interestingly, PP5 knock out (PP5-KO) MEFs display reduced capacity for adipocyte differentiation (lipid accumulation and lipogenesis, adipogenic genes), and introduction of PPARγ S112 mutant, but not wt, rescued the adipogenesis defect in PP5-KO cells [15]. Despite being both identified as phosphatases targeting S112 of PPARγ, PPM1B and PP5 seem to differ in their exact mode of action. First, we found PPM1B to interact with PPARγ in the absence of ligand (Fig. 2), while the PP5-PPARγ interaction was induced by ligand (1h rosiglitazone treatment) and lost again at longer time points [15]. Furthermore, in contrast to PPM1B, which co-localized with PPARγ in the nuclei of 3T3-L1 adipocytes (Fig. 2B), PP5 predominantly displayed perinuclear localization [15]. Additional studies are therefore required to establish the relative importance of PP5 and PPM1B in regulating PPARγ phosphorylation and activity.

While activation of PPARγ by strong agonists of the TZD class clearly inhibits insulin resistance, their use has been linked to adverse side effects such as undesired weight gain, fluid retention, peripheral edema, and potential increased risk of cardiac failure [45]. Modulation of PPARγ PTM could offer the possibility of more subtle therapeutic intervention and provide new ways of improving insulin sensitivity. A clear example of this is the recently identified phosphorylation site at serine 273 in PPARγ [10]. CDK5-mediated phosphorylation of this residue is induced by various cytokines, the levels of which are commonly increased in obesity, and leads to dysregulation of a subset of genes whose expression is altered in obesity including the insulin-sensitizing adipokine, adiponectin. Interestingly, S273 phosphorylation is blocked *in vivo* and *in vitro* by TZDs, but also by certain antidiabetic drugs that are weak PPARγ agonists or non-agonists [10,12]. These findings indicate that future PPARγ-based antidiabetic drugs should be selected based on inhibition of S273 phosphorylation rather than transcriptional agonism.

Alternatively, PPARγ-based antidiabetic drugs could be based on S112 phosphorylation. Indeed, *in vivo* evidence suggests that inhibition of S112 phosphorylation can contribute to metabolic health: PPARγ S112A knock-in mice display unaltered fat content and weight compared to wt animals, but are protected against diet-induced insulin resistance [46]. Activation of the PPAR S112 phosphatases PPM1B and PP5 might therefore potentially improve metabolic health. Although not much is known about upstream signalling cascades, the catalytic activity of both PPM1B and PP5 can be activated by unsaturated fatty acids [47,48,49]. More research is needed to explore the potential of modulators of PPARγ PTM, like the phosphatases PPM1B and PP5 as future targets for the development of insulin sensitizing drugs.

Acknowledgments

* This work was supported by in part by grant LSHC-CT-2004-503438 from the European Commission Transfog Consortium (A.B.B and N.J.F. vdB). We thank Drs. J. Bakema, P.J. Coffer, H.Th.M. Timmers and Y. Gao for helpful discussions and Dr. A. Koppen for critical reading of the mansucript.

Abbreviations used are: AF, activation function; DBD, DNA binding domain; GR, glucocorticoid receptor; HA, haemaglutinin; LBD, ligand binding domain; MEF, mouse embryo fibroblast; PEI, polyethylenimine; PP, protein phosphatase; PPAR, peroxisome proliferator activated receptor; PPRE, PPAR response element; PTM, posttranslational modification; TZD, thiazolidinedione.

References

1. Tontonoz P, Spiegelman BM (2008) Fat and beyond: the diverse biology of PPARgamma. AnnuRevBiochem 77: 289-312.
2. van Beekum O, Fleskens V, Kalkhoven E (2009) Posttranslational modifications of PPAR-gamma: fine-tuning the metabolic master regulator. Obesity(SilverSpring) 17: 213-219.
3. Floyd ZE, Stephens JM (2012) Controlling a master switch of adipocyte development and insulin sensitivity: covalent modifications of PPARgamma. Biochimica et biophysica acta 1822: 1090-1095.
4. Shao D, Rangwala SM, Bailey ST, Krakow SL, Reginato MJ, et al. (1998) Interdomain communication regulating ligand binding by PPAR-gamma. Nature 396: 377-380.
5. Yamashita D, Yamaguchi T, Shimizu M, Nakata N, Hirose F, et al. (2004) The transactivating function of peroxisome proliferator-activated receptor gamma is negatively regulated by SUMO conjugation in the amino-terminal domain. Genes Cells 9: 1017-1029.
6. Ohshima T, Koga H, Shimotohno K (2004) Transcriptional activity of peroxisome proliferator-activated receptor gamma is modulated by SUMO-1 modification. JBiolChem 279: 29551-29557.
7. Grimaldi B, Bellet MM, Katada S, Astarita G, Hirayama J, et al. (2010) PER2 controls lipid metabolism by direct regulation of PPARgamma. Cell metabolism 12: 509-520.
8. Compe E, Drane P, Laurent C, Diderich K, Braun C, et al. (2005) Dysregulation of the peroxisome proliferator-activated receptor target genes by XPD mutations. MolCell Biol 25: 6065-6076.
9. Iankova I, Petersen RK, Annicotte JS, Chavey C, Hansen JB, et al. (2006) Peroxisome proliferator-activated receptor gamma recruits the positive transcription elongation factor b complex to activate transcription and promote adipogenesis. MolEndocrinol 20: 1494-1505.
10. Choi JH, Banks AS, Estall JL, Kajimura S, Bostrom P, et al. (2010) Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5. Nature 466: 451-456.
11. Li P, Fan W, Xu J, Lu M, Yamamoto H, et al. (2011) Adipocyte NCoR knockout decreases PPARgamma phosphorylation and enhances PPARgamma activity and insulin sensitivity. Cell 147: 815-826.
12. Choi JH, Banks AS, Kamenecka TM, Busby SA, Chalmers MJ, et al. (2011) Antidiabetic actions of a non-agonist PPARgamma ligand blocking Cdk5-mediated phosphorylation. Nature 477: 477-481.
13. Anbalagan M, Huderson B, Murphy L, Rowan BG (2012) Post-translational modifications of nuclear receptors and human disease. Nuclear receptor signaling 10: e001.
14. Zhang Y, Leung DY, Nordeen SK, Goleva E (2009) Estrogen inhibits glucocorticoid action via protein phosphatase 5 (PP5)-mediated glucocorticoid receptor dephosphorylation. The Journal of biological chemistry 284: 24542-24552.

15. Hinds TD, Jr., Stechschulte LA, Cash HA, Whisler D, Banerjee A, et al. (2011) Protein phosphatase 5 mediates lipid metabolism through reciprocal control of glucocorticoid receptor and peroxisome proliferator-activated receptor-gamma (PPARgamma). The Journal of biological chemistry 286: 42911-42922.
16. Moorhead GBG, Trinkle-Mulcahy L, Ulke-Lemee A (2007) Emerging roles of nuclear protein phosphatases. NatRevMolCell Biol 8: 234-244.
17. Lammers T, Lavi S (2007) Role of type 2C protein phosphatases in growth regulation and in cellular stress signaling. Critical reviews in biochemistry and molecular biology 42: 437-461.
18. Klumpp S, Thissen MC, Krieglstein J (2006) Protein phosphatases types 2Calpha and 2Cbeta in apoptosis. Biochemical Society transactions 34: 1370-1375.
19. Ausubel FM, Brent R, Kingston R, Moore D, Seidman JJ, et al. (1993) Current Protocols in Molecular Biology. New York: John Wiley & Sons.
20. Kalkhoven E, Teunissen H, Houweling A, Verrijzer CP, Zantema A (2002) The PHD type zinc finger is an integral part of the CBP acetyltransferase domain. MolCell Biol 22: 1961-1970.
21. Kondo H, Shimomura I, Kishida K, Kuriyama H, Makino Y, et al. (2002) Human aquaporin adipose (AQPap) gene. Genomic structure, promoter analysis and functional mutation. EurJBiochem 269: 1814-1826.
22. Jeninga EH, van Beekum O, van Dijk AD, Hamers N, Hendriks-Stegeman BI, et al. (2007) Impaired peroxisome proliferator-activated receptor gamma function through mutation of a conserved salt bridge (R425C) in familial partial lipodystrophy. MolEndocrinol 21: 1049-1065.
23. van Beekum O, Brenkman AB, Grontved L, Hamers N, van den Broek NJ, et al. (2008) The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARgamma. Endocrinology 149: 1840-1849.
24. Lebbink RJ, de Ruiter T, Adelmeijer J, Brenkman AB, van Helvoort JM, et al. (2006) Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. JExpMed 203: 1419-1425.
25. van Beekum O, Gao Y, Berger R, Koppen A, Kalkhoven E (2012) A Novel RNAi Lethality Rescue Screen to Identify Regulators of Adipogenesis. PloS one 7: e37680.
26. Terasawa T, Kobayashi T, Murakami T, Ohnishi M, Kato S, et al. (1993) Molecular-Cloning of A Novel Isotype of Mg2+-Dependent Protein Phosphatase Beta (Type-2C-Beta) Enriched in Brain and Heart. ArchBiochemBiophys 307: 342-349.
27. Kusuda K, Kobayashi T, Ikeda S, Ohnishi M, Chida N, et al. (1998) Mutational analysis of the domain structure of mouse protein phosphatase 2Cbeta. BiochemJ 332 (Pt 1): 243-250.
28. Marley AE, Kline A, Crabtree G, Sullivan JE, Beri RK (1998) The cloning expression and tissue distribution of human PP2C beta. FEBS Lett 431: 121-124.
29. Wenk J, Mieskes G (1995) Cytosolic and nuclear localization of protein phosphatase 2C beta 1 in COS and BHK cells. European journal of cell biology 68: 377-386.
30. Hufnagel B, Dworak M, Soufi M, Mester Z, Zhu Y, et al. (2005) Unsaturated fatty acids isolated from human lipoproteins activate protein phosphatase type 2C beta and induce apoptosis in endothelial cells. Atherosclerosis 180: 245-254.

31. Seroussi E, Shani N, Ben Meir D, Chajut A, Divinski I, et al. (2001) Uniquely conserved non-translated regions are involved in generation of the two major transcripts of protein phosphatase 2C beta. JMolBiol 312: 439-451.
32. Hu E, Kim JB, Sarraf P, Spiegelman BM (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. Science 274: 2100-2103.
33. Hu ED, Kim JB, Sarraf P, Spiegelman BM (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR gamma. Science 274: 2100-2103.
34. Sasaki M, Ohnishi M, Tashiro F, Niwa H, Suzuki A, et al. (2007) Disruption of the mouse protein Ser/Thr phosphatase 2C beta gene leads to early pre-implantation lethality. MechDev 124: 489-499.
35. Sun W, Yu Y, Dotti G, Shen T, Tan X, et al. (2009) PPM1A and PPM1B act as IKKbeta phosphatases to terminate TNFalpha-induced IKKbeta-NF-kappaB activation. Cellular signalling 21: 95-102.
36. Hanada M, Ninomiya-Tsuji J, Komaki K, Ohnishi M, Katsura K, et al. (2001) Regulation of the TAK1 signaling pathway by protein phosphatase 2C. The Journal of biological chemistry 276: 5753-5759.
37. Klumpp S, Selke D, Krieglstein J (2003) Protein phosphatase type 2C dephosphorylates BAD. NeurochemInt 42: 555-560.
38. Cheng A, Ross K, Kaldis P, Solomon MJ (1999) Dephosphorylation of cyclin-dependent kinases by type 2C protein phosphatases. MolBiolCell 10: 3A-3A.
39. Park JH, Smith RJ, Shieh SY, Roeder RG (2011) The GAS41-PP2Cbeta complex dephosphorylates p53 at serine 366 and regulates its stability. The Journal of biological chemistry 286: 10911-10917.
40. Wang Y, Dow EC, Liang YY, Ramakrishnan R, Liu H, et al. (2008) Phosphatase PPM1A regulates phosphorylation of Thr-186 in the Cdk9 T-loop. The Journal of biological chemistry 283: 33578-33584.
41. Cho S, Schroeder S, Ott M (2010) CYCLINg through transcription: posttranslational modifications of P-TEFb regulate transcription elongation. Cell Cycle 9: 1697-1705.
42. Yien YY, Bieker JJ (2012) Functional Interactions between Erythroid Kruppel-like Factor (EKLF/KLF1) and Protein Phosphatase PPM1B/PP2Cbeta. The Journal of biological chemistry 287: 15193-15204.
43. Golden T, Swingle M, Honkanen RE (2008) The role of serine/threonine protein phosphatase type 5 (PP5) in the regulation of stress-induced signaling networks and cancer. Cancer metastasis reviews 27: 169-178.
44. Pantoja C, Huff JT, Yamamoto KR (2008) Glucocorticoid signaling defines a novel commitment state during adipogenesis in vitro. Molecular biology of the cell 19: 4032-4041.
45. Gervois P, Fruchart JC, Staels B (2007) Drug Insight: mechanisms of action and therapeutic applications for agonists of peroxisome proliferator-activated receptors. NatClinPractEndocrinolMetab 3: 145-156.
46. Rangwala SM, Rhoades B, Shapiro JS, Rich AS, Kim JK, et al. (2003) Genetic modulation of PPAR gamma phosphorylation regulates insulin sensitivity. DevCell 5: 657-663.

47. Klumpp S, Selke D, Hermesmeier J (1998) Protein phosphatase type 2C active at physiological Mg2+: stimulation by unsaturated fatty acids. FEBS Lett 437: 229-232.
48. Ramsey AJ, Chinkers M (2002) Identification of potential physiological activators of protein phosphatase 5. Biochemistry 41: 5625-5632.
49. Skinner J, Sinclair C, Romeo C, Armstrong D, Charbonneau H, et al. (1997) Purification of a fatty acid-stimulated protein-serine/threonine phosphatase from bovine brain and its identification as a homolog of protein phosphatase 5. The Journal of biological chemistry 272: 22464-22471.

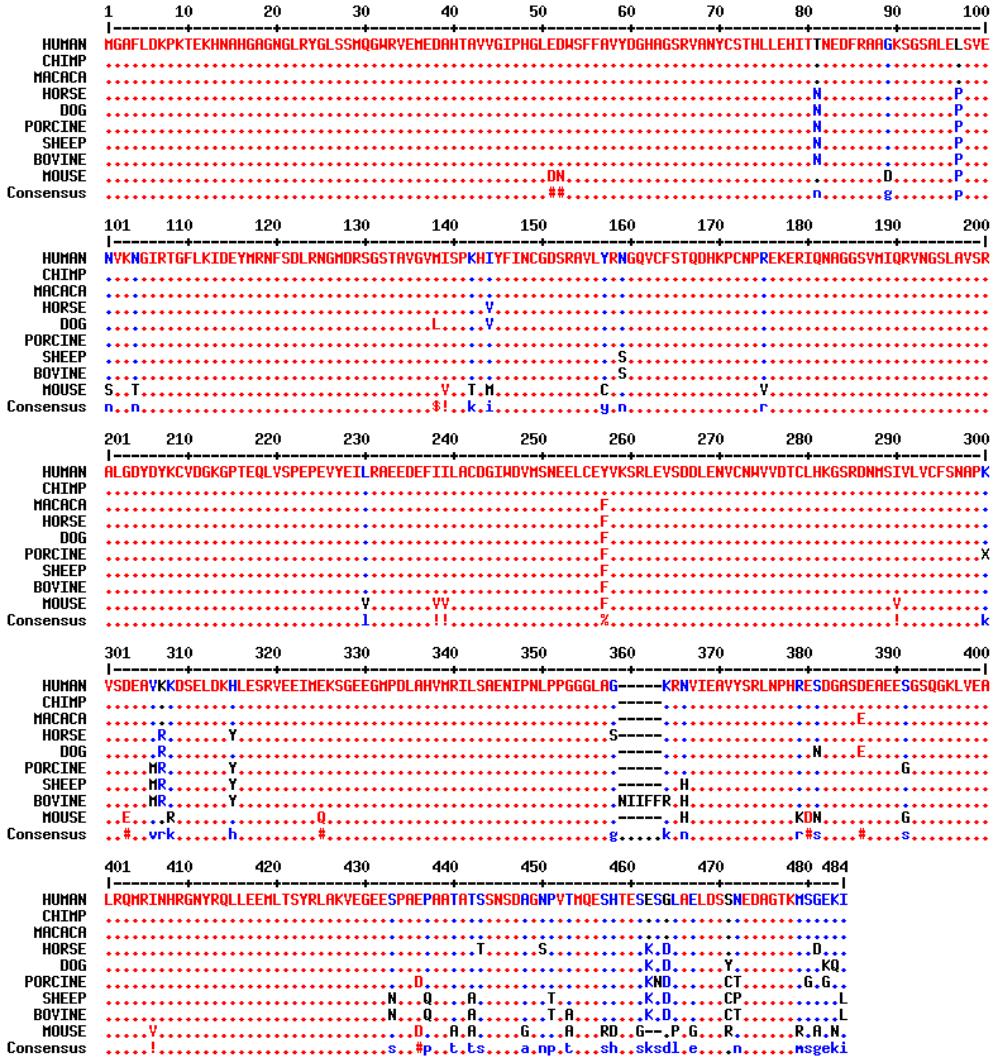
Supplementary Figures

Sequence	AA	Score	Charge
SGSALELSVENVK	91-103	88	2+
HIYFINCGDSR	143-153	50	2+
NGQVCFSTQDHKPCNPR	159-175	42	2+
IQNAGGSVMIQR	180-191	95	2+
VNGSLAVSR	192-200	65	2+
ILSAENIPNLPPGGGLAGK	341-359	64	2+
NVIEAVYSR	361-369	29	2+
IQNAGGSVMIQR	180-191	64	2+
IQNAGGSVMIQR	180-191	85	2+
VANYCSTHLLEHITTNEDFR	67-86	38	3+
SGSTAVGVMISPKHIYFINCGDSR	130-153	96	3+
IQNAGGSVMIQR	180-191	38	2+
SGSALELSVENVK	91-103	46	2+
SGSTAVGVMISPK	130-142	71	2+
IQNAGGSVMIQR	180-191	85	2+
IQNAGGSVMIQR	180-191	44	2+
ILSAENIPNLPPGGGLAGK	341-359	37	3+
QLLEEMLTSYR	409-419	69	2+

Supplementary Table S1 PPM1B peptides identified in the present study. Indicated are the peptide sequence, the amino acid (AA) positions, the ionscore and the charge of the peptides.

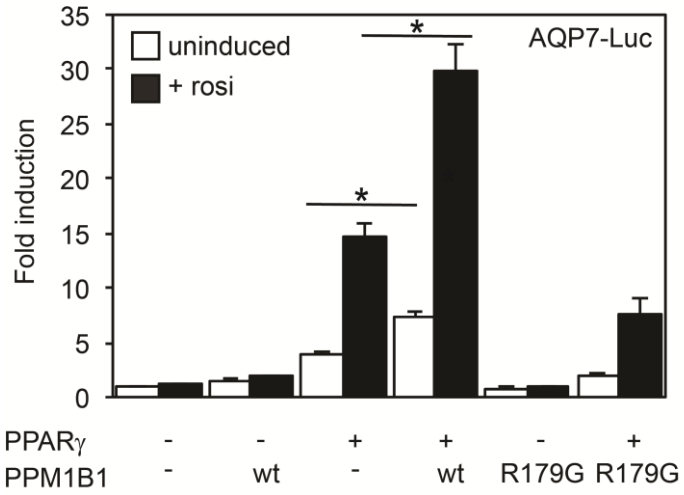
Mouse		*Human*	
Isoform	Acc. No.	Isoform	Acc. No.
1	NP_001152968.1	1	NP_002697.1
2	NP_035281.1	2	NP_808907.1
3	NP_001152969.1	3	NP_808908.1
4	NP_001152970.1	4	NP_001028728.1
		5	NP_001028729.1

Supplementary Table S2 NCBI accession numbers for isoforms of the mouse and human PPM1B protein



Supplementary Figure S1 Alignment of the isoform 1 PPM1B proteins

Aligment of human (NP_002697.1), chimp (XP_525747.1), Macaca (NP_001253131.1), horse (XP_001499388.1), dog (XP_851683.1), pig (XP_003125227.1), sheep (XP_004006035.1), cow, (O62830) and mouse (NP_001152968.1) proteins was generated with MultiAlin software (http://multalin.toulouse.inra.fr/multalin/multalin.html).



Supplementary Figure S2 PPM1B is a specific activator of PPARγ.
U2OS cells were transfected with expression vectors encoding PPARγ, wt PPM1B1, mutant PPM1B (R179G catalytic inactive mutant) and an AQP7-Luc reporter. Activation of the luciferase reporter in absence or presence of 1μM rosiglitazone is expressed as fold induction over that with empty vector in absence of ligand and normalized for Renilla luciferase activity. Results are averages of three independent experiments performed in duplo ± stand error of the means.

Chapter 3

THE SWI/SNF PROTEIN BAF57 REGULATES ADIPOGENESIS

Ismayil Tasdelen[1], Ronni Nielsen[2], Daphne Lelieveld[3], Marian Groot Koerkamp[4], Ruud Berger[1], Frank C.P. Holstege[4], David Egan[3], Susanne Mandrup[2], and Eric Kalkhoven[1§]

[1]Section Metabolic Diseases, Molecular Cancer Research, and Netherlands Metabolomics Centre, University Medical Centre Utrecht, Utrecht 3584 CG, The Netherlands, [2]Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense, Denmark, [3]Department of Cell Biology, University Medical Centre Utrecht, Utrecht 3584 CX, The Netherlands, [4]Molecular Cancer Research, University Medical Centre Utrecht, Utrecht 3584 CG, The Netherlands.

Manuscript in preparation

Abstract

Adipocyte differentiation is a tightly regulated process controlled by a cascade of transcriptional events, ultimately leading to up-regulation of the transcription factor PPARγ and the induction of multiple adipocyte-specific genes. These transcription factors operate in concert with chromatin remodelling factors and histone modifying enzymes in the epigenetic regulation of adipogenesis. Here we developed an siRNA-based knockdown platform to screen for novel epigenetic regulators in adipogenesis. We identified the Baf57 subunit of the SWI/SNF complex to be essential for adipogenesis. Knockdown of this SWI/SNF subunit led to impaired adipocyte differentiation, and reduced expression of PPARγ and several of its target genes. Baf57 was present at the *PPARG/Pparg* promoter at all stages of differentiation in both SGBS and 3T3-L1 cells. Collectively, these data provide evidence that Baf57 is a crucial player in adipogenesis through its regulation of *PPARG/Pparg* expression.

Introduction

Adipogenesis is a complex process defined by the morphological change of fibroblast-like pre-adipocytes into lipid-loaded adipocytes [1]. Many genes are involved at different stages and are under tight regulation by multiple transcription factors [2]. In this context, the nuclear receptor Peroxisome Proliferator-Activated Receptor gamma (PPARγ) is known to be a crucial key player. PPARγ is essential for adipogenesis because it drives expression of many genes that are directly involved in glucose and lipid metabolism and storage [3,4]. Moreover, PPARγ$^{-/-}$ mice are severely lipodystrophic, while PPARγ$^{+/-}$ mice have reduced amounts of adipose tissue [5,6,7,8]. PPARγ is also essential for the maintenance of adipose tissue, since conditional knockout of the *Pparg* gene resulted in reduced *in vivo* survival of mature adipocytes [9]. In general, unliganded PPARγ interacts with repressor proteins such as TAZ, RIP140, NCoR and SMRT, of which the latter two are associated with HDAC3-mediated gene inhibition. Upon ligand binding, PPARγ changes its structural conformation, dissociating the repressor complexes and subsequently allowing binding of co-activators, such as SRC/p160 and CBP/p300, HATs that acetylate histone tails and promote gene transcription [10,11].

Gene expression is subject to a complex interplay in which transcription factors (TF), transcriptional co-factors, histone modifying enzymes, chromatin remodelers, RNAs and RNA polymerase machinery play important roles [12]. Generally, TFs bind to enhancers in a concerted manner and -depending on the distance of the core promoter- chromatin looping may occur, and thereby allow regulation of gene transcription in a chromatin-wide context. In some cases the TF can also bind close to the TSS and attract RNA polymerase after recruitment of co-activators. In order to provide accessibility to compact chromatin, the so-called chromatin remodelers and histone modifying enzymes play important roles. Chromatin remodelers are generally large protein complexes that contain an ATPase subunit, which physically mobilize nucleosomes at the expense of ATP in order to provide access for the transcription apparatus and its regulators to DNA [12]. Histone-modifying enzymes on the other hand, which are recruited by transcription factors, either chemically tag histones at specific sites (“writers”) or erase such tags (“erasers”), thereby regulating DNA-histones interactions and also creating interaction surfaces for protein complexes that contribute to

transcriptional control ("readers")[12]. Collectively, all these factors play a role in gene regulation and expression.

One of the chromatin remodeling complexes is named SWI/SNF (switch/sucrose nonfermentable, originally identified in *Saccharomyces cerevisiae*), a large multi-protein complex capable of ATP-dependent chromatin remodeling leading to either gene activation or repression [10,13]. The SWI/SNF complex has many subunits; the core-subunit, BRG1 or BRM, harbours ATPase activity that drives chromatin remodeling at the expense of ATP. The amount and type of subunits representing the SWI/SNF complex depend on the organism and the cell type, but there are some proteins that are absolutely vital for the integrity of the complex; these are the ATPase, SNF5/Baf47, Baf155 and Baf170 [14]. In general, it is thought that the role of the additional subunits is to add specificity to the complex and thus it is possible to mediate different transcription cascades based on the cell-type it is present in. Baf250a for example is necessary for embryonic stemcell pluripotency and germ-layer formation [15], while Baf200 is involved in regulating expression of interferon responsive genes [16]. Research on the SWI/SNF complex has predominantly been carried out in the context of cancer development, in which SWI/SNF subunits appear to have intrinsic tumour suppressor activity [14].

Using an siRNA-based knockdown platform to screen for novel epigenetic regulators in adipogenesis in human SGBS pre-adipocytes, we have now identified the Baf57 subunit of the SWI/SNF complex to be essential for adipogenesis. Knockdown of this SWI/SNF subunit led to impaired adipocyte differentiation, and reduced expression of PPARγ and several of its target genes. Baf57 was present at the PPARG/Pparg promoter at all stages of differentiation in both SGBS and 3T3-L1 cells. Collectively, these data provide evidence that Baf57 is a crucial player in adipogenesis because it can regulate PPARG/Pparg expression.

Materials & Methods

Materials - Dharmacon siRNA oligonucleotides against PPARγ (L-003436-00), Baf57 (LU-017522-00-0002) and non-targeting siRNA (D-001810-10) were purchased from Thermo Scientific. For Western blotting primary antibodies against Baf57 (A300-810A, Bethyl), FABP4 (sc-18661, Santa-Cruz,) and β-actin (Ab8224, Abcam) were used. Oil-red-O (O0625) and Nile red (N3013) were from Sigma-Aldrich.

Cell culture, differentiation, siRNA transfection **-** The human pre-adipocyte cell line SGBS was cultured and differentiated as previously described [17,18]. For siRNA transfection, 4000 SGBS cells were plated in a single well of a 96-well plate five days prior to differentiation. Subsequently, differentiation cocktail was added before addition of the siRNA oligonucleotide containing mixture. siRNA transfection with lipofectamine RNAiMax (Life technologies) was carried out according to the manufacturer's instructions in which 0.3 µl of RNAiMax and a final concentration of 10 nM siRNA oligonucleotides was used.

Oil-red-O and Nile red staining **-** For Oil-red-O staining, SGBS cells were fixed for 10 minutes with 4% formaldehyde at room temperature. Afterwards cells were briefly washed once with 60% iso-propanol after which the Oil-red-O solution (3mg/ml in 60% isopropanol) was added for 10 minutes. Subsequently, the cells were washed twice with 60% isopropanol and then twice with water.

For Nile red staining, cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton-X for 2 minutes. Afterwards they were incubated with a Nile-red containing buffer (1mg Nile red in PBS) for 5 minutes, followed by washing twice with PBS.

siRNA library and screen **-** The siRNA screen was performed at the UMCU Cell Screening Center. The siRNA library, targeting expression of 529 chromatin associated genes, was purchased from Thermo Fischer Scientific Dharmacon (Lafayette, CO, custom made library, order numbers 245120-245146, 245170, 246203)[19]. The screen was performed in 96-well plates. SGBS cells were plated and treated for differentiation and transfection as described above. Each gene was targeted with a pool of four individual siRNA's and was tested in triplicates in adjacent wells. After seven days, the cells were washed and fixed with PFA for Nile

red and Hoechst staining and the plates were made ready for the automated microscope. The plates were imaged using a Thermo ArrayScan VTi automated microscope. The Cellomics Target Activation image analysis bioapplication was used to quantitate adipocytic differentiation. First, the algorithm identifies nuclei in the Hoechst channel. A mask is then generated around the nucleus and the intensity of Oil-Red-O staining in this mask is calculated. Based on the analysis of negative controls in pilot experiments and cutoff for the average pixel intensity in the Nile red channel was used to determine the percentage of Nile Red positive cells. This percentage of positive cells was normalized to the average of the samples in the plate. Afterwards, these outcomes are normalized to the average of each plate and each well would be given a score. If a particular well would deviate more than once than the standard deviation, it would get a score of 0.5. Should this be twice or three fold, the scores would be 1 and 2 respectively. The scores of the three replicates were added up. The genes that would have a total score of 3 or higher were selected for further analysis.

RNA isolation and QPCR **-** At the time of harvesting, cells were taken up in 1ml of Trizol reagent (Life technologies) and were subjected to RNA isolation and cDNA synthesis according to the manufacturer's instructions (Life technologies). Gene expression levels were determined by quantitative real time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to *36B4* expression. The primers used were as followed: human 36B4 forward primer,
5'- CGGGAAGGCTGTGGTGCTG-3', and reverse primer,
5'- GTGAACACAAAGCCCACATTCC-3'; human PPARγ forward primer,
5'- CCTATTGACCCAGAAAGCGATT-3' and reverse primer,
5'- CATTACGGAGAGATCCACGGA-3'; human BAF57 forward primer,
5'- CGACGAGAACATTCCGATG-3', and reverse primer,
5'- CCTTCTTCACCATTCTGTTGG-3'; human C/EBPα forward primer,
5'- CAACACTTGTATCTGGCCTCTG-3', and reverse primer,
5'- CCGAGCAAAACCAAAACAAA-3'; human KLF5 forward primer,
5'- CCACCACCCTGCCAGTTAAC-3', and reverse primer,
5'- TAAACTTTTGTGCAACCAGGGTAA-3'; human KROX20 forward primer,
5'- TTGACCAGATGAACGGAGTG-3' and reverse primer,
5'- TGGTTTCTAGGTGCAGAGACG-3'; human C/EBPβ forward primer,
5'- GCGACGAGTACAAGATCC-3' and reverse primer,

5’- AGCTGCTTGAACAAGTTCC-3’; human FABP4 forward primer,
5’- CCTTTAAAAATACTGAGATTTCCTTCA-3’ and reverse primer,
5’- GGACACCCCCATCTAAGGTT-3’; human LPL forward primer,
5’- ATGTGGCCCGGTTTATCA-3’ and reverse primer,
5’- CTGTATCCCAAGAGATGGACATT-3’; human ADIPOQ forward primer,
5’- CCTGGTGAGAAGGGTGAGAA-3’ and reverse primer,
5’- CACCGATGTCTCCCTTAGGA-3’.

Microarray **-** SGBS cells were differentiated and transfected with siRNA as described above. The non-targeting siRNA and siPPARγ were performed in duplicate whereas siBaf57 in quadruplicate. Five days after transfection cells were washed twice with PBS twice and lysed in 1.0ml Trizol (Invitrogen). Samples were treated similar to the RNA isolation procedure. DNAse treatment and purification was performed with RNeasy kit (Qiagen, Venlo, The Netherlands).
Microarrays used were Human Whole Genome Gene Expression Microarrays V1 (Agilent Technologies, Belgium) representing 41000 *H. sapiens* 60-mer probes in a 4x44K layout. RNA amplifications and labelling were performed on an automated system (Caliper Life Sciences NV/SA, Belgium) with 3 µg total RNA from each sample. Hybridizations were done on a hybridization station (HS4800PRO system with QuadChambers; Tecan Benelux B.V.B.A.) using 800 ng labelled cRNA per channel according to van Wageningen [20]. Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery), Loess normalization was performed [21] on mean spot-intensities. Gene-specific dye bias was corrected based on a within-set estimate as described in Margaritis *et al*.[22].
Data were analysed using MAANOVA [23]. In a fixed effect analysis, sample, array and dye effects were modelled. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Genes (based on unique ENSG gene IDs) with $p < 0.05$ after family wise error correction (FWER) were considered significantly changed. A fold change cut-off > ±2.0 was used.

Chromatin immunoprecipitations assays (ChIP) **-** ChIP experiments were performed according to standard protocols [3]. Lysed cells were sonicated using a Bioruptor (Diagenode) according to the manufacturer’s protocol, and chromatin was immunoprecipitated with Baf57 antibody. DNA enrichment was quantified by

real-time PCR (MX-3000, Stratagene) using SYBR green Master Mix (Sigma-Aldrich). Primer sequences are available upon request.

Results

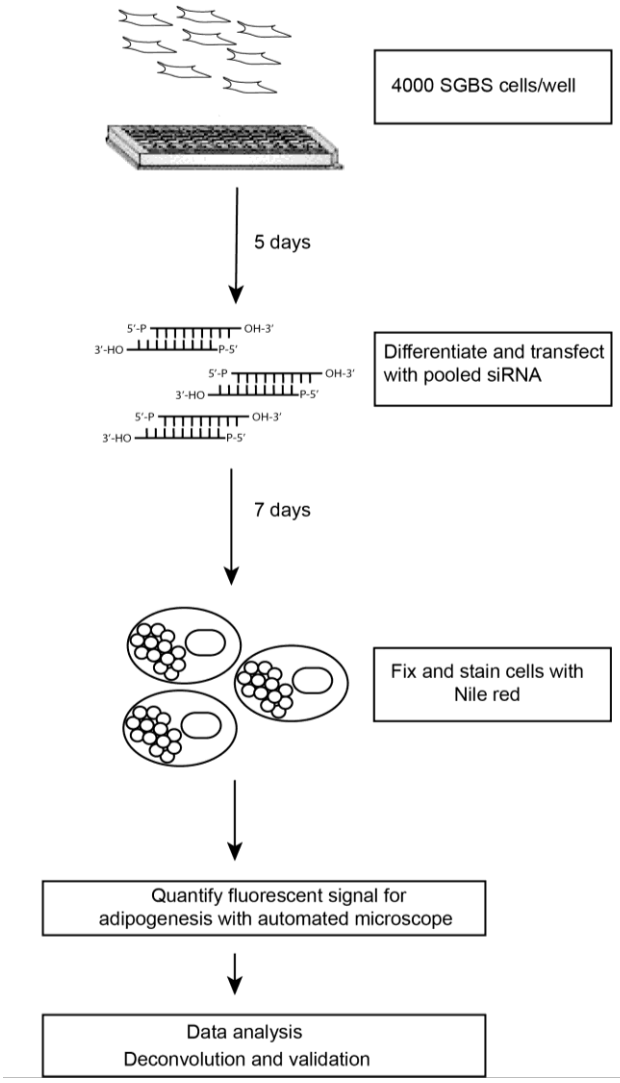
Development of an siRNA screen to identify novel epigenetic regulators of adipogenesis

In order to identify novel epigenetic regulators in adipogenesis, a loss-of-function screening platform was developed using an siRNA oligonucleotide library targeting 529 chromatin associated genes [19]. The siRNA library and screen were optimized using human SGBS cells, a pre-adipocyte cell line [17]. Importantly, 4000 cells were incubated 5 days prior to differentiation in a single well of 96-well plates (Fig. 1A). After incubation, the cells were differentiated and transfected with pooled siRNA oligonucleotides (four oligonucleotides per gene). At the end of differentiation the cells were stained for lipid loading using Nile red and an algorithm on the automated microscope was used to measure the amount of lipid loaded adipocytes. After the initial run, the hits were re-tested in order to filter out false positives. The remaining hits were analyzed and validated using single oligonucleotides, re-tested and subjected to statistical analysis (see Materials and Methods). Several genes previously implicated in adipogenesis, including Nipbl [24], Ogt [25], and Baf47 [26], were identified, thereby validating the screening procedure. In addition, the SWI/SNF subunit Baf57, also known as SMARCE1, was identified as a positive hit in our screen for epigenetic regulators of adipogenesis (Fig. 1B). Importantly, Baf57 knockdown does not impair the integrity of the SWI/SNF complex, despite co-depletion of Baf180 [27].

Baf57 is a valid hit of the screen and knockdown lowers PPARγ mRNA levels

To characterize the role of Baf57 in adipogenesis in more detail, we first examined its expression during adipogenesis. As shown in Fig. 2A, Baf57 protein was stably expressed during differentiation of SGBS cells into mature adipocytes. Next we identified the most potent oligonucleotide for Baf57 knockdown. For this, SGBS cells were transfected with four different siRNA oligonucleotides and incubated with differentiation cocktail. At the end of differentiation, the cells were fixed and the percentage of adipocytes was measured with the automated microscope (Fig. 2B). Baf57 oligonucleotide #7 was the most potent in impairing adipogenesis. This impairment was visualized by means of an Oil-red-O staining (Fig. 2D) and knockdown of PPARγ was done in parallel to demonstrate that the scope of adipogenic disruption was similar.

A



B

Gene	Score
BAF57	6
NIPBL	6
PHF12	6
SNF5	5
PRDM9	4.5
SIN3A	4
MBD1	3.5
OGT	3.5
DPF2	3
RAD21	3
ARID1A	2.5
AIRE	2.5
ARID2	2.5
PHF21A	2.5

Figure 1 Schematic representation of an siRNA-based screen for epigenetic regulators of adipogenesis. A) The screen was setup as described in materials & methods. In short, SGBS cells were seeded in 96-well plates prior to differentiation and siRNA transfection. After a period of incubation time, they were washed, fixed and stained with a fluorescent dye binding to lipids. Afterwards, the plates were analyzed in an automated microscope for analysis of the adipocyte population per well. B) The algorithm as described in materials & methods assigned points to each hit in the screen. The higher the score, the stronger the adipogenic disruption. The top of the list is shown, in which Baf57 scored amongst the highest.

Western Blot analysis revealed that oligonucleotide #7 specifically targeted Baf57 as its protein levels were reduced whereas no differences were seen in β-actin levels. To ascribe the abrogation of adipogenesis to the reduction of Baf57 levels, knockdown of Baf57 with all four oligonucleotides was performed (Fig. 2B). As expected, oligonucleotide #7 was most potent in lowering Baf57 mRNA. Since PPARγ is crucial for adipogenesis, we also wondered whether Baf57 knockdown affected PPARγ levels. Interestingly, this seemed to be the case since PPARγ levels were also lowered in cells with reduced Baf57 levels (Fig. 2B). These data indicate that Baf57 is necessary for adipogenesis and that knockdown of Baf57 leads to diminished PPARγ levels.

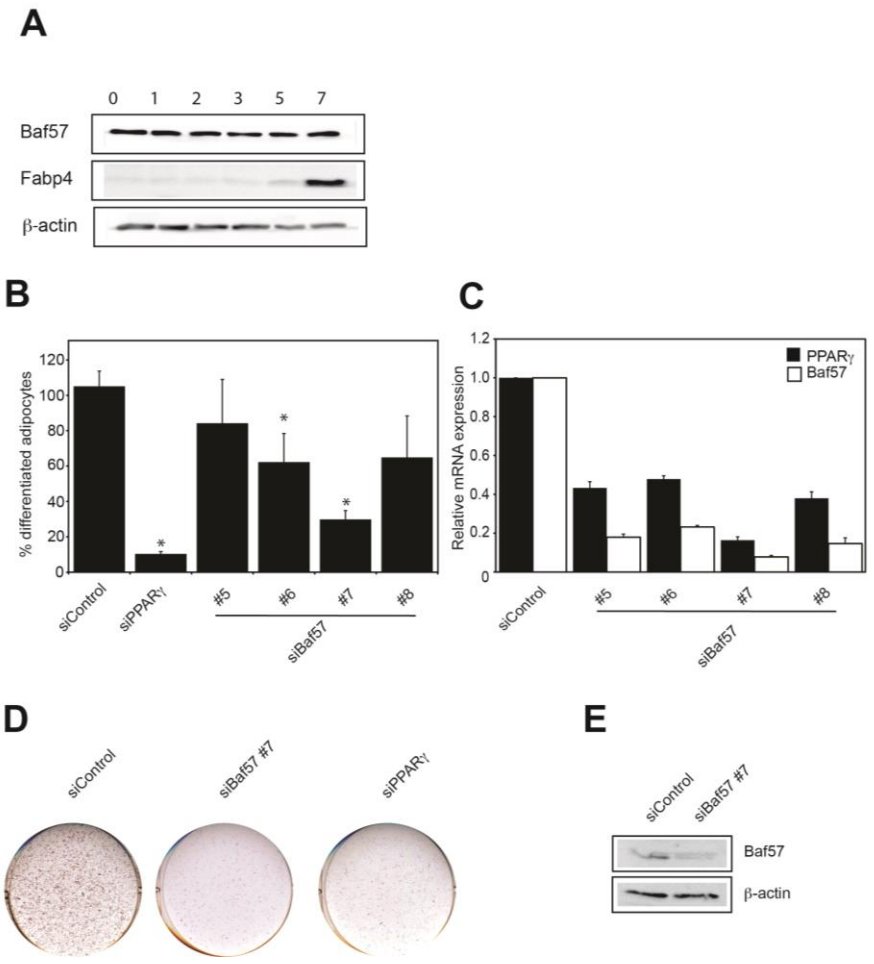


Figure 2 siRNA-mediated knockdown of Baf57 inhibits adipogenesis in SGBS cells.
A) Human SGBS pre-adipocytes were differentiated into mature adipocytes and samples were taken at different time points during differentiation. Protein expression of Baf57 and Fabp4 was determined by Western blot analysis. B) SGBS cells were transfected with 4 individiual siRNA oligonucleotides targeting Baf57 and differentiated. At day 7 cells were stained for lipid content (Nile red) and analysed by automated microscopy. Knockdown of PPARγ was included as a positive control. C) SGBS cells were treated as under B), except RNA was isolated at day 5 of differentiation and relative mRNA expression for Baf57 and PPARγ was assessed by qRT-PCR. D) SGBS cells were treated as under B), except that the cells were washed, fixed and stained with Oil-red-O dye. E) SGBS cells were treated as under B), except that cells were lysed at day 5 and subjected to Western Blot analysis.

Baf57 knockdown impairs adipogenic gene transcription similar to PPARγ knockdown

Having established that Baf57 is affecting PPARγ levels which are crucial for proper differentiation (Fig. 2), we decided to perform microarray analysis to analyze changes in gene transcription in more detail. In order to do this, we used SGBS cells that had undergone siRNA-mediated knockdown for Baf57, PPARγ and control siRNA. Microarray analysis revealed that the expression of 375 and 628 genes were altered after knock down of Baf57 or PPARγ, respectively (Fig. 3A). A significant overlap was found between the data sets, consisting of 153 genes that are altered upon Baf57 and PPARγ knock down. Importantly, knock down of Baf57 or PPARγ altered the expression of these commonly affected genes in the same direction (Fig. 3B). The gene set that is most significantly lowered by both PPARγ and Baf57 knockdown displays a clear overrepresentation of genes involved in lipid metabolism (GEO analysis; Supplemental Fig. 1) and encompasses many well-established PPARγ target genes that are normally upregulated during adipogenesis, including LPL, ADIPOQ (encoding adiponectin), PLIN1(encoding perilipin) and PCK1 (encoding PEPCK). Please note that in contrast to the data represented in Fig. 2, the *PPARG* gene was not affected, which was due to technical issues. In contrast to the genes downregulated by both PPARγ and Baf57 knockdown, genes that were upregulated upon knock down of either gene did not reveal a clear role in adipogenesis or metabolism (Fig. 3B). Also genes that were altered (either up- or downregulated) upon knockdown of only Baf57 did not have known functional roles in adipogenesis (Supplemental Fig. 2). Taken together, these findings suggest that the main role of Baf57 as an epigenetic regulator of adipogenesis lies in the PPARγ signalling pathway, either by reducing *PPARG* expression and/or by reducing the transcriptional activity of the PPARγ protein.

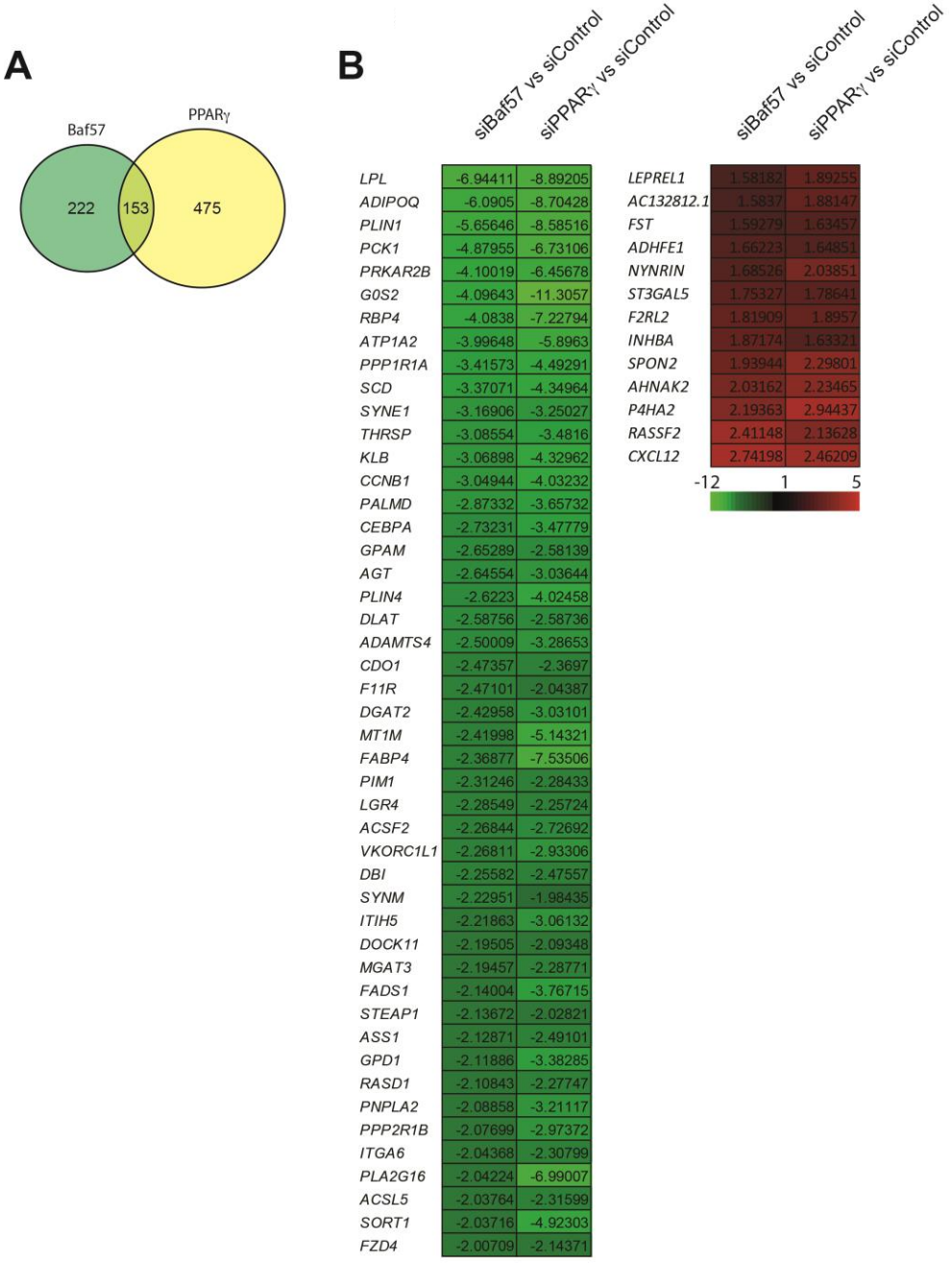


Figure 3 Gene regulation by Baf57 and PPARγ in human SGBS adipocytes.
A, Human SGBS pre-adipocytes were subjected to RNAi-mediated knock down with control, Baf57 and PPARγ-specific oligonucleotides. mRNA expression was assessed by microarray analysis. Shown are the number of genes with a $p<0.05$ after FWER correction and 5000 permutations which were

more then 2.0-fold changed after knock down of Baf57 or PPARγ compared to control cells. Data were obtained from experiments performed in quadruplicate (Baf57) or duplicate (PPARγ. B, Heat maps showing log2 fold change in gene expression after knockdown of Baf57 or PPARγ in human SGBS adipocytes. Red indicates increased gene expression compared to the control siRNA treatment, whereas green indicates decreased expression.

Baf57 regulates PPARγ expression

Since adipogenesis is a multistep process in which early and late factors play distinctive roles [2], we analyzed the expression of several genes in time to obtain more insight into the mechanisms by which Baf57 affects PPARγ signaling in adipogenesis. Three classes of genes were examined: i) early pro-adipogenic factors Klf5, Krox20 and C/EBPβ that play a role in activating PPARγ expression, ii) PPARγ itself and C/EBPα, late stage adipogenic transcription factors that regulate each others expression, and iii) the PPARγ target genes FABP4, LPL and ADIPOQ, that are induced during the final stages of differentiation. As shown in Figure 4, expression of Klf5, Krox20 and C/EBPβ was not significantly altered by either Baf57 or PPARγ knockdown. Baf57 knockdown leads to decreased mRNA levels of both PPARγ and C/EBPα at day 2 of differentiation already. As a possible consequence of that, expression of the PPARγ target genes FABP4, LPL and ADIPOQ was also blunted from day 2 onwards. Both of these latter effects were also observed upon PPARγ knockdown (Fig. 4). These data suggest a role for Baf57 in the regulation of the PPARG gene.

To support this view, we performed ChIP assays where we analyzed Baf57 recruitment to the *PPARG/Pparg* promoter in both human SGBS pre-adipocytes and 3T3-L1 cells. Baf57 was specifically detected at the *PPARG/Pparg* promoter already from day 0 onwards, with a slight increase observed in 3T3-L1 cells during differentiation (Fig. 5). As controls, C/EBPα and C/EBPβ were used in SGBS and 3T3-L1 cells respectively, as these proteins have previously been shown to be recruited to the *PPARG/Pparg* promoter in differentiation [28]. These data indicate that Baf57 is present at the *PPARG/Pparg* promoter and may therefore be directly involved in regulating its mRNA expression.

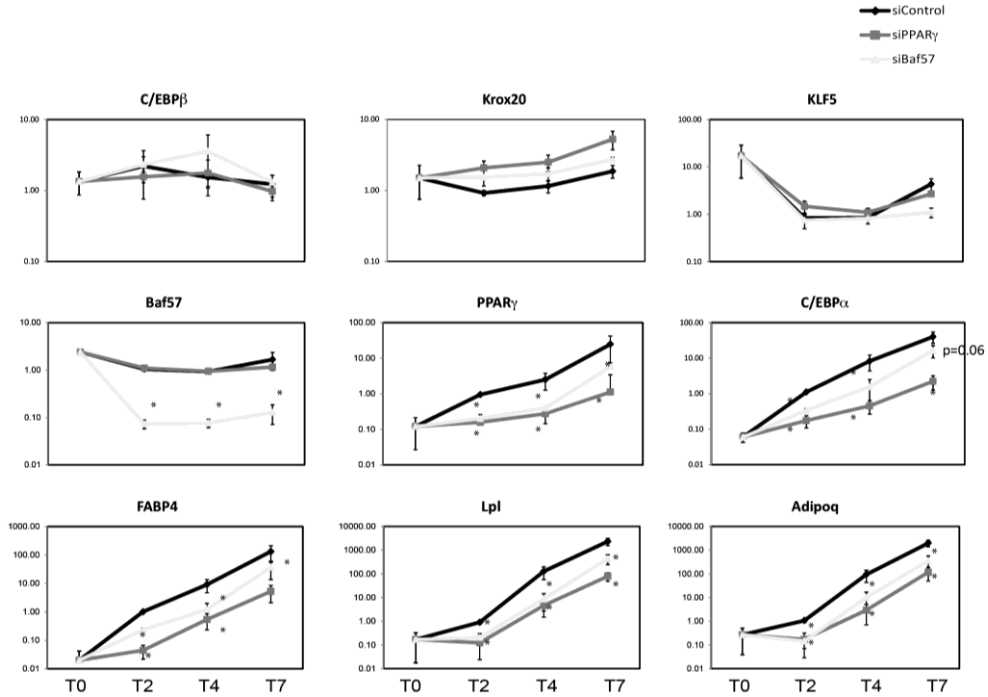


Figure 4 Expression of selected genes upon siRNA-mediated knockdown of Baf57 or PPARγ
SGBS cells were transfected with siRNA oligonucleotides targeting Baf57 (oligonucleotide #7), PPARγ, or non-targeting siRNA oligonucleotides after which they were differentiated. RNA was isolated at day 0 (non-transfected), day 2, 4 and 7 of differentiation and relative mRNA expression was assessed by qRT-PCR. Data are represented as mean ± SEM values normalized to a house keeping gene.

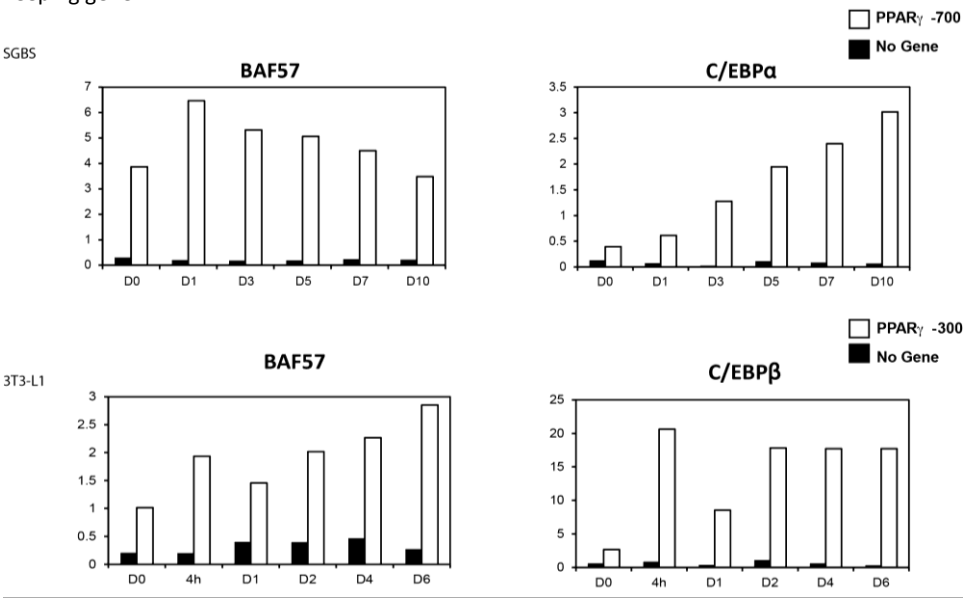


Figure 5 ChIP analysis for Baf57 on the *PPARG/Pparg* promoter in SGBS and 3T3-L1 cells.
Cells were differentiated and harvested at timepoints. The cells were IP'ed for Baf57 and subjected for PCR analysis. As a positive control, C/EBPα and C/EBPβ was used in SGBS and 3T3-L1 cells, respectively. For SGBS cells a region 700 bp upstream of the TSS was amplified, while a region 300 bp upstream of the TSS was amplified in 3T3-L1 cells. As a negative control an intergenic region on chromosome 15 was used

Discussion

Chromatin remodelers and histone modifying enzymes play important roles in many cellular processes because they are involved in transcriptional regulation. Adipogenesis is a complex process in which the transcriptome changes drastically. In this study we aimed to identify novel epigenetic regulators of adipogenesis by developing an siRNA based screening platform. We identified Baf57, a non-core subunit of the SWI/SNF chromatin remodeling complex, to be important for proper adipocyte differentiation. Knockdown of Baf57 leads to lower expression of PPARγ and PPARγ target genes, and Baf57 is present at the *PPARG/Pparg* promoter, suggesting that this SWI/SNF subunit plays a critical role in regulation of the *PPARG* gene.

Up to date several different epigenetic factors, including chromatin remodeling factors and histone modifying enzymes, have been implicated in adipogenesis [11,29,30]. The library used in the present study targeted 529 different chromatin-associated genes, including the majority of these genes. Several of these previously identified epigenetic factors scored as positive hits in our screen (e.g. SNF5; Fig. 1B), thereby validating the screening procedure. However, many other previously identified epigenetic factors were not identified in our screen. One possible reason for this is insufficient reduction of the target gene by RNAi-mediated knockdown; several of the epigenetic regulators in adipogenesis were identified through genetic inactivation, which is more effective. In addition, it should be noted that epigenetic regulators of adipogenesis have previously been identified in the murine 3T3-L1 cellular model system, which may differ is some aspects from the human SGBS adipocytes employed here [31].

A limited number of studies have indicated a role for SWI/SNF in adipogenesis. The Baf60c subunit for example was found to interact and enhance activity of PPARγ even though depletion did not seem to impair adipogenesis [32]. Similarly, SNF5, also referred to as Baf47, cooperates with C/EBPα and PPARγ elevating the transcriptional activity of the latter and thus leading to upregulation of adipocyte specific genes [26]. In contrast to Baf60c, knockdown of SNF5 abrogates differentiation [26]. In line with these observations, SNF5 was also identified as a positive regulator of SGBS adipogenesis in the current study (Fig. 1). In addition, SWI/SNF ATPase activity (BRG1 or hBRM) is necessary for

adipogenesis since it is capable of regulating PPARγ expression [33]. The complex appears to facilitate the preinitiation complex at the PPARγ promotor. Moreover, C/EBPα also requires recruitment of the SWI/SNF complex to adipocyte gene promotors in 3T3-L1 adipogenesis. This interaction is mediated via the transactivation element III (TE-III) of C/EBPα and the BRM subunit of the SWI/SNF complex. Take together, these findings indicate that the SWI/SNF complex as a whole is essential for adipogenesis- as exemplified by the essential role of the core subunits BRG1/hBRM and SNF5- and that more peripheral proteins like Baf60c and Baf57 may play essential roles through directly contacting specific transcription factors. In support of this view, Baf60a, but not BRG1, SNF5 or Baf57, interacts specifically with p53 leading to the upregulation of tumour suppressive genes [34]. Our current model would therefore be that the SWI/SNF complex is recruited to *PPARG* promoter through the interaction between its Baf57 subunit and sequence-specific transcription factors. A candidate for this might be GR, an early pro-adipogenic factor that is known to interact with Baf57 [35]. In order to address the role and recruitment of the SWI/SNF complex through Baf57 more extensively, Chip-Seq profiling of Baf57 during differentiation may be a useful approach. The Chip-Seq should also be performed when Baf57 is knocked down so that specific effects will be identified. Euskirchen et al. has investigated the genomic localization of the core SWI/SNF members (Baf155, Baf170, SNF5 and Brg1) in HeLa cells [36]. As expected, the genomic locations of the SWI/SNF members co-localize and have a preference of targeting chromatin near expressed geness together with RNA polymerase II. Interestingly, in some cases the core SWI/SNF members were present at different loci individually or as an "incomplete" SWI/SNF complex, in which several members were not present. This might suggests that even the core subunits of the complex can have different roles when they are not in the SWI/SNF complex or that different types of SWI/SNF complexes may exist simultaneously in the same cell.

Besides being implicated in PPARγ mediated adipogenesis here, the SWI/SNF complex is also associated with several other nuclear receptors including ER, GR, RXR and AR (reviewed in [32]). Baf57 is known to stimulate AR and ERα activity, by directly binding to them [37,38]. Moreover, Baf57 is also capable of binding to GR yet it is not known how this impacts GR activity [35]. In the case of TR, Baf57 enhances activity indirectly through BRG1 [39]. While our data suggest a critical role for BAF57 in regulation of *PPARG* expression, Baf57 may also affect

the transcriptional activity of PPARγ and can interact with PPARγ *in vitro* and overexpression of Baf57 in Baf57-negative BT-549 breast cancer cells results in increased PPARγ reporter activity (unpublished observations). Future studies are required to establish whether Baf57 plays a dual role in the regulation of adipogenesis: as a critical regulator of *PPARG* expression as well as a cofactor for the PPAR protein.

References

1. Rosen ED, MacDougald OA (2006) Adipocyte differentiation from the inside out. NatRevMolCell Biol 7: 885-896.
2. Farmer SR (2006) Transcriptional control of adipocyte formation. Cell Metab 4: 263-273.
3. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, et al. (2008) Genome-wide profiling of PPAR{gamma}:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev 22: 2953-2967.
4. Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, et al. (2008) PPAR{gamma} and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes Dev 22: 2941-2952.
5. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, et al. (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. MolCell 4: 585-595.
6. Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, et al. (1999) PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. MolCell 4: 597-609.
7. Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, et al. (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. MolCell 4: 611-617.
8. Nadra K, Quignodon L, Sardella C, Joye E, Mucciolo A, et al. (2010) PPARgamma in placental angiogenesis. Endocrinology 151: 4969-4981.
9. Imai T, Takakuwa R, Marchand S, Dentz E, Bornert JM, et al. (2004) Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. ProcNatlAcadSciUSA 101: 4543-4547.
10. Perissi V, Rosenfeld MG (2005) Controlling nuclear receptors: the circular logic of cofactor cycles. NatRevMolCell Biol 6: 542-554.
11. Sugii S, Evans RM (2011) Epigenetic codes of PPARgamma in metabolic disease. FEBS letters 585: 2121-2128.
12. Lee TI, Young RA (2013) Transcriptional regulation and its misregulation in disease. Cell 152: 1237-1251.
13. Euskirchen G, Auerbach RK, Snyder M (2012) SWI/SNF chromatin-remodeling factors: multiscale analyses and diverse functions. The Journal of biological chemistry 287: 30897-30905.
14. Wilson BG, Roberts CW (2011) SWI/SNF nucleosome remodellers and cancer. Nature reviews Cancer 11: 481-492.
15. Gao X, Tate P, Hu P, Tjian R, Skarnes WC, et al. (2008) ES cell pluripotency and germ-layer formation require the SWI/SNF chromatin remodeling component BAF250a. Proceedings of the National Academy of Sciences of the United States of America 105: 6656-6661.
16. Yan Z, Cui K, Murray DM, Ling C, Xue Y, et al. (2005) PBAF chromatin-remodeling complex requires a novel specificity subunit, BAF200, to regulate expression of selective interferon-responsive genes. Genes & development 19: 1662-1667.

17. Wabitsch M, Brenner RE, Melzner I, Braun M, Moller P, et al. (2001) Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. IntJObesRelat Metab Disord 25: 8-15.
18. Verrijn Stuart AA, Schipper HS, Tasdelen I, Egan DA, Prakken BJ, et al. (2012) Altered plasma adipokine levels and in vitro adipocyte differentiation in pediatric type 1 diabetes. The Journal of clinical endocrinology and metabolism 97: 463-472.
19. Baas R, Lelieveld D, van Teeffelen H, Lijnzaad P, Castelijns B, et al. (2013) A novel microscopy-based high-throughput screening method to identify proteins that regulate global histone modification levels. Journal of Biomolecular Screening: in press.
20. van Wageningen S, Kemmeren P, Lijnzaad P, Margaritis T, Benschop JJ, et al. (2010) Functional overlap and regulatory links shape genetic interactions between signaling pathways. Cell 143: 991-1004.
21. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, et al. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 30: e15.
22. Margaritis T, Lijnzaad P, van Leenen D, Bouwmeester D, Kemmeren P, et al. (2009) Adaptable gene-specific dye bias correction for two-channel DNA microarrays. Molecular systems biology 5: 266.
23. Wu YH, Kerr MK, Churchill GA (2002) MAANOVA: a software package for the analysis of spotted microarray experiments. The analysis of gene expression data: methods and software.
24. Kawauchi S, Calof AL, Santos R, Lopez-Burks ME, Young CM, et al. (2009) Multiple organ system defects and transcriptional dysregulation in the Nipbl(+/-) mouse, a model of Cornelia de Lange Syndrome. PLoS genetics 5: e1000650.
25. Whelan SA, Dias WB, Thiruneelakantapillai L, Lane MD, Hart GW (2010) Regulation of insulin receptor substrate 1 (IRS-1)/AKT kinase-mediated insulin signaling by O-Linked beta-N-acetylglucosamine in 3T3-L1 adipocytes. The Journal of biological chemistry 285: 5204-5211.
26. Caramel J, Medjkane S, Quignon F, Delattre O (2008) The requirement for SNF5/INI1 in adipocyte differentiation highlights new features of malignant rhabdoid tumors. Oncogene 27: 2035-2044.
27. Hah N, Kolkman A, Ruhl DD, Pijnappel WW, Heck AJ, et al. (2010) A role for BAF57 in cell cycle-dependent transcriptional regulation by the SWI/SNF chromatin remodeling complex. Cancer research 70: 4402-4411.
28. Siersbaek R, Nielsen R, John S, Sung MH, Baek S, et al. (2011) Extensive chromatin remodelling and establishment of transcription factor 'hotspots' during early adipogenesis. The EMBO journal 30: 1459-1472.
29. Ge K (2012) Epigenetic regulation of adipogenesis by histone methylation. Biochimica et biophysica acta 1819: 727-732.
30. Musri MM, Parrizas M (2012) Epigenetic regulation of adipogenesis. Current opinion in clinical nutrition and metabolic care 15: 342-349.
31. Poulos SP, Dodson MV, Hausman GJ (2010) Cell line models for differentiation: preadipocytes and adipocytes. ExpBiolMed(Maywood) 235: 1185-1193.

32. Debril MB, Gelman L, Fayard E, Annicotte JS, Rocchi S, et al. (2004) Transcription factors and nuclear receptors interact with the SWI/SNF complex through the BAF60c subunit. The Journal of biological chemistry 279: 16677-16686.
33. Salma N, Xiao HG, Mueller E, Imbalzano AN (2004) Temporal recruitment of transcription factors and SWI/SNF chromatin-remodeling enzymes during adipogenic induction of the peroxisome proliferator-activated receptor gamma nuclear hormone receptor. MolCell Biol 24: 4651-4663.
34. Oh J, Sohn DH, Ko M, Chung H, Jeon SH, et al. (2008) BAF60a interacts with p53 to recruit the SWI/SNF complex. The Journal of biological chemistry 283: 11924-11934.
35. Hsiao PW, Fryer CJ, Trotter KW, Wang W, Archer TK (2003) BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. Molecular and cellular biology 23: 6210-6220.
36. Euskirchen GM, Auerbach RK, Davidov E, Gianoulis TA, Zhong G, et al. (2011) Diverse roles and interactions of the SWI/SNF chromatin remodeling complex revealed using global approaches. PLoS genetics 7: e1002008.
37. Link KA, Burd CJ, Williams E, Marshall T, Rosson G, et al. (2005) BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. Molecular and cellular biology 25: 2200-2215.
38. Garcia-Pedrero JM, Kiskinis E, Parker MG, Belandia B (2006) The SWI/SNF chromatin remodeling subunit BAF57 is a critical regulator of estrogen receptor function in breast cancer cells. The Journal of biological chemistry 281: 22656-22664.
39. Heimeier RA, Hsia VS, Shi YB (2008) Participation of Brahma-related gene 1 (BRG1)-associated factor 57 and BRG1-containing chromatin remodeling complexes in thyroid hormone-dependent gene activation during vertebrate development. Molecular endocrinology 22: 1065-1077.

Supplementary Figures

GO term	P-value	Cluster frequency	Background frequency	Genes annotated to term
lipid metabolic process	4.19E-04	14 out of 81 (17.3%)	1761 out of 44669 annotated genes (3.9%)	PLA2G16, CEBPA, DGAT2, PLIN1, HADH, PNPLA2, AGT, SCD, FADS1, THRSP, FABP3, G0S2, PRDX6, APOC1
cellular lipid metabolic process	3.27E-03	11 out of 81 (13.6%)	1272 out of 44669 annotated genes (2.8%)	PLA2G16, DGAT2, PLIN1, HADH, PNPLA2, AGT, SCD, FADS1, FABP3, G0S2, PRDX6
acylglycerol acyl-chain remodeling	1.27E-02	2 out of 81 (2.5%)	5 out of 44669 annotated genes (0.0%)	DGAT2, PNPLA2
cellular response to oleic acid	1.90E-02	2 out of 81 (2.5%)	6 out of 44669 annotated genes (0.0%)	DGAT2, ASS1
positive regulation of cholesterol esterification	2.66E-02	2 out of 81 (2.5%)	7 out of 44669 annotated genes (0.0%)	AGT, APOC1
blood vessel development	2.96E-02	7 out of 81 (8.6%)	606 out of 44669 annotated genes (1.4%)	COL15A1, CSPG4, COL4A1, FZD4, AGT, MCAM, LAMA4
brown fat cell differentiation	3.53E-02	3 out of 81 (3.7%)	49 out of 44669 annotated genes (0.1%)	CEBPA, MRAP, LAMA4
vasculature development	4.37E-02	7 out of 81 (8.6%)	645 out of 44669 annotated genes (1.4%)	COL15A1, CSPG4, COL4A1, FZD4, AGT, MCAM, LAMA4

Figure S1. **GO analysis on the transcriptome of Baf57 knockdown SGBS cells**. The table indicates in which processes the clustered genes are involved.

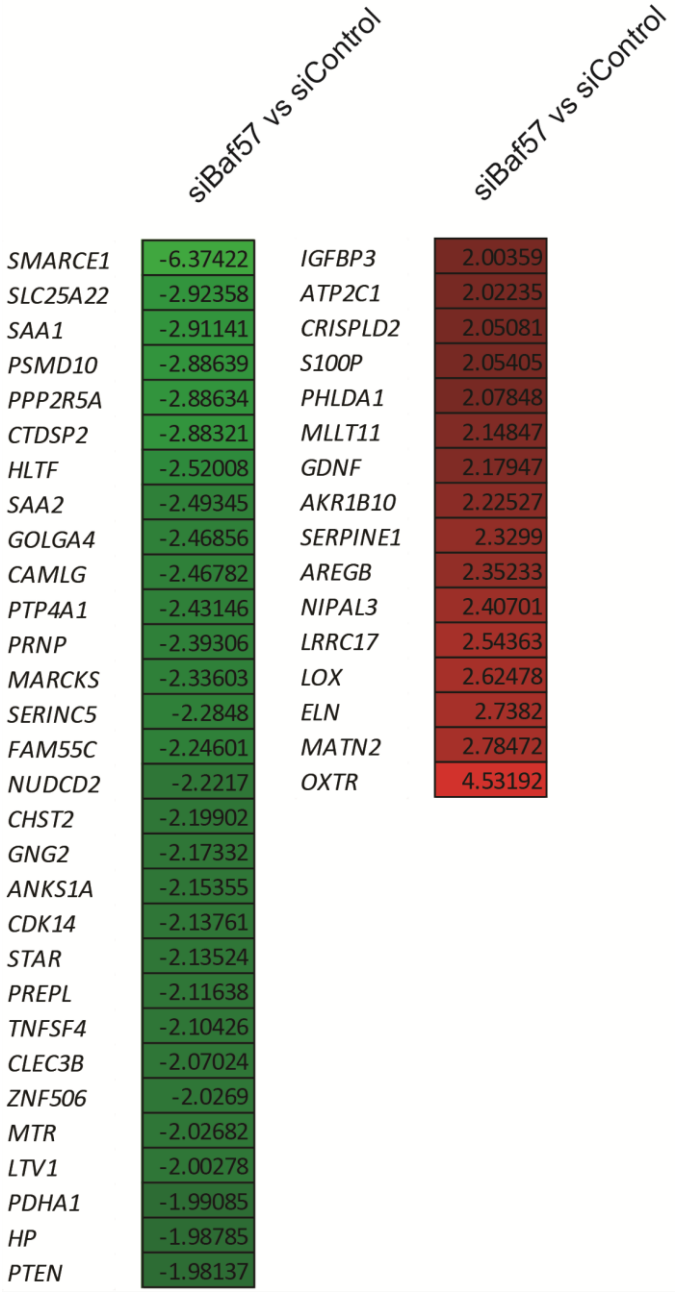


Figure S2 Heat map of genes that are affected by Baf57 knockdown only. Green indicates downregulation, whereas red indicates an increase in mRNA expression.

Chapter 4

REGULATION OF ADIPOGENESIS BY PHF12

Ismayil Tasdelen[1], Ronni Nielsen[2], Daphne Lelieveld[3], Dik van Leenen[4], Ruud Berger[1], Frank C.P. Holstege[4], David Egan[3], Susanne Mandrup[2], and Eric Kalkhoven[1§]

[1]Section Metabolic Diseases, Molecular Cancer Research, and Netherlands Metabolomics Centre, University Medical Centre Utrecht, Utrecht 3584 CG, The Netherlands, [2]Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense, Denmark, [3]Department of Cell Biology, University Medical Centre Utrecht, Utrecht 3584 CX, The Netherlands, [4]Molecular Cancer Research, University Medical Centre Utrecht, Utrecht 3584 CG, The Netherlands.

Manuscript in preparation

Abstract

Adipogenesis is regulated through a cascade of transcriptional events, ultimately resulting in induction of the adipogenic master regulator PPARγ. While several transcription factor, including STAT5A/B, SREBP1c and C/EBPα have been implicated in the regulation of the *PPARG* promoter, the role of epigenetic factors is not well-studied. Using an siRNA-based knockdown screen we identified PHD finger protein 12 (Phf12), a protein previously mainly associated with transcriptional repression, as a positive regulator of adipogenesis. The main role of Phf12 in the differentiation process may be through the transcriptional regulation of PPARγ expression: knockdown of Phf12 led to reduced expression of PPARγ and several of its target genes, and hence impaired adipocyte differentiation. Phf12 was present at the *PPARG* promoter, with increased occupancy in the course of differentiation, indicating that it may be directly involved in regulating PPARγ expression. These findings qualify Phf12 as a potential new regulator of adipocyte differentiation with gene activating rather than repressing activity.

Introduction

Adipogenesis is the conversion of pre-adipocytes into fully mature adipocytes, a complex differentiation process involving tightly regulated interplay between proteins at different subcellular levels. This process is vital for healthy metabolic homeostasis, since adipose tissue is known to function as an endocrine organ in addition to being a mere fat depot [1]. Adipose tissue dysfunction can lead to health problems such as diabetes type II and cardiovascular disorders [2,3]. It is therefore important to understand the molecular mechanisms underlying the process of adipogenesis and adipocyte biology. In the past decade, it has become clear that the nuclear receptor Peroxisome Proliferator Activated Receptor γ (PPARγ) is critical player in these processes. Experiments have shown that adipogenesis cannot take place without PPARγ, and overexpression of this protein in the fibroblast-like NIH-3T3 cell line is sufficient for adipocyte differentiation [4,5]. In addition, PPARγ is necessary for the maintenance of differentiated adipocytes and for upregulation of many adipocyte-specific genes. Examples of these genes are Fabp4, an intracellular lipid transporter, perilipin, a lipid droplet binding protein, and cd36, a membrane based fatty acid transporter [6,7,8]. However, the target gene repertoire of PPARγ also includes adipose-tissue specific hormones such as adiponectin and chemerin, which do not have a direct role in lipid accumulation but play a role in communication of adipose tissue and other organs [9,10]. These properties make PPARγ crucial for the proper function of adipose tissue.

In order to find novel regulators of adipogenesis we developed an siRNA-based library screen in which we tested 529 genes involved in epigenetic gene regulation. We report here the identification of the PHD finger 12 (PHF12) protein as a regulator of adipogenesis. Remarkably, Phf12 has mainly been linked to transcriptional repression and can be found associated with repressor proteins such as SIN3A/SIN3B, MRG15 and TLE [11,12,13]. So far, no link has been reported between Phf12 and any aspect of adipogenesis. In our study however, we found that knockdown of Phf12 and PPARγ resulted in similar transcriptome changes, suggesting that Phf12 may regulate PPARγ expression. Indeed, Phf12 is present at the *PPARG* locus throughout differentiation and knockdown of Phf12 lowers expression of PPARγ and PPARγ target genes. Taken together, these data indicate

that Phf12, an epigenetic regulator of gene expression that is mainly associated with transcriptional repression, is a novel positive regulator of adipogenesis.

Materials & Methods

Materials **-** siRNA against PPARγ (L-003436-00), Phf12 (LU-009736-00-0002) and non-coding siRNA (D-001810-10) were purchased from Dharmacon. For Western blot analysis, primary antibodies against Phf12 (A301-647A, Bethyl), FABP4 (Santa-Cruz, sc-18661) and β-actin (Ab8224, Abcam) were used. Oil-red-O (O0625) and Nile red (N3013) were from Sigma-Aldrich.

Cell culture, differentiation, siRNA transfection **-** The human pre-adipocyte cellline SGBS was cultured and differentiated as previously described (9,10). For siRNA transfection, 4000 SGBS cells were plated in a single well of a 96-well plate five days prior to differentiation. After incubation, the differentiation cocktail was added before the siRNA containing mixture. siRNA transfection with lipofectamine RNAiMax was performed according to the manufacturer's instructions in which 0.3 µl of RNAiMax and a final concentration of 10 nM siRNA was used.

Oil-red-O and Nile red staining - An Oil-red-O staining of SGBS cells was done as followed. The cells were fixed for 10 minutes with 4% formaldehyde at room temperature. Afterwards they were briefly washed once with 60% iso-propanol after which the Oil-red-O solution (3mg/ml in 60% isopropanol) was added for 10 minutes. Subsequently, the cells were washed twice in 60% isopropanol and then twice in water to remove all traces. For Nile red staining, the cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton-X for 2 minutes. Afterwards they were incubated with a Nile-red containing substance (1mg Nile red in PBS) for 5 minutes subsequently followed by washing twice with PBS.

siRNA library screening **-** The siRNA screen was performed at the UMCU Cell Screening Center. The siRNA library, targeting expression of 529 chromatin associated genes, was purchased from Thermo Fischer Scientific Dharmacon (Lafayette, CO, custom made library, order numbers 245120-245146, 245170, 246203)[19]. The screen was performed in 96-well plates. SGBS cells were plated and treated for differentiation and transfection as described above. Each gene was targeted with a pool of four individual siRNA's and was tested in triplicates in adjacent wells. After seven days, the cells were washed and fixed with PFA for Nile red and Hoechst staining and the plates were made ready for the automated

microscope. The plates were imaged using a Thermo ArrayScan VTi automated microscope. The Cellomics Target Activation image analysis bioapplication was used to quantitate adipocytic differentiation. First, the algorithm identifies nuclei in the Hoechst channel. A mask is then generated around the nucleus and the intensity of Oil-Red-O staining in this mask is calculated. Based on the analysis of negative controls in pilot experiments and cutoff for the average pixel intensity in the Nile red channel was used to determine the percentage of Nile Red positive cells. This percentage of positive cells was normalized to the average of the samples in the plate. Afterwards, these outcomes would be normalized to the average of each plate and each well would be given a score. If a particular well would deviate more than once than the standard deviation, it would get a score of 0.5. Should this be twice or three fold, the scores would be 1 and 2 respectively. The scores of the three replicates were added up. The genes that would have a total score of 3 or higher were selected for further analysis.

RNA isolation and QPCR - At the time of harvesting, cells were taken up in 1ml of Trizol reagent (Life technologies) and were subjected to RNA isolation and cDNA synthesis according to the manufacturer's instructions (Life technologies). Gene expression levels were determined by quantitative real time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to *36B4* expression. The primers used were as followed:
human 36B4 forward primer,
5'- CGGGAAGGCTGTGGTGCTG-3', and reverse primer,
5'- GTGAACACAAAGCCCACATTCC-3'; human PPARγ forward primer,
5'- CCTATTGACCCAGAAAGCGATT-3' and reverse primer,
5'- CATTACGGAGAGATCCACGGA-3'; human Phf12 forward primer,
5'- CCCGTCAAAGTCTGCTTCAC -3', and reverse primer,
5'- GTGAAACAGGAGAGGGCAAT-3'; human C/EBPα forward primer,
5'- CAACACTTGTATCTGGCCTCTG-3', and reverse primer,
5'- CCGAGCAAAACCAAAACAAA-3'; human KLF5 forward primer,
5'- CCACCACCCTGCCAGTTAAC-3', and reverse primer,
5'- TAAACTTTTGTGCAACCAGGGTAA-3'; human KROX20 forward primer,
5'- TTGACCAGATGAACGGAGTG-3' and reverse primer,
5'- TGGTTTCTAGGTGCAGAGACG-3'; human C/EBPβ forward primer,
5'- GCGACGAGTACAAGATCC-3' and reverse primer,

5’- AGCTGCTTGAACAAGTTCC-3’; human FABP4 forward primer,
5’- CCTTTAAAAATACTGAGATTTCCTTCA-3’ and reverse primer,
5’- GGACACCCCCATCTAAGGTT-3’; human LPL forward primer,
5’- ATGTGGCCCGGTTTATCA-3’ and reverse primer,
5’- CTGTATCCCAAGAGATGGACATT-3’; human ADIPOQ forward primer,
5’- CCTGGTGAGAAGGGTGAGAA-3’ and reverse primer,
5’- CACCGATGTCTCCCTTAGGA-3’.

Microarray **-** SGBS cells were differentiated and transfected with siRNA as described above. The non-targeting siRNA and siPPARγ were performed in duplicate whereas siBaf57 in quadruplicate. Five days after transfection cells were washed twice with PBS twice and lysed in 1.0ml Trizol (Invitrogen). Samples were treated similar to the RNA isolation procedure. DNAse treatment and purification was performed with RNeasy kit (Qiagen, Venlo, The Netherlands).
Microarrays used were Human Whole Genome Gene Expression Microarrays V1 (Agilent Technologies, Belgium) representing 41000 *H. sapiens* 60-mer probes in a 4x44K layout. RNA amplifications and labelling were performed on an automated system (Caliper Life Sciences NV/SA, Belgium) with 3 µg total RNA from each sample. Hybridizations were done on a hybridization station (HS4800PRO system with QuadChambers; Tecan Benelux B.V.B.A.) using 800 ng labelled cRNA per channel according to van Wageningen et al. [15]. Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery), Loess normalization was performed [16] on mean spot-intensities. Gene-specific dye bias was corrected based on a within-set estimate as described in Margaritis *et al*.[17].
Data were analysed using MAANOVA [18]. In a fixed effect analysis, sample, array and dye effects were modelled. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Genes (based on unique ENSG gene IDs) with $p < 0.05$ after family wise error correction (FWER) were considered significantly changed. A fold change cut-off > ±2.0 was used.

Chromatin immunoprecipitations assays (ChIP) **-** ChIP experiments were performed according to standard protocols [19]. Lysed cells were sonicated using a Bioruptor (Diagenode) according to the manufacturer’s protocol, and chromatin was immunoprecipitated with Baf57 antibody. DNA enrichment was quantified by

real-time PCR (MX-3000, Stratagene) using SYBR green Master Mix (Sigma-Aldrich). Primer sequences are available upon request.

Results

Knockdown of Phf12 inhibits adipogenesis and PPARγ mRNA levels

In the same siRNA-based screening platform in which we identified Baf57 as a positive regulator of adipogenesis, we also found knockdown of Phf12, a protein that is predominantly associated with gene repression (see also Discussion), to result in impaired adipogenesis. As no link has been reported between Phf12 and any aspect of adipogenesis so far, we first examined its expression during adipogenesis. Phf12 protein levels were unaltered during differentiation of SGBS pre-adipocytes into mature adipocytes (Fig. 1A).

Since the screen was performed with pooled siRNA oligonucleotides, we wished to verify the knockdown of Phf12 using single oligonucleotides. For this, SGBS cells were transfected with 3 different individual oligonucleotides against Phf12 (#5, 6 and 7) and incubated with differentiation cocktail. Automated microscopical analysis revealed that siRNA oligonucleotide #7 was the most potent in impairing adipogenesis (Fig. 1B) and reducing Phf12 mRNA levels (Fig. 1C). These findings were verified by Oil-red-O staining (Fig. 1D). Knockdown of PPARγ was performed in parallel as a positive control. Western Blot analysis revealed that siRNA oligonucleotide #7 also effectively reduced Phf12 protein levels (Fig. 1E). Since PPARγ is essential for adipogenesis, we also investigated whether Phf12 knockdown affected PPARγ mRNA levels. Interestingly, this seemed to be the case: knockdown of Phf12 by siRNA oligonucleotide #7 lowered PPARγ levels, with the other two siRNA oligonucleotide being less effective (Fig. 1C). These data indicate that Phf12 is crucial for adipogenesis and that knockdown of Phf12 leads to diminished PPARγ levels.

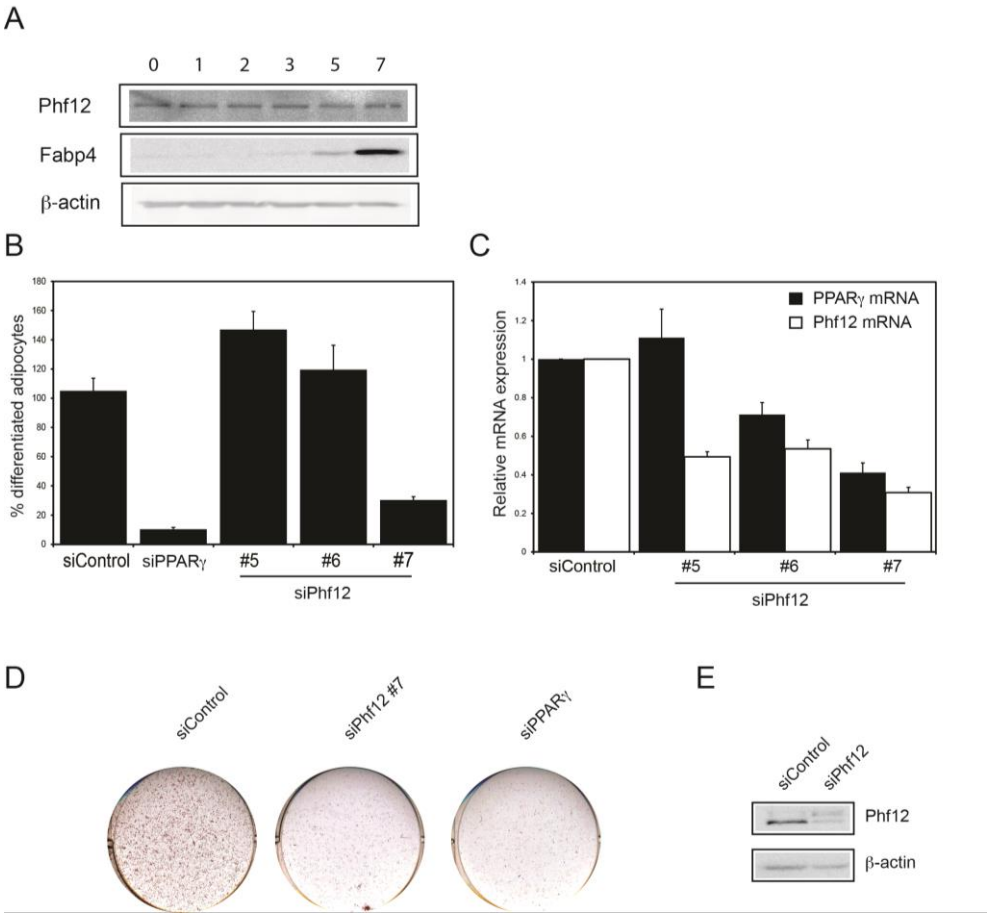


Figure 1 siRNA-mediated knockdown of Phf12 inhibits adipogenesis in SGBS cells.
A) Human SGBS pre-adipocytes were differentiated into mature adipocytes and samples were taken at different time points during differentiation. Protein expression of Phf12 and Fabp4 was determined by Western blot analysis. B) SGBS cells were transfected with 3 individual siRNA oligonucleotides targeting Phf12 and differentiated. At day 7 cells were stained for lipid content (Nile red) and analysed by automated microscopy. Knockdown of PPARγ was included as a positive control. C) SGBS cells were treated as under B), except RNA was isolated at day 5 of differentiation and relative mRNA expression for Phf12 and PPARγ was assessed by qRT-PCR. D) SGBS cells were treated as under B), except that the cells were washed, fixed and stained with Oil-red-O dye. E) SGBS cells were treated as under B), except that they were lysed at day 5 and subjected to Western Blot analysis.

Knockdown of Phf12 and PPARγ results in similar transcriptome changes
Having observed that Phf12 is affecting PPARγ levels, a protein that is crucial for proper differentiation, we decided to perform microarray analysis to analyze gene transcription changes in more detail. In order to do this, we compared the transcriptome of SGBS cells after knockdown of Phf12, or PPARγ to cells treated with control siRNA oligonucleotides. Microarray analysis revealed that the expression of 39 and 628 genes were altered after knock down of Phf12 or PPARγ, respectively (Fig. 2A). A significant overlap was found between the data sets, consisting of 30 genes that are altered upon Phf12 and PPARγ knock down. Importantly, knock down of Phf12 or PPARγ altered the expression of these commonly affected genes in the same direction (Fig. 2B). Figure 2b shows the top list of genes that were affected by both PPARγ and Phf12 knockdown. Most of these genes are well-established PPARγ target genes that are upregulated during adipogenesis (e.g. LPL, PCK1 (encoding PEPCK), ADIPOQ (encoding adiponectin) and PLIN1 (encoding perilipin)). Please note that in contrast to the data represented in Fig. 1C, the *PPARG* gene was not affected, which was due to technical issues. Interestingly, Phf12 knockdown only resulted in significant upregulation of 3 genes, two of which are shared by PPARγ (Figure 2A and B). This is a striking finding, since Phf12 is known to be a repressor in many other cellular systems, and knockdown would therefore be expected to result in upregulation of genes. The genes that are upregulated by both Phf12 and PPARγ knock down do not show a clear signature, which may be due to the small gene set. Genes that were affected only by Phf12 knockdown did not show a clear signature either (Supplemental Figure 1), indicating that Phf12 is essential for activation of the adipogenic gene program possible through regulation of PPARγ expression.

Phf12 affects PPARγ expression but not early adipogenic gene regulators
Since adipogenesis is a multistep process in which early and late factors play distinctive roles [20], we analyzed the expression of several genes in time to obtain more insight into the mechanisms by which Phf12 affects PPARγ signaling an adipogenesis. Three classes of genes were examined: i) early pro-adipogenic factors Klf5, Krox20 and C/EBPβ that play a role in activating PPARγ expression, ii) PPARγ itself and C/EBPα which are late stage adipogenic transcription factors that regulate each other's expression, and iii) the PPARγ target genes FABP4, LPL and ADIPOQ, that are induced during the final stages of differentiation.

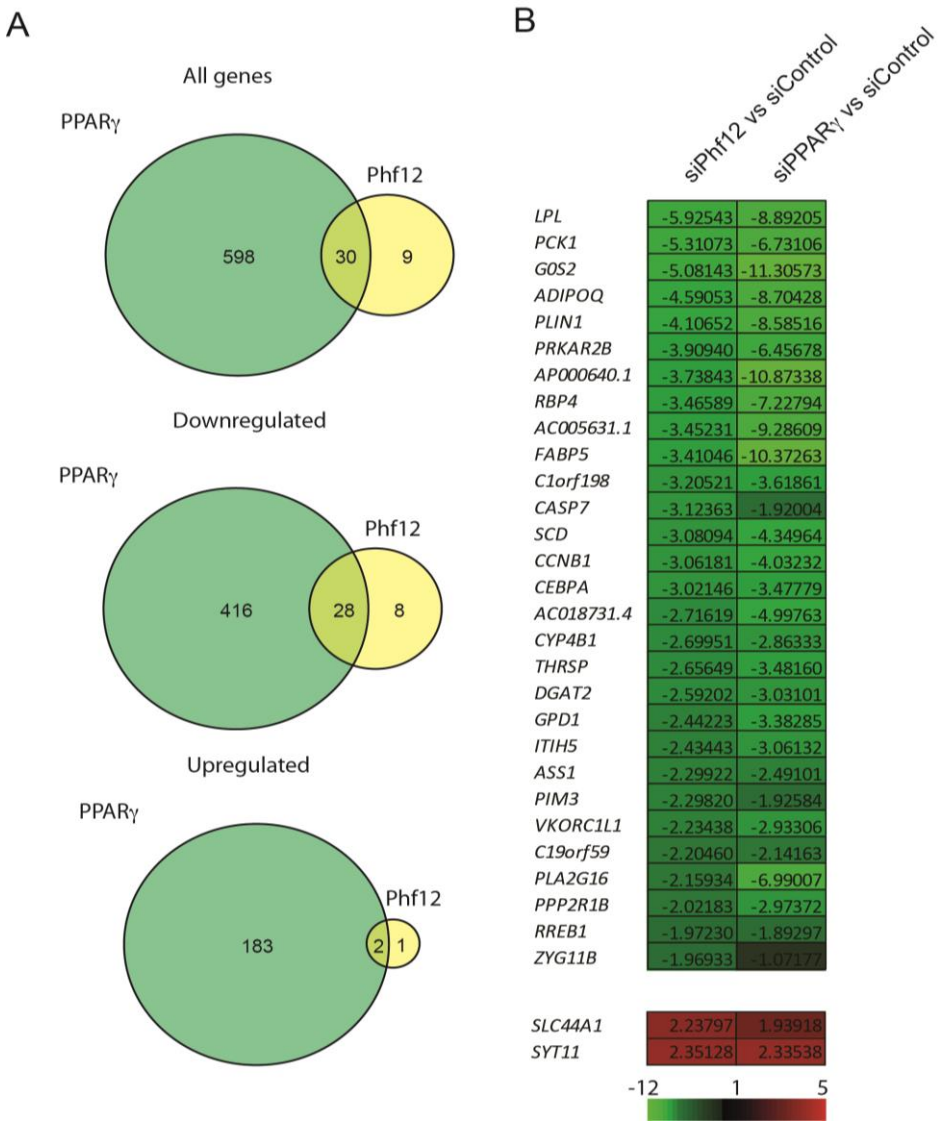


Figure 2 Gene regulation by Phf12 and PPARγ in human SGBS adipocytes.
A, Human SGBS pre-adipocytes were subjected to RNAi-mediated knock down with control, Phf12 and PPARγ-specific oligonucleotides. mRNA expression was assessed by microarray analysis. Shown are the number of genes with a $p<0.05$ after FWER correction and 5000 permutations which were more then 2.0-fold changed after knock down of Phf12 or PPARγ compared to control cells. Data were obtained from experiments performed in quadruplicate (Phf12) or duplicate (PPARγ). B, Heat maps showing log2 fold change in gene expression after knockdown of Phf12 or PPARγ in human SGBS adipocytes. Red indicates increased gene expression compared to the control siRNA treatment, whereas green indicates decreased expression.

As shown in Figure 3, expression of Klf5, Krox20 and C/EBPβ was not significantly altered by either Phf12 or PPARγ knockdown. Phf12 knockdown led to decreased mRNA levels of both PPARγ and C/EBPα at day 2 of differentiation already. As a possible consequence of that, expression of the PPARγ target genes FABP4, LPL and ADIPOQ was also blunted from day 2 onwards. Both of these latter effects were also observed upon PPARγ knockdown (Fig. 3). These data suggest a role for Phf12 in the regulation of the *PPARG* gene.

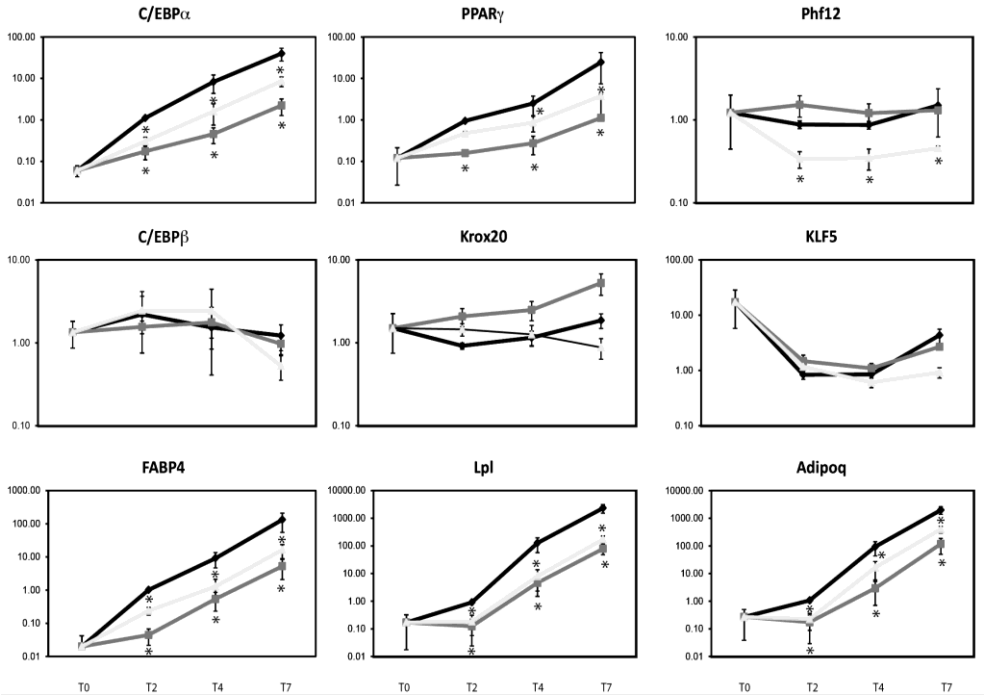


Figure 3 Expression of selected genes upon siRNA-mediated knockdown of Phf12 or PPARγ.
SGBS cells were transfected with siRNA oligonucleotides targeting Phf12 (oligonucleotide #7), PPARγ, or non-targeting siRNA oligonucleotides after which they were differentiated. RNA was isolated at day 0 (non-transfected), day 2, 4 and 7 of differentiation and relative mRNA expression was assessed by qRT-PCR. Data are represented as mean ± SEM values normalized to a house keeping gene.

Phf12 is present at the PPARγ promoter

Having established that Phf12 affects PPARγ levels but not early factors, we sought out to find a more direct link in regulation of the *PPARG* gene. To do this we performed ChIP assays where we analyzed the presence of Phf12 at the

PPARG promoter. We found that Phf12 was present at the promoter already from day 0 onwards (Fig. 4). This occupancy increased over the course of adipogenesis, despite the fact that overall Phf12 protein levels do not change in differentiation (Fig. 1A). As a control, C/EBPβ was used as it is known that this protein is present at the PPARγ promoter in differentiation [21]. These data indicate that Phf12 is present at the *PPARG* promoter and may therefore be involved in directly regulating its mRNA levels.

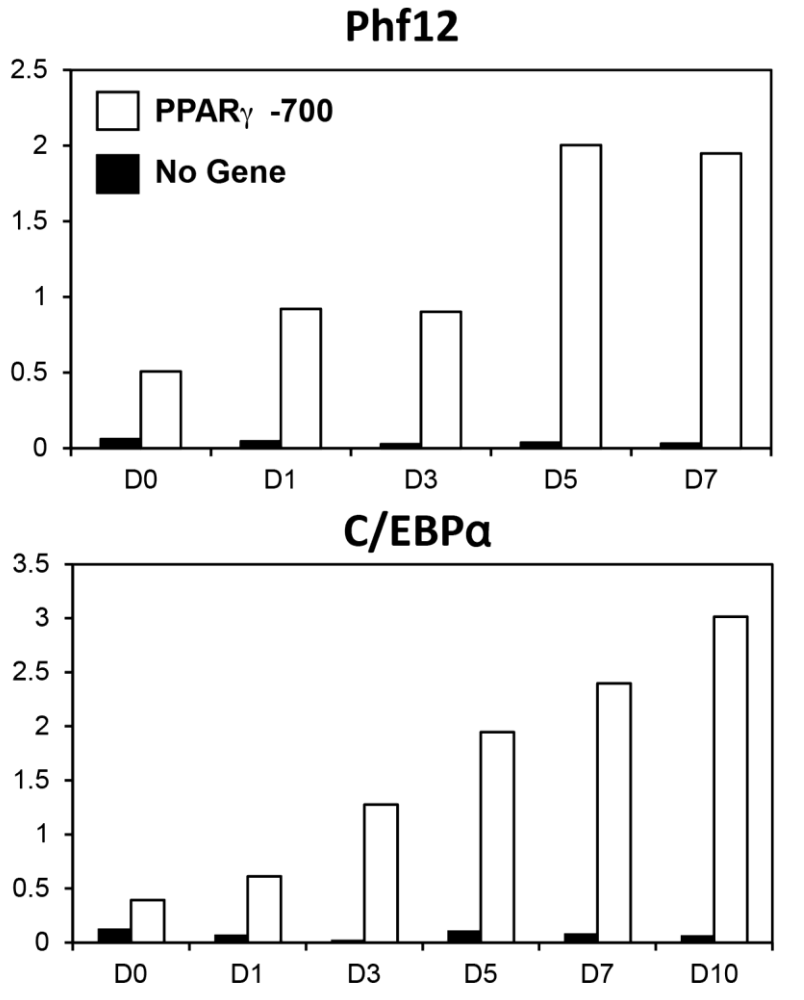


Figure 5 ChIP analysis for Phf12 on the *PPARG* promoter in SGBS cells.
Cells were differentiated and harvested at timepoints. The cells were IP'ed for Phf12 and subjected for PCR analysis. A region 700 bp upstream of the TSS was amplified, using C/EBPα as a positive control.

Discussion

Using an siRNA-based knockdown screen we identified Phf12 as an important regulator of adipogenesis, possibly through its regulation of PPARγ expression. Knockdown of Phf12 impaired adipogenesis and inhibited expression of PPARγ and PPARγ target genes. Moreover, Phf12 was present at the *PPARG* promoter, with increased occupancy in the course of differentiation, indicating that it may be directly involved in regulating PPARγ expression.

So far Phf12 had not been implicated in adipogenesis and little is known about the role of this protein in other biological systems. Phf12 can bind specific types of phosphoinoside with its polybasic region, suggesting that Phf12 is involved in phosphoinositide signalling cascades [22]. Importantly, Phf12 is mainly known for its association with different repressor proteins and complexes. It was shown in 2001 that Phf12 could bind to the TLE corepressor and the Sin3A-histone deacetylase complex in one system [12]. In this study it became clear that Phf12 would function as a transcriptional repressor when it was targeted to DNA. Remarkably, the authors reasoned that Phf12 might interact with other co-repressors because mutations in the Phf12 domain binding to Sin3A resulted in minimal transcriptional impairment. Support for this view came from studies in Drosophila, where Phf12 was found in a histone chaperone complex that was involved in the silencing of selective Notch genes in a H3K4 demethylating and H3 deacetylating manner [23]. While the exact role of Phf12 in this system was not established, it was shown that Phf12 was not directly responsible for demethylation and deacetylation activities. This complex was composed of other members such as such as Sin3A, EMSY and Mrg15 that associate with Phf12 in other biological systems. In contrast, Jelinic et al. showed that Phf12 was not binding to Sin3A, but rather to Sin3B [11]. Additionally Sin3B, Phf12, Mrg15 and HDAC1 form a complex that can bind to transcriptional active H3K4me3/H3K36me3 enriched promoters [13]. Upon knockdown of either Sin3B or Phf12, transcriptional inhibition of constitutively active genes such as GAPDH and RPL13a was relieved demonstrating the repressing nature of Phf12 in this biological context.

Phf12 is found to be overexpressed in certain types of breast cancer [24]. Interestingly, EMSY, a binding partner of Phf12 has also been found to be amplified in certain breast cancer lineages [25]. It is known that BRCA2 mutations

are often found in inherited ovarian and breast cancer, but this was not the case in sporadic (i.e. non-herited) breast cancer. It appears that in these types of cancer, EMSY is overexpressed and this leads to mass inhibition of the transcriptional activities of BRCA2 via exon 3 [25]. It is not clear whether EMSY performs this action by itself or in a complex, which would most likely be the case. Since EMSY (and Phf12) is also associated with HDAC1 and HDAC2, this is probably via histone deacetylation and possibly via H3K4me3 demethylation [26]. Vermeulen et al. investigated the associated proteins of Gatad1 and found Phf12, EMSY, Morf4L1, Jarid1a and Sin3B to be in a complex in HeLa cells [26]. It was revealed that Gatad1 localizes to H3K4me3 marks on the chromatin in this complex. Since Jarid1a is a H3K4me3-specific demethylase, this might be an additional mechanism by which the complex that Phf12 is part of, is capable of silencing genes.

It seems contradictory that Phf12 is necessary at the PPARγ promoter for its expression since Phf12 has mainly been linked to transcriptional repression. This role however might be locus-dependent. Interestingly, in a GAL4 setting using MRG15 as the repressor, Phf12 alleviated the repressing effects of MRG15 [12]. This demonstrates that Phf12 can have both a repressing and anti-repressing properties, which is also the case for its associated repressor protein EMSY. Similar to Phf12, EMSY is generally associated with basal promoter suppression function. Yet RARα activity was enhanced and HoxA1 and Sox genes were more expressed in the presence of EMSY [27]. This suggests that proteins like Phf12 and EMSY may perform dual and opposing roles, maybe related to the complex in which they are located. In agreement with this idea was our finding that Phf12 knockdown resulted in the induction of only 3 genes (Fig. 2). Whether Phf12 indeed can positively regulate transcription and whether this is a unique phenomenon in adipocytes requires further investigation.

References

1. Hajer GR, van Haeften TW, Visseren FL (2008) Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. European heart journal 29: 2959-2971.
2. Stevens J, Couper D, Pankow J, Folsom AR, Duncan BB, et al. (2001) Sensitivity and specificity of anthropometrics for the prediction of diabetes in a biracial cohort. Obesity research 9: 696-705.
3. Colditz GA, Willett WC, Rotnitzky A, Manson JE (1995) Weight gain as a risk factor for clinical diabetes mellitus in women. Annals of internal medicine 122: 481-486.
4. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM (2000) Transcriptional regulation of adipogenesis. Genes Dev 14: 1293-1307.
5. Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79: 1147-1156.
6. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM (1994) mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 8: 1224-1234.
7. Arimura N, Horiba T, Imagawa M, Shimizu M, Sato R (2004) The peroxisome proliferator-activated receptor gamma regulates expression of the perilipin gene in adipocytes. The Journal of biological chemistry 279: 10070-10076.
8. Sato O, Kuriki C, Fukui Y, Motojima K (2002) Dual promoter structure of mouse and human fatty acid translocase/CD36 genes and unique transcriptional activation by peroxisome proliferator-activated receptor alpha and gamma ligands. The Journal of biological chemistry 277: 15703-15711.
9. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, et al. (2003) Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 52: 1655-1663.
10. Muruganandan S, Parlee SD, Rourke JL, Ernst MC, Goralski KB, et al. (2011) Chemerin, a novel peroxisome proliferator-activated receptor gamma (PPARgamma) target gene that promotes mesenchymal stem cell adipogenesis. The Journal of biological chemistry 286: 23982-23995.
11. Jelinic P, Pellegrino J, David G (2011) A novel mammalian complex containing Sin3B mitigates histone acetylation and RNA polymerase II progression within transcribed loci. Molecular and cellular biology 31: 54-62.
12. Yochum GS, Ayer DE (2001) Pf1, a novel PHD zinc finger protein that links the TLE corepressor to the mSin3A-histone deacetylase complex. Molecular and cellular biology 21: 4110-4118.
13. Yochum GS, Ayer DE (2002) Role for the mortality factors MORF4, MRGX, and MRG15 in transcriptional repression via associations with Pf1, mSin3A, and Transducin-Like Enhancer of Split. Molecular and cellular biology 22: 7868-7876.
14. Baas R, Lelieveld D, van Teeffelen H, Lijnzaad P, Castelijns B, et al. (2013) A novel microscopy-based high-throughput screening method to identify proteins that regulate global histone modification levels. Journal of Biomolecular Screening: in press.

15. van Wageningen S, Kemmeren P, Lijnzaad P, Margaritis T, Benschop JJ, et al. (2010) Functional overlap and regulatory links shape genetic interactions between signaling pathways. Cell 143: 991-1004.
16. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, et al. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 30: e15.
17. Margaritis T, Lijnzaad P, van Leenen D, Bouwmeester D, Kemmeren P, et al. (2009) Adaptable gene-specific dye bias correction for two-channel DNA microarrays. Molecular systems biology 5: 266.
18. Wu YH, Kerr MK, Churchill GA (2002) MAANOVA: a software package for the analysis of spotted microarray experiments. The analysis of gene expression data: methods and software.
19. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, et al. (2008) Genome-wide profiling of PPAR{gamma}:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev 22: 2953-2967.
20. Farmer SR (2006) Transcriptional control of adipocyte formation. Cell Metab 4: 263-273.
21. Siersbaek R, Nielsen R, John S, Sung MH, Baek S, et al. (2011) Extensive chromatin remodelling and establishment of transcription factor 'hotspots' during early adipogenesis. The EMBO journal 30: 1459-1472.
22. Kaadige MR, Ayer DE (2006) The polybasic region that follows the plant homeodomain zinc finger 1 of Pf1 is necessary and sufficient for specific phosphoinositide binding. The Journal of biological chemistry 281: 28831-28836.
23. Moshkin YM, Kan TW, Goodfellow H, Bezstarosti K, Maeda RK, et al. (2009) Histone chaperones ASF1 and NAP1 differentially modulate removal of active histone marks by LID-RPD3 complexes during NOTCH silencing. Molecular cell 35: 782-793.
24. Malovannaya A, Lanz RB, Jung SY, Bulynko Y, Le NT, et al. (2011) Analysis of the human endogenous coregulator complexome. Cell 145: 787-799.
25. Hughes-Davies L, Huntsman D, Ruas M, Fuks F, Bye J, et al. (2003) EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. Cell 115: 523-535.
26. Vermeulen M, Eberl HC, Matarese F, Marks H, Denissov S, et al. (2010) Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers. Cell 142: 967-980.
27. Garapaty S, Xu CF, Trojer P, Mahajan MA, Neubert TA, et al. (2009) Identification and characterization of a novel nuclear protein complex involved in nuclear hormone receptor-mediated gene regulation. The Journal of biological chemistry 284: 7542-7552.

Supplementary Figures

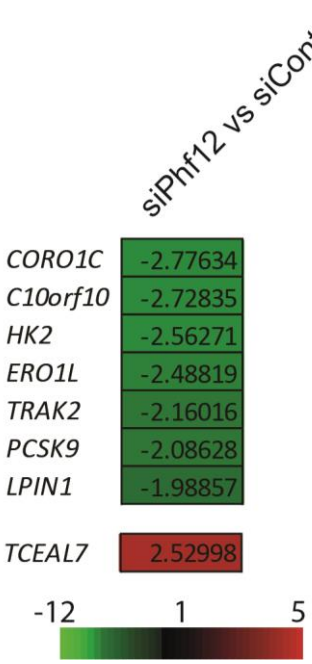


Figure S1 heat map of genes that have been affected by knockdown of Phf12 only.

Chapter 5

PPARγ REGULATES EXPRESSION OF CARBOHYDRATE SULFOTRANSFERASE 11 (CHST11/C4ST1), A REGULATOR OF LPL CELL SURFACE BINDING

Ismayil Tasdelen[1], Ruud Berger[1] and Eric Kalkhoven[1§]

From the [1]Department of Metabolic Diseases and Netherlands Metabolomics Center, University Medical Centre Utrecht, Utrecht, The Netherlands

PLoS One. 2013 May 16;8(5):e64284

Abstract

The transcription factor PPARγ is the key regulator of adipocyte differentiation, function and maintenance, and the cellular target of the insulin-sensitizing thiazolidinediones. Identification and functional characterization of genes regulated by PPARγ will therefore lead to a better understanding of adipocyte biology and may also contribute to the development of new anti-diabetic drugs. Here, we report carbohydrate sulfotransferase 11 (Chst11/C4st1) as a novel PPARγ target gene. Chst11 can sulphate chondroitin, a major glycosaminoglycan involved in development and disease. The Chst11 gene contains two functional intronic PPARγ binding sites, and is up-regulated at the mRNA and protein level during 3T3-L1 adipogenesis. Chst11 knockdown reduced intracellular lipid accumulation in mature adipocytes, which is due to a lowered activity of lipoprotein lipase, which may associate with the adipocyte cell surface through Chst11-mediated sulfation of chondroitin, rather than impaired adipogenesis. Besides directly inducing Lpl expression, PPARγ may therefore control lipid accumulation by elevating the levels of Chst11-mediated proteoglycan sulfation and thereby increasing the binding capacity for Lpl on the adipocyte cell surface.

Introduction

The close connections between obesity and its complications, such as type 2 diabetes and cardiovascular diseases, has firmly established white adipose tissue as a key regulator of whole body glucose and lipid metabolism [1]. White adipose tissue mainly regulates metabolism through storage of lipids (as triglycerides) and the secretion of so-called adipokines, which function in an endocrine or paracrine fashion. Several independent lines of research have firmly established the transcription factor peroxisome proliferator activator γ (PPARγ) as the master regulator of adipocyte differentiation, maintenance and function. For example, *in vitro* differentiation of fibroblasts into mature white adipocytes can be induced by introduction of PPARγ [2]. In addition, this protein directly regulates a large set of "adipocyte genes" involved in lipid and glucose metabolism [3,4]. Furthermore, PPARγ$^{-/-}$ mice are severely lipodystrophic, while PPAR γ$^{+/-}$ mice have reduced amounts of adipose tissue [5,6,7,8]. PPARγ is also essential for the maintenance of adipose tissue, since conditional knockout of the *Pparg* gene resulted in reduced *in vivo* survival of mature adipocytes [9]. Finally, human Familial partial lipodystrophy subtype 3 (FPLD3, MIM 604367) patients, harbouring heterozygous mutations in the *PPARG* gene, are characterized by aberrant fat distribution and metabolic disturbances, including insulin resistance and dyslipidaemia [10].

PPARγ activity can be stimulated by thiazolidinediones (TZDs), a class of anti-diabetic drugs that includes rosiglitazone [11]. Since elevated levels of serum free fatty acids promote insulin resistance [12], an important potential mechanism for the beneficial effects of TZDs is therefore the net partitioning of lipids in adipose tissue. This may partly be explained by the PPARγ-mediated stimulatory effect of TZDs on adipocyte differentiation, resulting in increased lipid storage capacity in adipose tissue. In addition, PPARγ also directly regulates genes involved in all different aspects of lipid handling, such as lipid uptake (e.g. lipoprotein lipase (Lpl) [13]), intracellular lipid transport (e.g. fatty acid binding protein 4 (Fabp4)[14]) and lipid storage (e.g. perilipin [15]), as well as anti-lipolytic genes (e.g. GPR81 [16]). While PPARγ is clearly a suitable pharmacological target, TZD use has unfortunately been linked to adverse side effects such as undesired weight gain, fluid retention, peripheral oedema, and potential increased risk of cardiac failure [11,17]. Interestingly, recent findings indicate that a more restricted modulation of PPARγ activity may provide a new way of improving

insulin sensitivity. A clear example of this is the recently identified phosphorylation site at serine 273 in PPARγ. CDK5-mediated phosphorylation of serine 273 in PPARγ leads to deregulation of a subset of genes whose expression is altered in obesity including the insulin-sensitizing adipokine, adiponectin [18]. Interestingly, S273 phosphorylation is blocked *in vivo* and *in vitro* by TZDs, but also by certain antidiabetic drugs that are weak PPARγ agonists or non-agonists [18,19]. These findings indicate that a comprehensive view on the mechanisms regulating PPARγ activity as well as its downstream target genes is required to develop the next generation of PPARγ-based antidiabetic drugs.

In the past few years, several genome wide PPARγ binding profiles have been generated in adipocytes, using either ChIP-ChIP [4,20,21], ChIPseq [3,22]} or ChIP-PET technology [23]. These global approaches have provided important new concepts, like the extensive crosstalk between PPARγ and C/EBPα as deduced from the overlap in their cistromes [3,4]. The binding profiles have also given important information on the single gene level, i.e. the identification of novel PPARγ target genes involved in lipid and glucose metabolism (e.g. Agpat2 and Hk2, respectively; [3]). Furthermore, genome wide binding profiles have helped to elucidate complex gene regulatory mechanisms, as exemplified by the genomic GPR81-GPR109A and UCP3-UCP2 regions, where single PPREs regulate multiple genes [16,24].

Using the PPARγ-RXR ChIPseq profile by Nielsen *et al.* [3] as a starting point, we identified the mouse chrondroitin-4-sulfotransferase 1 gene (*C4ST1/Chst11*) as a novel target of PPARγ. Chst11 is a Golgi-bound enzyme that catalyses the transfer of sulphate groups to the 4-O position of chondroitin sulphate (CS) and dermatan sulphate (DS). Membrane-bound sulphated proteoglycans are necessary for lipid accumulation in adipocytes, possibly because of their ability to interact with lipases like Lpl [25,26]. We found Chst11 mRNA and protein expression to be upregulated by PPARγ during 3T3-L1 adipogenesis and identified two functional intronic PPARγ binding sites in the Chst11 gene. In common with disruption of Lpl function [27], siRNA-mediated knock down of Chst11 resulted in reduced intracellular lipid accumulation in mature 3T3-L1 adipocytes. This effect is probably not due to inhibition of adipogenesis, as the expression of typical adipogenic genes such as *C/EBPa*, *Fabp4*, *Lpl* and adiponectin (*Adipoq*) was unaffected. Rather, knockdown of Chst11 inhibited the activity of Lpl. These findings suggest that PPARγ may

regulate Lpl-mediated lipid accumulation by two different mechanisms: it increases *Lpl* transcription directly [13], but can also indirectly regulate activity of the Lpl protein by elevating Chst11 expression and thereby increasing the number of docking sites for Lpl on the adipocyte cell surface.

Materials & Methods

Materials - Rosiglitazone and GW9662 were purchased from Alexis Biochemicals and Cayman Chemical, respectively. Heparin was purchased from LEO Pharma. Oil-red-o, dexamethasone and IBMX were from Sigma Aldrich. The Lpl activity kit was from Roar Biomedical. Anti-PPARγ (sc-7196) from Santa Cruz Biotechnology was used for ChIP assays. Anti-PPARγ (sc-7273) antibody from Santa Cruz Biotechnology and anti-Chst11 (ab57225) antibody from Abcam were used for immunofluorescence.

Plasmids - The regions surrounding peak 1 and 2 in the intron of the Chst11 gene were subcloned using the following primers. Peak 1, forward: 5'-CGGGGTACCCCGTGAAGACTAAGATAACATAG-3' and reverse: 5'-CCGCTCGAGCGGACACACACACACATTCAGCTC-3'; Peak 2, forward: 5'-CGGGGTACCCCG TTCCCGCTCTGGGAAAAAAAG-3' and reverse: 5'-CCGCTCGAGCGGGACCTGGTCTTCCCTGTCTTGATG-3'. The products were cut with KpnI and XhoI and ligated into the TK-Pgl3 vector [28]. All other plasmids have been described before [29,30].

Cell culture, differentiation and reporter assays - The human osteosarcoma cell line U2OS (ATCC, Manassas, VA) was maintained in DMEM Glutamax (Dulbecco) containing 10% foetal calf serum (Life Technologies, Inc., Rockville, MD), penicillin and streptomycin (both 100 µg/ml; Life Technologies). The murine 3T3-L1 cell line (ATCC, Manassas, VA) was cultured in the same media but with 10% bovine serum (Life Technologies) and penicillin and streptomycin (both 100µg/ml; Life Technologies). For differentiation, 3T3-L1 cells were grown to confluence and after 2 days incubated with culture medium containing dexamethasone (250 nM), 3-isobutyl-1-methylxanthine (500 µM) and insulin (170 nM) for 2 days. On day 3, medium was changed for culture medium supplemented with insulin (170 nM) and left for a week. Subsequently, cells were stained with Oil-red-O, or lysed and subjected to Western blot analysis as described before at day 5 after differentiation [16,29,30]. For Western blot analyses, differentiated 3T3-L1 cells were lysed in RIPA lysis buffer (200 mm Tris-HCl, pH 8.0; 0,1% SDS, 1% Triton X-100; 10 mm EDTA; 150 mm NaCl; 1% sodium deoxycholate containing protease inhibitors). Cell lysates were subjected to SDS-PAGE, and transferred to

Immobilon membranes (Millipore). ECL Plus (PerkinElmer Life Sciences) was used for detection on an ImageQuant LAS 4000 (GE Lifesciences).
Reporter assays were performed essentially as described before [29,30]. In short, cells were transfected in 24-well plates with 1µg reporter plasmid, 10ng PPARγ expression construct, and 2ng pCMV-Renilla reporter plasmid (Promega). The next day, cells were washed twice with PBS and subsequently maintained in medium in absence or presence of rosiglitazone for 24 h. After incubation, cells were washed twice with PBS and harvested in passive lysisbuffer (Promega) and assayed for luciferase activity according to the manufacturer's protocol (Promega Dual-Luciferase Reporter Assay System) and for Renilla luciferase activity to correct for transfection efficiency. The relative light units were measured by a CentroLB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Chromatin immunoprecipitation (ChIP) - Chromatin immunoprecipitation (ChIP) was performed exactly as decribed before [30]. Quantitative PCR was performed with primers against mouse Fabp4 PPRE (5'-GAGAGCAAATGGAGTTCCCAGA-3'; 5'-TTGGGCTGTGACACTTCCAC-3'), Chst11 peak 1 (5'-ACAGGCTTGCTTTGGCAC-3'; 5'-ACACTCACTACTCACAATCTGT-3'), Chst11 peak 2 (5'-CTCATCCAACCTGGGTTTTGG-3'; 5'-GAGTTCCTAGACTTGAAGAACTATG-3') and the mouse beta globin gene as control (5'-CCTGCCCTCTCTATCCTGTG-3'; 5'- GCAAATGTGTTGCCAAAAAG-3').

siRNA transfection - 3T3-L1 cells were transfected with siRNA oligonucleotides as described before [30] using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol. The siRNA oligonucleotides used were siControl (D-001810-10-20, Dharmacon), siPPARγ (L-040712-00-0010, Dharmacon), siChst11 (J-040396-11-0020, Dharmacon), siLpl (L-042649-01-0005, Dharmacon).

RNA isolation and QPCR - 3T3-L1 fibroblasts were differentiated as described above. Four independent samples of total RNA were extracted at different time points using TRIzol reagent (Invitrogen). cDNA was synthesized using the superscript first strand synthesis system (Invitrogen) according to manufacturer's protocol. Gene expression levels were determined by quantitative real time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to *TFIIb* expression.

The primers used were as follows: murine TFIIb forward primer, 5'-TCCTCCTCAGACCGCTTTT-3', and reverse primer, 5'-CCTGGTTCATCATCGCTAATC-3'; murine PPARγ forward primer, 5'- CGCTGATGCACTGCCTATGA-3' and reverse primer, 5'-AGAGGTCCACAGAGCTGATTCC-3'; murine Chst11 forward primer, 5'-GTCCCCTGCAGGAGCTCTA-3', and reverse primer, 5'-CTCATCTGGTGCAGGATGG-3'; murine Fapb4 forward primer, 5'-CGCAGACGACAGGAAGGT-3', and reverse primer, 5'-TTCCATCCCACTTCTGCAC-3'; murine Adipoq forward primer, 5'-GGAACTTGTGCAGGTTGGAT-3', and reverse primer, 5'- TCTCCAGGCTCTCCTTTCCT-3'; murine Lpl forward primer, 5'- TTTGTGAAATGCCATGACAAG-3' and reverse primer, 5'- TCAAACACCCAAACAAGGGTA-3'; murine C/EBPα forward primer, 5'-AAACAACGCAACGTGGAGA-3' and reverse primer, 5'-GCGGTCATTGTCACTGGTC-3'.

Immunofluorescence - For immunofluorescence staining, 3T3-L1 cells were plated on glass coverslips and differentiated for 5 days. Subsequently, cells were fixed with 4% paraformaldehyde (20', RT) and permeabilized in PBS supplemented with 0.2% Triton for 5 minutes. After 30' incubation in blocking buffer (2% BSA in PBS), cells were stained with primary antibodies for 2h at room temperature, and then incubated with secondary fluorochrome-conjugated antibodies. After several washes, coverslips were incubated Nile-Red and Hoechst, washed with PBS, mounted in Mowiol and analysed with an LSM710 Met confocal microscope (Carl Zeiss, Jena, Germany).

Lpl activity assays - Lpl activity was measured according to the manufacturers instructions. In short, cells were grown and differentiated in 6-well plates. Media were removed and cells were incubated with 50 units of heparin in 500ul of PBS (30', 37**°** C). Supernatants were collected and debris was removed by centrifugation (12.000 rpm, 10'). Supernatants (25 µl) were incubated with assay buffer mix (175 µl) for 45' and analysed on a Victor3 (Perkin Elmer) at wavelengths 355/460.

Results

Chst11 is a novel PPARγ target gene

To identify novel PPARγ target genes we thoroughly analysed ChIPseq data generated by Nielsen *et al.* [3]. We focused on loci that displayed PPARγ/RXRα binding at day 6 with little or no binding of these transcription factors at day 0 of 3T3-L1 adipogenesis. Using these criteria we identified the carbohydrate sulfotransferase 11 *Chst11*, also known as C4st1, as a potential direct PPARγ target gene. Chst11 can sulphate chondroitin sulphate-proteoglycans (CSPG), which plays an important role in development and disease ([31,32]; see also Discussion). Two intronic binding sites for PPARγ were observed on day 2 of adipogenesis, with increased binding during later stages of adipogenesis (day 4 and 6; Fig. 1A). These binding sites were also present in the binding profiles of RXRα, which is required for PPARγ to bind to DNA. Please note that these sites do not overlap with the regulatory elements previously identified in the Chst11 locus, some of which confer regulation by TGFβ [33] .

The recruitment of PPARγ to the two intronic binding sites in the Chst11 gene in mature adipocytes (day 6) was confirmed by ChIP-PCR (Fig. 1B). As a positive control the well-characterized PPARγ/RXRα enhancer bindingsite in the Fabp4 gene was used. In addition, the recruitment in preadipocytes (day 0), in which PPARγ expression is low, was negligible and PPARγ was not detected on an arbitrary region of the beta-globin gene, which served as a negative control (Fig. 1B). Having established that PPARγ is recruited to site 1 and 2 upon differentiation (Fig. 1A and B), we wished to investigate whether these intronic binding sites could act as functional enhancers. Therefore, both regions were cloned in a promoter-containing reporter plasmid (TK-pGL3). The activity of these reporters was determined in human osteosarcoma (U2OS) cells, which express negligible levels of endogenous PPARγ protein, but display a robust response upon overexpression of the protein [29]. As shown in Figure 1C and D, transfection of cells with an expression vector encoding PPARγ2 activated the reporter genes containing PPARγ/RXRα binding sites of the Chst11 gene compared to empty vector control (pCDNA), most markedly for binding site 1. Activation of PPARγ by the synthetic ligand rosiglitazone further enhanced the PPARγ-mediated activation of both reporters (Fig. 1C and D), indicating that the two intronic PPARγ/RXRα binding sites in the Chst11 gene can act as functional enhancers.

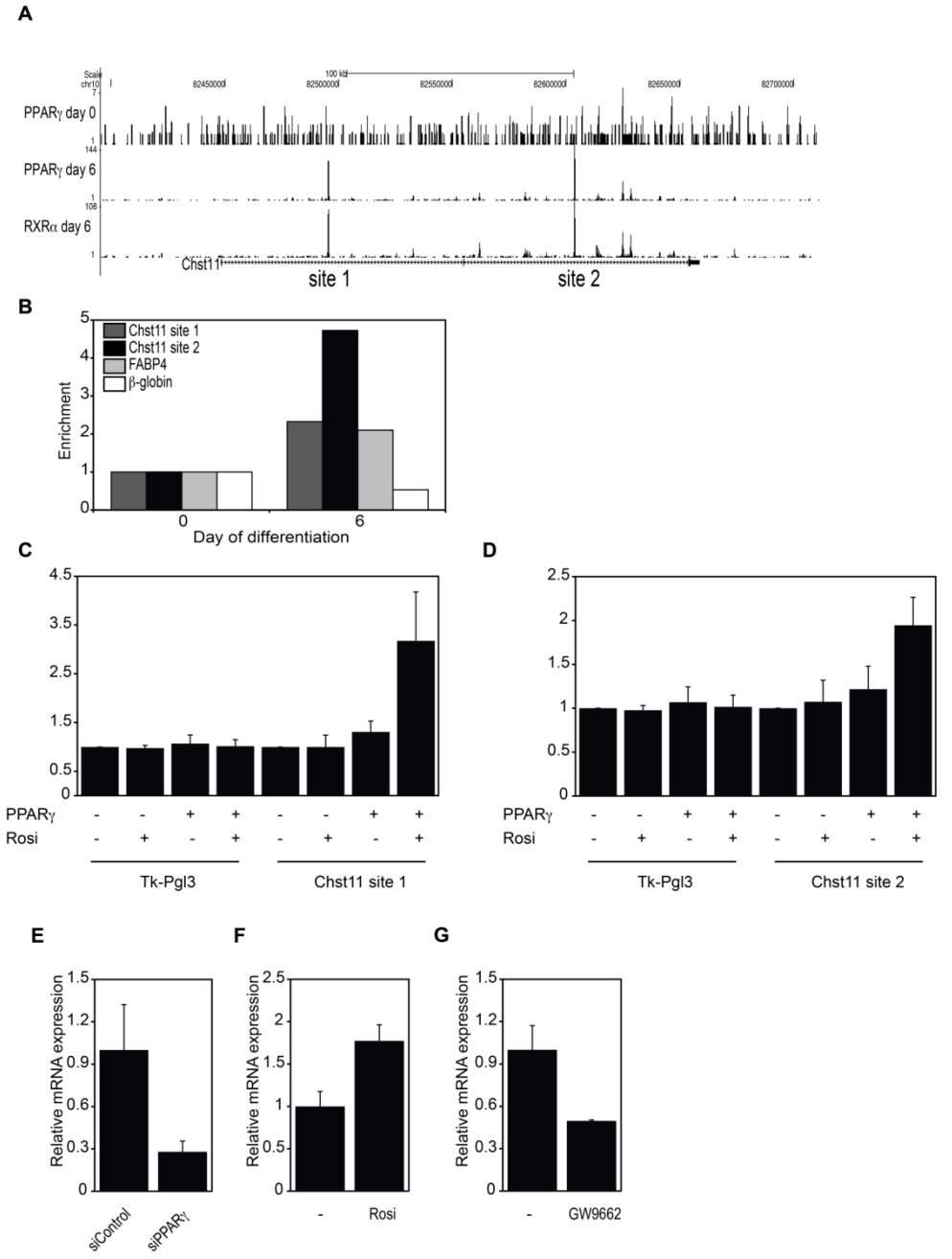


Figure 1: Chst11 is a novel PPARγ target gene (A) Chip-Seq data of PPARγ and RXRα occupancy on the Chst11 gene according to Nielssen et al. [3]. UCSC Genome Browser tracks at day 0, 1, 2 4 and 6 of differentiation are shown. Please note differences in y-axes. Two intronic PPARγ-RXRα binding

sites were designated site 1 and 2. (B) ChIP-PCR on 3T3-L1 preadipocytes and adipocytes. Chromatin was prepared on day 0 and day 6 of differentiation and subjected to immunoprecipitation using antibodies against PPARγ. Enriched DNA was analysed using quantitative PCR with primers located at site 1 and 2 in the Chst11 gene (dark gray and blacks bars, respectively). As a positive control, primers located at the Fabp4 PPREs (~5500 bp from transcription start site; light gray bars) were used, primers located in the globin locus were used as a negative control (white bars). Results are shown relative to normalized ChIP recovery data of day 0 and results are representative of at least 3 independent experiments. (C and D) U2OS cells were cotransfected with a reporter construct (TKpGL3) containing Chst11 site 1 or site 2 sequences, or the parental reporter construct, together with empty (pCDNA) or PPARγ encoding expression vectors. Activation of the luciferase reporter in the absence or presence of 1μM rosiglitazone is expressed as fold induction over that with empty reporter cotransfected with pCDNA in the absence of rosiglitazone after normalisation for Renilla luciferase activity. The error bars display SEM and significance is shown by the asterisks ($p<0.05$), n=3 (E) Chst11 mRNA expression in 3T3-L1 adipocytes that had been treated with control or PPARγ siRNA from the start of differentiation and analyzed at day 5. Relative mRNA expression levels were related to control siRNA treated cells and normalized for the TFIIb reference gene. The error bars display SEM and significance is shown by the asterisks ($p<0.05$), n=3 (F) Chst11 mRNA expression in 3T3-L1 adipocytes and the effect of rosiglitazone treatment (1μM, 24h). Relative mRNA expression levels were normalized for the TFIIb reference gene. (G) As in panel F, but after treatment with the PPARγ antagonist GW9662 (10 μM, 24h).

To corroborate these findings, we investigated whether modulation of endogenous PPARγ protein affected Chst11 expression. First, siRNA-mediated knock down of PPARγ was performed. PPARγ knock down resulted in a significant reduction of Chst11 mRNA expression in mature adipocytes (Fig. 1E). Furthermore, treatment of mature adipocytes with the PPARγ agonist rosiglitazone resulted in increased Chst11 levels (Fig. 1F), while incubation with the antagonist GW9662 lowered Chst11 expression (Fig. 1G). Taken together, these data classify Chst11 as a novel direct PPARγ target gene.

Chst11 is expressed in mature adipocytes

Next, we examined Chst11 mRNA and protein expression in adipogenesis. Using quantitative RT-PCR, Chst11 mRNA levels were found to increase 8 fold and reached its climax at day 2 of differentiation after which the levels were stable (Fig. 2A). As a control, expression of PPARγ was analysed, which increased steadily over the course of differentiation (Fig. 2B). To investigate Chst11 protein expression and localization, immunofluorescent staining of Chst11 was combined with fluorescent staining for intracellular lipid droplets (Nile red) to mark differentiated cells. As shown in Fig. 2C, mature adipocytes (Nile Red positive) also stained positive for Chst11 protein, while Chst11 could not be detected in preadipocytes (Nile Red negative). Chst11 displayed cytoplasmic localization in

mature adipocytes, in agreement with other cell types [31]. In summary, these data indicate that Chst11 is induced during adipogenesis, both at the mRNA and protein level.

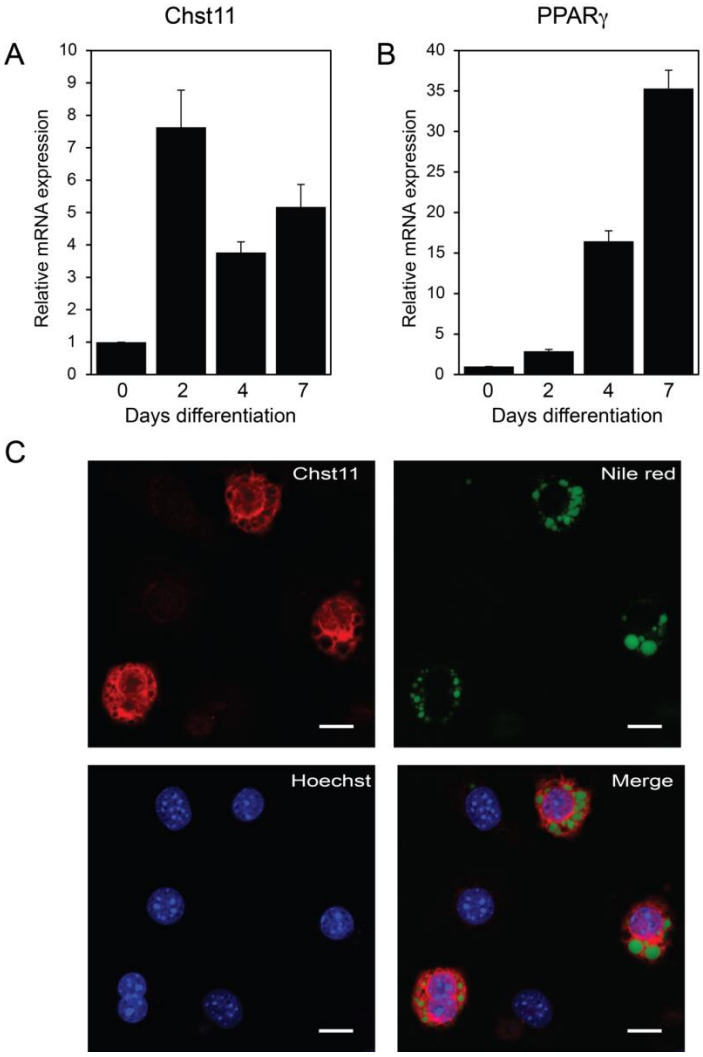
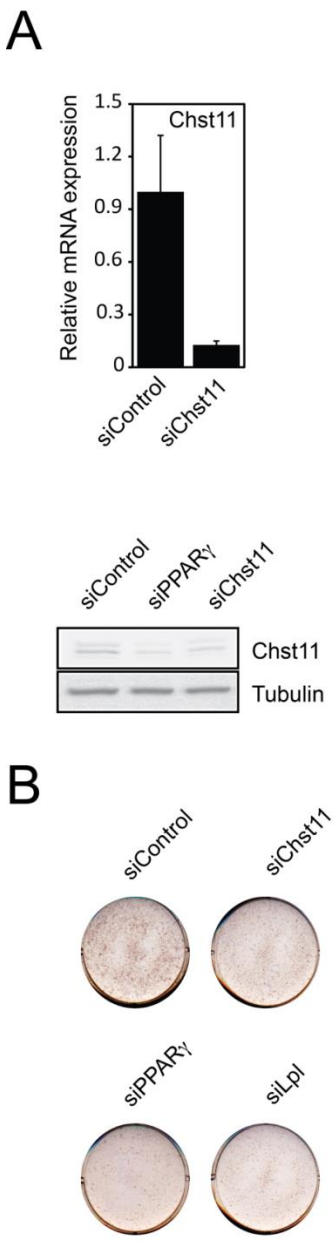


Figure 2: Chst11 is expressed in mature 3T3-L1 adipocytes (A) and (B): mRNA expression profiles of Chst11 and PPARγ in adipogenesis of 3T3-L1 cells during different days. Data are represented as described in Figure 1E. (C) Representative confocal microscopy images of 3T3-L1 (pre)adipocytes. Endogenous Chst11 (red) was visualized utilizing specific antibodies, differentiated cells were identified with Nile Red (green), Hoechst was used to visualize the nuclei (blue).

Knockdown of Chst11 leads to decreased intracellular lipid accumulation, but not adipogenesis

Having established that PPARγ regulates Chst11 levels (Fig. 1) and that Chst11 is expressed on the mRNA and protein level in mature adipocytes (Fig. 2), we wished to investigate the role of Chst11 in adipocytes. To address this, siRNA-mediated knockdown was performed in 3T3-L1 cells, and cells were subsequently subjected to adipogenic culture conditions. siRNA-mediated knockdown reduced Chst11 mRNA and protein (Fig 3A). Chst11 knock down led to decreased lipid accumulation, as assessed by Oil-red-O staining of intracellular lipids, a phenomenon also observed upon Lpl knock down (Fig. 3B) [27]. Reduced lipid staining may be the result of reduced adipogenesis or only reduced lipid accumulation. To distinguish between these possibilities the effect of Chst11 knock down on the expression of *C/EBPα* and *PPARγ*, two key regulators of adipogenesis, was examined. Only marginal differences were observed between cells that had been treated with Chst11 siRNA oligonucleotides or scrambled siRNA oligonucleotides (Fig 3C). PPARγ protein levels were also not affected dramatically by Chst11 knockdown. Expression of the genes encoding Lpl, adiponectin (Adipoq) and FABP4, which are all up-regulated in adipogenesis, was also unaffected by Chst11 knock down. Knock down of PPARγ, which has repeatedly been reported to block adipogenesis (e.g. [8] [34]), however significantly inhibited the expression of all these genes. These findings therefore indicate that Chst11 specifically regulates lipid accumulation rather than adipogenesis.

Figure 3: Knockdown of Chst11 leads to decreased lipid accumulation but not adipogenesis
(A) 3T3-L1 cells were treated with siRNA oligonucleotides targeting Chst11 or control (scrambled) oligonucleotides and Chst11 mRNA and protein expression levels were determined at day 5 after differentiation by qPCR (upper panel) and Western blotting (lower panel), respectively. The error bars display SEM and significance is shown by the asterisks ($p<0.05$), n=3 (B) 3T3-L1 cells were treated with siRNA oligonucleotides targeting PPARγ, Chst11, Lpl, or control (scrambled) oligonucleotides, fixed and stained for lipid accumulation using Oil-red-O. Pictures are representative of 3 independent experiments. (C) 3T3-L1 cells were treated with siRNA oligonucleotides targeting Chst11, PPARγ or control (scrambled) oligonucleotides and mRNA expression levels of PPARγ, C/EBPα, Lpl, adiponectin (adipoq) and Fabp4 were determined after 5 days of differentiation. Relative mRNA expression levels were related to control siRNA treated cells and normalized for the TFIIb reference gene. Samples were also generated for a western blot in which PPARγ expression was shown upon Chst11 and PPARγ knockdown. The error bars display SEM and significance is shown by the asterisks ($p<0.05$), n=3.


A
Relative mRNA expression
1.5
1.2
0.9
0.6
0.3
0
Chst11
siControl
siChst11
siControl
siPPARγ
siChst11
Chst11
Tubulin
B
siControl
siChst11
siPPARγ
siLpl
C
Relative expression
PPARγ
C/EBPα
Lpl
Adipoq
FABP4
siControl
siChst11
siPPARγ

Knockdown of Chst11 leads to decreased Lpl activity

Since Chst11 knock down reduced lipid accumulation (Fig. 3B) and given that Lpl is required for lipid accumulation in 3T3-L1 adipocytes (Fig. 3B) [27] we hypothesized that Chst11 activity may regulate Lpl activity in mature adipocytes. In favour of this hypothesis is the finding that Lpl requires a stable negatively charged docking site on HSPGs for binding to the cell surface of endothelial cells [35], which can be provided by Chst11-mediated sulfation of CS in adipocytes. To test this possibility, 3T3-L1 cells were differentiated and subjected to scrambled, PPARγ and Chst11 siRNA-mediated knock down, after which Lpl was released from the adipocyte cell surface by heparin treatment [36], and enzymatic activity was determined as a measure for cell-surface-associated Lpl. Upon knock down of Chst11 a significant reduction in Lpl activity was observed (Fig. 4; $p<0.05$). As a control, knock down of PPARγ, which regulates the expression of both Chst11 (current study) and Lpl [13], was performed. As expected, reduced PPARγ expression also resulted in impaired Lpl activity (Fig. 4). These data support a model in which PPARγ regulates Chst11 expression, leading to increased sulphated CS chains which can then form docking sites for Lpl, ultimately contributing to lipid accumulation in mature adipocytes.

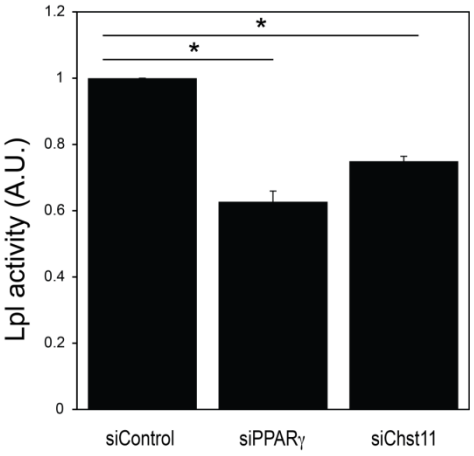


Figure 4: Knockdown of Chst11 leads to decreased Lpl activity

3T3-L1 cells were treated with siRNA oligonucleotides targeting Chst11, PPARγ or control (scrambled) oligonucleotides and Lpl activity was analysed. The error bars display SEM and significance is shown by the asterisks ($p<0.05$), n=3

Discussion

Multiple extracellular signalling pathways have been implicated in adipogenesis, ultimately leading to upregulation of the transcription factor PPARγ, the master regulator of adipogenesis [37]. PPARγ directly regulates the expression of genes involved in various aspects of lipid handling, including Lpl [38]. Here we have identified the enzyme Chst11, which harbours sulfotransferase activity, as a novel PPARγ target gene. Knockdown of Chst11 does not affect the adipogenic gene program, but rather inhibits lipid accumulation, possibly through inhibition of the cell surface binding of Lpl. These findings suggest that the central role of PPARγ in lipid metabolism in adipocytes extends beyond the regulation of genes directly involved in lipid handling, and includes genes like Chst11, which play a more indirect role. Chst11 can sulphate the 4-O position of GalNac residues in chondroitin sulphate (CS), one of the major classes of glycosaminoglycans (GAG) [39,40]. GAGs are sulphated repeating disaccharide units, which together with core proteins can form proteoglycans like heparan- and chondroitin sulphate proteoglycan (HSPG/CSPG), and function in cell-cell communication, adhesion, and protein presentation [41]. In addition, CSPG/HSPG are necessary for lipid accumulation in adipocytes [25]. Sulfation of GAGs by sulfotranferases like Chst11 adds negative charge to proteoglycans, which is important for interactions with various other proteins like growth factors, apolipoproteins, extracellular matrix, and plasma proteins [41]. Importantly, sulphated and thus negatively charged HSPG can function as a docking site for Lpl in endothelial cells [35,42]. Furthermore, macrophages produce an oversulfated CSPG that can also bind Lpl [43], and CSPG is the dominant proteoglycan on the adipocyte cell surface [25,44]. When combined with our current data, these findings suggest that the main role of Chst11 in adipocytes may be to increase the amount of sulphated, negatively charged CS chains that could then act as binding sites for Lpl activity on the adipocyte cell surface. Additional experiments are required to establish the sulphation status of CSPG, and its role in Lpl binding in adipocytes.

Lpl is best known for its role in endothelium, where secreted Lpl travels through the capillary walls towards the luminal space of the endothelium, and binds to the proteoglycans that are anchored into vascular endothelial cells [42,45,46,47]. The transport of Lpl from adipocytes and myocytes, which produce high Lpl levels, to the capillary walls has just recently been unravelled. The

GPIHBP1 protein, which is present on both apical and basolateral surface of endothelial cells, is capable of transporting Lpl from basolateral to the apical surface of the cells [48]. At the luminal side, Lpl can help to cleave lipids off chylomicrons and VLDL particles, so that the fatty acids can enter through the capillaries towards the adipocytes where it can be stored. Lpl molecules that are internalized by endothelial cells are recycled back to the cell surface [26]. Although Lpl binds to the surface of adipocytes with 5-10 fold higher efficiency compared to endothelial cells [49], its role in adipocytes is less well-defined. Multiple studies have provided evidence that lipolysis of lipoproteins at the endothelium by Lpl can loosen the junctions between endothelial cells so that large lipid enriched lipoproteins can pass through them [45,50,51]. In this event, Lpl should also be present at the cell surface of adipocytes to deplete the lipoproteins of their lipids. Surface-bound Lpl activity, which may be positively regulated by sulfotransferases like Chst11, can therefore be regarded as a back-up system for efficient take-up of lipids from lipoproteins. As genetic inactivation of Chst11 is associated with severe developmental abnormalities [31], adipose tissue-specific Chst11 knock-out mice will need to be generated to establish the precise role for Chst11 in lipid accumulation in adipocytes *in vivo*.

Acknowledgements

The authors thank Dr. Ronni Nielsen and Dr. Susanne Mandrup for help with analysis of ChIPseq data, Benthe Freyee for technical assistance, and Dr. Sander Kersten for critically reading the manuscript.

References

1. Rosen ED, Spiegelman BM (2006) Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444: 847-853.
2. Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79: 1147-1156.
3. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, et al. (2008) Genome-wide profiling of PPAR{gamma}:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev 22: 2953-2967.
4. Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, et al. (2008) PPAR{gamma} and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes Dev 22: 2941-2952.
5. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, et al. (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. MolCell 4: 585-595.
6. Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, et al. (1999) PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. MolCell 4: 597-609.
7. Nadra K, Quignodon L, Sardella C, Joye E, Mucciolo A, et al. (2010) PPARgamma in placental angiogenesis. Endocrinology 151: 4969-4981.
8. Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, et al. (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. MolCell 4: 611-617.
9. Imai T, Takakuwa R, Marchand S, Dentz E, Bornert JM, et al. (2004) Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. ProcNatlAcadSciUSA 101: 4543-4547.
10. Hegele RA (2005) Lessons from human mutations in PPARgamma. IntJObes(Lond) 29 Suppl 1:S31-5.: S31-S35.
11. Yki-Jarvinen H (2004) Thiazolidinediones. NEnglJMed 351: 1106-1118.
12. Boden G (1997) Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 46: 3-10.
13. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, et al. (1996) PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO J 15: 5336-5348.
14. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM (1994) mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 8: 1224-1234.
15. Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, et al. (2004) Adipose tissue expression of the lipid droplet-associating proteins S3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-gamma. Diabetes 53: 1243-1252.
16. Jeninga EH, Bugge A, Nielsen R, Kersten S, Hamers N, et al. (2009) Peroxisome proliferator-activated receptor gamma regulates expression of the anti-lipolytic G-protein-coupled receptor 81 (GPR81/Gpr81). JBiolChem 284: 26385-26393.

17. Gervois P, Fruchart JC, Staels B (2007) Drug Insight: mechanisms of action and therapeutic applications for agonists of peroxisome proliferator-activated receptors. NatClinPractEndocrinolMetab 3: 145-156.
18. Choi JH, Banks AS, Estall JL, Kajimura S, Bostrom P, et al. (2010) Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5. Nature 466: 451-456.
19. Choi JH Banks, AS, Kamenecka TM, Busby SA, Chalmers MJ, Kumar N, Kuruvilla DS, Shin Y, He Y, Bruning JB, Marciano DP, Cameron MD, Laznik D, Jurczak MJ, Schürer SC, Vidović D, Shulman GI, Spiegelman BM, Griffin PR (2011) Antidiabetic actions of a non-agonist PPARγ ligand blocking Cdk5-mediated phosphorylation. Nature 466: 451-456.
20. Nakachi Y, Yagi K, Nikaido I, Bono H, Tonouchi M, et al. (2008) Identification of novel PPARgamma target genes by integrated analysis of ChIP-on-chip and microarray expression data during adipocyte differentiation. BiochemBiophysResCommun 372: 362-366.
21. Wakabayashi K, Okamura M, Tsutsumi S, Nishikawa NS, Tanaka T, et al. (2009) The peroxisome proliferator-activated receptor gamma/retinoid X receptor alpha heterodimer targets the histone modification enzyme PR-Set7/Setd8 gene and regulates adipogenesis through a positive feedback loop. Mol Cell Biol 29: 3544-3555.
22. Lefterova MI, Steger DJ, Zhuo D, Qatanani M, Mullican SE, et al. (2010) Cell-specific determinants of peroxisome proliferator-activated receptor gamma function in adipocytes and macrophages. Mol Cell Biol 30: 2078-2089.
23. Hamza MS, Pott S, Vega VB, Thomsen JS, Kandhadayar GS, et al. (2009) De-novo identification of PPARgamma/RXR binding sites and direct targets during adipogenesis. PLoS One 4: e4907.
24. Bugge A, Siersbaek M, Madsen MS, Gondor A, Rougier C, et al. (2010) A novel intronic peroxisome proliferator-activated receptor gamma enhancer in the uncoupling protein (UCP) 3 gene as a regulator of both UCP2 and -3 expression in adipocytes. J Biol Chem 285: 17310-17317.
25. Wilsie LC, Chanchani S, Navaratna D, Orlando RA (2005) Cell surface heparan sulfate proteoglycans contribute to intracellular lipid accumulation in adipocytes. Lipids Health Dis 4: 2.
26. Misra KB, Kim KC, Cho S, Low MG, Bensadoun A (1994) Purification and characterization of adipocyte heparan sulfate proteoglycans with affinity for lipoprotein lipase. J Biol Chem 269: 23838-23844.
27. Gonzales AM, Orlando RA (2007) Role of adipocyte-derived lipoprotein lipase in adipocyte hypertrophy. Nutr Metab (Lond) 4: 22.
28. Kalkhoven E, Teunissen H, Houweling A, Verrijzer CP, Zantema A (2002) The PHD type zinc finger is an integral part of the CBP acetyltransferase domain. MolCell Biol 22: 1961-1970.
29. Jeninga EH, van Beekum O, van Dijk AD, Hamers N, Hendriks-Stegeman BI, et al. (2007) Impaired peroxisome proliferator-activated receptor gamma function through mutation of a conserved salt bridge (R425C) in familial partial lipodystrophy. MolEndocrinol 21: 1049-1065.

30. van Beekum O, Brenkman AB, Grontved L, Hamers N, van den Broek NJ, et al. (2008) The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARgamma. Endocrinology 149: 1840-1849.
31. Kluppel M, Wight TN, Chan C, Hinek A, Wrana JL (2005) Maintenance of chondroitin sulfation balance by chondroitin-4-sulfotransferase 1 is required for chondrocyte development and growth factor signaling during cartilage morphogenesis. Development 132: 3989-4003.
32. Kluppel M (2010) The roles of chondroitin-4-sulfotransferase-1 in development and disease. Prog Mol Biol Transl Sci 93: 113-132.
33. Willis CM, Wrana JL, Kluppel M (2009) Identification and characterization of TGFbeta-dependent and -independent cis-regulatory modules in the C4ST-1/CHST11 locus. Genet Mol Res 8: 1331-1343.
34. Koppen A, Houtman R, Pijnenburg D, Jeninga EH, Ruijtenbeek R, et al. (2009) Nuclear receptor-coregulator interaction profiling identifies TRIP3 as a novel PPARgamma cofactor. MolCell Proteomics 8: 2212-2226.
35. Lookene A, Chevreuil O, Ostergaard P, Olivecrona G (1996) Interaction of lipoprotein lipase with heparin fragments and with heparan sulfate: stoichiometry, stabilization, and kinetics. Biochemistry 35: 12155-12163.
36. Weinstein MM, Yin L, Beigneux AP, Davies BS, Gin P, et al. (2008) Abnormal patterns of lipoprotein lipase release into the plasma in GPIHBP1-deficient mice. J Biol Chem 283: 34511-34518.
37. Rosen ED, MacDougald OA (2006) Adipocyte differentiation from the inside out. NatRevMolCell Biol 7: 885-896.
38. Lehrke M, Lazar MA (2005) The many faces of PPARgamma. Cell 123: 993-999.
39. Yamauchi S, Hirahara Y, Usui H, Takeda Y, Hoshino M, et al. (1999) Purification and characterization of chondroitin 4-sulfotransferase from the culture medium of a rat chondrosarcoma cell line. J Biol Chem 274: 2456-2463.
40. Hiraoka N, Nakagawa H, Ong E, Akama TO, Fukuda MN, et al. (2000) Molecular cloning and expression of two distinct human chondroitin 4-O-sulfotransferases that belong to the HNK-1 sulfotransferase gene family. J Biol Chem 275: 20188-20196.
41. Bishop JR, Schuksz M, Esko JD (2007) Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature 446: 1030-1037.
42. Mead JR, Irvine SA, Ramji DP (2002) Lipoprotein lipase: structure, function, regulation, and role in disease. J Mol Med (Berl) 80: 753-769.
43. Edwards IJ, Xu H, Obunike JC, Goldberg IJ, Wagner WD (1995) Differentiated macrophages synthesize a heparan sulfate proteoglycan and an oversulfated chondroitin sulfate proteoglycan that bind lipoprotein lipase. Arterioscler Thromb Vasc Biol 15: 400-409.
44. Calvo JC, Rodbard D, Katki A, Chernick S, Yanagishita M (1991) Differentiation of 3T3-L1 preadipocytes with 3-isobutyl-1-methylxanthine and dexamethasone stimulates cell-associated and soluble chondroitin 4-sulfate proteoglycans. J Biol Chem 266: 11237-11244.
45. Saxena U, Klein MG, Goldberg IJ (1991) Transport of lipoprotein lipase across endothelial cells. Proc Natl Acad Sci U S A 88: 2254-2258.

46. Obunike JC, Lutz EP, Li Z, Paka L, Katopodis T, et al. (2001) Transcytosis of lipoprotein lipase across cultured endothelial cells requires both heparan sulfate proteoglycans and the very low density lipoprotein receptor. J Biol Chem 276: 8934-8941.
47. Goldberg IJ (1996) Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. J Lipid Res 37: 693-707.
48. Davies BS, Beigneux AP, Barnes RH, 2nd, Tu Y, Gin P, et al. (2010) GPIHBP1 is responsible for the entry of lipoprotein lipase into capillaries. Cell Metab 12: 42-52.
49. Saxena U, Klein MG, Goldberg IJ (1991) Identification and characterization of the endothelial cell surface lipoprotein lipase receptor. J Biol Chem 266: 17516-17521.
50. Rutledge JC, Woo MM, Rezai AA, Curtiss LK, Goldberg IJ (1997) Lipoprotein lipase increases lipoprotein binding to the artery wall and increases endothelial layer permeability by formation of lipolysis products. Circ Res 80: 819-828.
51. Hennig B, Shasby DM, Fulton AB, Spector AA (1984) Exposure to free fatty acid increases the transfer of albumin across cultured endothelial monolayers. Arteriosclerosis 4: 489-497.

Chapter 6

General Discussion

PPARγ based interventions

In this thesis we have investigated different aspects of the function of PPARγ in adipogenesis. PPARγ is a central player in adipogenesis as we have discussed in Chapter 1. In the past, PPARγ ligands were used in the clinic for treatment of T2DM. The mechanism of action revolves around the so called “lipid steal” hypothesis. T2DM is associated with increased levels of TG and FFA in the plasma consequently leading to deposition of lipids in tissues other than AT such as liver and skeletal muscle. The storage of lipids in muscle is associated with insulin resistance and lowers systemic glucose disposal. Activation of PPARγ and its target genes would lead to higher fat-storage capacity of adipose tissue, so the plasma FFA would decrease and sequestered away from tissues in which TG accumulation would cause insulin resistance [1]. Importantly, this is not the only cause of insulin resistance. Lipid overload in AT itself leads to an altered profile of AT-resident immune cells -e.g. macrophage infiltration and skewing towards a pro-inflammatory M1 phenotype- which results in a pro-inflammatory cytokine profile [2]. Crosstalk between immune cells and adipocytes also alters the secretome of the adipocytes leading to lower amounts of adiponectin among others, which has deleterious effects on the metabolic homeostasis and insulin resistance. Activation of PPARγ can counteract many of these phenomena as it results in increased adipogenesis, it endows adipocytes with lipid loading capacities and it is responsible for the expression of adipokines such as adiponectin and chemerin. However, while indeed being effective in patients with metabolic disorders, PPARγ has lost as a lot of its attractiveness as a drug target in recent years. The reason for this is that serious life-threatening side effects occur when full agonists of PPARγ such as TZDs are used. Since PPARγ1 is expressed in many tissues, side effects may occur in various tissues through this receptor instead of solely through PPARγ2 in adipose tissue. TZD-associated side effects include increased risk of congestive heart failure, myocardial infarction, cardiovascular disease, bladder cancer, weight gain, fluid retention, bone fractures and edema [3,4,5]. There are some side effects that are shared by all TZDs, including weight gain, fluid retention, bone fractures and oedema [4]. However, rosiglitazone increases the risk of congestive heart failure, myocardial infarction, cardiovascular disease and all-cause mortality [3], while pioglitazone, another full agonist, does not share the cardiovascular risks of rosiglitazone. However, pioglitazone has been linked

with congestive heart failure and bladder cancer [3]. The mechanisms behind the different and overlapping effects of these compounds is not completely understood (Table 1).

Some of the side effects can be explained through our current knowledge on the role of PPARγ in cellular functions and in whole body physiology. For example, weight gain can be explained by the fact that PPARγ is activated leading to increased adipocyte differentiation [1]. The increase in bone fractures is most likely due to a simultaneous increase in osteoclast differentiation and decrease in osteoblast differentiation [4]. Some side effects seem to be connected to one another. For example, it is thought that the negative impact of TZDs on the heart is an indirect effect mainly caused by fluid retention in the kidney rather than a direct activation of heart endogenous PPARγ [3]. Still, explaining this side effect is complex since increased PPARγ expression in the heart has been observed in patients with metabolic syndrome [3].

While full PPARγ agonists may thereby not be the best way to fight metabolic diseases, studies with TZDs have clearly indicated that i) insulin resistance can be ameliorated by improving adipose tissue function, ii) activation of the PPARγ pathway results in improved adipose tissue function. This makes PPARγ still an interesting drug target, but partial agonists that have a weaker, subtler effect in specific tissues, may be a better way to treat T2DM patients [6]. Alternatively, one could think of modulating the PPARγ pathway on different levels, including 1) *PPARG* gene regulation, 2) post translational modifications of PPARγ, which can for example affect PPARγ activity or protein turnover, 3) direct regulation of specific PPARγ target genes (Figure 1). In addition, while studies have mainly focused on the function and therapeutic effects of PPARγ in WAT, it should be noted that this transcription factor is also required for the formation of BAT. This is of special interest as recent studies indicate that the formation of BAT will have positive effects for metabolic health [7]. Importantly, many of these features are inherently connected to each other. These aspects will be discussed below based on the findings presented in this thesis, with the aim to highlight different possibilities of PPARγ-based intervention strategies.

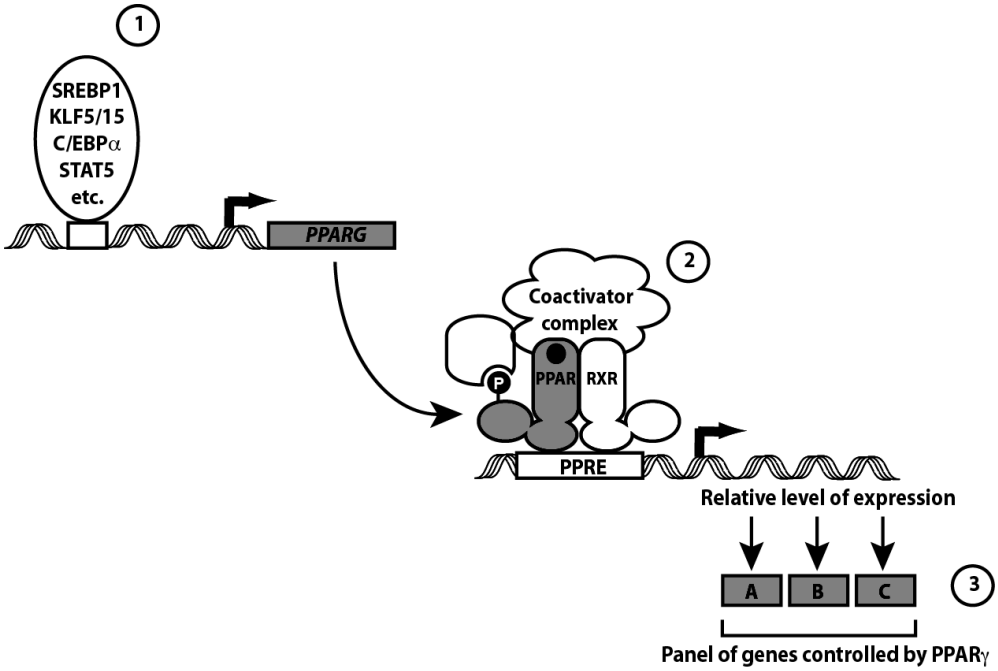


Figure 1 Schematic representation of different levels of therapeutic intervention in the PPARγ pathway. Indicated are 1) *PPARG* gene regulation, 2) post translational modifications of PPARγ, which can for example affect PPARγ activity or protein turnover, 3) direct regulation of specific PPARγ target genes.

Regulation of *PPARG* expression

Regulation of the *PPARG* gene itself in specific tissues can in principle be an effective method to treat metabolic disorders. A number of transcription factors have been implicated in the regulation of the *PPARG* gene including SREBP1c [8], C/EBPα [9,10], STAT5 [11] and Zfp423 [12]. It should be noted that direct binding to the *PPARG* locus has not been formally proven for all factors (e.g Zfp423). More importantly, these transcription factors all play multiple roles in various different biological processes: SREBP1c for example is a key regulator of cholesterol biosynthesis and uptake and synthesis of fatty acids [13], while C/EBPα is not only a key factor in adipogenesis but also in hematopoiesis [14]. Their complex roles in multiple biological processes makes the transcription factors that regulate *PPARG* expression unattractive drug targets.

Besides transcription factors that bind directly to the DNA in the *PPARG* locus, a number of non-DNA binding proteins have been identified as regulators of *PPARG* expression, including PTIP [15]. PTIP is a component of a histone methyltransferase (HMT) complex that contains two H3K4 methyltransferases (MLL3 and -4) and one H3K27 demethylase (UTX) [16,17,18]. Since both methylation of H3K4 and demethylation of H3K27 associate with gene activation, this complex is probably involved in gene activation. Deletion of *PTIP* impairs the enrichment of H3K4 methylation and RNA polymerase II on the promotors of PPARγ and also C/EBPα, resulting in impaired adipogenesis in MEFs [15]. Furthermore, in Chapters 2 and 3 we show that Baf57, which is part of the SWI/SNF chromatin remodeling complex, and Phf12 (also called Pf1), which is implicated in HDAC-mediated gene repression [19], are able to regulate PPARγ expression. As the PTIP-, Baf57- and Phf12-containing complexes all harbor enzymatic activities, pharmacological manipulation is in principle feasible as a means to increase *PPARG* expression. However, the biological role of these 3 complexes is obviously not limited to the regulation of *PPARG* expression, and altering their activities will result in many undesirable effects. Taken together, both transcription factors (e.g. SREBP1c, C/EBPα, STAT5, Zfp423) and transcriptional coregulators (e.g. PTIP, Baf57, Phf12) that have currently been implicated in the regulation of *PPARG* expression are unattractive drug targets, given the plethora of biological processes in which they function. Strategies to increase *PPARG* expression as a means to fight metabolic diseases may only become interesting again when very specific regulators of *PPARG* expression are identified.

Modulation of PPARγ PTM

In this thesis we have shown that PPARγ activity can be modulated on different levels. In general, the activity of a given transcription factor may differ between cell types and tissues through PTMs that can for example affect the stability of the protein, change its subcellular localization or alter its affinity for specific transcriptional co-activators or co-repressors. Differences in the expression and/or activity of the enzymes responsible for the PTM, including kinases, phosphatases, (de)acetyltransferases and E3-ligases, can therefore be responsible for the net transcriptional output of a given transcription factor. Interfering with

the PTM pathways may therefore present a way to subtly modulate PPARγ activity in the target tissue(s) while undesired effects can be minimized [20].

The effect of PTMs on PPARγ was already evident in 1998, when Shao et al. demonstrated that overexpression of the PPARγ S112A mutant, which can no longer be phosphorylated on residue serine 112, in pre-adipocytes induced spontaneous differentiation in the absence of any ligand [21]. In that same set of experiments the PPARγ S112D mutant, which mimicks PPARγ phosphorylated on S112, displayed less adipogenic capabilities compared to wild-type PPARγ. Surprisingly, while these data suggest that S112 phosphorylation impairs PPARγ activity, other reports suggest a stimulatory effect (reviewed in [20]). Multiple kinases for S112 phosphorylation have been identified, and the kinase involved plus the cellular background may determine the net outcome of S112 phosphorylation [20]). Targeting S112 kinases is therefore currently a difficult strategy to modulate PPARγ activity. Recently two new players have been identified that are linked to S112 phoshorylation, the phosphatase PPM1B and the circadian protein PER2.

Serine 112 phosphorylation and PPM1B

In chapter 2 we showed that PPM1B, a phosphatase of PPARγ, is able to stimulate PPARγ activity by dephosphorylating serine 112. Interestingly, loss of PPM1B abbrogated mRNA transcription of specific PPARγ target genes such as adiponectin, lpl and lipin but not cd36. Stimulation of PPM1B activity might therefore ameliorate certain aspects of the metabolic syndrome. Especially since the effect of PPM1B is quite subtle and selective, side effects might be limited. The catalytic activity of PPM1B can be induced by unsaturated fatty acids [22,23], but at the moment no small molecule agonist for PPM1B has been identified. However, synthetic inhibitors for PPM1D, a member of the same family as PPM1B, have been developed and can be used to slow down growth of breast cancer cells and reduce tumour growth in mice [24]. Furthermore, diabetic rats treated with d-chiro-inositol-galactosamine, an allosteric activator of PPM1A, displayed improved insulin signaling and decreased blood sugar levels [24]. More recently another study showed that activation of PPM1A by a chemical compound in rats prevented liver fibrosis [25]. Together these findings suggest that, like PPM1A and PPM1D, the phosphatase PPM1B can in principle be targeted by a small molecule

activator. As soon as one is available, it would be interesting to see the effect of PPM1B agonists on PPARγ activity both *in vitro* and *in vivo*.

It should be noted that besides PPARγ, PPM1B can also dephosphorylate a number of other proteins, including Cdk9, TAK1, IKKβ, cdk2, cdk6 ([26] and references therein). In a complex with Gas41, PPM1B is also able to stabilize p53 by dephosphorylation [27]. While this raises a potential problem of specificity when considering PPM1B as a potential drug target, it may also be an advantage. Interestingly, TAK1 is part of a complex that is downstream of IL-1 and TNFα signaling which subsequently leads to NFkB-mediated suppression of PPARγ activation. This pathway occurs in pluripotent mescenchymal stem cells and favours the formation of osteoblasts rather than adipocytes [28]. In line with the PPARγ-activating property of PPM1B, this phosphatase is known to dephosphorylate and thereby inhibit TAK1, thereby possibly inhibiting its PPARγ suppressive action. While it remains to be investigated whether the same pathway is operational in adipocytes, PPM1B-mediated inhibition of TAK1 may also help to maintain high PPARγ activity in these cells. In addition, IKKβ, an activator of NFκB, is also a target of PPM1B [29]. NFκB inhibits PPARγ expression and it was found that IKKβ inhibitors blocked TNFα mediated downregulation of adiponectin secretion while increasing the phosphorylation of AKT necessary for insulin signaling [30]. It was shown in HEK293T cells that PPM1B and PPM1A were capable of dephosphorylating IKKβ, leading to an abbrogation of TNFα-induced IKKβ-NFκB activation [29]. In adipocytes PPM1B could clearly favour PPARγ expression and activation in this manner, but no research has been done on the inhibiting activities of PPM1B in the TNFα/IKKβ signaling pathways in these cells so far. Based on the role of PPM1B in the NFκB inflammatory pathway, activating this phosphatase by means of a small molecule drug may not only ameliorate insulin resistance by modulating PPARγ activity, but may also lower the inflammatory pathways in adipose tissue that result from TNFα/NFκB signaling.

Serine 112 phosphorylation and PER2

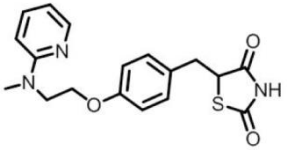
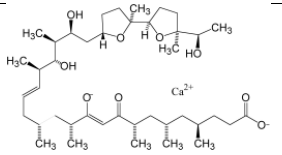
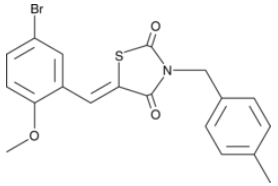
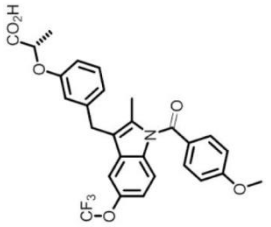
The PER2 gene is a member of the Period family of genes and is expressed in a circadian pattern in several cell types, including adipocytes [31]. PER2 inhibits PPARγ through blocking of PPARγ recruitment to target promoters [32]. This inhibitory action of PER2 depeends on S112 phosphorylation, but PER2 does not

directly affect the phosphorylated status of PPARγ. Importantly, PER2-mediated inhibition of PPARγ activity is at least partly tissue specific: PER2 deletion induces expression of BAT-specific genes in WAT, but not in WAT or the liver. Moreover, PER2-/- mice display an altered lipid profile and increased oxidative capacity in WAT. Presumably the phosphorylated state of PPARγ is at the base of these differences since PPARγ is less phosphorylated in BAT [32]. It is unclear if and how the inhibitory action of PER2 is related to the sumoylation of lysine 107, which is also controlled by serine 112 phosphorylation and associated with reduced PPARγ activity (reviewed in [20]).

Serine 273 phosphorylation, Cdk5 and PPARγ ligands

Recently, a second phosphorylation site on PPARγ was identified, serine 273, which can be phosphorylated by CDK5. Serine 273 phosphorylation by CDK5 dysregulated expression of a selective subset of PPARγ genes, which included adipsin and adiponectin, but not LPL, FABP4 or Glut4 [33,34]. While both HFD and TNFα can activate CDK5, which leads to increased S273 phosphorylation, rosiglitazone treatment leads to decreased S273 phosphorylation, and increased transcriptional activity [33,34].

Table 1 Selected PPARγ ligands

Compound		Full/ partial	Mode of action	Structural target	Effects
Rosiglitazone		Full	Increases S112 phosphorylation. Increases ubiquitination. Faster protein turnover. Blocks S273 phosphorylation	helix 7 and 12	Increases insulin mediated glucose uptake, hepatic/adipose insulin sensitivity. Reduction of plasma insulin
Ionomycin		Partial	Blocks S273 phosphorylation	helix 7 and 10	Improves hyperglycaemia in diabetic mice. Reduction of plasma insulin
GQ-16		Partial	Blocks S273 phosphorylation	β-sheet and helix 3	Improves insulin sensitivity.
MRL24		Partial	Blocks S273 phosphorylation	β-sheet and helix 3	Reduction hyperglycaemia

Interestingly, MRL24, a weak PPARγ agonist with little adipogenic potential, was able to block S273 phosphorylation as efficiently as rosiglitazone, and selectively induce increased adipsin and adiponectin expression. The mechanisms underlying this specificity in target gene regulation remain unclear at this moment. Most likely, the phosphorylation at serine 273 together with other PTMs is associated with different co-activators and repressors. Interestingly, mice with an adipose tissue specific deletion of NCoR have enhanced PPARγ activity and insulin sensitivity. The authors state that NCoR enhances the ability of PPARγ to associate with CDK5 [35]. Adipose specific NCoR depleted mice are not affected by TZD treatment suggesting that TZDs main role of action within the adipose tissue is by dissociating PPARγ from NCor leading to less S273 phosphorylation [35]. Whether the same mechanism is also operational for weaker, partial agonists like MRL24 needs to be determined. Interestingly, compounds other than MRL24 have been reported that may operate through similar yet slightly different mechanisms (Table 1). For example, GQ-16 is reported to be a novel ligand for PPARγ which promotes insulin sensitization by inhibition of CDK5-mediated phosphorylation [36]. Moreover, side effects such as weight gain and edema were absent when diabetic mice were treated with this compound. Very recently, another PPARγ ligand that inhibits S273 phosphorylation was identified, the antibiotic ionomycin, which effectively improved hyperglycaemia in diabetic mice with minimal side effects [37]. It should be noted that, similar to GQ -16, this molecule has very little adipogenic capacity, so the sequestration of lipids in adipose tissue should be investigated as well. There are structural differences and mode of operation between these compounds. Whereas TZDs mainly act on helix 7 and 12, weak agonist such as MRL24, GQ-16 and nTZDpa act on helix 3 and the beta sheet; ionomycin seems to affect helix 7 and 10, which may explain the different features in vitro and in vivo ([33,36,37], see also below). In case of all these new compounds it remains to be investigated in humans whether their therapeutic effects are still potent enough: the “lipid steal” effect (i.e. sequestering of lipids) is probably reduced compared to TZDs.

Regulation of specific PPARγ target genes

As discussed above, certain PPARγ PTMs will affect PPARγ in such a manner that this nuclear receptor will only alter the expression of a subset of genes. If such an

intervention, e.g. through compounds like MRL24, is still associated with side effects, a third level of intervention can be considered: direct modulation of specific PPARγ target genes rather than targeting PPARγ itself. A very good candidate for this is the *ADIPOQ* gene, which encodes adiponectin [38]. This adipokine, which is a well-established direct target gene of PPARγ [39], is known to decrease the degree of insulin resistance. The hormone is present in high, medium, and low molecular weight forms (HMW, MMW, and LMW) in plasma of which the HMW form has the most potent insulin-sensitizing activity [38]. An important part of the antidiabetic effect of adiponectin occurs through AMPK and PPARα pathways in the liver [38]. Two compounds have been found to increase endogenous adiponectin levels and/or its multimerization: resveratrol and emodin. Resveratrol, a natural compound that is present in grapes, is found to promote adiponectin multimerization via a Sirt1-dependent mechanism [40]. In addition emodin, an active ingredient of Chinese herbs, is reported to activate AMPK, PPARγ and increases the ratio of HMW adiponectin to total adiponectin in 3T1-L1 adipocytes [41]. These products may therefore have beneficial effects on patients suffering from T2DM or other metabolic disorders. On the other hand, a synthetic ligand for the adiponectin receptors may have similar effects. Interestingly, a small-molecule adiponection receptor agonist was recently developed which ameliorates insulin resistance and glucose intolerance in mice [42]. In addition, this compound, named AdipoRon, also enhances lifespan in genetically obese *db/db* mice.

It should be noted however that increased expression of PPARγ target genes does not necessarily lead to beneficial effects. For example, mice that had mutations in the fabp4 gene did not develop insulin resistance or diabetes in contrast to control mice, suggesting that inactivation of this classical PPARγ target genes protects against metabolic disorders [43].The mechanism behind this surprising effect is that obese fabp4-/- animals failed to express TNFα in adipose tissue, which is linked to insulin resistance. To go even further, a small molecule inhibitor of fabp4 was shown to be an effective therapeutic agent against severe atherosclerosis and T2DM in mouse models [44]. In conclusion, small molecule activators (e.g AdipoRon) or inhibitors (e.g. Fabp4) that are directed against PPARγ target genes might be able to ameliorate the effects of metabolic disorders in humans.

In chapter 5, we have identified Chst11 as a novel PPARγ target gene and shown that depletion of Chst11 in adipocytes leads to reduced intracellular lipid accumulation. Therefore, stimulation of Chst11 expression or activity might be an interesting target in diabetic patients: increased Chst11 activity in adipocytes may result in increased lipid storage in adipose tissue and net partitioning away from liver and muscle ("lipid steal hypothesis"). Although no specific agonist has been reported for Chst11, other strategies to increase Chst11 expression or activity might exist. Chst11 appears to be a downstream target of BMP signaling pathway in embryoid bodies [45]]. In addition, TGFβ was also demonstrated to regulate Chst11 expression in the human context [46]. While stimulating these pathways in adipocytes may be a way to increase Chst11 expression, both growth factors have multiple roles in other biological processes and targeting them specifically to adipose tissue may not be feasible. Alternatively, the activity of Chst11 may be altered by focusing on its glycosylation status, as Chst11 is a protein that is heavily glycosylated, a modification that is necessary for the protein to be functionally active [47]. Treatment of recombinant Chst11 with N-glycosidase F caused a firm decrease in activity. This means that maintaining the glycosylated state of Chst11 will keep the protein from turning inactive. Chst11 from the culture medium of rat chondrosarcoma cells was glycosylated for approximately 35%. For intracellular Chst11 in human adipocytes this is yet to be determined. However, while targeting Chst11 in adipose tissue may be possible, its expression is highest in the lung, placenta, spleen, thymus and peripheral leukocytes [48]. Therefore, it is realistic to assume that many side effects will be reported when Chst11 is a target of pharmacological intervention.

Browning of WAT

Formation of brown or beige/brite fat is associated with anti-obesity and anti-diabetic effects in several mouse models (reviewed in [7]). It is known that Fgf21 and TZDs are insulin sensitizers with browning properties that also promote PPARγ sumoylation [49,50]. Strikingly, full agonism of PPARγ is required to activate a thermogenic brown fat gene program in subcutaneous white fat [51]. This observation was made when the effects of rosiglitazone were compared with weaker agonists such as MRL24, nTZDpa, Mbx-102 and BVT.13 of which the latter four did not strongly induce brown genes such as Ucp1 and Cidea [51]. An

essential protein in this process is the transcriptional regulator PRDM16, since depletion of PRMD16 abbrogated the effects of rosiglitazone. More specifically, rosiglitazone stabilizes PRDM16 and increases its half-life 3-fold [51]. PRMD16 is both sufficient and essential for brown fat differentiation since it drives the expression of brown-fat genes while PRDM16 knockdown impairs BAT differentiation [52,53,54]. Furthermore, recently it has been shown that it is necessary for PPARγ to be deacetylated at Lys268 and Lys293 for PRDM16 to bind [49]. The deacetylation of Lys268 and Lys293 occurs via Sirt1 and leads to selective induction of BAT genes and repression of visceral WAT genes associated with insulin resistance [49]. Interestingly, Villanueava et al. have recently identified the groucho family member TLE3 as a white adipose-selective co-factor that acts reciprocally with the brown-selective PRDM16 for induction of the brown fat gene program [55,56]. TLE3 counters PRDM16 by blocking its interaction with PPARγ, an thereby supressing brown-selective genes while inducing white adipogenic genes. Mice deficient in TLE3 show enhanced thermogenesis in inguinal white adipose depots and are protected from age-dependent loss of BAT function. Together these findings indicate that perhaps compounds that could raise PRMD16 and/or Sirt1 or lower TLE3 levels in in pre-adipocytes would promote the formation of BAT leading to an improved metabolic state.

Final remarks

While activation of PPARγ has clear anti-diabetic effects, its use is accompanied by undesired and even harmful side effects, probably due to the full agonistic action of the TZD class of ligands. Partial agonists may overcome some of these issues, but their efficacy in humans remains to be established. Alternative pathways to alter PPARγ activity include interventions on the level of 1) *PPARG* gene regulation, 2) post translational modifications of PPARγ, which can for example affect PPARγ activity or protein turnover, 3) direct regulation of specific PPARγ target genes. Altering the expression of a subset of PPARγ target genes by manipulation of PPARγ PTMs may be the best way of using PPARγ as an anti-diabetic drug target. A full activation of PPARγ should be avoided due to the side effects described in the introduction. However, modulating PPARγ in the right tissue and cell type through the desired and specific PTM should be the goal we

should strive for. This way, only a select set of target genes that have beneficial effects on the metabolic state will be expressed. It should be noted that there will always be variation in PPARγ treatment because this gene is prone to sequence variation. Wolford et al. identified sequence variation in *PPARG* that may contribute to different insulin-sensitizing responses to troglitazone therapy in women with previous gestational diabetes [57].

Finally, it should be noted that, while targeting PPARγ and adipose tissue has proven anti-diabetic effects, alternative interventions may be developed that originate from other tissues, but still improve adipose function. PGC1α, a co-activator of PPARγ, is responsible for the secretion of a peptide that drives brown-fat-like development of white fat and thermogenesis [58]. This peptide, irisin, is secreted by the muscle and acts on white adipose cells to stimulate UCP1 and other brown fat gene expression. The authors show that short treatment of obese mice with irisin improves glucose homeostasis and causes a small weight loss. However, the exact mode of action of this myokine in adipocytes is unknown and more research is necessary.

References

1. Tontonoz P, Spiegelman BM (2008) Fat and beyond: the diverse biology of PPARgamma. AnnuRevBiochem 77: 289-312.
2. Tilg H, Moschen AR (2006) Adipocytokines: mediators linking adipose tissue, inflammation and immunity. NatRevImmunol 6: 772-783.
3. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, et al. (2013) PPARgamma signaling and metabolism: the good, the bad and the future. Nature medicine 19: 557-566.
4. Kahn BB, McGraw TE (2010) Rosiglitazone, PPARgamma, and type 2 diabetes. The New England journal of medicine 363: 2667-2669.
5. Yki-Jarvinen H (2004) Thiazolidinediones. NEnglJMed 351: 1106-1118.
6. Knouff C, Auwerx J (2004) Peroxisome proliferator-activated receptor-gamma calls for activation in moderation: lessons from genetics and pharmacology. EndocrRev 25: 899-918.
7. Harms M, Seale P (2013) Brown and beige fat: development, function and therapeutic potential. Nature medicine 19: 1252-1263.
8. Fajas L, Schoonjans K, Gelman L, Kim JB, Najib J, et al. (1999) Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism. MolCell Biol 19: 5495-5503.
9. Clarke SL, Robinson CE, Gimble JM (1997) CAAT/enhancer binding proteins directly modulate transcription from the peroxisome proliferator-activated receptor gamma 2 promoter. Biochemical and biophysical research communications 240: 99-103.
10. Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, et al. (2002) C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 16: 22-26.
11. Kawai M, Namba N, Mushiake S, Etani Y, Nishimura R, et al. (2007) Growth hormone stimulates adipogenesis of 3T3-L1 cells through activation of the Stat5A/5B-PPARgamma pathway. Journal of molecular endocrinology 38: 19-34.
12. Gupta RK, Arany Z, Seale P, Mepani RJ, Ye L, et al. (2010) Transcriptional control of preadipocyte determination by Zfp423. Nature 464: 619-623.
13. Raghow R, Yellaturu C, Deng X, Park EA, Elam MB (2008) SREBPs: the crossroads of physiological and pathological lipid homeostasis. Trends EndocrinolMetab 19: 65-73.
14. Koschmieder S, Rosenbauer F, Steidl U, Owens BM, Tenen DG (2005) Role of transcription factors C/EBPalpha and PU.1 in normal hematopoiesis and leukemia. International journal of hematology 81: 368-377.
15. Cho YW, Hong S, Jin Q, Wang L, Lee JE, et al. (2009) Histone methylation regulator PTIP is required for PPARgamma and C/EBPalpha expression and adipogenesis. Cell metabolism 10: 27-39.
16. Cho YW, Hong T, Hong S, Guo H, Yu H, et al. (2007) PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. The Journal of biological chemistry 282: 20395-20406.

17. Issaeva I, Zonis Y, Rozovskaia T, Orlovsky K, Croce CM, et al. (2007) Knockdown of ALR (MLL2) reveals ALR target genes and leads to alterations in cell adhesion and growth. Molecular and cellular biology 27: 1889-1903.
18. Patel SR, Kim D, Levitan I, Dressler GR (2007) The BRCT-domain containing protein PTIP links PAX2 to a histone H3, lysine 4 methyltransferase complex. Developmental cell 13: 580-592.
19. Yochum GS, Ayer DE (2001) Pf1, a novel PHD zinc finger protein that links the TLE corepressor to the mSin3A-histone deacetylase complex. Molecular and cellular biology 21: 4110-4118.
20. van Beekum O, Fleskens V, Kalkhoven E (2009) Posttranslational modifications of PPAR-gamma: fine-tuning the metabolic master regulator. Obesity(SilverSpring) 17: 213-219.
21. Shao DL, Rangwala SM, Bailey ST, Krakow SL, Reginato MJ, et al. (1998) Interdomain communication regulating ligand binding by PPAR-gamma. Nature 396: 377-380.
22. Klumpp S, Selke D, Hermesmeier J (1998) Protein phosphatase type 2C active at physiological Mg2+: stimulation by unsaturated fatty acids. FEBS Lett 437: 229-232.
23. Skinner J, Sinclair C, Romeo C, Armstrong D, Charbonneau H, et al. (1997) Purification of a fatty acid-stimulated protein-serine/threonine phosphatase from bovine brain and its identification as a homolog of protein phosphatase 5. The Journal of biological chemistry 272: 22464-22471.
24. McConnell JL, Wadzinski BE (2009) Targeting protein serine/threonine phosphatases for drug development. Molecular pharmacology 75: 1249-1261.
25. Wang L, Wang X, Chen J, Yang Z, Yu L, et al. (2010) Activation of protein serine/threonine phosphatase PP2Calpha efficiently prevents liver fibrosis. PloS one 5: e14230.
26. Tasdelen I, van Beekum O, Gorbenko O, Fleskens V, van den Broek NJ, et al. (2013) The serine/threonine phosphatase PPM1B (PP2Cbeta) selectively modulates PPARgamma activity. The Biochemical journal 451: 45-53.
27. Ofek P, Ben-Meir D, Kariv-Inbal Z, Oren M, Lavi S (2003) Cell cycle regulation and p53 activation by protein phosphatase 2C alpha. The Journal of biological chemistry 278: 14299-14305.
28. Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, et al. (2003) Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. NatCell Biol 5: 224-230.
29. Sun W, Yu Y, Dotti G, Shen T, Tan X, et al. (2009) PPM1A and PPM1B act as IKKbeta phosphatases to terminate TNFalpha-induced IKKbeta-NF-kappaB activation. Cellular signalling 21: 95-102.
30. Nguyen MT, Satoh H, Favelyukis S, Babendure JL, Imamura T, et al. (2005) JNK and tumor necrosis factor-alpha mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. The Journal of biological chemistry 280: 35361-35371.
31. Otway DT, Frost G, Johnston JD (2009) Circadian rhythmicity in murine pre-adipocyte and adipocyte cells. Chronobiology international 26: 1340-1354.

32. Grimaldi B, Bellet MM, Katada S, Astarita G, Hirayama J, et al. (2010) PER2 controls lipid metabolism by direct regulation of PPARgamma. Cell metabolism 12: 509-520.
33. Choi JH, Banks AS, Estall JL, Kajimura S, Bostrom P, et al. (2010) Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5. Nature 466: 451-456.
34. Choi JH, Banks AS, Kamenecka TM, Busby SA, Chalmers MJ, et al. (2011) Antidiabetic actions of a non-agonist PPARgamma ligand blocking Cdk5-mediated phosphorylation. Nature 477: 477-481.
35. Li P, Fan W, Xu J, Lu M, Yamamoto H, et al. (2011) Adipocyte NCoR knockout decreases PPARgamma phosphorylation and enhances PPARgamma activity and insulin sensitivity. Cell 147: 815-826.
36. Amato AA, Rajagopalan S, Lin JZ, Carvalho BM, Figueira AC, et al. (2012) GQ-16, a novel peroxisome proliferator-activated receptor gamma (PPARgamma) ligand, promotes insulin sensitization without weight gain. The Journal of biological chemistry 287: 28169-28179.
37. Zheng W, Feng X, Qiu L, Pan Z, Wang R, et al. (2013) Identification of the antibiotic ionomycin as an unexpected peroxisome proliferator-activated receptor gamma (PPARgamma) ligand with a unique binding mode and effective glucose-lowering activity in a mouse model of diabetes. Diabetologia 56: 401-411.
38. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, et al. (2006) Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. The Journal of clinical investigation 116: 1784-1792.
39. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, et al. (2003) Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 52: 1655-1663.
40. Wang A, Liu M, Liu X, Dong LQ, Glickman RD, et al. (2011) Up-regulation of adiponectin by resveratrol: the essential roles of the Akt/FOXO1 and AMP-activated protein kinase signaling pathways and DsbA-L. The Journal of biological chemistry 286: 60-66.
41. Chen Z, Zhang L, Yi J, Yang Z, Zhang Z, et al. (2012) Promotion of adiponectin multimerization by emodin: a novel AMPK activator with PPARgamma-agonist activity. Journal of cellular biochemistry 113: 3547-3558.
42. Okada-Iwabu M, Yamauchi T, Iwabu M, Honma T, Hamagami KI, et al. (2013) A small-molecule AdipoR agonist for type 2 diabetes and short life in obesity. Nature.
43. Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, et al. (1996) Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. Science 274: 1377-1379.
44. Furuhashi M, Tuncman G, Gorgun CZ, Makowski L, Atsumi G, et al. (2007) Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. Nature 447: 959-965.
45. Kluppel M, Vallis KA, Wrana JL (2002) A high-throughput induction gene trap approach defines C4ST as a target of BMP signaling. Mechanisms of development 118: 77-89.

46. Willis CM, Wrana JL, Kluppel M (2009) Identification and characterization of TGFbeta-dependent and -independent cis-regulatory modules in the C4ST-1/CHST11 locus. Genetics and molecular research : GMR 8: 1331-1343.
47. Yusa A, Kitajima K, Habuchi O (2005) N-linked oligosaccharides are required to produce and stabilize the active form of chondroitin 4-sulphotransferase-1. The Biochemical journal 388: 115-121.
48. Kluppel M (2010) The roles of chondroitin-4-sulfotransferase-1 in development and disease. Progress in molecular biology and translational science 93: 113-132.
49. Qiang L, Wang L, Kon N, Zhao W, Lee S, et al. (2012) Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Ppargamma. Cell 150: 620-632.
50. Dutchak PA, Katafuchi T, Bookout AL, Choi JH, Yu RT, et al. (2012) Fibroblast growth factor-21 regulates PPARgamma activity and the antidiabetic actions of thiazolidinediones. Cell 148: 556-567.
51. Ohno H, Shinoda K, Spiegelman BM, Kajimura S (2012) PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. Cell metabolism 15: 395-404.
52. Seale P, Bjork B, Yang W, Kajimura S, Chin S, et al. (2008) PRDM16 controls a brown fat/skeletal muscle switch. Nature 454: 961-967.
53. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, et al. (2007) Transcriptional control of brown fat determination by PRDM16. Cell metabolism 6: 38-54.
54. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, et al. (2011) Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. The Journal of clinical investigation 121: 96-105.
55. Villanueva CJ, Vergnes L, Wang J, Drew BG, Hong C, et al. (2013) Adipose subtype-selective recruitment of TLE3 or Prdm16 by PPARgamma specifies lipid storage versus thermogenic gene programs. Cell metabolism 17: 423-435.
56. Villanueva CJ, Waki H, Godio C, Nielsen R, Chou WL, et al. (2011) TLE3 Is a Dual-Function Transcriptional Coregulator of Adipogenesis. Cell Metab 13: 413-427.
57. Wolford JK, Yeatts KA, Dhanjal SK, Black MH, Xiang AH, et al. (2005) Sequence variation in PPARG may underlie differential response to troglitazone. Diabetes 54: 3319-3325.
58. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, et al. (2012) A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 481: 463-468.

Chapter 7

Summary en Samenvatting

Summary

Obesity is regarded as a major public health problem by the World Health Organization. Obesity is characterized by an increase in adipose tissue, both through an increase in the amount of fat stored per adipocyte, and the generation of new adipocytes through differentiation of pre-adipocyte (adipogenesis), a process in which the transcription factor PPARγ plays a significant role. In **Chapter 1**, we described different aspects of PPARγ, adipose tissue and adipogenesis. PPARγ is necessary for adipogenesis, maintenance of adipose tissue and is able to regulate many "adipocyte genes" that are necessary for lipid and glucose metabolism. Many aspects of PPARγ are under intense study, here we focused on the modulation of the expression and activity this factor.

It is known that PPARγ is subject to post-translational modifications (PTM) such as phosphorylation, ubiquitination, acetylation and sumoylation. All of these marks have an effect on the activity of PPARγ. However, not much was known about the opposite process: de-modification**.** In **Chapter 2**, we identify PPM1B to be a phosphatase for PPARγ. PPM1B was capable of directly binding to PPARγ and dephosphorylating a serine residue at position 112. In general, S112 phosphorylation lowers PPARγ activity, subsequently leading to decreased target gene expression. Knocking down PPM1B indicated that this was indeed the case for a subset of genes. This led to the conclusion that PPARγ (de)phosphorylation at S112 had subtle effects which could be seen in terms of specific target gene expression, but not on adipogenesis as a whole. Interestingly, a second phosphorylation site was recently identified on PPARγ, but no link has been made between S273 phosphorylation and PPM1B.

Adipogenesis occurs in at least two waves; genes such as C/EBPβ, C/EBPδ, KLF5, CREB, SREBP-1c and Krox20 come up early in adipogenesis and ultimately lead to the expression of PPARγ and C/EBPα, which can than regulate genes that have a direct role in glucose and lipid metabolism. We investigated which other proteins would be capable of regulating the *PPARG* gene and thus adipogenesis. In **Chapter 3** we developed an siRNA based knockdown screen and subsequently found Baf57, a subunit of the SWI/SNF chromatin remodeling complex, to regulate *PPARG* expression and adipogenesis. Knockdown of Baf57 impairs adipogenesis; transcriptome analysis reveals that the genes that are inhibited are to a large extent the same as the genes that are inhibited by PPARγ knockdown.

The genes that are affected are the so-called late adipocyte genes, rather than the early genes mentioned above. Further analysis indicated that Baf57 directly binds to the *PPARG* promotor to induce its expression in both human and murine adipocyte cell lines. A similar case is Phf12, a “chromatin-associated” factor that has mainly been implicated in transcriptional repression, which is investigated in **Chapter 4**. Phf12 was also discovered in the siRNA based knockdown screen from Chapter 3, and is also necessary for adipogenesis. Phf12 is also capable of binding to the *PPARG* promotor to induce expression of this protein and knockdown of Phf12 shows that it only affects the expression of late adipocyte genes.

Regulation of a large set of “adipocyte genes” is an important role of PPARγ in adipogenesis. In **Chapter 5**, we show that Chst11 is a novel target gene of PPARγ. We identified two loci on the Chst11 promotor that are PPARγ responsive and show that PPARγ inhibition or knockdown also leads to decreased Chst11 levels. Chst11 is expressed early in differentiation and is only present in mature adipocytes. Knockdown of this protein leads to lower intracellular triglyceride accumulation in differentiating adipocytes. This does not appear to be due to an impairment of adipogenesis itself, since hallmark genes such as LPL, adiponectin (Adipoq), C/EBPα and PPARγ are not altered in expression. The mechanism by which Chst11 regulates triglyceride regulation is probably by increasing the amount of negatively charged sulphated CS chains on the adipocyte cell surface that can form docking sites for Lpl, which can then facilitate the uptake of fatty acids in adipocytes.

In **Chapter 6** we discuss PPARγ as a potential target for clinical intervention. Modulation of PPARγ may be used in order to ameliorate obesity if done properly. Full agonists of PPARγ lead to many severe side effects in humans, but subtle modulation through post translational modifications (PTM) or PPARγ associated proteins may provide an alternative answer for treatment of obesity.

Samenvatting

Obesitas is een steeds groter wordend gezondheidsprobleem in de westerse maatschappij. Door verscheidene (leefstijl) factoren begint deze ziekte meer mensen te overkomen. Obesitas is een verhoging van het percentage lichaamsvet dat komt door een toename in het aantal vetcellen (adipocyten) en het groter worden van adipocyten. Het proces dat ervoor zorgt dat voorloper cellen zich ontwikkelen tot volwaardige adipocyten die in staat zijn vet op te slaan, heet adipogenese. Er zijn erg veel eiwitten die een rol spelen in dit proces, maar voor PPARγ is duidelijk een centrale rol weggelegd. PPARγ is belangrijk vanwege een aantal redenen, waaronder het reguleren van genen die een directe rol hebben in vet- en suiker metabolisme. Adipocyten die geen PPARγ meer hebben sterven af en zonder PPARγ kunnen de pre-adipocyten sowieso niet differentieren tot adipocyten. Door deze belangrijke functies van PPARγ wordt er veel onderzoek gedaan naar dit eiwit. Deze worden onder andere in **Hoofdstuk 1** besproken.

Net zoals vele andere eiwitten, kan er een fosfaat groep op PPARγ geplaatst worden door een eiwit dat een kinase heet. De positie van deze fosfaat groep (een post translationele modificatie, PTM) is van belang voor de activiteit en werking van het eiwit. Dit geldt ook voor PPARγ. Hoewel al vele kinases bekend waren voor PPARγ, waren er nog bijna geen eiwitten bekend dat deze fosfaat groep konden verwijderen (phosphatase). In **Hoofdsstuk 2** laten we zien dat PPM1B een dergelijke phosphatase van PPARγ is; PPM1B is in staat om een fosfaat groep op een serine op positie 112 (S112) te verwijderen. Een fosfaat groep op S112 leidt er vaak toe dat PPARγ minder actief wordt, dus verwijdering van dit label zou er voor moeten zorgen dat die inactiviteit ongedaan gemaakt wordt. De activiteit van PPARγ kan worden gemeten door te bepalen hoe dit eiwit (target) genen kan reguleren. Verwijdering van PPM1B uit de adipocyt zorgt er inderdaad voor dat PPARγ target genen minder tot expressie komen. Het opmerkelijke hieraan is dat deze reductie niet voor alle target genen van PPARγ geldt en er dus sprake is van selectie. Deze selectieve aanpak van PPARγ kan mogelijk gebruikt worden voor therapeutische doeleinden. PPARγ heeft vele target genen die allerlei functies hebben in vet en glucose metabolisme.

Behalve dat PPARγ zelf geactiveerd kan worden en target genen kan induceren, hebben we ook onderzoek gedaan naar eiwitten die PPARγ expressie kunnen reguleren. PPARγ heeft een relatief late rol in adipogenese. Dit begint met

de inductie van andere genen zoals c/ebpβ en c/ebpδ die uiteindelijk leiden tot de expressie van PPARγ. Via een screen die heel selectief meer dan 500 verschillende genen inactiveert met behulp van siRNA's hebben we de eiwitten Baf57 en Phf12 kunnen identificeren als nieuwe spelers in adipogenese. Deze 2 factoren worden in **Hoofdstuk 3 en 4** respectievelijk besproken. Beide eiwitten komen voor in adipocyten en zijn nodig voor adipogenese. Het verwijderen van deze eiwitten zorgt voor een veranderd genen patroon dat erg vergelijkbaar is met het verwijderen van PPARγ zelf. Daarom vermoedden we dat Baf57 en Phf12 in staat waren om PPARγ zelf direct te kunnen reguleren. Dit bleek inderdaad het geval te zijn; vroege adipocyt genen zoals c/ebpβ en c/ebpδ werden niet aangetast door het verwijderen van Baf57 en Phf12, maar PPARγ en haar target genen wel. Beide eiwitten waren in staat om te binden aan het gebied rondom het PPARγ gen en spelen dus waarschijnlijk een directe rol in de regulatie van deze "adipogene hoofdregulator".

In **Hoofdstuk 5** laten we zien dat we een nieuw target gen van PPARγ gevonden hebben. Rondom het gen Chst11 blijken gebieden in het DNA te zijn waar PPARγ aan kan binden en ervoor kan zorgen dat Chst11 tot expressie komt. Dit eiwit is alleen aanwezig in volwassen adipocyten en het inactiveren van PPARγ leidt ook tot verminderde expressie van Chst11. Het verwijderen van Chst11 uit adipocyten leidt er toe dat adipocyten minder in staat zijn om vetten op te slaan. Dit komt hoogstwaarschijnlijk omdat Chst11 ervoor zorgt dat bepaalde suikermoleculen een sulfaat groep krijgen. Door deze sulfaat groep krijgen ze een negatieve lading en dat vormt een soort anker van een ander eiwit, LPL. LPL is in staat om vetzuren af te knippen van eiwitten dat aan vetten binden, waardoor een cel in staat kan zijn om deze vetzuren te importeren.

Zoals blijkt uit dit proefschrift is de rol van PPARγ in adipogenese and adipocyt biologie complex en kan deze op verschillende niveau's gestuurd worden. In **Hoofdstuk 6**, bediscussiëren we verschillende mogelijkheden om PPARγ als aangrijpingspunt van klinische interventie te gebruiken. Dit was tot voor kort een aantrekkelijk concept, maar therapeutische liganden zoals rosiglitazone, die PPARγ tot maximale activiteit stimuleerden werden verboden omdat ze zorgden voor vele gevaarlijke bijwerkingen zoals hartproblemen en vochtophoping. In hoofdstuk 6 laten we zien dat PPARγ activiteit beter gemoduleerd kan worden met behulp van partiële liganden (dat wil zeggen liganden die niet voor maximale activiteit zorgen), PTMs of co-regulatoren. Met

behulp van deze strategieën kan het subtiel sturen van PPARγ een alternatieve methode zijn om obesitas en obesitas gerelateerde ziektes te behandelen.

Dankwoord

So exalt [Allah] with praise of your Lord
and be of those who prostrate [to Him].
15:98, Quran

Ik wil beginnen te zeggen dat ik erg blij ben dat ik de afgelopen 4.5 jaar als PhD student heb mogen werken. Ik heb tijdens deze periode veel geleerd over de biochemie, over mijzelf en heb fantastische mensen leren kennen. Voor dat laatste wil ik nu een moment nemen om ze te bedanken.

Allereerst co-promotor dr. Kalkhoven, beste **Eric**. Heel erg bedankt dat je me hebt aangenomen voor deze positie en voor je begeleiding tijdens het gehele PhD traject. Ik heb de vrijheid in het onderzoek die je mij gaf altijd erg gewaardeerd. Door jou heb ik mijzelf verder kunnen ontwikkelen als wetenschapper. Door je laagdrempeligheid, je positieve houding, behulpzaamheid en je oprechte interesse in het onderzoek ben je een super begeleider geweest voor mij. Ik kan dan ook niet anders zeggen dan te hopen dat mijn toekomstige bazen/begeleiders op jou zullen lijken in dat opzicht.

Geachte prof. Berger, beste **Ruud**, ik ben jou ook erg dankbaar voor de interesse die je in mijn project hebt getoond. Hoewel je niet dagelijks betrokken was bij mijn werk ben ik wel blij dat je hebt meegedacht over mijn projecten.

Paranimfen Henk en Mariette. De reden dat ik wilde dat jullie mijn paranimfen zouden zijn is omdat jullie mij door en door kennen. Jullie hebben mij in mijn goede, maar ook minder goede tijden gezien. **Mariette**, ik was altijd onder de indruk van jouw doorzettingsvermogen en intelligentie. Je kwam onze groep binnenwandelen als een student dat net van de collegebanken kwam. Voordat ik het wist deed je superlange experimenten tot 03.00 in de nacht en fietste gewoon in het donker weer terug naar huis, om de dag daarop vervolgens weer om 09.00 te beginnen. Klagen hierover kwam niet eens in je op, supervrouw! Wist je trouwens dat ik jouw stageverslagen nog heb gebruikt voor het schrijven van deze thesis? Dan dr. Schipper, **Henk**. Ik denk dat ik kan zeggen dat jij de persoon bent die mij het meest heeft geinspireerd van iedereen uit mijn PhD periode. Je bent slim, ijverig, grappig, loyaal aan je principes (behalve de muizen experimenten uit je PhD periode dan ☺), sociaal en een supergoede vader. Zelden heb ik iemand ontmoet die zoveel goede eigenschappen in zich verenigd heeft. Bedankt voor alle steun, hulp, gezelligheid en bedankt dat ik uit je enorme vriendenkring jouw paranimf mocht zijn. Dan Dr. Faber, **Daniel**, mijn backup paranimf ☺. Ik leerde je pas echt goed kennen toen we 2 jaar lang elke dag gingen carpoolen. Toen leerde ik ook dat we een aantal gezamenlijke interesses hadden. Wetenschap, drop, games, films en de politiek. Ik mis vooral onze gesprekken over dat laatste, maar

ben blij dat ik de muziek die je in de auto draaide niet meer hoef te horen ☺. Ik wil je bedanken voor alle advies en leuke momenten die we zowel in het lab als in de auto gehad hebben. Heb je trouwens gemerkt dat het BovenIJ ziekenhuis het al 2 jaar veel beter doet in de top100 van beste ziekenhuizen? Hier heb jij zeker een bijdrage aan geleverd!?

Dan de andere (ex) leden van de adipogroep. **Nicole**!!! Ik zal je vrolijke en leuke houding nooit vergeten. Wanneer ik down was kon ik altijd bij je terecht voor een pep-talk. Dank je wel hiervoor! Dr. Flockenstoff, oftewel **Arjen** ☺, ik waardeer al het advies dat je me hebt gegeven en moet vaak met een grote grijns terugdenken aan alle leuke grappen die je hebt gemaakt (waaronder die ene van Tupac Shakur). **Yuan Gao**, 我的女友 , I cannot remember how much I have laughed because of you. Your practical attitude and insight in the biology are something that I was impressed by. I wish you all the best in China. **Maryam**, thank you for the very nice time in Keystone. You are a hardworking and nice person, I'm sure you will get far in science. By the way, I really did wonder how your Iranian chicken with saffran and rice would taste like☺. **Inkie**, hoewel we binnen de groep ver uit elkaar zaten qua onderzoeksdomein, weet ik dat je erg goed bezig bent. Jou wil ik bedanken om je gezelligheid en je georganiseerdheid, want jij wist altijd waar we nog stocks verborgen hadden ☺. **Annemarie**, ik vond het erg leuk om MS gerelateerde kennis te leren van jou, bedankt daarvoor.

Verder wil ik ook de oud-collega's van het WKZ bedanken voor hun gezelligheid en behulpzaamheid. **Annemieke Kolkman**, jou zal ik herinneren als 1 van de mensen die mij het meest aan het lachen heeft gemaakt. In tijden van stress wist je het weer goed te maken door anekdotes te vertellen die zelf had ervaren tijdens je eigen PhD periode. Bedankt! Ook **Stan (the man)**, **Ellen, Rina, Rafaella, Willianne, Peter, Sjors, Stiaan, Wouter, Ingrid, Noortje, Danielle, Mariette Sprong, Edwin, Samira, Sophia** en **Niels** wil ik bedanken voor de leuke tijd in het lab. **Vittoria, Alexandra**, thank you very much for the nice moments in the lab. **Truus**, onze werkrelatie is HET bewijs dat jong en oud het wel degelijk gezellig kunnen hebben in het lab. Ik zal onze gesprekken over normen en waarden missen. Verder wil **Arjan, Jo, Joost en Nanda** bedanken voor hun input tijdens de wekelijkse meetings. **Monique de Gooijer**, ik vond het altijd erg leuk om je te zien. Het ga je goed! **Benthe**, **Micha**, ik vond het erg leuk om jullie te mogen begeleiden tijdens jullie stage en ik hoop dat jullie het ook leuk vonden.

De collega's van de **Burgering/Bos/Timmers/Vermeulen/Holstege** groep wil ik hierbij ook bedanken voor hun behulpzaamheid. Ik wil **Roy** en **Petra** apart bedanken omdat wij als co-screeners tips hebben uitgewisseld. **Irem**, bir gün sende tezini yazip bittirirsin, buna emin ol. Sana bol sans dilemek isterim gelecek

kariyerinde. **David**, thank you for your help when I was setting up the screen for all the bottlenecks that I have encountered. **Marian**, bedankt voor je microarray analyses, mijn papers zijn daardoor een stuk beter geworden. **Christina**, wat hebben we toch heerlijk gesproken over Japan, dat we beiden zo een geweldig land vonden. Ik moet toegeven dat ik de zon + winter, jouw favoriete weertype, nu steeds meer begin te waarderen.

Tenslotte wil ik ook mijn familie en vrienden bedanken voor alle steun. **Sinan, Adem**, bedankt voor de leuke tijd. Door de bioscoop bezoekjes en andere uitjes kon ik even opladen om daarna verder te knallen aan mijn project. **Souffian**, Japan was een geweldig land toch? Ik vond onze reis dat we ondernamen vóór het einde van mijn aanstelling in het UMC echt top en was daardoor weer helemaal opgeladen! Bedankt daarvoor. **Azra, Hay-Yeng, Tineke**, ik vond de gezamenlijke etentjes erg leuk. Als ik het even niet zag zitten op het werk kon ik even bij jullie terecht. Bedankt! Zonder de steun van mijn vader en moeder zou ik nooit zo ver zijn gekomen. Hersey icin tessekkurler. Verder wil ik ook mijn broer **Kemal** en zus **Gulcan** bedanken. Ik heb hun interesse in mijn werk altijd gewaardeerd.

Als laatst wil mijn vrouw *Ebru* en zoon *Mikail Baran* bedanken. Jullie zijn de belangrijkste reden waarom ik dit project tot een goed einde heb gebracht. Bedankt voor alle steun, jullie zijn mijn meest waardevolle schatten.

About the author

Curriculum Vitae

Ismayil Tasdelen was born the 25th of October in 1984 in Amsterdam, the Netherlands. He finished secondary school (Gymnasium, Amstellyceum in Amsterdam) in 2003. In that same year he started his bachelor in Chemistry in the Vrije Universiteit in Amsterdam. After finishing this he continued with his master Biomolecular Sciences at the same university. He did an internship at the department of immunology under supervision of dr. Irma van Die and also did an internship in Germany at the R&D department of Sanofi-Aventis under supervision of dr. Dirk Bald and dr. Jochen Kruip. In 2008 he finished his master degree and started at UMC Utrecht in the group of dr. Eric Kalkhoven as a PhD student. For this position he set up a screen to identify novel regulators in adipogenesis and also investigated other PPARγ related proteins and genes. The results of these studies are shown in this thesis.

List of Publications

van Stijn CM, van den Broek M, van de Weerd R, Visser M, **Taşdelen I,** Tefsen B, van Die I**.** Regulation of expression and secretion of galectin-3 in human monocyte-derived dendritic cells. *Mol Immunol. 2009 Oct;46(16):3292-9. Epub 2009 Aug 21.*

Verrijn Stuart AA*, Schipper HS*, **Tasdelen I,** Egan DA, Prakken BJ, Kalkhoven E, de Jager W**.** Altered plasma adipokine levels and in vitro adipocyte differentiation in pediatric type 1 diabetes. *J Clin Endocrinol Metab. 2012 Feb;97(2):463-72. Epub 2011 Nov 23.*
*These authors contributed equally

Tasdelen I, Kalkhoven E**.** PPARγ regulates expression of carbohydrate sulfotransferase 11 (Chst11/C4ST1), a modulator of Lpl activity. *PLoS One 2013 May 16;8(5):e64284.*

Tasdelen I, van Beekum O, Gorbenko O, Fleskens V, van den Broek NJF, Koppen A, Hamers N, Berger R, Coffer PJ, Brenkman AB, Kalkhoven E. The serine/threonine phosphatase PPM1B (PP2Cβ) regulates PPARγ activity. *Biochem J. 2013 Jan 15*

Gao Y, Koppen A, Rakhshandehroo M, **Tasdelen I**, van de Graaf SF, van Loosdregt J, van Beekum O, Hamers N, van Leenen D, Berkers CR, Berger R, Holstege FC, Coffer PJ, Brenkman AB, Ovaa H, Kalkhoven E. Early adipogenesis is regulated through USP7-mediated deubiquitination of the histone acetyltransferase TIP60. *Nat. Commun. 2013*